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Transgenic cotton: factors influencing *Agrobacterium*-mediated transformation and regeneration

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Abstract

Various aspects of transformation and regeneration processes were examined in efforts to improve the efficiency of production of transgenic cotton (*Gossypium hirsutum* L.). Green fluorescent protein (GFP) proved to be a valuable tool in elucidating the timing and localization of transient gene expression and in visualizing conversion of transient events to stable transformation events. By day 4 after infection, there was maximal transient activity in the cells at the cut edge of *Agrobacterium*-infected cotyledon disks. We were able to visualize conversion of some of these events to stable transformation by day 8. The effects of *Agrobacterium* strains, acetosyringone, and temperature on stable transformation were also evaluated. Strain LBA4404 proved to be significantly better than EHA105. Acetosyringone increased significantly the stable transformation efficiency in cotton. Cocultivation at 21 °C, compared to 25 °C, consistently resulted in higher transformation frequencies. GFP expression in stably transformed callus was useful in studying the efficiency of selection during early stages of culture. We found that the survival of individual callus lines on selection medium was influenced by their original size and initial transgene expression status. Larger-size calluses and calluses expressing the transgene (GFP) had a higher rate of survival. Survival could be improved by an additional two-week culture on medium high in cytokinin and low in auxin before transfer to a medium to induce embryogenesis. However, this treatment delayed embryogenesis. Various other important aspects of the regeneration process are described and an overall scheme for producing transgenic cotton is presented.

Abbreviations: AS – Acetosyringone, GFP – green fluorescent protein

Introduction

Cotton is an important source of fiber, feed, and edible oil. It is the fourth largest crop in terms of economic value in the USA and is grown in more than eighty other countries. Worldwide, it ranks sixth in terms of acreage planted, with 33 million hectares devoted to cotton in 1999. During that year, 52 million metric tons of seed cotton and 18 million metric tons of lint were produced throughout the world (FAO statistics). Genetic engineering will play a major role in further improving the yield and quality of fiber, feed, and oil and in increasing the production efficiency and reducing both the monetary and environmental cost of production. First reports on successful transformation of cotton appeared as early as 1987 (Firoozabady et al. 1987; Umbeck et al. 1987). Cotton (Bt cotton) is also one of the first transgenic crops to be planted on large scale in the USA where it has been a commercial success. Despite the economic importance of cotton and early successes in producing transgenic cotton (only four years after the first reports on transforming the model species, tobacco, with chimeric genes), there are less than twenty published reports on cotton transformation. One of the main reasons for this is that production of transgenic cotton, which requires transformation of appropriate tissue followed by regeneration, remains extremely difficult with the regeneration

aspect being more troublesome. Recalcitrance problems with cotton relate to the rather long tissue culture duration before regeneration occurs, the unpredictability of tissue culture, and a high degree of genotype dependence. The two studies mentioned above utilized *Agrobacterium*-mediated transformation of cotton tissue followed by regeneration via embryogenesis. Some attempts have been made to transform cells directly in the shoot apex via either the gene gun or *Agrobacterium* (McCabe and Martinell 1993; Zapata et al. 1999), but these methods suffer from extremely low transformation efficiencies. Others have used particle bombardment of suspension cultures of cotton (Finer and McMullen 1990; Rajasekaran et al. 2000). This method suffers from the fact that additional time is required for establishment of suspension cultures and that high-copy-number transgene integration usually takes place with the gene gun method. Therefore, *Agrobacterium*-mediated transformation followed by somatic embryogenesis (Firoozabady et al. 1987; Umbeck et al. 1987; Lyon et al. 1993; Thomas et al. 1995) remains the method of choice for most laboratories. As stated earlier, it is the regeneration aspect of the transformation process that remains more difficult and several reports suggest that it can take 10 to 12 months to regenerate cotton plants from the best regenerable genotypes. This length of time adds substantially to the cost of producing transgenic cotton and can result in a higher rate of somaclonal variation. There are no standard protocols established for cotton transformation. While some laboratories obtain regeneration directly from transgenic callus lines (Firoozabady et al. 1987; Umbeck et al. 1987), others report a combination of callus and suspension cultures to obtain somatic embryos (Cousins et al. 1991). In addition, a variety of hormonal regimes were used by different groups. Because most of the difficulties in obtaining transgenic cotton are associated largely with regeneration, it will be beneficial to improve the transformation efficiencies so that a large number of independent transgenic callus lines can be obtained from which good regenerable lines can be selected. We have examined several factors that can significantly affect the efficiencies of transformation and regeneration. We have also made use of the green fluorescent protein (GFP) gene from jellyfish to gain insight into various aspects of the transformation process and used it to mark the progression of transformation and regeneration.

Materials and methods

Plant material

Cotton (*Gossypium hirsutum* L.) cv. Coker 312 seeds were obtained from Seedco, Lubbock, TX. Seeds of genotypes TAM-94L25, TAM-89E51 and TAM-94WE37S were kindly provided by Dr C. Wayne Smith (Texas A&M University). The seeds were surface-sterilized by first treating with 70% ethanol for 1 min followed by two washes with sterile distilled water. This was followed by a 20 min treatment with 100 ml of 20% bleach with a drop of Tween 20. Seeds were washed three times and germinated on MS0 medium (MS salts, 2% glucose pH 5.8, solidified with 0.2% Phytagel) at 28 °C, under a 16 h photoperiod (70 μmol m^{-2} s^{-1}) in jars. Hypocotyl segments (5–6 mm) excised from 10–12-day old seedlings were used for transformation and regeneration studies (Figures 1 and 2 and Table 4). Cotyledon disks (7 mm diameter), excised from 10–12-day old seedlings, were used in experiments to study the factors influencing transformation (Tables 1, 2 and 3). Transformation efficiency differed widely among disks obtained from cotyledons from different seedlings. In order to avoid this variability, each cotyledon was split into two halves along the middle rib. Disks cut with a cork borer from one half were utilized for one treatment and those from the other half for the other treatment in the comparison studies (EHA105 vs. LBA4404; 21 vs. 25 °C; + acetosyringone vs. – acetosyringone).

Bacterial strains and plasmids

The *Agrobacterium tumefaciens*-disarmed helper strain LBA4404 (Ooms et al. 1982) harboring pBINmGFP5-ER with *nptII* as a selection marker was used for transformation and regeneration experiments. The binary vector pBINmGFP5-ER (obtained from J. Haseloff, MRC, Cambridge, UK) harbors a modified version of GFP, with higher gene expression, enhanced fluorescence, and ER targeting signal, driven by CaMV 35S promoter. The supervirulent helper strain EHA105 (Hood et al. 1993) or LBA4404 each harboring pCNL56 (Li et al. 1992; kindly provided by S.B. Gelvin) were used for cotyledon disk transformation experiments. The plasmid pCNL56 carries *uidA* as the reporter gene and the *nptII* gene as the selectable marker gene. The binary vectors were mobilized into *Agrobacterium* by the heat shock method (An et al. 1988). The *Agrobacterium* strains were grown on YEP

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medium (Chilton et al. 1974) plates containing rifampicin (10 mg/l) and kanamycin (100 mg/l). Five single colonies were inoculated individually in 2 ml YEP medium containing rifampicin (10 mg/l) and kanamycin (100 mg/l) in tubes and were grown for 36 h at 28 °C with 200 rpm shaking. Cells from 5 tubes were pooled, harvested by centrifugation, and resuspended in 10 ml of pre-induction medium (1% glucose, 7.5 mM MES, 2 mM sodium phosphate buffer pH 5.6, AB salts (Chilton et al. 1974) containing 100 μM acetosyringone (Aldrich)). The culture was grown for 24 h at 28 °C in a 125 ml flask on a shaker at 200 rpm to A_{600} 1.6–1.9. Just prior to co-cultivation, additional acetosyringone was added to the culture at a final concentration of 100 μM.

Cotton transformation and regeneration

The hypocotyl segments or cotyledon disks excised from 10–12-day old seedlings were placed over a filter paper on P1-AS medium (modified G2 medium from Firoozabady and DeBoer 1993; MS salts, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 5 mg/l N^6-(2-isopentenyl)adenine, 0.1 mg/l naphthaleneacetic acid, 3% glucose, 1 g/l $MgCl_2.6H_2O$, 100 μM acetosyringone, pH 5.8, solidified with 0.2% Phytagel). The hypocotyl segments were laid horizontally and the cotyledon disks were placed with their adaxial side facing downward on the filter. A 5 μl portion of pre-induced *Agrobacterium* suspension was applied to each cut side of the hypocotyl segments and 10 μl was applied around the cut edge of cotyledon disks. Co-cultivation was carried out at 25 °C (or at 21 °C, wherever stated) for three days under a 16 h photoperiod (70 μmol m^{-2} s^{-1}). The explants were then transferred to P1-c4k50 medium containing 50 mg/l kanamycin and 400 mg/l carbenicillin (same as P1-AS except that acetosyringone was replaced with antibiotics) and incubated at 28 °C, under a 16 h photoperiod (70 μmol m^{-2} s^{-1}) for 20–25 days. The kanamycin-resistant calluses growing around the edges of the cotyledons (Figure 1I), which represent individual stably transformed callus lines, were scored at 21 days after co-cultivation by visualizing under a stereo microscope (Olympus, model-SZH10). Regeneration studies reported here were carried out with hypocotyl explants of cv. Coker 312. In the case of transformation of hypocotyl segments, 1–3 mm kanamycin-resistant calluses growing at the cut edges were excised under a stereo microscope and transferred to P7-c4k50 medium (modified G3 medium from Firoozabady and DeBoer 1993; MS salts, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 0.1 mg/l N^6-(2-isopentenyl) adenine, 5 mg/l naphthaleneacetic acid, 3% glucose, 1 g/l $MgCl_2.6H_2O$, 50 mg/l kanamycin and 400 mg/l carbenicillin, pH 5.8, solidified with 0.2% Phytagel). These calluses were maintained on this medium for 12 weeks (16 h photoperiod, 2–10 μmol $m^{-2}s^{-1}$) with regular subculture to fresh medium every four weeks.

After three subcultures on P7-c4k50, the calluses were transferred to MSBOK medium (MS salts, 100 mg/l myo-inositol, B-5 organics, additional 1.9 g/l KNO_3, 3% glucose, 1 g/l $MgCl_2.6H_2O$, 25 mg/l kanamycin and 200 mg/l carbenicillin, pH 5.8, solidified with 0.2% Phytagel) and maintained on this medium (16 h photoperiod, 10 μmol m^{-2} s^{-1}) with monthly subculture until somatic embryos developed. After the first two rounds of culture on MSBOK medium, kanamycin was not included in this medium for subsequent rounds of subculture. The somatic embryos, at least 7–8 mm in length, were transferred onto a filter paper on EG3 medium (Firrozabady and DeBoer 1993; 1/2× MS salts, 0.5% glucose, 100 mg/l myo-inositol, 0.4 mg/l thiamine, pH 5.9, solidified with 0.2% Phytagel) for germination and incubated at 25 °C under a 16 h photoperiod (70 μmol m^{-2} s^{-1}). After germination, the plantlets (2–3 cm long shoots with true leaves) with roots were transferred to MS3 (1/2× MS salts, 0.5% glucose, 0.14 mg/l thiamine, 0.1 mg/l pyridoxine, 0.1 mg/l nicotinic acid, pH 5.8, solidified with 0.08% Phytagel and 0.4% Difco Bacto agar) medium in jars and incubated at 25 °C, under 16 h photoperiod (70 μmol $m^{-2}s^{-1}$). Plantlets with 5–7 cm long shoots with a good root system were transferred to soil (Figure 1U) and kept under high humidity on a laboratory bench for 2 weeks. The plants were then gradually hardened in growth chambers (28 °C, 16 h photoperiod, 200 μmol m^{-2} s^{-1}) for 2–3 weeks. They were then transferred to large pots in Metro-mix700 soil and grown to maturity in the greenhouse (Figure 1V).

Fluorescence microscopy

Green fluorescent protein expression in cotyledon disks, hypocotyl segments, calluses, developing embryos and germinated somatic embryos was visualized with a Zeiss M^2BIO Fluorescence Combination Zoom Stereo/Compound microscope. The microscope is equipped with a GFP filter set comprising an exciter filter (BP 470/40 nm), a dichromatic beam splitter (495 nm) and a barrier filter (LP 500 nm). When

needed, a band-pass interference filter (BP 525/50 nm) slider was substituted for the LP 500 nm barrier filter slider to block the red autofluorescence from chlorophyll. The light source was an HBO 100 W mercury lamp. Photographs were taken with a Zeiss MC 80 automatic exposure photomicrographic system and Kodak Elite II 400ASA daylight film.

Molecular analyses

Genomic DNA was isolated from young leaves of control and transgenic cotton plants (Paterson et al. 1993). PCR analysis for detection of the *nptII* gene was carried out with the primers 5′-TCGGCTATGACTGGGCACAACAGA-3′ (forward) and 5′-AAGAAGGCGATAGAAGGCGATGCG-3′ (reverse) that were part of the coding region of the *nptII* gene. About 50 ng of template genomic DNA was subjected to 35 cycles of amplification in a 25 μl reaction. PCR products were analyzed by gel electrophoresis on 1% agarose gels. The procedures for Southern blot analysis were according to Sambrook *et al*. (1989) and the manufacturer's recommendations. A 10 μg portion of genomic DNA was used for each of the restriction enzyme digests and PCR-amplified *nptII* gene was used as the radioactive probe.

Results

Transient and stable transformation

Several aspects of the cotton transformation and regeneration processes were investigated in this study. Some of these studies were made possible because of the use of the visual, non-destructive GFP-based reporter system which allowed periodic monitoring of transformation events without having to remove the tissue from the culture plates and without adversely affecting the tissue growth. Another advantage of this reporter system was that there was no apparent secretion or intercellular diffusion of GFP and the fluorescence appeared to be confined to the transformed cells. GFP enabled us to visualize transient transformation events followed by conversion of a small number of these to stable integration events that were recognized by their ability to divide and grow in the presence of kanamycin, eventually developing into calluses. After transformation with pBINmGFP-ER, transient expression in cotton cotyledons was visible as early as 45 h after *Agrobacterium* infection and reached maximum levels by day 4 (Figure 1A, B). The majority of the cells showing transient expression were close to the wounded edge. Most disks showed a ring of transient GFP activity all around the edge (Figure 1A). *Agrobacterium* emitted a dull, pale green fluorescence as observed under our fluorescence microscope; however, this was quite distinct from the bright green fluorescence of GFP-expressing cotton cells. Interestingly, transient expression was first visible (at 45 h) in the epidermal cells on the abaxial side close to the wounded edge (results not shown). It was not until day 4 that the transient activity appeared in the epidermal cells on the adaxial side, within the mesophyll cells, and in the vascular region (Figure 1A, B). Because of the way hypocotyl segments were cultured, it was not possible to monitor the timing of transient expression at the cut surface without reorienting them. However, as shown in Figure 1EE, where the hypocotyl was reoriented with the cut surface facing up for photography, the transient activity was predominant in the stele area at the cut surface in the hypocotyls. Very few of the transient transformation events were observed in the unwounded parts of either the cotyledons or hypocotyls. Even these transformations were probably at the sites where unintentional wounding occurred while handling the explants. Some comparative studies carried out with tobacco leaf disks showed that the number of transiently transformed cells was higher in cotton cotyledons compared to tobacco leaf disks (results not shown). The results on transient transformation of cotyledon and hypocotyl cells suggest that *Agrobacterium*-mediated transfer of T-DNA to cotton cells is highly efficient.

Although the most dramatic and the earliest transient GFP activity was seen in the abaxial epidermal cells, cell division activity was only seen in cells (mesophyll and vascular) located between the two epidermal cell layers. As early as day 6 after infection, some of these cells could be seen dividing, eventually giving rise to individual callus lines. It was not until day 8 that we were able to capture such an event clearly on a photomicrograph (Figure 1C, D). The fluorescent cluster of cells represents an individual transformation event where at some point in time, stable integration of the T-DNA had presumably occurred, followed by cell division. Figure 1F shows a hypocotyl segment 4 weeks after initiating selection on kanamycin medium after transformation with pBINmGFP-ER. Bright-field and fluorescence images (Figure 1F, FF) clearly show stably transformed callus tissue on both cut surfaces. It should be noted that in this report, a kanamycin-resistant callus is considered

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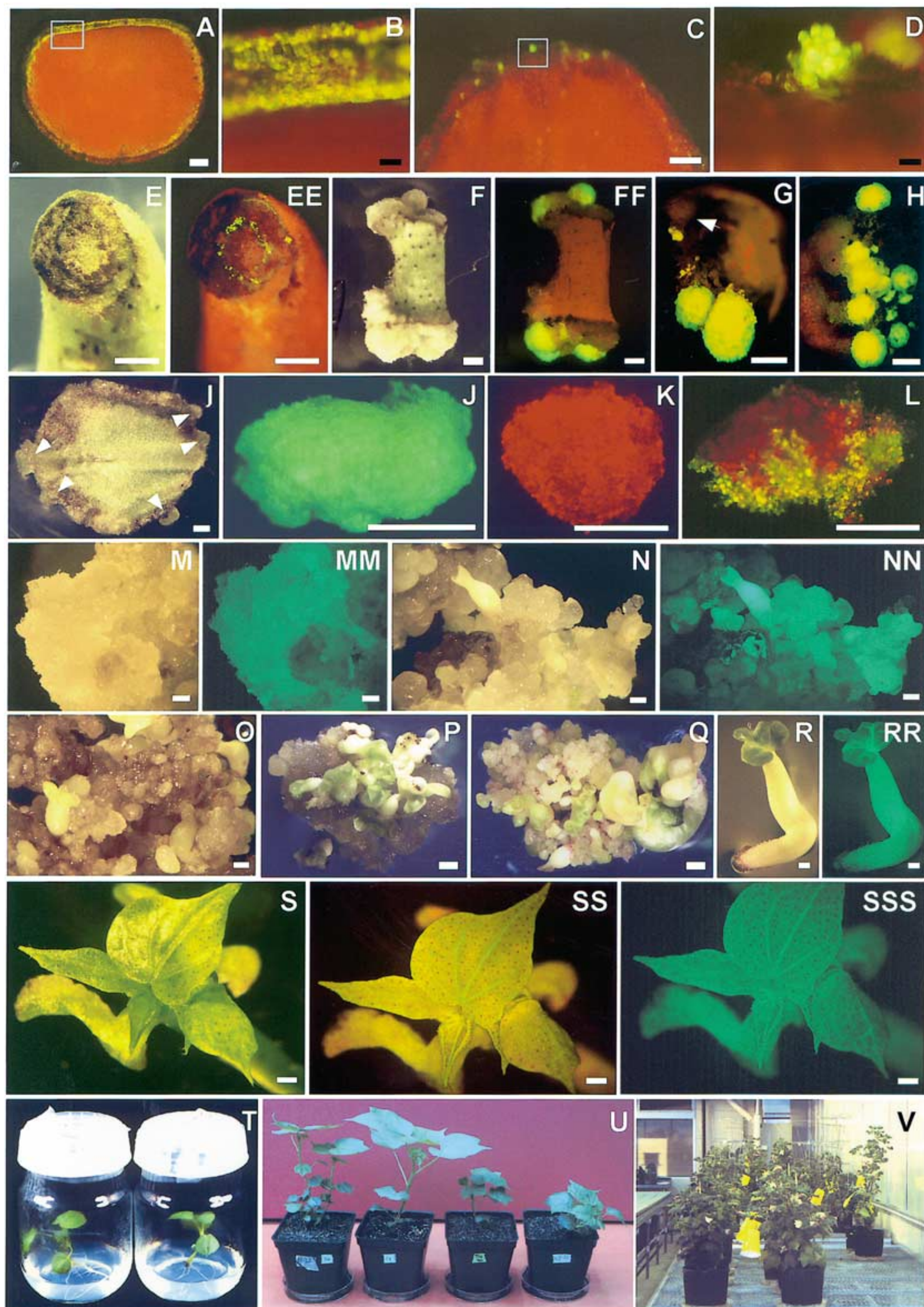

Figure 1.

Figure 1. Various stages of transformation and regeneration in cotton. A. Cotyledon disk showing transient GFP expression in the cells at the cut surface, 86 h after infection. Note the red auto-fluorescence from the chloroplast throughout the disc. A chlorophyll fluorescence blocking filter was not used for most of the fluorescence micrographs for better visual effects. B. Higher magnification fluorescence image of the area in the inset in A, showing transient GFP expression in the cells of both epidermal layers and the cells in the middle of these two layers, at the cut edge. Note that this part of cotyledon disc has curled up (curling usually occurs in most cotyledons) making it possible to visualize both epidermal layers and the cells in between the two layers at the edge of cotyledon disk. C. Cotyledon disk showing a number of individual stable transformation events, 8 days after infection. D. Higher magnification image of an area shown in the inset in C, showing an individual, stable transformation event in the form of a cluster of GFP-expressing cells that are able to divide in the presence of kanamycin. E. Light micrograph of a hypocotyl segment 5 days after infection. EE. Corresponding fluorescence image showing a ring of transient transformation events in cells in the stele region. F. Light micrograph of a hypocotyl segment, 4 weeks after transformation, showing growth of stably transformed individual calluses at both the cut surfaces. FF. Corresponding fluorescence image showing stable transformed calluses that are expressing GFP. G. Hypocotyl segment, 4 weeks after transformation, with its original cut surface facing the objective, showing two individual GFP expressing stable transgenic events and one non-GFP-expressing event (arrow). H. Another hypocotyl segment, 4 weeks after transformation, with its original cut surface facing the objective, showing at least seven individual GFP-expressing stable transgenic events. I. Light micrograph of a cotyledon disk, 20 days after transformation, showing five individual stable transgenic calluses (arrows). J. GFP-expressing individual callus line, 3 weeks after excision from the original hypocotyl explant. K. Fluorescence image of an individual callus line not expressing GFP, 3 weeks after excision from the original hypocotyl explant. Note that the red fluorescence is from chloroplasts. L. Fluorescence image of a callus line, three weeks after excision from the original hypocotyl explant, showing sectors with (green) and without (red) GFP expression. M and MM. Light and fluorescence images, respectively, of part of a callus line growing on P7-c4k50 medium, 8 weeks after excision. N and NN. Light and fluorescence images, respectively, of part of an embryogenic callus line growing on MSBOK medium, 18 weeks after transformation. O. A 20-week old embryogenic line showing intense browning as well as good embryoids. P and Q. 22 weeks old culture lines showing embryo formation. R and RR. Light and fluorescence images, respectively, of a somatic embryo after transfer to EG3 medium. The image shown in RR was taken with the chlorophyll blocking filter. S, SS and SSS. Light and fluorescence images of a germinated embryo. Note that SS is a fluorescence image without the chlorophyll fluorescence blocking filter and shows greenish yellow fluorescence. The SSS image was taken with the chlorophyll fluorescence blocking filter. T. Germinated embryo developed into a plantlet with good roots and leaves. U. Transgenic plants in soil. V. Transgenic plants in the greenhouse (from other non-GFP experiments). White and black (B and D) bars represent 1 mm and 100 μm, respectively.

a stably transformed line. As will be described below, PCR and Southern blot analyses on DNA from several plants obtained from kanamycin-resistant callus lines did show the presence and integration of the *nptII* gene, respectively. Several independent transformation events were visible in the form of distinct fluorescent foci (Figure 1FF). Figures 1G and 1H show two different, transformed hypocotyls that were oriented with their callus-growing sides facing the microscope objective for the purpose of photography. Figure 1G shows at least two and Figure 1H more than seven distinct, independent transformation events that resulted in kanamycin-resistant, GFP-expressing calluses. In addition, a callus visible in Figure 1G (indicated by arrowhead) probably represents a transformation event where GFP gene may be silenced or did not integrate completely as it was close to the left border of T-DNA. Three to four weeks after transformation, the calluses growing at the edge of a hypocotyl were usually excised under white light with the aid of a stereo microscope and cultured as individual transgenic events. In some cases where a callus line that appeared as an independent transgenic event when seen under white light, that line was actually chimeric when seen with fluorescence microscopy. Figure 1L is an example of such a callus that was excised from the original hypocotyl explant about three weeks prior to photography. Figure 1J, K and L shows three different callus lines excised from hypocotyl segments and kept on kanamycin medium for about three weeks. The callus shown in Figure 1J is expressing GFP in most of its cells, whereas the callus in Figure 1K is either not expressing the GFP gene or may be the result of an event where GFP integration did not occur. The red fluorescence is due to chlorophyll that is often present in calluses of this age. The chimeric nature of the callus in Figure 1L may be due either to gene silencing in certain parts of the callus or the fact that transformation of two cells that were very close to each other occurred, and the resulting progeny from one expressing GFP merging with the other non-expressing GFP. Figure 1I shows a cotyledon disk that was transformed 3 weeks prior to photography. Five distinct callus lines (arrows) at the cut surface are clearly visible. The kanamycin-resistant, growing calluses on the cut surface of a cotyledon disk could be distinguished more easily from each other microscopically under white light compared to those growing on the cut surface of a hypocotyl segment. For this reason, cotyledon disks were utilized to evaluate the factors affecting stable transformation efficiencies.

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Factors affecting Agrobacterium*-mediated transformation of cotton*

In order to optimize conditions for cotton transformation, the effect of several parameters known to influence *Agrobacterium*-mediated DNA transfer were compared. Disks (7 mm) cut from cotton cotyledons with a cork borer were chosen as the explants because, as stated above, they produced distinct kanamycin-resistant calluses at the cut edge which facilitated scoring. Hypocotyl segments were not used for this set of investigations because it was difficult to distinguish independently transformed callus lines since they tend to grow close to each other making it hard to score independent events. We observed that the cotyledons (as well as hypocotyls) from different seedlings of the same age showed a wide range of response to *Agrobacterium*-mediated transformation. For example, the number of kanamycin-resistant calluses growing on a cotyledon could range from 0 to 21. To minimize the variation introduced by the explants, we performed the experiments in the following way: (1) the disks obtained from either side of the midrib of a single cotyledon were utilized for each of the two comparative treatments; (2) a large sample size of 90–120 disk explants was tested for each treatment to account for the variability in cotyledons obtained from different seedlings. Transformation efficiency was expressed as mean number of kanamycin-resistant calluses per cotyledon disk. Total number (n) of cotyledons scored and the percentage of cotyledon disks that showed stable transformation are also presented in the tables. In some experiments the number of cotyledons scored is less than 90 because of loss of some samples through contamination.

Comparison of the efficiency of LBA4404 and EHA105 for transformation

We compared the efficiency of strains LBA4404 and EHA105, harboring the binary plasmid pCNL56, in transforming cotton. The bacterial cultures were grown to A_{600} 1.6–1.9 and the co-cultivation was performed on P1-AS medium. Experiments were performed in duplicate and the transformation efficiency was measured as the mean number of kanamycin-resistant calluses per cotyledon disk. The results from this set of studies are presented in Table 1. The transformation efficiency of the strain LBA4404 was significantly higher (>2-fold) than that of EHA105 in the cotton cultivar Coker 312. A similar comparison was performed with three other cotton genotypes (TAM-94L25, TAM-89E51, and TAM-94WE37S). Again, strain LBA4404 was found to be significantly better than EHA105 in all the varieties tested. In addition, the overall percentage of disks transformed was higher with LBA4404. Strain EHA105 was found to overgrow on some of the explants during selection. This overgrowth of bacteria interfered with callus growth on selection medium. The problem of bacterial overgrowth was solved by including cefotaxime (100 mg/l) in addition to carbenicillin (250 mg/l) in the selection medium in all the experiments involving cotyledon disks.

Effect of inclusion of acetosyringone during co-cultivation

Acetosyringone is one of the phenolic compounds secreted by wounded plant tissues and is known to be a potent inducer of *Agrobacterium vir* genes (Stachel et al. 1985). Several reports on monocots and some reports on certain dicots suggest that acetosyringone pre-induction of *Agrobacterium* and/or inclusion of acetosyringone in the co-cultivation medium can enhance significantly *Agrobacterium*-mediated transformation (Sheikholeslam and Weeks 1987; Owens and Smigocki 1988; Rashid et al. 1996; Levee et al. 1999; Sunilkumar et al. 1999). In fact, acetosyringone is used routinely for monocot transformations (Aldemita and Hodges 1996; Ishida et al. 1996; Cheng et al. 1997; Hiei et al. 1997). None of the earlier investigations on cotton transformation report the use of acetosyringone. In our experiments, acetosyringone was included at a final concentration of 100 μM during the final stage of bacterial growth and during cocultivation (see Materials and methods for details). For the control treatment, transformation was performed by completely omitting acetosyringone from every step. Strain LBA4404 harboring pCNL56 was used for this comparative study. The results in Table 2 show that acetosyringone improves significantly the transformation efficiencies. The mean number of kanamycin-resistant calluses was 2–3-fold higher when acetosyringone was included in the medium for transformation of cv. Coker 312. In the other three cotton cultivars (TAM-94L25, TAM-89E51, and TAM-94WE37S) a significant increase in the number of kanamycin-resistant calluses was observed when transformation was performed in the presence of acetosyringone. In addition, the overall percentage of disks transformed was higher when acetosyringone

Table 1. Comparison of LBA4404 and EHA105 on transformation efficiencies in cotton.

Strains	Kanamycin-resistant calluses per cotyledon disk							
	Coker 312		TAM-94L25		TAM-94WE37S		TAM-89E51	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
LBA4404	2.7±0.4**	2.2±0.3**	3.6±0.3***	2.1±0.2***	2.7±0.3^{ns}	4.1±0.4**	2.8±0.2**	2.5±0.2***
	n =58	n =103	n =109	n =119	n =94	n =108	n =83	n =120
	(77%)	(56%)	(79%)	(72%)	(83%)	(87%)	(94%)	(81%)
EHA 105	1.2±0.2	1.0±0.2	2.0±0.2	0.9±0.1	2.0±0.2	2.8±0.3	1.9±0.2	1.3±0.1
	n =54	n =102	n =112	n =120	n =96	n =102	n =94	n =115
	(62%)	(34%)	(67%)	(45%)	(77%)	(77%)	(71%)	(55%)

The data represent mean number of kanamycin-resistant calluses per cotyledon disk ± standard error of the mean (n = number of cotyledon disks scored). The difference between the two treatments is: $^{**}p < 0.01$, $^{***}p < 0.001$ or ^{ns} not significant ($p > 0.05$) as tested by ANOVA. The numbers in parenthesis represent the percentage of disks showing at least one kanamycin-resistant callus.

Table 2. Effect of acetosyringone on transformation efficiencies in cotton. Acetosyringone (AS) was added (+) to bacterial culture 24 h prior to and immediately prior to infection, as well as in the co-cultivation medium at a final concentration of 100 μM. Control treatment (–) was performed by completely omitting AS from all steps.

Asetosyrin-gone	Kanamycin-resistant calluses per cotyledon disk						
	Coker 312		TAM-94L25		TAM-94WE37S		TAM-89^{E}51
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1
+	2.2±0.3***	4.7±0.4***	2.6±0.3**	1.5±0.2***	2.1±0.2***	1.6±0.2***	2.0±0.2***
	n =90	n =86	n =109	n =118	n =89	n =100	n =101
	(71%)	(92%)	(71%)	(46%)	(75%)	(66%)	(71%)
–	0.8±0.1	1.0±0.2	1.5±0.2	0.6±0.1	0.6±0.1	0.6±0.1	0.8±0.1
	n =96	n=86	n =113	n =119	n =94	n =107	n =105
	(50%)	(47%)	(60%)	(43%)	(41%)	(43%)	(51%)

The data represent mean number of kanamycin-resistant calluses per cotyledon disk ± standard error of the mean (n = number of cotyledon disks scored). The difference between the two treatments is: $^{**}p < 0.01$ or $^{***}p < 0.001$ as tested by ANOVA. The numbers in parenthesis represent the percentage of disks showing at least one kanamycin-resistant callus.

was used. These results suggest that acetosyringone can be used to obtain significant improvements in transformation of cotton. All of the other experiments described in this report were performed with acetosyringone treatment during the final stages of bacterial growth and during co-cultivation.

Effect of co-cultivation temperature

Co-cultivation at low temperature has been shown to enhance the *Agrobacterium*-mediated transformation efficiency in *Phaseolus acutifolius* and *Nicotiana tabacum* (Dillen et al. 1997). In earlier investigations on cotton transformation, co-cultivation was carried out at 25–28 °C. In order to examine the influence of low co-cultivation temperature on cotton transformation, we compared the effect of co-cultivation temperatures of 21 °C and 25 °C in cotton cultivar Coker 312. *Agrobacterium* strain LBA4404 harboring the binary vector pCNL56 was used for these transformations. Results of this study are presented in Table 3. We consistently observed higher transformation efficiencies when the co-cultivation was carried out at 21 °C.

Influence of initial size on the survival of individual callus lines on selection medium

Some of our earlier experiments showed that, after excision from the original tissue explant (hypocotyl or cotyledon), a significant number of individual callus lines were lost during the selection process on kanamycin medium over the next two rounds of subculture. This could be due to a cell mass insufficient to survive the selection process or due to the presence of escapes. In order to understand the causes of this loss, we carried out a large-scale experiment (with 368 individual callus lines) where we studied the relationship between callus size, GFP expression

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Table 3. Effect of co-cultivation temperature on transformation efficiencies in cotton (Coker 312).

Temperature	Kanamycin-resistant calluses per cotyledon disk		
	Exp. 1	Exp. 2	Exp. 3
21 °C	5.5±0.4***	5.6±0.4*	4.7±0.4^{ns}
	n =106	n =114	n =101
	(95%)	(90%)	(87%)
25 °C	3.6±0.3	4.4±0.3	3.8±0.3
	n =108	n =110	n =98
	(88%)	(83%)	(86%)

The data represent mean number of kanamycin-resistant calluses per cotyledon disk ± standard error of the mean (n = number of cotyledon disks scored). The difference between the two treatments is: $^{*}p < 0.05$, $^{***}p < 0.001$ or ^{ns} not significant ($p > 0.05$) as tested by ANOVA. The numbers in parenthesis represent the percentage of disks showing at least one kanamycin-resistant callus.

and survival during the course of two months under kanamycin selection. The results from this experiment are shown in Figure 2. Calluses growing at the cut surface of hypocotyl explants were excised and transferred to P7-c4k50 medium. After transfer to this selection medium, the calluses were scored for GFP fluorescence and their sizes were measured and recorded. The highest percentage of calluses were GFP-positive (50%), but 30% were chimeric and about 20% were not expressing GFP. The calluses were separated into three categories according to their sizes, small (<1 mm–1 mm), medium (>1 mm–2 mm), and large (>2 mm–3 mm), and their survival was followed over the next two months. The results show clearly that there was a drop in the number of surviving calluses in all of the three size categories. However, larger calluses had a higher survival rate: the survival rate was 68.2% with large calluses (pooled from all GFP expression categories) compared to 50.2% for the medium-size and 25.6% for the small calluses. The relationship between callus size and survival held true regardless of GFP expression status. Another interesting fact to emerge from this study was that, regardless of fluorescence status (GFP expression), there was a decrease in survival of calluses over two months on kanamycin medium. However, the percentage of surviving calluses was higher (50.6%) in GFP-positive calluses than in chimeric calluses (32.4%) or GFP-negative calluses (30.6%). Even when the size of the calluses was <1 mm, the survival rate was higher among GFP-positive calluses (33.8%) than in chimeras (22.4%) and GFP –ve calluses (16.2%). The high death rate among GFP-negative calluses indicates the presence of calluses that are either not carrying (escapes) or have stopped expressing the selectable marker gene. The fact that even in the case of GFP-expressing lines the small-sized calluses died at a much higher rate than their larger counterparts suggests that a certain cell mass is required for surviving the selection/proliferation process. Once individual callus lines have survived the first two months on selection medium, they continue to grow, albeit at different rates.

Embryogenesis and regeneration

Excised calluses, during their first month on P1 or P7, grow as somewhat hard, compact, light green callus. However, after 2–3 rounds on P7 medium this tissue becomes friable and turns to a pale cream color as shown in Figure 1M. Either during the second or the third round on P7 medium or sometime during subsequent culture on MSBOK medium, callus lines become embryogenic. Somatic embryos begin to appear on the friable callus (Figure 1N, O). Some of our earlier experiments had suggested that in the individual callus lines, survival and timing of embryogenesis was different depending on whether or not, after excision, there was a two-week passage through P1-c4k50 medium before culture on P7-c4k50 began. In order to investigate the timing of embryogenesis, we carried out another large-scale experiment involving 1156 individual callus lines excised from hypocotyl segments that were cultured on P1-c4k50 selection medium for one month after transformation. This experiment was carried out in two different culture rooms (under two different culture conditions) with two culture protocols. In condition A, light intensity was 5 μmol m^{-2} s^{-1} and temperature was 24 °C, whereas in condition B, light intensity was 2 μmol m^{-2}s^{-1} and temperature was 28 °C. Photoperiod was 16L:8D under both conditions. Protocol 1 involved passage of excised callus lines through P1-c4k50 (2 weeks) → P7-c4k50 (4 weeks) → P7-c4k50 (4 weeks) → P7-c4k50 (4 weeks) → MSBOK (4 weeks) → MSBOK (4 weeks). Protocol 2 involved passage of individual excised callus lines through P7-c4k50 (4 weeks) → P7-c4k50 (4 weeks) → P7-c4k50 (4 weeks) → MSBOK (4 weeks) → MSBOK (4 weeks). Survival was recorded three months after the individual callus lines were excised from the hypocotyls and the embryogenic response of these lines was evaluated at the 3, 4, and 5 months. Results from this experiment are shown in Table 4. As expected, a number of callus

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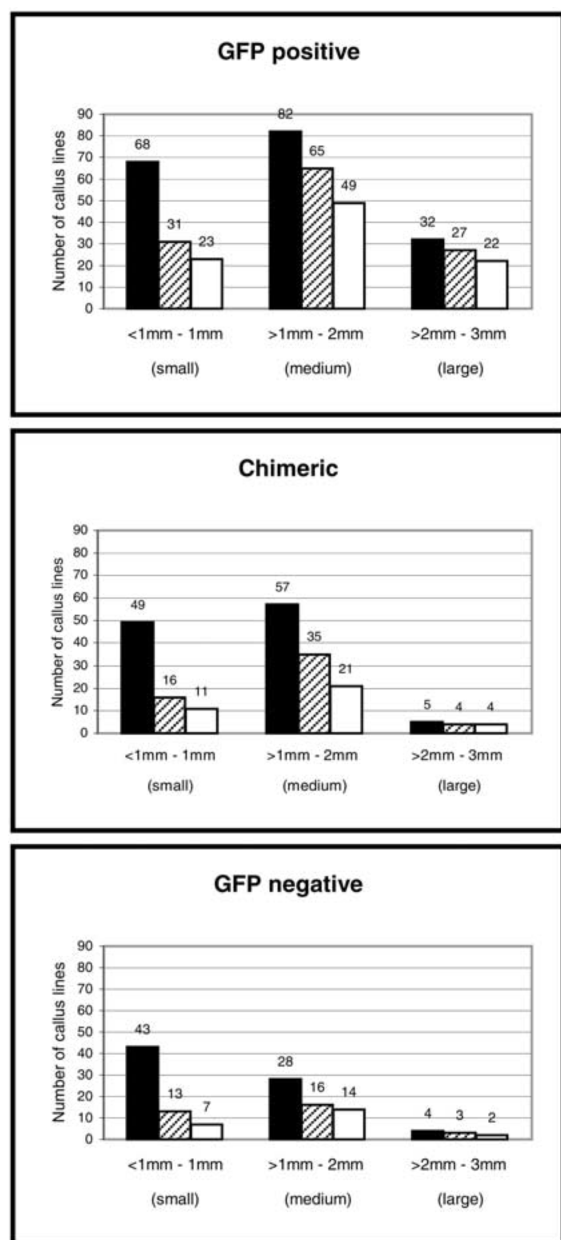

Figure 2. Survival of excised, individual callus lines over two months under selection and callus proliferation conditions. After excision from hypocotyl explants, the individual callus lines were divided into three size categories, small (<1 mm–1 mm), medium (>1 mm–2 mm), and large (>2 mm–3 mm) and their fluorescence status was noted. Their survival was monitored over the next two months. A solid bar represents their initial number, a hatched bar is the number that survived after one month and an empty bar is the number that survived after two months. The numbers above the bar represent the number of callus lines in that category.

lines died after 3 months; however, survival of callus lines was much higher in protocol 1 than in protocol 2, under either condition. Also, as expected, the percentage of embryogenic calluses increased with time as indicated by the results from 3-, 4-, and 5-month time points. This increase was seen with both protocols. Interestingly, the embryogenic response of the surviving culture lines was much faster in protocol 2 than in protocol 1, under either culture conditions. These results suggest that the culture protocol has an impact both on survival and embryogenic response. The results also suggest that under culture condition B, with a 28 °C temperature and lower light intensity, the survival rate of the calluses is a little higher and there is some improvement in the embryogenic response. Thus, an extra two-week passage through high-cytokinin/low-auxin medium (P1) before culture on embryogenesis medium (P7) increased survival but delayed embryogenesis.

As discussed in earlier reports, embryos at various stages of development can be seen to emerge from embryogenic callus lines (Figure 1P, Q). Some culture lines do become dark and in some cases die. However, the lines that became dark were still capable of producing good, healthy somatic embryos (Figure 1O). Once the embryos reach a size of 7–8 mm (Figure 1R), they were transferred to EG3 medium in tall plates for germination. In majority of cases, we found that the embryos rooted poorly and the part of the embryo in contact with the medium produced callus. Placing the embryos on filter paper laid over EG3 medium overcame both these problems. Germinated embryos (Figure 1S) with roots and a few leaves were then transferred to jars on MS3 media (Figure 1T). We observed that the growth of the plantlets, including the root system, was better on MS3 compared to the media suggested by earlier reports (results not shown). Plants with well-developed root system and leaves were transferred to soil.

Selection efficiency

DNA samples from transgenic cotton plants obtained from kanamycin-resistant cultures were tested for the presence of the *nptII* coding sequence by PCR analysis. The results showed presence of the expected 722 bp fragment in all of the 22 plants tested (Figure 3). Two of these plants (lanes 1 and 2) were pBINmGFP5-ER transformants and the remaining were transformed with various other binary vectors containing *nptII* as the selectable marker gene. The

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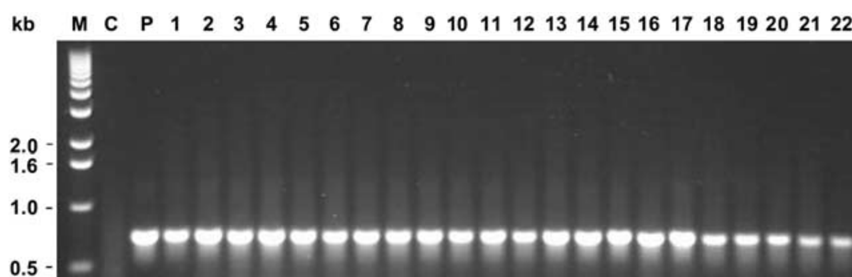

Figure 3. PCR analysis of genomic DNA to detect the presence of the *nptII*-coding region. Lanes: M, molecular weight markers; C, untransformed control plant; P, positive control pCNL56; 1–22, cotton plants regenerated from 22 independent, kanamycin-resistant callus lines showing amplification of the predicted 722 bp *nptII*-specific sequence. Two of these plants (1 and 2) were pBINmGFP5-ER transformants and the remaining were transformed with various other binary vectors containing *nptII* as the selectable marker gene.

Table 4. Effect of two different culture protocols on embryogenesis in cotton (Coker 312).

	Culture condition A		Culture condition B	
	protocol 1	protocol 2	protocol 1	protocol 2
Number excised	280	302	266	308
Number (percentage) surviving after 3 months	119 (42.5%)	73 (24.2%)	181(68.0%)	108 (35.1%)
Number (percentage) of lines embryogenic after 3 months	15 (5.4%)	36 (11.9%)	10 (3.8%)	64 (20.8%)
Number (percentage) of lines embryogenic after 4 months	35 (12.5%)	41 (13.6%)	44 (16.5%)	71 (23.1%)
Number (percentage) of lines embryogenic after 5 months	51 (18.2%)	44 (14.6%)	55 (20.7%)	71 (23.1%)

See Results section for details on culture conditions and protocols.

results from PCR analysis presented in this report and molecular analyses carried out on several other transgenic plants suggest that our selection system is quite effective in eliminating escapes. Southern blot analysis carried out on DNA from plants obtained from a different set of experiments showed integration of one to as many as five copies of transgene per genome, however, half of the transformants had single-copy integration (Figure 4). The results from Southern blot analysis confirm the integration of T-DNA into cotton genome and also rule out the possibility of *Agrobacterium* contamination of tissue resulting in false-positives.

Discussion

The purpose of this study was to investigate all stages of cotton transformation, including transient expression, stable transformation, callus proliferation, embryogenesis, and plant regeneration. GFP proved to be a powerful tool in understanding various aspects of transformation and early stages of the regeneration process. To our knowledge, this is the first report demonstrating expression of GFP in cotton. We have gained valuable insight into various aspects of transformation and regeneration of cotton that will make production of transgenic cotton easier and more efficient.

The timing and localization of transient transformation events were illustrated clearly by transformation with the GFP gene. T-DNA transfer, as assessed by GFP expression, initiated in the abaxial epidermal cells of a cotyledon at about 45 h after infection and was followed by transformation of adaxial epidermal cells and the mesophyll and vascular cells sandwiched in between the two epidermal layers by day 4. This is an interesting and unexpected result because in the way the co-cultivation is carried out, the bacterial cells would be in closer contact with the adaxial side of the explant and the cut edge. It should be noted

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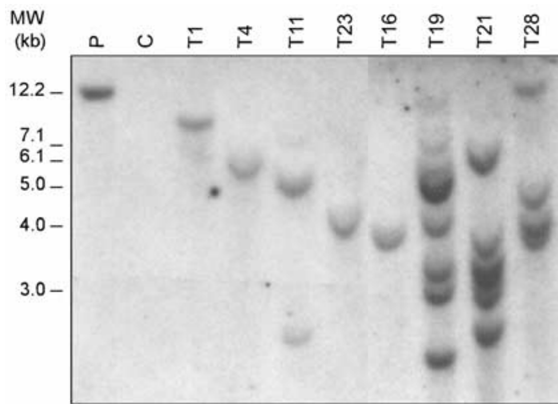

Figure 4. Southern blot analysis of genomic DNA from leaves of control (C) and transgenic (T#) cotton plants. Genomic DNA (10 μg) was digested with *Bam*HI (C, T1, T4, T11 and T23) or *Eco*RI (T16, T19, T21 and T28) and the *nptII* gene was used as the probe. There is a single *Bam*HI and a single *Eco*RI site within the T-DNA that lie outside the *nptII*-coding region. Lane P is a linearized pBI121-based binary vector used for transformation.

that the cotyledon disks were placed on the filter paper with their abaxial side facing up (see Materials and methods for details). Perhaps, the physiological state of the abaxial epidermal cells is more favorable for *Agrobacterium* infection, leading to faster T-DNA transfer. After four days, however, the epidermal cells on the adaxial side as well as the cells in the middle of the two epidermal layers (mesophyll and vascular) showed strong transient activity. As expected, most of the transient activity was confined to an area close to the wound. Our results on visualization of transient transformation suggest that T-DNA transfer to cotton cells is not a rate-limiting step; in fact, it is highly efficient.

Stable transformation events in the form of kanamycin-resistant callus growth were seen 3–4 weeks after transformation in both hypocotyls and cotyledons. Callus lines, excised from explants, when cultured individually on kanamycin medium, do not all survive. Results presented in Figure 2 indicate that callus size had a strong influence on its survival. Calluses of smaller size (at the time of excision) had a lower rate of survival. However, for a regular transformation experiment, it is impractical to wait for all the calluses on the explant to grow to a large enough size before excision in order to increase their chance of survival because this leads to the possible merging of two growing calluses. This merging will result in mixing of independent transformation events. In the case of calluses not expressing GFP, it is possible that some of the calluses died because either they were escapes or the *nptII* gene became silenced. The low survival rate of smaller size callus lines may be due to the requirement for certain critical cell density for continued independent growth after excision from the original explant. We believe that this situation is similar to the requirement of a critical minimum cell density reported for growth of cells or protoplasts (Nagata and Takebe 1971; Raveh et al. 1973; Shneyour et al. 1984). However, this requirement could, to a large extent, be compensated for by the use of feeder or nurse cells to support the growth of small cell clusters or protoplasts (Raveh et al. 1973; Horsch and Jones 1980; Shneyour et al. 1984; Imbrie-Milligan and Hodges 1986). In fact, use of feeder or nurse cells is routine in investigations where protoplasts are plated at low density but are then required to divide to form cell clusters that can then be used to regenerate plants, for example, in protoplast regeneration or protoplast-mediated transformation experiments (Rhodes et al. 1988; Lee et al. 1989; Peng et al. 1992; Rathore et al. 1993). Although we have not investigated this, it may be possible to minimize loss in cotton callus lines by using feeder cells during early stages of callus growth. This may be a better alternative than the additional two-week passage on P1 medium, described in Results, which will delay the onset of embryogenesis.

Individual transgenic lines are usually isolated by carefully dissecting out kanamycin-resistant calluses growing on either hypocotyl (1F) or cotyledon explants under white light with the aid of a stereo microscope. The 368 lines used for the experiment shown in Figure 2 were obtained in this way from transformed hypocotyl segments. However, fluorescence microscopy revealed that only 50% of the calluses were expressing GFP, while the remaining calluses were either chimeric (30%) or not expressing GFP (20%). Over the next two months during the selection and proliferation phase, 41% of the calluses died. Of the surviving calluses, GFP-expressing calluses formed the larger (61%) percentage compared to 23% for chimeric and 15% for non-expressing lines. These results suggest that there was an enrichment of the lines that were expressing GFP among the surviving calluses. The reduction in chimeric and non-expressing lines would be a desired result in any transformation experiment with another gene of interest. The higher rate of elimination of individual lines in these two categories may possibly be the result of gradual silencing of the selectable marker gene or because of lower expression level of the selectable marker gene or gradual death of escapes (in case of

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non-GFP expressing lines). Since regeneration in cotton occurs through embryogenesis, even the chimeric calluses will be expected to produce plants that carry and express the gene of interest. Assuming that further gene silencing does not occur, we estimate that of all the transgenic cotton plants obtained, at least 60–70% will express the gene of interest.

There are no reports on systematic optimization of *Agrobacteium*-mediated transformation of cotton. Several factors can have a profound influence on *Agrobacterium*-mediated transformation of a given plant species. In this study, we have evaluated transformation efficiencies with two commonly used *Agrobacterium* strains, effects of two different co-ultivation temperatures, and the effect of acetosyringone on transformation.

The choice of *Agrobacterium* strain can have a major effect on transformation efficiencies. For example, when using a 'regular' binary vector, the supervirulent strain A281 or its derivatives EHA101 and EHA105, has been found to be more suitable for transformation of cereals (Raineri et al. 1990; Rashid et al. 1996) and certain dicots (Meurer et al. 1998; Donaldson and Simmonds 2000) compared to LBA4404 (derived from Ach5). Comparison of LBA4404 and EHA105 showed consistently that transformation with LBA4404 yielded higher transformation efficiencies in four different cotton genotypes. Moreover, a higher percentage of discs showed stable transformation when LBA4404 was used. The two original studies on cotton transformation (Firoozabady et al. 1987; Umbeck et al. 1987) were carried out with LBA4404.

The fact that transformation is possible without acetosyringone suggests that wounded cotton tissue does produce certain *vir*-inducing factors needed for *Agrobacterium*-mediated transformation. However, the results shown in Table 2 indicate that acetosyringone improved stable transformation efficiencies 2–4-fold in all four varieties of cotton tested. Acetosyringone not only increased the average number of stable transformation events per explant but also increased the percentage of disks showing stable transformation. Our results with acetosyringone are consistent with the findings of several other studies in a wide range of species, i.e., maize (Shen et al. 1993), *Antirrhinum majus* and soybean (Godwin et al. 1991), and white pine (Levee et al. 1999). Acetosyringone has been found to be beneficial even in the case of tobacco transformation, where efficiency was increased threefold when it was included in co-cultivation medium (Sunilkumar et al. 1999).

Co-cultivation with *Agrobacterium* at lower temperature has been shown to improve *Agrobacterium*-mediated gene transfer to plant cells. Dillen *et al.* (1997) reported that in *Phaseolus acutifolius* and *Nicotiana tabacum*, irrespective of the type of helper plasmid, the transient GUS activity decreased at cocultivation temperatures above 22 °C. The transient activity was lower at 25 °C with a further drop at 27 °C, becoming undetectable at 29 °C. Fullner *et al.* (1996) showed that low temperatures promoted pilus assembly leading to increased number of pili on the cell surface. It has been proposed that the low-temperature increase in transformation efficiency may be due to better functioning of the VirB-VirD4 part of the T-DNA transfer machinery (Fullner and Nester 1996; Fullner et al. 1996). In the published cotton transformation reports, co-cultivation temperatures ranged from 25 °C to 28 °C. In the present investigation, results from three separate experiments with Coker 312 showed consistent improvement in transformation efficiencies when co-cultivation was carried out at 21 °C. Our results on stable transformation of cotton are in line with the previous report of Dillen et al. (1997) which showed improvement in transient transformation efficiencies at low temperature.

As pointed out earlier, one of the biggest limiting steps in obtaining transgenic cotton is regeneration from callus lines. The limitation with regeneration relates to survival of the culture lines and the length of time it takes to obtain embryogenic callus. In a large-scale experiment, we evaluated the relationship between callus survival and timing of embryogenesis in response to two slightly different media regimes. Results shown in Table 4 show that just one extra round of a two-week culture on high-cytokinin/low-auxin medium, after excision, significantly improved survival of calluses. However, this treatment delayed the onset of embryogenesis. It is possible that the higher concentration of cytokinin in the medium is necessary for the survival and proliferation of cells within the individual callus soon after excision. Transfer and culture of callus lines to low-cytokinin/high-auxin medium, immediately after excision, reduced the survival but substantially improved both the timing and the extent of embryogenic response in surviving calluses. A higher auxin-to-cytokinin ratio appears to result in friable callus state (Figure 1M) much earlier. It is this friable callus that eventually turns embryogenic.

Several studies described in this report were made possible because of the use of GFP. Unlike the *uidA*

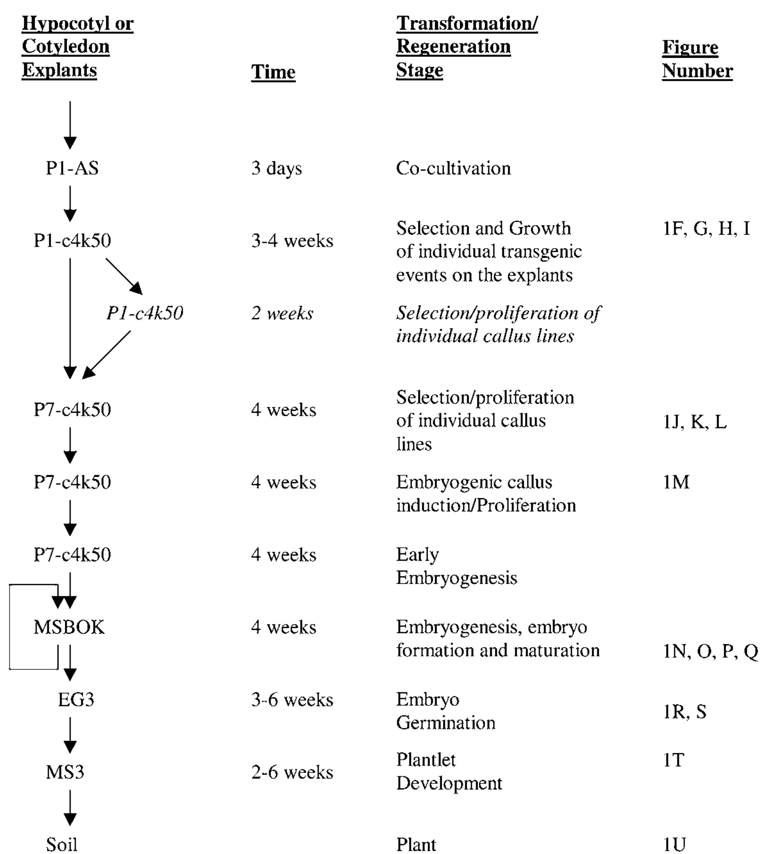

Figure 5. Transformation and regeneration scheme for cotton, *G. hirsutum*.

gene, without the intron, that can sometimes be expressed in the *Agrobacterium*, thus leading to false-positives, GFP did not confer additional fluorescence to the *Agrobacterium*. In fact, the GFP-carrying *Agrobacterium* did not fluoresce differently or any brighter than the *Agrobacterium* carrying other transgenes (results not shown). The growth and proliferation of an individual transgenic event could easily be tracked visually without disturbing the tissue in any way. It was possible to follow an individual transformation event from the transient stage to stable integration, to a proliferating callus line, to embryogenesis, and to plant formation. Early scoring of transgene-expressing lines, identification of chimeric lines, and following their fate would have been more difficult with any other reporter gene. Also, estimation of efficiencies in obtaining transgene-expressing plants would have been difficult without the use of GFP. During the callus proliferation and embryogenesis stages, we found GFP to be neither toxic nor inhibitory (Figures 1MM, NN, RR, SS and SSS). Some GFP-expressing plants had been transferred to soil. These results suggest that GFP can be utilized as a reporter gene in cotton for other studies, such as promoter analyses, to visualize subcellular localization of proteins by using fusion constructs, etc.

On the basis of results presented in this report, and over 50 other transformation experiments carried out in our laboratory, we have established a protocol for cotton (Coker 312) transformation based on easily available MS media formulations. The method is depicted in Figure 5 in a step-by-step manner. Co-cultivation of hypocotyl or cotyledon sections is carried out with pre-induced LBA4404 strain on acetosyringone-containing medium for three days at 21 °C. Selection and culture are carried out as shown in Figure 5. Protocol 1 delays the regeneration process and is no longer used in our laboratory. During the third subculture on P7-c4k50 and first subculture on MSBOK, it is important to select a callus that is friable and pale cream in color that grows at the outer edge, close to the medium. If the callus does not be-

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come embryogenic by the first round on MSBOK, it is repeatedly subcultured on the same medium until embryogenesis occurs. Embryos 7–8 mm long are transferred to EG3 medium for germination. Embryos tended to form better root systems and a fewer embryos formed callus at the base if they were cultured on filter paper on EG3 medium. Germinated embryos are grown in jars to develop good roots and leaves before transfer to soil. Results from molecular analyses suggest that selection on 50 mg/l kanamycin is effective in eliminating escapes. We believe that the protocol described here can be adapted for other regenerable cotton cultivars.

Our GFP-based transient transformation studies suggest that cotton is highly responsive to *Agrobacterium* infection under the conditions used in this investigation. We have shown that stable transformation efficiencies can be increased several fold by modifying co-cultivation conditions. In addition, we have made improvements and refinements in culture and regeneration protocols that will make the production of transgenic cotton plants easier and more efficient. By using the protocol described above, we have established transgenic cotton in soil in less than six months, after *Agrobacterium*-mediated transformation of hypocotyl segments, instead of 10–12 months when using the protocols described in earlier reports. This represents a considerable saving in time and resources in the production of transgenic cotton.

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