

Agrobacterium-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower

R CHAKRABARTY, N VISWAKARMA, S R BHAT, P B KIRTI*, B D SINGH** and V L CHOPRA[†]

National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

*Department of Plant Sciences, University of Hyderabad, Hyderabad 500 046, India

**School of Biotechnology, Banaras Hindu University, Varanasi 221 005, India

[†]Corresponding author (Fax, 91-11-5826054; Email, nass@vsnl.com)

A number of factors that are known to influence genetic transformation were evaluated to optimize *Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1. The binary vector p35SGUSINT mobilized into *Agrobacterium* strain GV2260 was used for transformation and transient GUS expression was used as the basis for identifying the most appropriate conditions for transformation. Explant age, preculture period, bacterial strain and density were found to be critical determinants of transformation efficiency. Using the optimized protocol, the synthetic *cryIA(b)* gene was mobilized into cauliflower. Molecular analyses of transgenics established the integration and expression of the transgene. Insect bioassays indicated the effectiveness of the transgene against infestation by diamondback moth (*Plutella xylostella*) larvae.

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1. Introduction

Brassica oleracea var. *botrytis* (cauliflower) is an important vegetable crop grown for its edible inflorescence (curd). It is highly vulnerable to insect-pests that cause about 20–30% yield loss (Estruch *et al* 1997). Massive quantities of insecticides are used especially in mid and late-season crop in the north Indian plains where incidence of pests is high. Traditional breeding methods have significantly enhanced productivity and quality of the crop, but insect-pests, for which no source of resistance is available, continue to cause severe damage. Environment and health concerns associated with the use of pesticides call for alternative methods of pest control. Genetic engineering of plants offers practical solutions by incorporating genes for resistance from unrelated sources.

Insecticidal crystal proteins (Cry) of *Bacillus thuringiensis* (Bt) have been used since long to control insect-pests (Tabashnik 1997). In recent years transgenic cultivars expressing the *cry* gene have been shown to defend

insect attack in a wide variety of crops (Cheng *et al* 1998; Xiang *et al* 2000). *Plutella xylostella* (diamondback moth) larvae extensively feed on the leaves and curd of cauliflower, leading to reduced yield and poor quality of produce. Engineering cauliflower with the *cry* gene for resistance against the diamondback moth is an attractive proposition.

Genetic transformation of *B. oleracea* has been reported both by direct DNA uptake by protoplasts (Mukhopadhyay *et al* 1991) and by *Agrobacterium*-mediated gene transfer (Henzi *et al* 2000; Pius and Achar 2000). Among *B. oleracea*, cauliflower is reported to be the least amenable to genetic transformation (Puddephat *et al* 1996; Passelgue and Kerlan 1996). In an exercise aimed at developing Bt-transgenic cauliflower, we first optimized transformation conditions by altering parameters such as bacterial strain, explant age and co-cultivation conditions for the selection and regeneration of transformed cells. We employed *Agrobacterium* strains carrying the binary vector p35SGUSINT for transformation and identified optimal conditions based on transient GUS

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expression. The standardized protocol was then used to mobilize *cryIA(b)* gene (Fujimoto *et al* 1993) into cauliflower cv. Pusa Snowball K-1 which is the main late-season variety in northern India occupying nearly 40% of the area under this category.

2. Materials and methods

2.1 Plant material and culture conditions

Seeds of cauliflower variety Pusa Snowball K-1 used for transformation were obtained from the National Seeds Corporation, New Delhi. Seeds, surface sterilized in 0.1% HgCl_2 and 0.1% SDS for 10 min, were placed on half-strength MS (Murashige and Skoog 1962) medium with 0.8% agar for germination. The incubation conditions for *in vitro* culture, unless stated otherwise, were $25 \pm 1^\circ\text{C}$ and 16 h photoperiod of approximately $28 \mu\text{Em}^{-2} \text{s}^{-1}$.

2.2 Bacterial strains and binary plasmid vectors

The *Agrobacterium* strains were grown in minimal medium (Chilton *et al* 1974) at 28°C with continuous shaking under appropriate antibiotic selection. The disarmed *A. tumefaciens* strains, GV2260 (25 mg/l rifampicin, 100 mg/l carbenicillin and 50 mg/l kanamycin), LBA4404 (25 mg/l rifampicin and 50 mg/l kanamycin), A208 (10 mg/l rifampicin and 50 mg/l kanamycin) and EHA105 (50 mg/l kanamycin), carrying the binary plasmid vector p35SGUSINT (Vancanneyt *et al* 1990) were used in the preliminary transformation experiments. The synthetic *cryIA(b)* gene containing the first intron of cas-

tor bean catalase-1 gene and *ocs* terminator (Fujimoto *et al* 1993) was sub-cloned in the binary plasmid vector pBinAR (Hofgen and Willmitzer 1990), designated as pBinAR-Bt (figure 1), and mobilized into the *A. tumefaciens* strain GV2260.

2.3 Plant transformation

The transformation parameters were optimized for hypocotyl explants using the bacterial strain GV2260 harbouring the p35SGUSINT binary plasmid vector. Parameters were tested, one at a time, in a sequential order. The optimized conditions determined in earlier experiments were used in subsequent experiments. The following parameters (and treatments) were tested in the order stated; density of bacterial culture (0.5 OD_{600} and its dilutions 1 : 10, 1 : 20), seedling age (4, 7 and 10 d), duration of pre-culture (0, 1, 2 and 4 d), transformation enhancers (acetosyringone 0, 50 and 100 μM ; 5-azacytidine 0, 100 μM), co-cultivation temperature (22°C and 26°C), co-cultivation duration (2, 4 d), disarmed *Agrobacterium* strains (GV2260, LBA4404, A208 and EHA105).

For testing the first parameter, hypocotyl explants from 7-day-old germinated seedlings were pre-cultured for a day on pre-culture (PI) medium (MS with 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.25% phytigel) and co-cultivated at 26°C for 2 days. The explants were then transferred to MS medium with 2.0 mg/l BAP, 0.5 mg/l IAA, 250 mg/l cefotaxime (Hoechst) and 0.25% phytigel (MSI-C). After 2–3 days of growth in MSI-C medium, transformation was assessed using the GUS histochemical assay (Jefferson 1987).

Explant mortality, number of explants expressing GUS and GUS spots/explant were considered while stan-

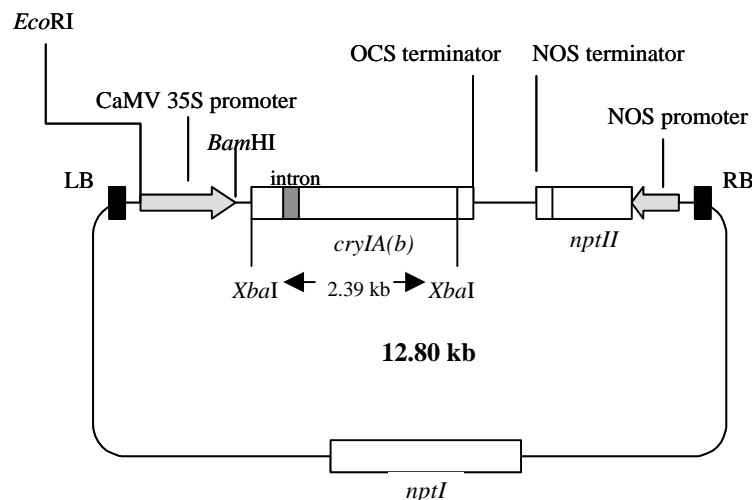


Figure 1. Schematic representation of the binary plasmid vector pBinAR-Bt.

standardizing the transformation parameters. GUS expression results were statistically analysed. Further, the optimized protocol was used to evaluate efficiency of other *A. tumefaciens* strains. Next, the *Agrobacterium* strains mobilized with pBinAR-Bt binary plasmid construct were used to transform the hypocotyl explants. Putative shoots that regenerated in MSI-CK selection medium (MS with 20 mg/l kanamycin) were further subjected to molecular analyses.

2.4 Molecular analyses of transformants

Total plant genomic DNA was isolated from leaves according to the method described by Doyle and Doyle (1990). Gene-specific primers were used to amplify by PCR a 700 and a 1000 bp fragment of *nptII* and *cryIA(b)* gene, respectively. Amplification conditions were, 1 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C; and a final extension of 5 min at 72°C. PCR products were visualized after electrophoresis in a 1% agarose gel.

Total genomic DNA was digested with *EcoRV* (Promega) whose restriction site is absent in the T-DNA. The plasmid DNA (pBinAR-Bt) was linearized by digesting with *BamHI* (Promega) and used as the positive control. The restricted DNA was electrophoresed on 0.8% agarose gel and transferred on to nylon N⁺ membrane (Amersham) according to the manufacturer's instructions. Southern hybridization was carried out with a [³²P]dCTP-labelled *XbaI* (2.39 kb) fragment of the *cryIA(b)* gene (figure 1) according to Church and Gilbert (1984).

Total RNA was isolated from young, well-expanded leaves (Chomczynski and Sacchi 1987). The RNAs were electrophoresed on 1.2% formaldehyde/MOPS (3-[N-morpholino] propane sulphonic acid) gel and transferred on to nylon N⁺ membrane (Amersham) according to the manufacturer's instructions. The membrane was then hybridized with [³²P]dCTP-labelled *XbaI* (2.39 kb) fragment of the *cryIA(b)* gene. All the solutions and glassware were treated with 0.1% DEPC. RT-PCR was performed using gene specific primers that amplify a 1000 bp fragment of the *cryIA(b)* coding region. The RT-PCR reaction was set as per the technical bulletin provided with "Access RT-PCR" kit (Promega). Amplification conditions were: 45 min at 48°C; 30 cycles of 2 min 94°C, 30 s at 65°C, 2 min at 72°C and the final step of 5 min at 72°C. RT-PCR products were visualized by electrophoresis in a 1% agarose gel.

Total cellular protein (60 µg) from transformed and untransformed control plants and Bt protein (2 µg) as positive control were fractionated in SDS-PAGE (10% polyacrylamide) and transferred onto PVDF membrane (Amersham) using an electro-blotting apparatus (Atto,

Japan) as per the method described by Ausubel *et al* (1994) and the manufacturer. The membrane was blocked for 2 h (37°C) using 2% BSA (fraction V) in Tris-buffered saline (TBS), pH 7.0. Further, it was incubated with rabbit anti-CryIA(b) serum (1 : 10000 dilution) overnight at room temperature. Next the membrane was incubated in goat-rabbit IgG coupled to alkaline phosphatase (Sigma) for 2 h (37°C). Finally the membrane was developed in BCIP/NBT (Sigma) till the bands became visible. The membrane was washed with washing solution containing 0.1% Tween-20 (ICN) in TBS for 10 min after each stage.

2.5 Leaf-disc bioassays

Bt-transgenics confirmed for the integration and expression of the *cryIA(b)* sequence were subjected to insect bioassay to assess their tolerance to the diamondback moth (*P. xylostella*). The positive control for the experiment was prepared by surface coating leaf discs of untransformed plants with the CryIA(b) protein obtained from the total cellular extract of *E. coli* (DH5a) harbouring the expression vector for *cryIA(b)* gene. The actual concentration of the toxin protein was not known. Leaves from untransformed plants served as negative control. Leaf discs of 25 mm diameter were excised from young leaves and placed on moistened filter paper in petri-dishes. On each leaf disc, a total of 10 s instar (4–5 day-old) larvae of *P. xylostella* were released. Three replicates were maintained for each treatment. Larval mortality was recorded at 24 h and 48 h. Growth of larvae (length and weight) was recorded after 48 h of feeding. Rearing conditions of 26–28°C and 60% relative humidity were maintained.

Corrected percentage mortality was calculated according to the formula:

$$\text{Percent mortality (corrected)} = \frac{T - C}{100 - C} \times 100$$

where, *T* = percent mortality in transgenics and *C* = percent mortality in untransformed control plants.

3. Results

3.1 Optimization of plant transformation conditions

Exposure of hypocotyl explants to an undiluted culture of *Agrobacterium* (OD₆₀₀ = 0.5) resulted in severe necrosis of the explants. Diluted culture (1 : 10 and 1 : 20 dilution) reduced necrosis to a great extent (table 1). Only 7–11.6% of the explants showed 1–2 GUS spots per explant. The GUS response varied significantly among the

treatments. The maximum GUS response was obtained with 1 : 20 dilution for 30 min. While standardizing the seedling age, it was observed that explants from 7-day-old seedlings performed better than 10-day-old seedling explants. Necrotic reaction on explants from 4-day-old seedlings was so high that none could survive the treatment. The maximum GUS response was obser-

ved with 7-day-old seedlings (11.3%). Therefore, subsequent experiments were carried out with 7-day-old seedlings.

Hypocotyls were hypersensitive to the bacterial culture when no pre-culture was allowed. A short pre-culture period (1–2 days) lowered explant mortality and improved GUS expression. Extending pre-culture beyond 2

Table 1. Effect of transformation conditions on mortality and GUS expression in hypocotyl explants exposed to GV2260 with p35SGUSINT.

Transformation parameters	Explant mortality (%)	GUS spots per explant	Explants showing GUS response (%)	Remarks
Bacterial inoculum density				
undiluted culture (OD ₆₀₀ 0.5)				
30 min	89.7	Nil	Nil	Tissue necrosis and mortality
1 : 10 dilution, 10 min	60.3	1–2	7.6	Reduced necrosis
1 : 10 dilution, 30 min	57.0	1–2	10.3	Bacterial overgrowth visible
1 : 20 dilution, 30 min*	24.0	1–3	11.6	No bacterial over growth
SEM±	–	–	0.29	
CD at 5%	–	–	0.95	
Seedling age				
4 days	100.0	Nil	Nil	Hypersensitive response, necrosis
7 days*	25.0	1–2	11.3	Healthy growth at the cut ends
10 days	14.3	1–2	8.6	
SEM±	–	–	0.28	
CD at 5%	–	–	0.96	
Pre incubation period				
0 day	97.6	Nil	Nil	Hypersensitive response, necrosis
1 day	35.6	1–2	12.0	Reduced hypersensitive reaction
2 days*	28.3	1–2	12.0	Reduced hypersensitive reaction
4 days	6.3	1	8.3	Poor <i>Agrobacterium</i> infection
SEM ±	–	–	0.16	
CD at 5%	–	–	0.53	
Transformation enhancers at the time of infection				
50 µM acetosyringone*	29.0	1–4	15.3	Improved GUS expression
100 µM acetosyringone	43.3	1-multiple	15.0	Tissue browning at cut ends
50 µM acetosyringone + 5-azacytidine (100 µM)	27.0	1-multiple	14.6	
SEM ±	–	–	0.43	
CD at 5%	–	–	1.49	
Co-cultivation				
Temperature				
26°C for 2 days	31.6	1–3	14	Necrosis, bacterial overgrowth
22°C for 2 days*	15	1–3	17.3	Low explant mortality
Duration				
2 days at 22°C	16.6	1–3	17	Good explant vigor
3 days at 22°C*	22	1-multiple	19.6	Low necrosis
4 days at 22°C	96.3	Nil	Nil	Explants failed to survive
Anti-ethylene agent				
3.5 mg/l AgNO ₃ *	5.6	1-multiple	20	Visible improvement in explant condition
SEM ±	–	–	0.45	
CD at 5%	–	–	1.39	

*Chosen condition for subsequent experiments. The mortality percentage and GUS expression are averages of three replicates, each with 50 explants.

days was not beneficial. Substantial improvement in GUS expression was achieved with the addition of acetosyringone. Treatment at 50 μM concentration gave GUS expression in 15.3% explants with 1–4 GUS spots per explant. Though more GUS spots were observed when 100 μM acetosyringone was used, this treatment was found unsuitable due to a high degree of tissue browning and mortality. No visible difference in GUS expression was observed when 5-azacytidine (100 μM) was used in conjunction with acetosyringone. While evaluating the co-cultivation temperature and duration, it was found that 22°C was better than 26°C because explant mortality was low (15%) and percent of explants showing GUS expression was higher (17.3%). At 5% level of critical difference, the treatment difference was significant. The experiment failed completely when duration of co-cultivation (at 22°C) was increased to 4 days. A 3-day co-cultivation period was a better option over 2 or 4 days. The bacterial growth around explants could be controlled with 250 mg/l cefotaxime. The addition of 3.5 mg/l antiethylene agent (AgNO_3), from the pre-culture stage onwards, substantially improved explant health and reduced the mortality to 5.6%, though no statistical significance was observed between the treatments. Employing combinations of the above-optimized parameters gave GUS expression in about 20% explants (figure 2).

The results of transformation with four *A. tumefaciens* strains (table 2) clearly indicated the superiority of GV2260 over LBA4404, A208 or EHA105. Transformation frequency as evidenced by GUS staining ranged from 2.5 to 22.6%.

3.2 Plant transformation with pBin-AR-Bt

Shoot bud differentiation was drastically reduced in explants that were subjected to *Agrobacterium* treatment. Most of the shoots that regenerated turned white/violet under kanamycin selection. Following co-cultivation,

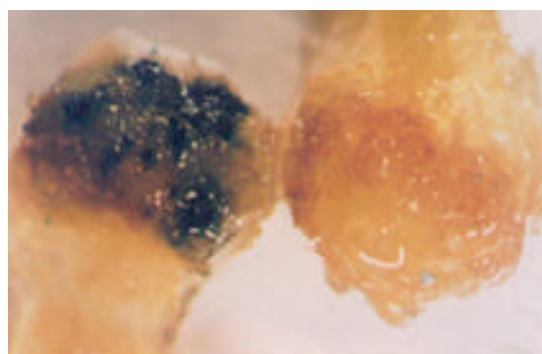


Figure 2. GUS expression at a cut end of a hypocotyl explant subjected to *Agrobacterium* transformation (left), while no staining is seen in the control (right).

explants were cultured in a medium lacking a selection agent for varying periods (delay period), before transfer to a medium containing kanamycin. When a short delay period was given, no green shoots could be recovered. Therefore a 7–10 day delay period was employed. Under these conditions, a few green calli regenerated and gave rise to hyperhydric shoots during the initial stages. Upon subculture, normal shoots were recovered. Maximum regeneration frequency of 25% was recovered with GV2260 whereas with LBA4404, A208 and EHA105 only 8.3, 0.7 and 0.7% respectively of explants gave rise to green shoots. Further, only about 4.8% of the green shoots established roots when sub-cultured in root initiation (RI) medium [MS medium, 0.1 mg/l BAP and 1.0 mg/l IAA and 0.8% (w/v) agar] containing kanamycin (20 mg/l). This indicated that the delay period had allowed differentiation of shoot buds from both transformed and non-transformed cells.

3.3 Molecular analyses of Bt-transgenics

A total of five well established Bt-transformants isolated through the described selection regime were subjected to molecular analyses. PCR with *nptII* and *cryIA(b)* gene-specific primers (figure 3A, B) amplified 0.7 and 1.0 kb fragments respectively, from genomic DNA of kanamycin resistant shoots, thereby indicating the presence of the transgenes in the regenerated plants. Southern analysis revealed integration of the transgene at one to three loci in independent transgenic plants (figure 3C). This is the minimal estimate of transgene copy number because in the absence of *EcoRV* site in the T-DNA, Southern hybridization cannot detect closely linked or tandemly repeated insertions, if any. All five transgenics showed the expected 1 kb amplified fragment in RT-PCR (figure 3D) demonstrating transcriptionally active *cryIA(b)* gene in the plant genome. The 2.2 kb (figure 3F) mRNA transcript (in the RNA blot) obtained after splicing of a 0.19 kb intron from the Bt-gene further sup-

Table 2. GUS expression in hypocotyl explants transformed with different *A. tumefaciens* strains.

<i>Agrobacterium</i> strains	GUS spots/explant	Percentage response* mean \pm SD
GV2260	1-multiple	22.6 \pm 0.21
LBA4404	1–3	13.6 \pm 0.08
A208	1	2.5 \pm 0.22
EHA105	1–2	7.3 \pm 0.19
SEM \pm	–	0.13
CD at 5%	–	0.41

*The data derived from three replicate experiments with 50 explants per treatment.

ported the RT-PCR results. All the transformants showed the presence of 81.3 kDa Bt toxin protein in the Western blot (figure 3E).

3.4 Leaf-disc bioassays

The Bt-transgenic plants showing detectable levels of Cry protein in the Western blot analysis were assessed for their tolerance to the second instar larvae of *P. xylos-*

tella. The larvae showed different feeding patterns and growth on control and transgenic leaf discs. The length and weight of the larvae fed on leaf discs of transgenic plants were significantly less than those fed on untransformed (control) plants (table 3). Among the transgenics, plant number 4 supported the least growth of the larvae (3.4 ± 1.02 mm) and plant 1 recorded the maximum (4.60 ± 0.49 mm). No significant difference in larval growth was observed between plant 3 (having single lo-

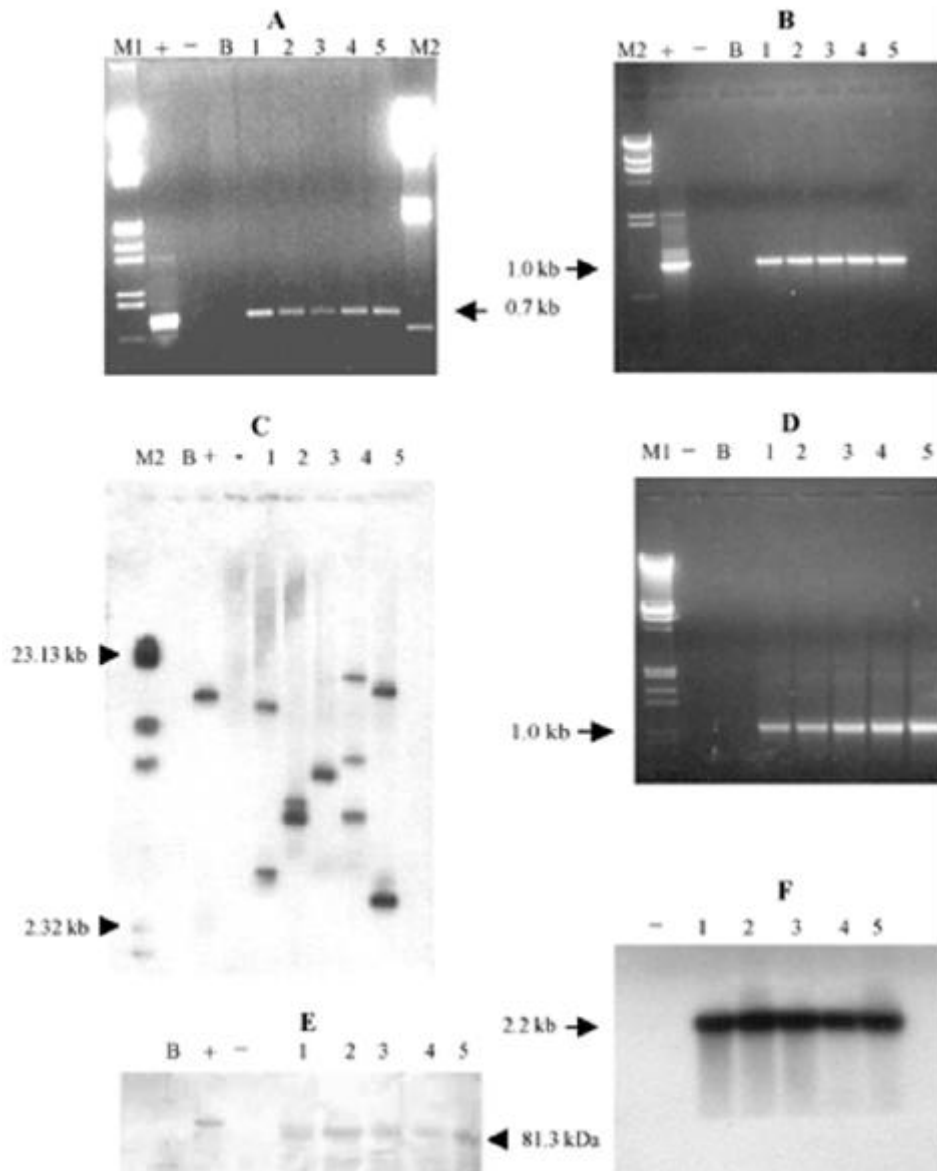


Figure 3. (A, B) PCR of Bt-transgenic showing amplified fragments with *nptII* and *cryIA(b)* gene specific primers. (C) Southern hybridization pattern of the Bt-transgenic plants with *cryIA(b)* probe. (D) The 1.0 kb RT-PCR amplicon obtained with *cryIA(b)* gene specific primers. (E) Western blot showing Bt-protein (81.3 kDa) in transgenic plants. (F) RNA blot probed with *cryIA(b)* sequence. M1, *I*HindIII/*EcoRI*; M2, *I*HindIII; B, blank; +, positive control; -, negative control; 1 to 5, independent Bt-transformants.

cus insertion of the transgene) and plant 4 (having at least 1 copy of the transgene). The maximum corrected mortality of *P. xylostella* larvae when fed on leaf discs of transgenic cauliflower was 85.7% after 48 h. It was observed that the Bt-transformants (plant 1, 2 and 5) with the transgene inserted at two loci demonstrated varied average mortality percentage, ranging from 66.6% to 80.0% at 48 h.

4. Discussion

Agrobacterium-mediated transformation involves interaction between two biological systems and is affected by various physiological conditions (Bhalla and Smith 1998). Considering the large number of factors involved, it is not possible to conduct a factorial experiment and hence interaction effects, if any, cannot be assessed from our experiments. In general, our results confirmed earlier observations that lowering bacterial density (Srivastava *et al* 1988; Henzi *et al* 2000) and preculture of explant on callus inducing medium (Sangwan *et al* 1992; Ovesna *et al* 1993) help to improve transformation frequency. The phenolic compound acetosyringone has been known to induce *vir* gene (Shimoda *et al* 1990) and increase transformation frequency. Although addition of acetosyringone to bacterial culture medium was not beneficial, its presence during co-culture stage was found to improve transformation efficiency (Henzi *et al* 2000). Similarly, the demethylating agent, 5-azacytidine has been reported to improve transformation and transgene expression (Palmgren *et al* 1993). However, we did not observe any improvement in transformation frequency upon addition of 5-azacytidine. Results of our experiments on co-cultivation temperature and duration are also in agreement with the earlier report of Metz *et al* (1995).

The results of transformation with a Bt-gene construct have validated our contention that the transient GUS assay

approach is a reliable method to optimize conditions of transformation. However, the selection regime had to be worked out separately. While delayed application of the selective agent leads to excessive escapes (Metz *et al* 1995), too early selection pressure adversely affects shoot regeneration (Van Wordragen and Dons 1992) even from transformed calli (Ding *et al* 1998). We found that a delay period of 7 days was necessary to recover transgenics.

Molecular analyses established the transgenic status of the shoots recovered in selection medium. Only one plant was found to carry single site integration of the transgene. There was, however, no clear relation between number of transgene insertions and transgene expression. Multiple site integrations of transgenes were widely reported in most species including *Brassica* (Berthomieu *et al* 1994; Metz *et al* 1995). Bioassays with diamondback moth larvae confirmed the effectiveness of the Bt crystal proteins against insect feeding.

5. Conclusion

Our results show that by fine-tuning conditions of transformation, even a recalcitrant crop like *B. oleracea* can be transformed. The transient GUS assay approach was found to be an easy and reliable way of establishing optimal conditions for transformation. Optimal conditions for transformation of hypocotyl explants were 2-days of preculture, 2-days of co-cultivation, 7–10 days delay period followed by culture in regeneration medium containing 20 mg/l kanamycin. Although the frequency of transformation was still low as compared to model species, the protocol is repeatable and can be used to mobilize genes of agronomic importance into elite cultivars. The transgenic cauliflower of the popular variety Pusa Snowball K-1 generated from the present exercise shows promise of practical utility in pest management.

Table 3. Growth and mortality of *P. xylostella* larvae (II instar) fed on leaf disc of cauliflower.

Plants	Growth		Mortality		
	Length mean \pm SD (mm)	Weight mean \pm SD (mg)	Average mortality (%)	Corrected mortality (%)	Transgene copy number
Control (+)	2.4 \pm 0.49	0.52 \pm 0.07	100.0 \pm 0.00	–	–
Control (–)	6.0 \pm 0.63	3.50 \pm 0.16	6.6 \pm 0.47	0.0	0
Plant 1	4.6 \pm 0.49	1.46 \pm 0.14	70.0 \pm 0.82	67.8	2
Plant 2	4.0 \pm 0.63	1.42 \pm 0.13	66.6 \pm 0.47	64.2	2
Plant 3	3.8 \pm 0.75	1.30 \pm 0.09	76.6 \pm 1.24	74.9	1
Plant 4	3.4 \pm 1.02	1.30 \pm 0.06	86.6 \pm 0.94	85.7	3
Plant 5	3.6 \pm 0.49	1.34 \pm 0.12	80.0 \pm 1.41	78.6	2
SEM \pm	0.33	0.15	0.63	–	–
CD at 5%	0.96	0.45	1.91	–	–

The growth data recorded are the average of five replicates (larvae) randomly selected from a total of ten larvae and the mortality data are the average of three replicates with ten 2nd instar larvae in each set.

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