Plant Cell Rep (2003) 21:955–960
DOI 10.1007/s00299-003-0613-x

CELL BIOLOGY AND MORPHOGENESIS

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Slow desiccation leads to high-frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310 FR)

Received: 17 September 2002 / Revised: 27 January 2003 / Accepted: 28 January 2003 / Published online: 12 April 2003

Abstract In *Agrobacterium*-mediated genetic transformation of cotton (*Gossypium hirsutum* L. cv. Coker 310FR) the frequency at which somatic embryos were converted to plantlets was significantly improved by subjecting the embryos to slow physical desiccation. We used *Agrobacterium* strain GV3101 containing the binary vector pGSFR with the *nos-nptII* gene for in vitro selection and the 35S *gus-int* fragment as a reporter to optimize the transformation protocol. Although the concentration of kanamycin was reduced during embryogenesis and embryo maturation, even at the lower levels somatic embryos were predominantly abnormal, showing hypertrophy and reduced or fused cotyledons or poor radicle ends. A majority of these embryos (more than 75%) were β-glucuronidase (GUS)-positive. Embryos with an abnormal appearance showed a very poor conversion to plantlets. However, these embryos, when subjected to slow physical desiccation followed by transfer to fresh medium, regenerated single or multiple shoots from the cotyledonary end. These shoots could be grafted on wild-type seedling stocks in vitro, which, following their transfer to soil, developed normally and set seeds. Regenerated plants tested positive for the transgene by Southern analysis. An overall scheme for the high-frequency production of cotton transgenics from both normal and abnormal appearing somatic embryos is presented.

Keywords Cotton · *Gossypium hirsutum* · Somatic embryogenesis · Desiccation

Abbreviations *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *BA:* Benzylaminopurine · *GA_3:* Gibberellic acid · *GUS:* β-Glucuronidase · *NAA:* α-Naphthaleneacetic acid · *nptII:* Neomycin phosphotransferase II · *WT:* Wild type

Introduction

The *nptII* gene, which confers resistance to kanamycin, has been used very extensively for in vitro selection of transformed tissues in cotton (Firoozabady et al. 1987; Umbeck et al. 1987; Perlak et al. 1990; Cousins et al. 1991; Rajasekaran et al. 1996; Sunilkumar and Rathore 2001; Zhang et al.2001). A number of investigations have been reported on the development of transgenics in cotton by means of *Agrobacterium* mediated transformation of seedling-derived explants using callus to somatic embryogenesis pathway of in vitro regeneration (Firoozabady et al. 1987; Perlak et al. 1990; Cousins et al. 1991; Sunilkumar and Rathore 2001; Zhang et al. 2001). In these investigations Coker lines were predominantly used although in a few cases other genotypes were used. In most of these studies, the levels of kanamycin were either reduced (Firoozabady et al. 1987; Sunilkumar and Rathore 2001) or completely removed (Cousins et al. 1991) during the induction and maturation of the somatic embryos as high levels of kanamycin were reported to interfere with embryo development.

We reported earlier the development of Coker 310FR (FR stands for fully regenerating) lines which readily regenerate through somatic embryogenesis and plantlets

Communicated by R.J. Rose

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can be recovered by the germination of somatic embryos. When transferred to half-strength MS medium (Murashige and Skoog 1962) supplemented with charcoal and kinetin, properly developed embryos with distinct cotyledons and a radicle end showed an embryo-to-plantlet conversion frequency of up to 44.4% (Kumar et al. 1998). This 310FR line was tested for *Agrobacterium*-mediated genetic transformation using *nos-nptII* as a marker gene and 35S *gus-int* as a reporter gene. In preliminary experiments it was observed that even low levels of kanamycin in the medium during induction and maturation of the somatic embryos interfered with their proper development. Such embryos could be converted into plantlets at very low frequencies (0.1–1%) using the medium and protocol described earlier by Kumar et al. (1998) for the conversion of well-formed somatic embryos to plantlets. We tested a number of other media and physical conditions—GRMgn medium (Firoozabady et al. 1987), air drying and SEGM medium (Voo et al. 1991), cold treatment of embryos (4°C for 20 days) and combinations of high percentages of agarose, charcoal and kinetin—but no reproducible increase in embryo-to-plantlet conversion could be achieved.

In this communication, we report that an additional step to the protocol, one of slow desiccation of the embryos that have differentiated on selection medium, leads to a very high frequency conversion of the embryos to plantlets. Based on these observations, we describe an efficient and robust protocol for the routine development of transgenics in cotton.

Materials and methods

The cotton (*Gossypium hirsutum* L.) Coker 310FR line (Kumar et al. 1998) used for the genetic transformation studies was maintained by selfing and has been regularly tested for purity for the trait of regeneration in vitro. For genetic transformation, *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell 1986) containing the binary vector pGSFR, modified from pGSFR780A (Deblaere et al. 1987) with the *nos-nptII* gene and 35S *gus*-int gene (Vancannyet et al. 1990), was used.

Delinted seeds of Coker 310FR were surface-sterilized following Hemphill et al. (1998) and germinated on half-strength MS medium [MSB medium (Table 1)]. Hypocotyl (4–5 mm) and cotyledonary explants (approx. 1 cm^2 area) from 7-day-old seedlings were used for *Agrobacterium* infections (Firoozabady et al. 1987), and explants were co-cultivated for 48 h at 22°C. Augmentin (250 mg/l) (Medreich Sterilab) and kanamycin (50 mg/l) were used as a bacteriostatic agent and selective agent, respectively.

The regeneration protocol of Kumar et al. (1998) (Fig. 1; steps A, B, D–F) was followed for the induction of somatic embryos from transformed calli. The media used in this study are described in Table 1. All of the cultures were grown in 9-cm-diameter disposable petri dishes (Tarsons, India) under controlled environmental conditions [28±2°C; 750 lux light intensity at the culture level; 16/8-h (light/dark) photoperiod]. For all the culture steps (Fig. 1) petri dishes were placed on perforated shelves (Fig. 2) to prevent the condensation of water on the lids of the petri dishes, which leads to vitrification of the somatic embryos.

Modifications to the original regeneration protocol were made at steps G and H (Fig. 1). At step G, embryos were allowed to elongate at the least 1 cm in the same petri dish along with callus.

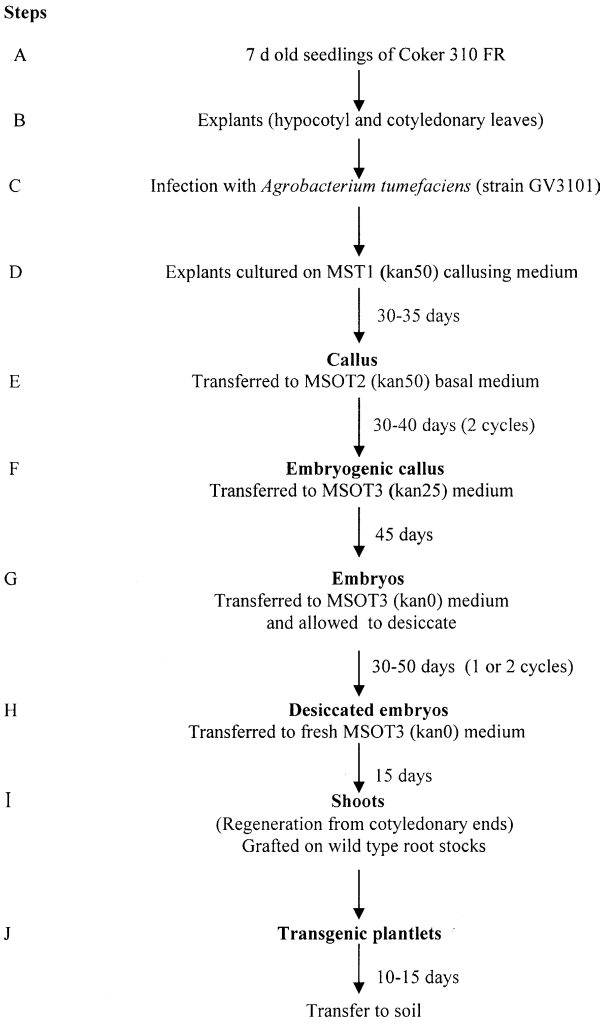

Fig. 1 Protocol for high-frequency genetic transformation of cotton (*Gossypium hirsutum* L. cv. Coker 310FR), including culture regimes described earlier, with the additional steps that allow conversion of transformed embryos to plantlets. At *step G* petri dishes are sealed with Micropore tape to enable rapid depletion of water in the medium; in the other steps Nescofilm was used. The entire scheme from explants to potted transgenics takes around 9 months. Augmentin was used at *steps D*, *E* and *F* at a concentration of 250, 100 and 50 mg/l, respectively

Elongated embryos (approx. 1 cm in length) irrespective of their age and morphology were transferred to MSOT3 medium without kanamycin, and the petri dishes were sealed with Micropore tape (3M Health Care, USA). For all of the other steps (Fig. 1, A–F) Nescofilm (Azwell, Osaka, Japan) was used. Cultures were grown on MSOT3 medium for 30–40 days. The desiccated embryos were transferred to fresh MSOT3 medium without kanamycin, and the step was repeated until the shoots emerged. These emerged shoots were excised from the embryo and tested for direct root formation on MS medium supplemented with 2 mg/l IBA or grafted (Lou and Gould 1999) on 4-day-old rootstocks. WT seedlings grown in 4-cm-diameter tubes were used as rootstocks for in vitro grafting. When the secondary roots emerged, the seedlings were taken out of the tube and decapitated. A 0.5-cm-deep incision was made at the top of the rootstock and the rootstock carefully inserted back into the same tube in an upright position. Transgenic shoots (approx. 1.5 cm long) were excised from the embryos. The base of the excised shoot was cut into a wedge shape and the shoot placed in

Table 1 Composition of the various media used

S. no.	Medium	Salts[a] (MS)	B5 Vitamins[a]	Carbohydrate source	Phytohormones	Gelling agent	pH
1.	1/2-strength MSB	0.5×	0.5×	Sucrose (2%)	–	Agar[d] (0.7%)	5.80
2.	MST1	1×	1×	Glucose[b] (3%)	2,4-D (100 μg/l); kinetin[c] (500 μg/l)	Phytagel[a] (0.2%)	5.80
3.	MSOT2	1×	1×	Glucose[b] (3%)	–	Phytagel[a] (0.2%)	5.80
4.	MSOT3	1×; extra KNO_3 (1.9 gm/l)	1×	Glucose[b] (3%)	–	Phytagel[a] (0.2%)	5.80

[a] Obtained from Sigma
[b] Glucose solution was filter-sterilized and added after autoclaving
[c] Filter-sterilized kinetin was added after autoclaving
[d] HIMEDIA (RM301) agar was used

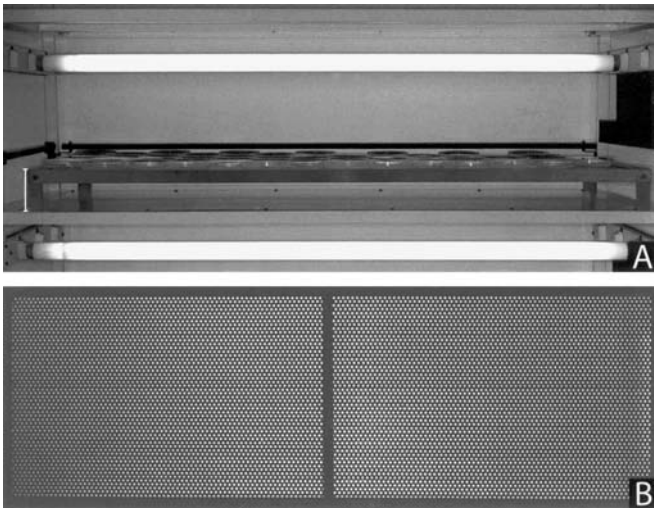

Fig. 2 **A** A shelf of a culture trolley showing the arrangements of culture plates. The *white bar* represents the height of the perforated stand (length=115 cm, width=40 cm, height=8 cm). The arrangement shown avoids the condensation of water on the lids of the petri dishes containing the cultures as direct heating of the bottom surface of the petri dishes from the lights placed on the lower shelf is eliminated. **B** Surface view of the perforated stand that is used to avoid condensation of water on the petri dish lids

the incision made at the top of the rootstock. When the grafted shoots had produced two new leaves (10–15 days), the plants were transferred to pots (soil and soilrite, 1:1 ratio) for hardening under controlled conditions [32°C; 70% relative humidity; 16/8-h (light/dark) photoperiod; 2,000 lux fluorescent light] in a growth chamber (Conviron, Canada). Approximately 20 cm-tall plants were transplanted to 30 cm diameter pots and transferred to the greenhouse [32±4°C; 16/8-h (light/dark) photoperiod; approx. 70% relative humidity].

Staining for the expression of reporter gene *gus* was carried out following Jefferson et al. (1987). DNA for Southern hybridization was isolated from plants using the Plant DNeasy kit (Qiagen, Hilden, Germany). Southern hybridization was carried out according to Sambrook et al. (1989).

Results and discussion

After 10–15 days of culture on MST1 medium (Table 1) supplemented with 50 mg/l kanamycin, callus tissues were observed at the cut ends of the hypocotyl and cotyledonary explants. In transformation experiments, around 78% of explants produced callus (Table 2) as compared to 100% callus formation in control experiments. We randomly chose explants with callus from different transformation experiments and checked these for GUS expression. All of the explants tested stained blue, indicating that the transformation frequency was very high. Approximately 30-day-old callus tissues from MST1 medium with 50 mg/l kanamycin were transferred to MSOT2 medium (Table 1) supplemented with 50 mg/l kanamycin. After 30–40 days of growth on MSOT2 medium, around 45% of the transferred calli gave rise to white and yellowish granular embryogenic callus (Table 2).

In initial experiments (data not provided), embryogenic callus tissues cultured on MSOT2 medium were plated on MSOT3 medium (Table 1) supplemented with 50 mg/l kanamycin. However, poor embryo induction was observed at this level of selection. Following the recommendations of earlier reported protocols (Firoozabady et al. 1987; Cousins et al. 1991; Sunilkumar and Rathore 2001), the concentration of kanamycin in MSOT3 was

Table 2 Frequency of embryogenic callus formation and somatic embryogenesis

Transformation experiment no.	Number of explants	Percentage callus formation[a]	Percentage embryogenic callus formation[a]	Percentage calli producing embryos[a]
1.	86	97.6	50.0	17.4
2.	121	80.9	15.7	8.2
3.	92	60.8	35.8	10.8
4.	120	73.3	39.1	15.8

[a] All percentages are based on the number of explants inoculated

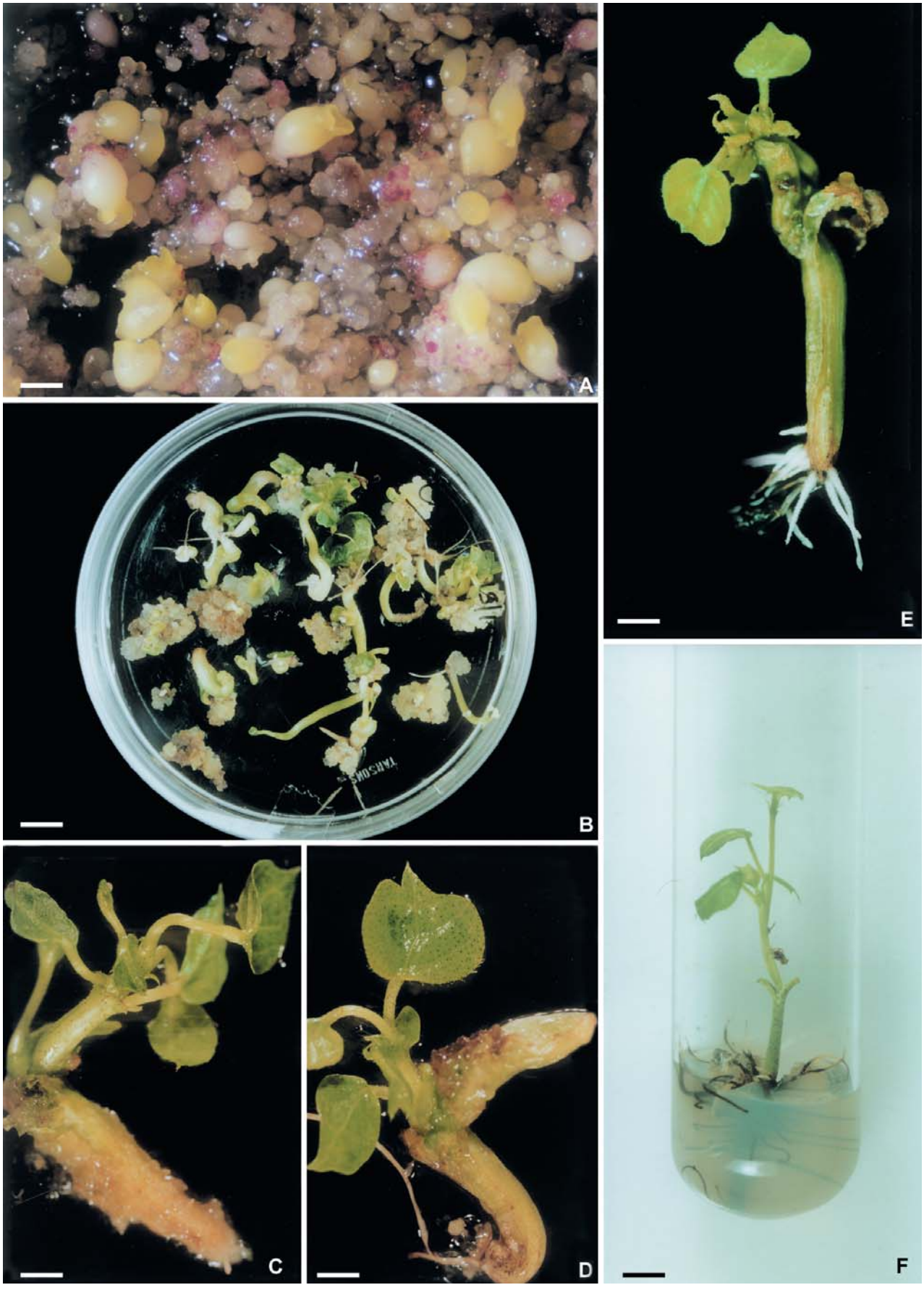
A
B
C
D
E
F

Table 3 Shoot recovery in genetic transformation experiments on Coker 310 FR

Number of embryos per experiment subjected to desiccation	Percentage of embryos giving rise to shoots on MSOT3 medium	Percentage survival of grafted shoots[a] in vitro
197	54.3	95.2
54	55.5	93.3
94	62.7	89.7
37	48.6	94.4

[a]Approximately 95% of the grafted shoots could be established in soil after 15 days of growth in vitro

reduced to 25 mg/l. At this selection level, 39% of the embryogenic calli (around 13% of the initial explants) gave rise to somatic embryos (Table 2). The number of embryos obtained from each embryogenic callus varied. Regenerated embryos were checked for GUS expression, and more than 75% of embryos stained uniformly, indicating that the transformation and selection procedures were effective. During the early stages of embryo development, globular- and torpedo-stage embryos on MSOT3 medium with 25 mg/l kanamycin appeared to be similar to embryos that regenerated from control cultures (Fig. 3A). However, at maturity (after 45 days of growth on MSOT3 medium), a majority of the former showed abnormal morphologies, including a hypertrophied appearance and ill-formed cotyledonary or radicle ends. Some of the embryos had no visible cotyledons but produced roots from the radicle ends.

In four separate experiments, elongated embryos of approximately 1 cm length were allowed to undergo slow physical desiccation at step G of the protocol for 30–40 days on MSOT3 medium without kanamycin (Fig. 3B). After about 40 days of culture, the initial medium was gradually reduced to a thin layer, bringing about slow desiccation. Upon transfer to fresh MSOT3 medium (Fig. 1, step H), on average, 51% of the embryos subjected to slow desiccation (Table 3) differentiated into single or multiple shoots from the cotyledonary ends (Fig. 3C–E). Some of the embryos that did not regenerate shoots immediately after transfer to fresh medium responded after an additional 20 days of culture on the same medium. Regenerated shoots had healthy leaves and sturdy stems.

Fig. 3A–F Various stages of embryogenesis and shoot recovery from transformed somatic embryos of cotton (*G. hirsutum* L. cv. Coker 310FR). **A** Transformed embryogenic callus on MSOT3 medium with 25 mg/l of kanamycin. Embryo development at this stage is normal and comparable to development observed in control cultures (*bar* 1 mm). **B** Mature embryos transferred to MSOT3 medium undergoing slow desiccation. Note that most of the embryos though elongated are abnormal and do not have any first leaves. Many have rooted at the radicle ends (*bar* 8 mm). **C** A hypertrophied embryo giving rise to robust shoots from the cotyledonary ends following transfer to fresh MSOT3 medium (*bar* 4 mm). **D**. An embryo with aberrant cotyledons showing shoot morphogenesis from the cotyledonary end (*bar* 4 mm). **E** An elongated embryo with multiple roots at the radicle end differentiating shoots with well-defined leaves. The shoot can be excised and grafted to WT stock, and the embryo explant would be discarded (*bar* 4 mm). **F** An in vitro graft of a transgenic shoot on a WT seedling stock, 15 days after grafting (*bar* 8 mm)

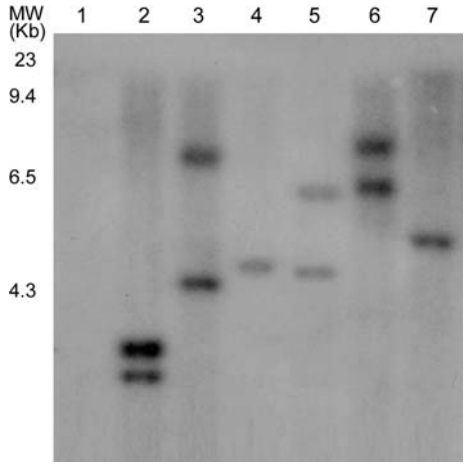

Fig. 4 Southern blot analysis of genomic DNA from leaves of untransformed control (*lane 1*) and transgenic cotton plants (*lanes 2–7*). Genomic DNA from all the transgenic and control lines were digested with *Eco*RV (unique site within the T-DNA borders) and probed with the *nptII* gene for analysis of integration towards the left border of the T-DNA

Regenerated shoots excised from the embryo explant were grown either on MS medium with 2 mg/l IBA or grafted on WT rootstock (Fig. 3F). Less than 8.5% of the shoots rooted directly on IBA medium, while 89–95% of the grafted shoots gave rise to plantlets. Grafted plants could be transferred to soil with an average success of 94%; all were fertile and set seeds. Southern analysis of a subset of plants showed single or multiple T-DNA integration in the plant genome (Fig. 4). These results clearly indicate the transgenic nature of the shoots obtained through this modified protocol of regeneration of transgenic plants from transformed cotton somatic embryos (Fig. 1).

In an earlier study, Voo et al. (1991) investigated the effects of physical and chemical desiccation on germination frequencies and plantlet formation from cotton somatic embryos. The highest frequency of plantlet formation (40%) was observed when the embryos were subjected to physical desiccation. The desiccation treatment applied in this study involved air-drying of embryos inside a laminar flow cabinet and incubation of embryos in sealed petri dishes without any medium. However, this investigation was performed on untransformed embryos of Coker 312. An earlier investigation carried out by us (Kumar et al. 1998) showed similar conversion frequen-

cies without a desiccation treatment. The difficulty seems to arise in genetic transformation experiments where kanamycin selection is applied. In other studies on genetic transformation of cotton, except for the earlier work of Firoozabady et al. (1987), most of the authors (Finer and McMullen 1990; Cousins et al. 1991; Rajasekaran et al. 2000; Sunilkumar and Rathore 2001) have recorded poor embryo formation on selection media and conversion frequencies are not mentioned.

The regeneration of abnormal embryos and low-frequency plantlet recovery have been a limitation for the development of cotton transgenics. We report here that very reproducible results can be obtained with respect to the development of transgenic plantlets from transformed somatic embryos of cotton (cv. Coker 310FR) by including a step of slow physical desiccation in the protocol. The inclusion of this slow-desiccation step resulted in the regeneration of sturdy shoots from the cotyledonary ends of both normal- and abnormal-looking somatic embryos at a very high frequency.

Acknowledgements This investigation was supported by a research grant from the Department of Biotechnology, India. BC and SK were supported by Research Fellowships from the Council of Scientific and Industrial Research (CSIR). The technical assistance of Amarjeet Kumar Singh is acknowledged.

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