

## Transient expression of $\beta$ -glucuronidase in embryo axes of cotton by *Agrobacterium* and particle bombardment methods

A.K. BANERJEE, D.C. AGRAWAL, S.M. NALAWADE and K.V. KRISHNAMURTHY\*

Plant Tissue Culture Division, National Chemical Laboratory, Pune-411 008, India

### Abstract

Transient expression of  $\beta$ -glucuronidase (GUS) in zygotic embryo axes of two cotton (*Gossypium hirsutum* L.) cultivars NHH-44 and DCH-32 was induced by *Agrobacterium* mediated transformation or by particle bombardment. For *Agrobacterium* transformation, disarmed *A. tumefaciens* strain GV 2260/p35SGUSINT was used. In cv. NHH-44, the maximum frequency of transient expression (14.28 %) was achieved on spotting *Agrobacterium* paste on the apical regions of the split embryo axes. The method resulted in a transformed callus line, which showed strong GUS activity. Integration of NPTII gene was confirmed by Southern analysis. Transgene expression by particle bombardment was achieved with p35SGUSINT and pIBGUS plasmids independently. The maximum frequency of GUS expression in 29.16 % explants was observed in cultivar NHH-44 with gold microcarriers (1.1  $\mu$ m) when bombarded once with rupture disc of 7586 kPa at target cell distance of 6 cm. A transformed callus line was obtained when explants were bombarded with p35SGUSINT and cultured on Murashige and Skoog's medium supplemented with B<sub>5</sub> vitamins, 0.1 mg dm<sup>-3</sup> 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, 0.01 mg dm<sup>-3</sup>  $\alpha$ -naphthaleneacetic acid, 3 % glucose + 50 mg dm<sup>-3</sup> kanamycin. High GUS activity was observed in callus tissue as well as in somatic embryo like structures achieved in liquid shake cultures.

*Additional key words:* *Gossypium hirsutum*, micrografting, transgenic plants.

### Introduction

Although more than 30 crop species have been genetically modified, the technology of gene transfer for most species including cotton is not efficient and limited by recalcitrance of cultivars to *in vitro* methods (Zapata *et al.* 1999). In cotton, insect control protein genes of *Bacillus thuringiensis* var. *kurstaki* HD-1(Cry 1A(b) and Cry 1A(c) have been expressed in some American cultivars (Perlack *et al.* 1990). The attempts to prepare

genetically modified cotton plants have been reviewed recently (Wilkins *et al.* 2001).

The present study was undertaken to standardize conditions which affects the transfer and expression of reporter gene (GUS) in embryo axis explants of cotton cultivars NHH-44 and DCH-32 by *Agrobacterium* and biolistic methods of plant transformation.

### Materials and methods

**Explant preparation and treatment with *Agrobacterium*:** Delinted seeds of two Indian cultivars of cotton (*Gossypium hirsutum* L.), NHH-44 and DCH-32, were

surface sterilized and germinated aseptically in dark for 48 h. Embryo axes were excised and their radicles were discarded leaving 2 mm long structures (plumular axes)

Received 26 June 2001, accepted 4 December 2001.

**Abbreviations:** BAP - 6-benzylaminopurine; NAA -  $\alpha$ -naphthaleneacetic acid; SDS - sodium dodecyl sulphate; SSC - sodium chloride and sodium citrate solution; TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea.

**Acknowledgments:** The financial assistance in the form of research grant by the Department of Biotechnology (DBT) and senior research fellowships to Dr. A.K. Banerjee and Dr. S.M. Nalawade by CSIR, Govt. of India is gratefully acknowledged.

\*Corresponding author; fax: (+91) 20 589 3438, e-mail: kvk@ems.ncl.res.in

which were used as explants (Agrawal *et al.* 1997, 1998).

The explants were split longitudinally and treated with *Agrobacterium tumefaciens* strain C58CI/GV2260 carrying the plasmid p35SGUSINT. The plasmid is a pBin 19 derivative and contains the  $\beta$ -glucuronidase (GUS) gene with a ST-LS1-derived intron under the control of CaMV 35S promoter (Vancanneyt *et al.* 1990). Culture of *Agrobacterium* strain was grown overnight at 28 °C in liquid YEB medium containing kanamycin 50 mg dm<sup>-3</sup>. The culture was spun at 1 649 g 10 min at room temperature. The pellet obtained was resuspended in 3 cm<sup>3</sup> of liquid medium (suspension S1) containing Murashige and Skoog's (1962) salts + Gamborg's (Gamborg *et al.* 1968; B<sub>5</sub>) vitamins + 0.1 mg dm<sup>-3</sup> 6-benzylaminopurine (BAP) + 0.02 mg dm<sup>-3</sup>  $\alpha$ -naphthaleneacetic acid (NAA) + 2 % sucrose (hereinafter referred as MS1) or in 0.2 cm<sup>3</sup> of liquid medium (suspension S2) containing MS salts + B<sub>5</sub> vitamins + 0.1 mg dm<sup>-3</sup> 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) + 0.01 mg dm<sup>-3</sup> NAA + 3 % glucose (hereinafter referred as MS2). Treatment of explants was carried out by two methods: dipping batches of 40 split embryo axes in *Agrobacterium* suspension (S1) for 3 - 4 s, 5, 10, 20 and 30 min (method A) or spotting 0.002 cm<sup>3</sup> of *Agrobacterium* suspension (S2) on the individual split embryo axis (method B). Treated explants were incubated for 72 h.

The explants treated by dipping (method A) were blotted dry and co-cultivated on agar solidified medium MS1 for 72 h. These were then washed thrice with sterile double distilled water containing 500 mg dm<sup>-3</sup> of *Claforan* (Russel, Mumbai, India), blotted dry and cultured on MS1 medium containing 50 mg dm<sup>-3</sup> kanamycin + 500 mg dm<sup>-3</sup> *Claforan*. Explants not treated with *Agrobacterium* and cultured on medium containing 50 mg dm<sup>-3</sup> kanamycin served as controls. Cultures were incubated under 16-h photoperiod (irradiance of 27  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 25 °C for 4 weeks. Explants treated by spotting (method B) were co-cultivated at 30 °C at a irradiance of 27  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 48 h. After that, these were washed as above and cultured on semisolid medium MS2 containing 50 mg dm<sup>-3</sup> kanamycin and 500 mg dm<sup>-3</sup> *Claforan*. These were incubated for 4 weeks as mentioned above. To develop plantlets from the slow growing putatively transformed shoots which survived on above selection medium for 3 months, *in vitro* micrografting was carried out (Banerjee *et al.* 2000).

**Southern analysis:** To carry out radioactive Southern hybridization, DNA fractions (15  $\mu\text{g}$ ) from negative control (non transformed cotton callus tissue), transformed callus of cotton, putatively transformed plants, and positive control (transformed tobacco) were digested with *Hind*III for overnight at 37 °C. Digested DNA samples were electrophoresed on a 1 % agarose gel. The gel was blotted onto *Hybond* positively charged

nylon membrane (*Boehringer*, Mannheim, Germany) and DNA was capillary transferred to membrane by standard alkali transfer method. DNA was fixed by baking the membrane for 2 h at 80 °C. Pre-hybridization was carried out for 6 h at 65 °C and then boiled radiolabelled probe (The 0.85-kb NPTII fragment was labeled with  $\alpha^{32}\text{P}$  by standard random prime labelling method.) was added to the hybridization solution. Hybridization at 65 °C continued for 24 h. The membrane was washed for 15 min at RT in 2  $\times$  SSC containing 0.1 % SDS. Thereafter the membrane was given 2 washes for 30 min each with 0.5  $\times$  SSC containing 0.1 % SDS. at 60 °C. Autoradiography was carried out by exposing the membrane to X-ray film at -70 °C for 5 d.

**Particle bombardment method:** Embryo axes of cotton cultivar NHH-44 and DCH-32 excised as earlier were arranged (40 explants per dish) on a sterile filter paper moistened with 1 cm<sup>3</sup> of liquid MS1 medium in a pre-sterilized plastic Petri dish (55 mm dia). The explants were arranged in a manner that their apical regions face the trajectory of DNA coated microcarriers.

For bombardment, Dupont (USA) biolistic *PDS-1000/He Particle Delivery System* was used. The plasmid DNA (1  $\mu\text{g dm}^{-3}$ ) (p35SGUSINT or pIBGUS obtained from two different cell lines of *E. coli* HB101) was coated on gold microcarriers (of size 1.1  $\mu\text{m}$ ) using the method described earlier (Chlan *et al.* 1995). Rupture disks of 7586 or 8966 kPa, number of bombardments (once or twice) and target cell distance (6 or 8 cm) were used as variables in the study. For each bombardment, chamber vacuum was raised to 28 Hg before firing. After bombardment, the dishes were incubated in dark for 24 h. Thereafter, the explants were divided into two groups and each group was transferred to MS1 and MS2 semi-solid media containing kanamycin (50 mg dm<sup>-3</sup>) and then were incubated for 12 weeks at 30 °C under a irradiance of 27  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

Randomly chosen samples of calli formed from the bombarded embryo axis explants cultured on MS2 supplemented with 50 mg dm<sup>-3</sup> kanamycin were tested for GUS assay. Callus from GUS positive line was transferred to 50 cm<sup>3</sup> of liquid MS salts supplemented with B<sub>5</sub> vitamins, 3 % sucrose, 1 mg dm<sup>-3</sup> TDZ, and 50 mg dm<sup>-3</sup> kanamycin in 250 cm<sup>3</sup> flasks. These liquid cultures were rotated on a shaker under continues light (11.7  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 30 d. After that, 25 cm<sup>3</sup> of fresh medium was added to each flask at one month intervals. After six months, the TDZ concentration in the medium was reduced from 1.0 mg dm<sup>-3</sup> to 0.1 mg dm<sup>-3</sup>.

**GUS assay:** Random samples of embryo axes both from treatments of *Agrobacterium* and particle bombardment were taken after 3 weeks or 72 h of incubation, respectively. The GUS assay was carried out as described

by (Stomp 1992). Blue loci, indicative of transient GUS expression, were counted 24 h after addition of the X-Gluc substrate solution. The somatic embryo like

structures obtained in the liquid cultures were also tested for the GUS assay.

## Results and discussion

The  $\beta$ -glucuronidase (GUS) has been proven useful for optimizing DNA delivery conditions and detecting the tissues receiving foreign genes transferred by *Agrobacterium* or microprojectile (Christou 1996). Kanamycin concentration of 50 mg  $\text{dm}^{-3}$  was found to be lethal for split and non-split embryo axes. The percentage of explants showing GUS expression varied depending on the period and mode of *Agrobacterium* treatment employed (Table 1). The maximum split embryo axes (14.28 %) showed GUS activity when *Agrobacterium* suspension (S2) was spotted on the apical region of embryo axis of NHH-44 and on culture of explants on semi-solid medium MS2 for 3 weeks. Treatment of split embryo axis with *Agrobacterium* suspension (S1) for 5, 10 and 20 min resulted in 0.98, 1.28 and 1.62 % of explants showing GUS expression. (Table 1). Split embryo axes dipped for 3 - 4 s and co-cultivated for 72 h at 30 °C resulted in 5.88 % of explants showing GUS expression (Table 1).

Embryo axis explants spotted with *Agrobacterium* suspension (S2) and cultured on semi-solid medium (MS2) after washing and blotting induced callus at the cut ends after 12 d of incubation. The induced callus showed high GUS expression (Fig. 1A). The transformed callus could be multiplied and is being maintained on the selection medium for differentiation into plants.

Though, Southern analysis (Fig. 1D) confirmed the integration of NPT II gene in cotton callus tissue and tobacco plant (used as a positive control), however, both the putative transformants of cotton cultivar NHH-44 did not show any bands. Absence of NPTII gene in putative transformants suggest that these may be escapes of the transformation event surviving on selection pressure for a period of 3 months. It is also evident (Fig. 1D) that transformed callus of cotton cultivar NHH-44 and transgenic tobacco treated with *Agrobacterium* strain pGV2260 harboring the p35SGUSINT received multiple copy of the NPT II gene.

Table 1.  $\beta$ -glucuronidase expression in split embryo axis of cotton (*G. hirsutum* L.) cultivar NHH-44 after inoculation with *Agrobacterium tumefaciens* strain GV2260 / p35SGUSINT. Explants were tested for GUS assays after 3 week of culture. Survival of shoots was tested after 3 and 12 weeks of culture.

Number of explants	Mode of treatment	Treatment period [min]	Number of explants tested	GUS positive explants		Survived transformants	
				number	[%]	3 weeks	12 weeks
2450	dipping	30	70	-	0.00	68	0.00
5389	dipping	20	123	2	1.62	143	0.00
3245	dipping	10	78	1	1.28	129	3.00
2680	dipping	5	102	1	0.98	83	2.00
586	dipping	3 - 4 s	34	2	5.88	28	2.00
450	spotting	-	28	4	14.28	21	2.00

**GUS expression after single bombardment:** Between the two constructs pIBGUS and p35SGUSINT, more than 3 fold frequency of GUS expression was obtained with the later. The plasmid p35SGUSINT resulted in the maximum percentage of explants showing GUS expression (29.16 %) in cultivar NHH-44 irrespective of target distance and rupture disks (Table 2). The plasmid pIBGUSINT on the other hand resulted in the lowest percentage (12 %) of explants having blue loci when bombarded at a target distance of 6 cm with 7586 kPa rupture disks. Blue loci were observed in different regions of the embryo axes (Fig. 1B-E). Embryo axes of cultivar DCH-32 when bombarded with gold microcarriers coated

with plasmid p35SGUSINT using 7586 kPa and 6 cm target distance resulted in 23.68 % of GUS expression, while 25.80 % of explants showed GUS expression when bombarded with 8966 kPa rupture disk and at 8 cm target distance. Since pIBGUSINT with cultivar NHH-44 resulted in the minimum percentage of explants (only 12 %) showing GUS expression, the construct was not used with cultivar DCH-32.

**GUS expression after double bombardment:** Double bombardment of gold microcarriers coated with plasmid p35SGUSINT marginally decreased the percentage of explants showing GUS expression (26.82 %) compared to

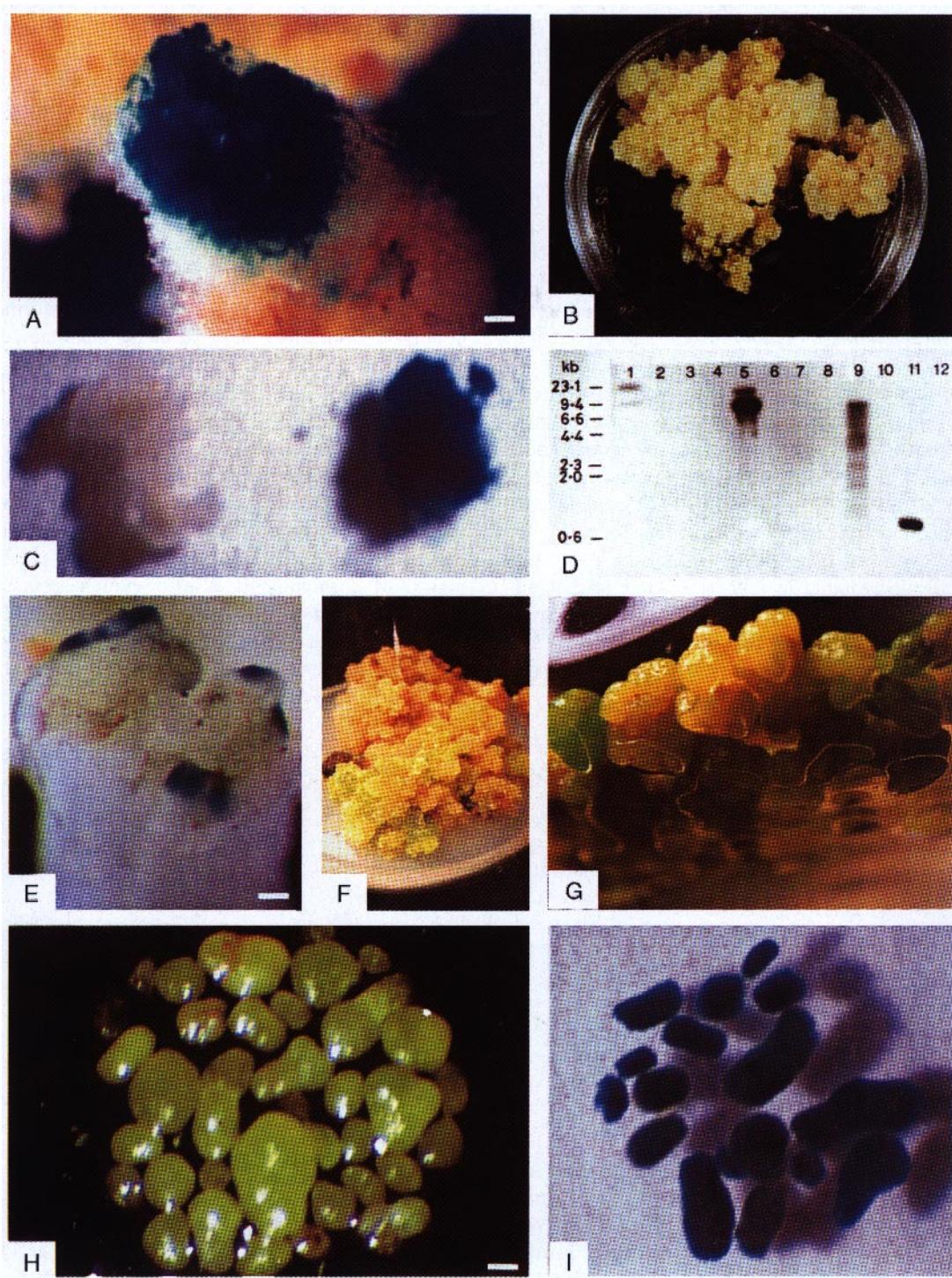


Fig. 1. *A* - GUS expression in callus induced in split embryo axis treated with *Agrobacterium* (bar = 222  $\mu$ m). *B* - Proliferation of transformed callus. *C* - Transformed (blue) and non-transformed (white) callus; *D* - Southern analysis: agarose gel electrophoresis of *Hind*III digested DNA from control callus (lane 3), transformed callus (lane 5), putatively transformed plants of cultivar NHH-44 (lanes 7, 8) and transformed tobacco (lane 9); lane 1 is  $\lambda$ - DNA *Hind*III digest molecular mass marker; lane 11 is the 0.85 kb fragment of NPTII from pHp23; probe used  $^{32}$ P labelled 0.85 kb PTII from plasmid pHp23. *E* - GUS expression in apical region of embryo axis explant after particle bombardment (bar = 166  $\mu$ m). *F* - Proliferation of transformed callus on semi solid medium. *G* - Differentiating transformed callus in liquid culture. *H* - Differentiating structures resembling early stages of somatic embryos (bar = 535  $\mu$ m). *I* - Structures showing GUS expression.

Table 2. Transient GUS expression in embryo axes after single bombardment with gold microcarriers (TD - target distance, RD - rupture disk).

Cotton cultivar	Number of axes bombarded	Plasmid DNA	RD [kPa]	TD [cm]	Number of axes tested for GUS assay	Number of axes showing GUS expression	Axes showing GUS expression [%]
NHH-44	960	p35GUSINT	8966	8	29	6	20.68
NHH-44	1275	p35GUSINT	7586	6	48	14	29.16
NHH-44	1450	PIBGUSINT	7586	6	25	3	12.00
DCH-32	1060	p35GUSINT	7586	6	38	9	23.68
DCH-32	840	p35GUSINT	8966	8	31	8	25.80

Table 3. Transient GUS expression in embryo axes of cultivar NHH-44 after double bombardment with (gold microcarriers, rupture disk - 7586 kPa, target distance - 6 cm).

Number of axes bombarded	Plasmid DNA	Number of axes tested for GUS assay	Number of axes showing GUS expression	Axes showing GUS expression [%]
594	p35GUSINT	41	11	26.82
300	PIBGUSINT	21	4	19.04

single bombardment (29.16 %) (Tables 2, 3). However, with plasmid pIBGUSINT an increase in the percentage of explants (from 12.0 to 19.04 %) having blue loci was observed (Tables 2, 3).

In an earlier study with rice, Jain *et al.* (1996) obtained higher frequency of GUS expression by using gold microcarriers compared to tungsten. Gold microcarriers are generally preferred for biolistic bombardment due to their size uniformity, spherical shape, inert nature and non-toxicity to plant tissues.

There are several reports on integration of chimeric genes in plants via particle bombardment mediated transformation (Christou 1996). Due to high regeneration potential and ease in tissue culture, embryo axis has earlier been used as an explant for gene transfer by particle bombardment method in soybean (McCabe *et al.* 1988), peanut (Brar *et al.* 1994), cotton (McCabe and Martinell 1993, Chlan *et al.* 1995) and sunflower (Hunold *et al.* 1995). Transient gene expression and optimization of different parameters of particle bombardment has also been reported in pearl millet (Taylor and Vasil 1991), cassava (Schopke *et al.* 1997), sunflower (Hunold *et al.* 1995), maize (Vain *et al.* 1993), rice (Jain *et al.* 1996), and peanut (Lacorte *et al.* 1997).

**Culture of embryo axes after single bombardment:** As in the present study, incubation of bombarded explants in dark for 24 h before they are shifted to light is considered necessary for the healing of tissues (McCabe and Martinell 1993, Chlan *et al.* 1995).

Embryo axes from cultivars NHH-44 and DCH-32 developed single shoots (80 and 75 %, respectively) when bombarded with gold microcarriers coated with plasmid

p35GUSINT, 7586 kPa rupture disks, 6 cm target distance and after two weeks of incubation. Out of 1275 embryo axes of NHH-44 bombarded, only 7 survived on kanamycin selection medium and induced shoots. In case of cultivar DCH-32, out of 1060 explants only 5 survived and induced shoots.

Leaf samples from these shoots were found to be GUS negative. Embryo axes when bombarded with plasmid pIBGUSINT under identical conditions though developed single shoots on basal medium supplemented with phosphinothricin (10 mg dm<sup>-3</sup>), turned brown after one month of culture. Explants from cultivars NHH-44 and DCH-32 when bombarded with gold microcarriers coated with plasmid p35GUSINT, 8966 kPa rupture disks, 8 cm target distance did not survive after three transfers in a medium containing kanamycin.

**Culture of embryo axes after double bombardment:** Bombardment of explants twice with gold particles decreased the shoot formation from embryo axes. Explants from cultivar NHH-44 developed single shoots (44.94 %) when bombarded twice with gold microcarriers coated with plasmid p35GUSINT, 7586 kPa rupture disks, 6 cm target distance and after two weeks of incubation. Out of 594 explants bombarded, only 2 survived and developed single shoots. The leaf samples of these shoots were found to be GUS negative. Embryo axis explants when bombarded with plasmid pIBGUSINT under identical conditions but cultured on basal medium containing 10 mg dm<sup>-3</sup> phosphinothricin developed single shoots (46.33 %) after 2 weeks of incubation. On further transfer of these shoots to fresh selection medium, shoots turned brown and died.

Shoots which survived on selection pressure both from single or double bombardments could be escapes of transformation event since none of them showed GUS expression. In an earlier study on cotton transformation, McCabe and Martinell (1993) have reported that frequency of transformation differs among the cultivars and the number of germline plants produced was one per one thousand bombarded explants.

The callus formation in bombarded embryo axis explants was observed after one month of incubation on MS salts + B5 vitamins supplemented with 0.1 mg dm<sup>-3</sup> TDZ + 0.01 mg dm<sup>-3</sup> NAA + 3 % glucose + 50 mg dm<sup>-3</sup> kanamycin. The GUS positive callus proliferated on the semi solid medium (Fig. 1F) supplemented with 1.0 mg dm<sup>-3</sup> TDZ and kanamycin. Enhancement in the callus growth was observed on its transfer to liquid medium. On reducing the TDZ concentration to 1/10 in the medium, structures resembling to early stages of somatic embryos were developed (Fig. 1G, H). Samples structures tested for the GUS assay stained dark blue (Fig. 1I). Recovery

of transgenic plants from these somatic embryo like structures requires attention at the moment.

The present study demonstrates the usefulness of *Agrobacterium* strain GV 2260 (p35GUSINT) in the transfer of  $\beta$ -glucuronidase gene into cotton tissues both by infection as well as biolistic methods. Use of embryo axis as an explant has several advantages: 1) Due to its smaller size, it is amicable to both *Agrobacterium* as well as particle bombardment mediated transformation techniques, 2) the explant requires the least time to develop into a single shoot (10 - 15 d) compared to several months in case of plant regeneration via callus phase, 3) somaclonal variation can be avoided if callus phase can be bypassed, 4) and regeneration through embryo axis is genotype independent. The present investigation has application in development of transgenic cotton. The results will also facilitate the use of particle bombardment study of gene regulation in cotton by identifying factors that influence levels of transgene expression.

## References

Agrawal, D.C., Banerjee, A.K., Kolala, R.R., Dhage, A.B., Nalawade, S.M., Kulkarni, A.V., Hazra, S., Krishnamurthy, K.V.: *In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). - Plant Cell Rep. **16**: 647-652, 1997.

Agrawal, D.C., Banerjee, A.K., Kedari, P.H., Jacob, S., Hazra, S., Krishnamurthy, K.V.: Effect of cefotaxime on the growth of excised embryo axes of six cultivars of cotton (*Gossypium hirsutum* L.). - J. Plant Physiol. **152**: 580-582, 1998.

Banerjee, A.K., Agrawal, D.C., Nalawade, S.M., Krishnamurthy, K.V.: Recovery of *in vitro* cotton shoots through micrografting. - Curr. Sci. **78**: 623-626, 2000.

Brar, G.S., Barry, A.C., Carole, L.V., Johnson, G.W.: Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilizing ACCELL technology. - Plant J. **5**: 745-753, 1994.

Christou, P.: Transformation technology. - Trends Plant Sci. **1**: 423-431, 1996.

Chian, C.A., Lin, J., Cary, J.W., Cleveland, T.E.: A procedure for biolistic transformation and regeneration of transgenic cotton from meristematic tissue. - Plant mol. Biol. Rep. **13**: 31-37, 1995.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension culture of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Hunold, R., Burrus, M., Bronner, R., Duret, J., Hahne, G.: Transient gene expression in sunflower (*Helianthus annuus* L.) following microprojectile bombardment. - Plant Sci. **105**: 95-109, 1995.

Jain, R.K., Jain, S., Wang, B., Wu, R.: Optimization of biolistic method for transient gene expression and production of agronomically useful transgenic Basmati rice plants. - Plant Cell Rep. **15**: 963-968, 1996.

Lacorte, C., Aragao, F.J.L., Almeida, E.R., Mansur, E., Rech, E.L.: Transient expression of GUS and the 2S albumin gene from Brazil nut in peanut (*Arachis hypogaea* L.) seed explants using particle bombardment. - Plant Cell Rep. **16**: 619-623, 1997.

McCabe, D.E., Martinell, B.J.: Transformation of elite cotton cultivars via particle bombardment of meristems. - Bio/Technology **11**: 596-598, 1993.

McCabe, D.E., Swain, W. F., Martinell, B. J., Christou, P.: Stable transformation of soybean (*Glycine max*) by particle acceleration. - Bio/Technology **6**: 923-926, 1988.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Perlack, F.J., Deaton, R.W., Armstrong, T.A., Fuchs, R.L., Sims, S.R., Greenplate, J.T., Fischhoff, D.A.: Insect resistant cotton plants. - Bio/Technology **8**: 939-943, 1990.

Schopke, C., Taylor, N.J., Carcamo, R., Beachy, R.N.: Optimization of parameters for particle bombardment of embryogenic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis. - Plant Cell Rep. **16**: 526-530, 1997.

Stomp, A.M.: GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. - Academic Press, San Diego 1992.

Taylor, M.G., Vasil, I.K.: Histology of, and physical factors affecting, transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) R. Br.) embryos following microprojectile bombardment. - Plant Cell Rep. **10**: 120-125, 1991.

Vain, P., McMullen, M.D., Finer, J.J.: Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. - Plant Cell Rep. **12**: 84-88, 1993.

---

TRANSIENT EXPRESSION OF GUS IN EMBRYO AXES

Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L., Rocha-Sosa, M.: Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated transformation. - Mol. gen. Genet. **220**: 245-250, 1990.

Wilkins, T.A., Rajasekaran, K., Anderson, D.M.: Cotton Biotechnology. - Crit. Rev. Plant Sci. **19**: 511-550, 2001.  
Zapata, C., Park, S.H., El-Zik, K.M., Smith, R.H.: Transformation of a Texas cotton cultivar by using *Agrobacterium* and the shoot apex. - Theor. appl. Genet. **98**: 252-296, 1999.