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High-frequency stable transformation of cotton (*Gossypium hirsutum* L.) by particle bombardment of embryogenic cell suspension cultures

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Abstract Stable transformation of cotton (*Gossypium hirsutum* L.) at a high frequency has been obtained by particle bombardment of embryogenic cell suspension cultures. Transient and stable expression of the β-glucuronidase (GUS) gene was monitored in cell suspension cultures. Transient expression, measured 48 h after bombardment, was abundant, and stable expression was observed in over 4% of the transiently expressing cells. The high efficiency of stable expression is due to the multiple bombardment of rapidly dividing cell suspension cultures and the selection for transformed cells by gradually increasing the concentrations of the antibiotic Geneticin (G418). Southern analysis indicated a minimum transgene copy number of one to four in randomly selected plants. Fertile plants were obtained from transformed cell cultures less than 3 months old. However, transgenic and control plants from cell cultures older than 6 months produced plants with abnormal morphology and a high degree of sterility.

Key words Biolistic transformation · Cotton · Embryogenic cell suspension · *Gossypium hirsutum* · Stable expression

Abbreviations *CaMV*: Cauliflower mosaic virus · *ELISA*: Enzyme linked immunosorbent assay · *G418*: Geneticin · *GUS*: β-Glucuronidase · *NAA*: α–Naphthaleneacetic acid · *NPT II*: Neomycin phosphotransferase II · *MS*: Murashige and Skoog (1962)

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Introduction

Transgenic cotton plants have been produced by biolistic bombardment of organized shoot-ip meristems (McCabe and Martinell 1993; Chlan et al. 1995; Keller et al. 1997) and regenerable embryogenic cell cultures (Finer and McMullen 1990; Rajasekaran et al. 1996b), and by *Agrobacterium*-mediated transformation (Firoozabady et al. 1987; Umbeck et al. 1987; Perlak et al. 1990; Cousins et al. 1991; Bayley et al. 1992; Rajasekaran et al. 1996b). The latter two methods have proven to be the more reliable and efficient in obtaining germ-line transformation at relatively high frequencies. The major drawback of *Agrobacterium*-mediated transformation is that it is a very time-consuming and labor-intensive procedure due to the long time (6–9 months) involved in obtaining embryogenic callus from transformed, competent cells. An availability of highly regenerable embryogenic cell cultures for transformation would reduce the time involved in regenerating transgenic cotton plants. Embryogenic cell cultures also serve as an ideal target material for biolistic transformation (Christou 1992) and can be made readily available from cryopreserved cultures (Rajasekaran 1996). Finer and McMullen (1990) demonstrated the successful recovery of transgenic cotton plants by biolistic bombardment of embryogenic suspension cultures and reported a transient to stable conversion frequency of about 0.7%. Here we report an increased biolistic stable transformation frequency (4% of the transiently expressing cells). The increased frequency was obtained by multiple bombardments of embryogenic cell suspension cultures during the rapid growth phase followed by a gradual increase in selection pressure for the identification of stably transformed cells. We also report the importance of using young or cryopreserved cultures to obtain fertile, regenerated plants.

Materials and methods

Initiation and maintenance of embryogenic cell suspension cultures

Cell suspension cultures of vars. Acala B1654 and Coker 315 were initiated from finely dispersed embryogenic callus cultures in liquid medium agitated (120 rpm, 27° ± 2 °C) on a gyratory shaker (New Brunswick G-10, Edison, N.J.) according to the procedures of Rangan and Rajasekaran (1996). The embryogenic cell suspension cultures consisted of aggregates of four to ten cells and were subcultured every week in fresh liquid MS medium (Murashige and Skoog 1962) containing 100 mg l^{-1} myo-inositol and 2 mg l^{-1} NAA. Cryopreserved, young cell suspension cultures (less than 3 months old) of both varieties (Rajasekaran 1996) were also used for biolistic transformation after about 2–3 weeks of regrowth.

Growth measurements

Equal amounts of embryogenic cells (200 mg fresh weight) were used to inoculate 40 ml of liquid medium. Fresh weight increases were monitored every 2 days over a period of 10 days in ten flasks. Cells were collected by vacuum filtration onto sterile filter paper, and fresh weights were determined under aseptic conditions. Cells were then returned to the same flasks containing the original medium.

Plasmid DNA

The plasmid pRH205–89 (Fig. 1), used in all experiments, contained the GUS gene [obtained as a 3-kb *Bgl*II/*Eco*RI fragment from plasmid pAGUS1-TN2 (Skuzeski et al. 1990)] and the CaMV 35S : NPT II gene (Yenofsky et al. 1990) subcloned into pUC19.

Biolistic bombardment of embryogenic cell suspension cultures

The plasmid DNA used in the biolistic experiments was coated onto 1.0-μm gold particles using $CaCl_2$ and spermidine as described previously (Sanford et al. 1993). Transformation of cell suspension cultures was carried out using the PDS 1000/Helium biolistic device (Bio-Rad, Richmond, Calif.).

Cell suspensions (cell aggregate size < 840 μm) from young and old cultures, collected during different phases of growth, were vacuum-deposited as a thin layer onto moist filter paper (Whatman no. 1; 3.5 cm in diameter) placed on top of a 120 μm nylon screen (Nitex, Tetco, Depew, N.Y.) in sterile petri dishes (5.5 cm in diameter). Approximately 1×10^6 cells were transferred to each dish and were kept moist until bombardment. A 400-mesh nylon screen was placed over the surface of the suspensions to serve as a baffle. In experiments that involved multiple bombardment (three bombardments at 3, 5 and 7 days after subculture), nylon screens along with cells were returned to the petri plates with filter papers (Whatman no. 1) moistened with liquid medium after every bombardment. The optimal bombardment conditions (Rajasekaran et al. 1996b) included the use of 1450 psi rupture disks, a distance between the stopping screen and the cell suspensions of 7.5 cm, a macrocarrier travel distance of 10 mm and a vacuum in the sample chamber of 28 inches Hg (95 kPa).

Following particle bombardment, the cell suspension cultures were grown for 1 week without any selection in maintenance medium. During this week, aliquots of cells were drawn to determine the extent of transient expression of the GUS gene by means of histochemical staining for up to 4 h according to Jefferson (1987) except that the staining solution contained 20% methanol to inhibit endogenous GUS activity (Martin et al. 1992). The number of GUS-expressing blue cells or aggregates was counted and reported as percentage of the total number of cells plated (Rajasekaran et al. 1996a). Aggregates were counted as one transformation event to account for their possible origin from a single transformed cell. Subsequently, in each experiment, half of the cells were directly exposed to a high level of antibiotic Geneticin (G418 at 50 μg ml^{-1}; Gibco-BRL, Rockville, Md.) for 3 weeks. The other half of the cells was treated with gradually increasing antibiotic concentrations. The starting concentration of G418 was 10 μg ml^{-1}, and this was increased in 10 μg ml^{-1}-increments at weekly intervals to obtain a final concentration of 50 μg ml^{-1} after 5 weeks (Table 1). For antibiotic selection, bombarded cells along with nylon screens were transferred every week to fresh plates with filter papers pre-moistened with the nutrient medium and G418. The number of independent stable transformation events was determined after 3 weeks of selection (G418 at 50 μg ml^{-1} following single bombardment) or 5 weeks of gradual selection (following multiple bombardments) when all the non-transformed cells were totally bleached (Table 1). In addition to histochemical staining, several randomly selected antibiotic resistant colonies in every experiment were tested for the presence of NPTII protein by ELISA (Firoozabady et al. 1987). No escapes were found using either direct or gradual selection regimes.

Plants from antibiotic-resistant clones were regenerated on solidified agar medium according to the procedures of Rangan and Rajasekaran (1996). Pollination and crossing studies using both transformed and non-transformed plants as male or female parents were carried out in a greenhouse (28° ± 2 °C).

Southern blot analyses

Southern blots were carried out according to the methods of Klessig and Berry (1983). The transgenic plant leaf DNA was digested with *Eco*RI or *Bam*HI and hybridized with a [^{32}P]-labeled nick-translated GUS probe (Maniatis et al. 1992) representing the coding region obtained from pRH20589.

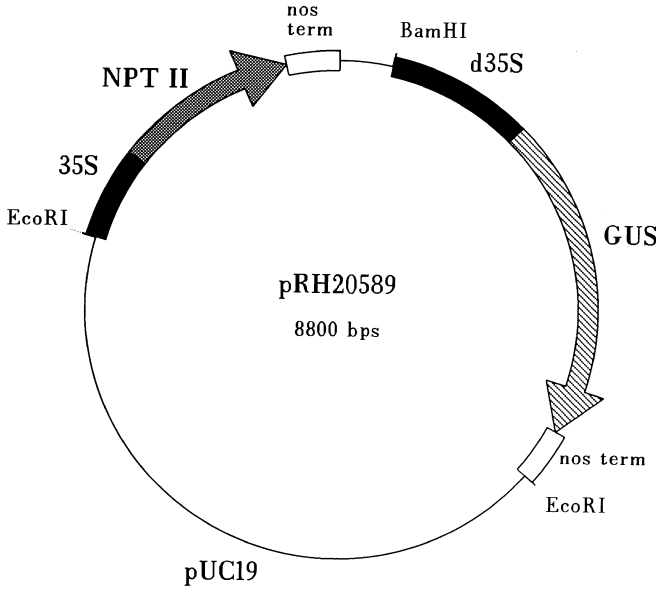

Fig. 1 pRH 20589 plasmid map

Table 1 Scheme for transformation of cotton embryogenic cell suspension cultures by multiple biolistic bombardments and antibiotic selection

Week 0	Day 1	Weekly subculture of cell suspensions
Week 0	Day 3	1st bombardment
Week 0	Day 5	2nd bombardment
Week 0	Day 7	3rd bombardment
Week 1	No selection; maintain in fresh liquid medium; Determine transient expression	
Week 2	*Direct selection*	*Gradual selection*
	50 μg ml^{-1} G418	10 μg ml^{-1} G418
Week 3	50 μg ml^{-1} G418 – Non-transformed cells bleached	20 μg ml^{-1} G418
Week 4	50 μg ml^{-1} G418 – Determine stable expression[a]	30 μg ml^{-1} G418
Week 5		40 μg ml^{-1} G418 – Non transformed cells bleached
Week 6		50 μg ml^{-1} G418 – Determine stable expression[a]

[a] Somatic embryos were developed on agar medium in the presence of 50 μg mg^{-1} G418 from actively growing, antibiotic-resistant embryogenic aggregates. No selection pressure was applied during germination of somatic embryos into plantlets

Results and discussion

Growth measurements

Freshly subcultured cell suspension cultures of var. Acala B1654 showed a lag phase of about 2 days prior to active growth. A rapid increase in cell growth, as measured by fresh weight, was observed between days 3 and 8 (Fig. 2). On average, the doubling of fresh weight occurred every 2 days during the rapid phase of growth. A similar growth curve was observed with the embryogenic cell suspension cultures of var. Coker 315 (not shown). The liquid medium slowly turned brown after 7 to 8 days and thereafter the cultures showed little or no fresh weight increase.

G418 selection for transformed cells

Cells were subjected to antibiotic selection by placing the nylon screen support on filter papers premoistened with the liquid medium and G418. Preliminary experiments indicated that control cells, bombarded with gold particles only, were bleached within 2 weeks of culture in medium containing 50 μg ml^{-1} G418. Under the gradual selection regime (Table 1), control cells were bleached after 4 weeks of selection when the concentration of G418 reached 40 μg ml^{-1}. For counting stably transformed cells, we stained randomly selected plates for GUS expression, and the positive cells were counted using a stereomicroscope. The cells were retained on their original nylon screens during selection, staining and counting. This method of quantification avoided potential overestimation of transformed colonies due to clonal duplication in liquid shake cultures. Moreover, the number of antibiotic resistance cells closely matched that of cells that were determined to be GUS-positive by histochemical staining.

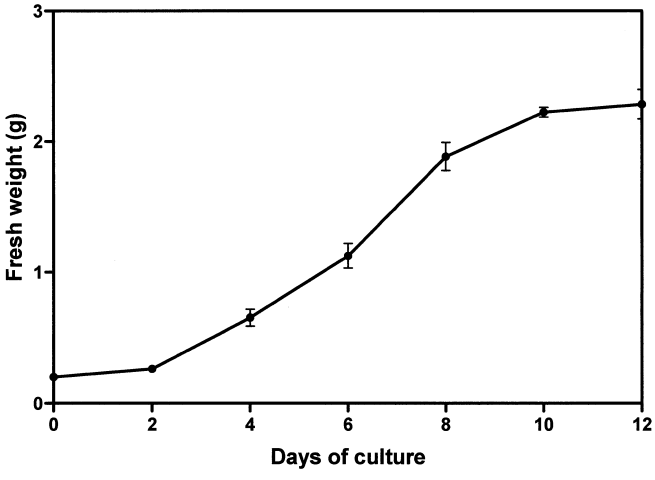

Fig. 2 Growth of B1654 embryogenic cell suspension cultures as measured by fresh weight (mean of ten replicates)

Biolistic transformation of embryogenic cell suspension cultures

Biolistic transformations of embryogenic cell suspension cultures of both vars. Acala B1654 and Coker 315 were carried out in parallel, and there was no difference in the transformability of these two varieties, similar to our earlier observations (Rajasekaran et al 1996b). Transient expression was abundant in biolistically bombarded embryogenic cell cultures of both varieties. This is similar to earlier observations on multiple bombardment of sugarcane suspension cells (Franks and Birch 1991) or cotton meristems (Chlan et al. 1995). Approximately 5000 cells transiently expressed the GUS gene in every sample of 10^6 cells that was subjected to one bombardment during the rapid growth of cell suspension cultures (3, 5 or 7after subculture). However, stable expression was largely dependent on active growth of the cell suspension. For example, no antibiotic-resistant colonies were obtained when cells were bombarded once after the first day of subculture and selected directly at a high concentration of antibiotic, and an average of only 4 transformed colonies was obtained after gradual anti-

Table 2 Effect of time and number of bombardments and selection regime on transformation of cotton (cv. Acala B1654) embryogenic cell suspension cultures older than 6 months

Time of bombardment	Number of bombardments	G418 selection method[a]	Number of colonies with stable GUS expression[b]
Day 1	1	Direct	0
Day 1	1	Gradual	4.3 ± 2.0
Day 5	1	Direct	56.7 ± 16.5
Day 5	1	Gradual	127.4 ± 21.2
Days 3,5 and 7	3	Direct	72.6 ± 35.1
Days 3,5 and 7	3	Gradual	658.8 ± 72.5

[a] Direct selection with a high level of antibiotic (50 μg ml^{-1} G418) for 3 weeks. Under the gradual selection regime, the initial concentration of G418 was 10 μg ml^{-1}, which was increased in 10 μg ml^{-1}-increments at weekly intervals to obtain a final concentration of 50 μg ml^{-1} after 5 weeks

[b] Per 10^6 cells; average of three experiments

biotic selection (Table 2). However, a significant increase in the number of stable transformants was obtained when actively growing cells (more than 3 days after subculture) were bombarded once and selected at 50 μg ml^{-1} G418. For example, cells that were bombarded once at 5 days after subculture (representing the rapid growth phase) produced an average of 56 stable transformants per 10^6 cells (Table 2). In the same experiment, when gradual selection with G418 was applied, the number of stably transformed colonies increased 2.5-fold. It has been well-documented that cell division and proliferation, characteristics of rapidly dividing cell cultures, are required for stable expression of the introduced genes (An et al. 1988; Paszty and Lurquin 1987; deKathen and Jacobsen 1995; Villemont et al. 1997). The increased number of transformants in the present study is likely due to peak receptivity of introduced DNA by rapidly dividing cells and additive effects of multiple bombardments. In addition, it appears that direct selection at high levels of G418 is too toxic for the transformed cells to survive during the early stages of selection.

Multiple bombardments of cell cultures during the rapid growth phase, at 3, 5 and 7 days after subculture, resulted in at least a three-fold increase in the frequency of transient expression (15,000 per 10^6 cells). However, when cells were subjected to direct selection at a high concentration of G418 (50 μg ml^{-1}), there was not a significant increase in the number of stable transformants. Conversely, a fivefold increase in the level of stable transformation was obtained from multiple bombardments, as compared to single bombardment, when cells were subsequently selected by a gradually increasing G418 concentration. Thus, multiple bombardments and gradual selection resulted in a transient to stable conversion frequency of over 4%, i.e., 659 stable lines from 15,000 transiently expressing cells (Table 2, Fig. 3). The number of transformants was always higher when gradual selection was used in comparison with direct selection at 50 μg ml^{-1} G418.

Regeneration of plants from transformed cell cultures

GUS expression was observed in all plant parts – leaves, vascular bundles, pollen, stigma, ovules, young fibers and root tips of plants regenerated from transformed cells. Stable GUS expression in a young leaf from a transgenic plant is shown in Fig. 4. Tissues from non-transformed plants did not stain blue upon GUS histochemical staining.

More than 80% of the regenerated plants from young cell cultures, either non-transformed or transformed, were both female- and male-fertile (Table 3). On the other hand, plants regenerated from older cultures (>6 months old) showed reduced growth and exhibited a high degree of abnormality and sterility. The problem of reduced pollen fertility and reduced number of stamens was common among both non-transformed and transformed regenerants, similar to previous observations on cotton (Trolinder and Goodin 1987; Rajasekaran et al. 1996a). However, some seeds were obtained using selected, relatively vigorous R_0 transgenic plants from older suspension cultures as male parents in backcrosses to B1654, indicating pollen fertility (Table 3). This was only accomplished by pooling all of the anthers from several flowers of the R_0 plant and using these to pollinate a

Table 3 Regeneration of non-transformed and transgenic plants (cv. Acala B1654) from embryogenic cell suspension cultures

Age of cell cultures	Regenerated plants	Number of plants transferred to soil	Number of fertile plants
<3 months (cryopreserved)	Non-transformed	20	20[a]
	Transformed	12	10[a]
>6 months (not cryopreserved)	Non-transformed	52	10[b]
	Transformed	90	13[b]

[a] Both male and female fertile

[b] Pollen fertile, although the number of stamens were significantly reduced in transgenic plants and had to be bulked for effective pollination to compensate for reduced fertility; female fertility was not demonstrated in regenerated R_0 plants due to lack of boll set

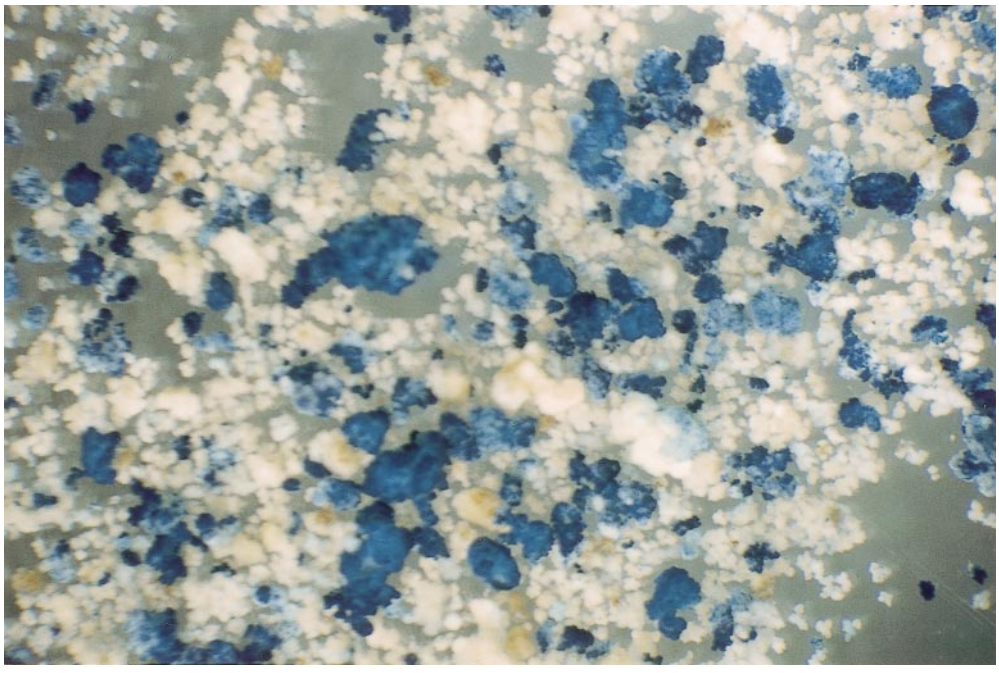

Fig. 3 Stable expression of GUS gene in bombarded embryogenic cell suspension cultures. Cells that were subjected to three bombardments and gradual selection (see Table 1) were stained for GUS expression (*blue* coloration) after 5 weeks of selection. Photographed after 2 h after staining

single B1654 flower. As expected, progeny seedlings from such outcrosses were positive in either GUS or NPT II assays in a 1:1 ratio. Due to poor vigor and growth of the R_0 plants, boll set was rarely observed when they were used as female parents.

Southern analysis of transformed plants

Southern analysis of leaf DNA from transgenic plants, derived from cells that were bombarded once, is presented in Fig. 5. Hybridization of the GUS probe with a 5.4-kb *Eco*RI fragment was detected in 9 randomly selected transgenic plants (Fig. 5B). This is consistent with the restriction map of pRH205–89 (Fig. 1), which has two *Eco*RI sites, separated by 5.4 kb, that flank the GUS gene. The GUS probe hybridized with *Bam*HI fragments of undefined sizes, as expected. Since pRH205–89 contains only one *Bam*HI site, the other site is defined by the plant genome. However, the presence of one uniform fragment of about 8.8 kb (Fig. 5A) indicates arrays of integrated plasmid DNA.

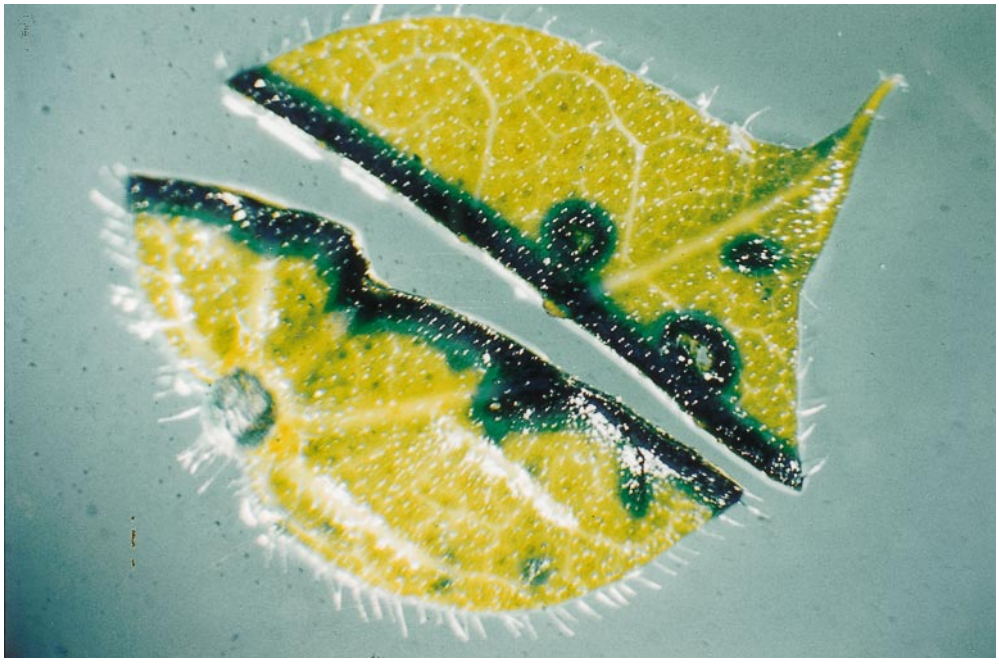

Fig. 4 Fragments of a young leaf from a transgenic plantlet of cv. Acala B1654 showing GUS expression (*dark-blue* coloration) along the cut ends (due to slow penetration of the substrate). Photographed 4 h after staining

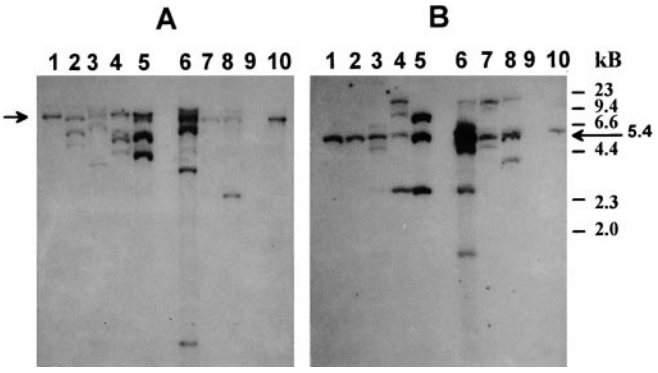

Fig. 5A,B Southern blot hybridization with the GUS probe with *Bam*HI (**Panel A**) and *Eco*RI (**Panel B**) digests of genomic leaf DNA (10 μ g/lane) from transgenic plants. *Lane 9* Non-transformed control; *Lanes 1–5*, *6–8* and *10* transformed plants. The *lane between 5* and *6* was used as marker lane in both the blots, hence no hybridization. *Arrow* in **Panel A** indicates the presence of concatemers of the plasmid DNA, about 8.8 kb, and *arrow* in **Panel B** indicates the location of the expected 5.4-kb *Eco*RI fragment. *Hind*III-digested λ-DNA (in kilobases) was used as a molecular size standard

Similar observations on the presence of concatemers of the plasmid have been previously observed in transgenic plants and cells obtained by biolistic transformation of cotton (Finer and McMullen 1990), soybean (Finer and McMullen 1991) and maize (Klein et al. 1989). Southern analysis of randomly selected transformed plants from a single bombardment (Fig. 5) or multiple bombardments (not shown) revealed a minimum transgene copy number of one to four, based on *Bam*HI digests of genomic DNA (Fig. 5A). The presence of multiple copies of the introduced gene is common among transgenic plants obtained via microprojectile bombardment (McCabe et al. 1988; Christou et al. 1989; Gordon-Kamm et al. 1990; Livingstone and Birch 1999; Altpeter et al. 1999). Cotton plants derived from a parallel experiment on multiple-bombardment of cotton embryogenic cells with a mutant form of a native acetohydroxyacid synthase gene or the GUS gene were also found to have transgene copy numbers of between one and four (Rajasekaran et al. 1996b).

It has been demonstrated in the present study that stable transformation of embryogenic cell suspension cultures of cotton can be obtained at a high frequency (over 4% of transiently expressing cells) using multiple biolistic bombardments of cultures during the rapid growth phase and by gradually increasing the selection pressure. However, as shown in the present study (Table 3), care should be taken to avoid using long-term cultures (more than 6 months), which are known to accumulate somatic mutations and cytogenetic abnormalities leading to undesirable morphological and fertility-related problems in regenerated cotton plants (Trolinder and Goodin 1987; Stelly et al. 1989; Rajasekaran et al. 1996a, 1996b). We also demonstrated that it is possible to overcome the problems with long-term cell cultures by using freshly initiated or cryopreserved embryogenic cell cultures (Table 3, Rajasekaran 1996).

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