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| <b>(54) Title:</b> AGROBACTERIUM-MEDIATED TRANSFORMATION OF COTTON WITH NOVEL EXPLANTS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| <b>(57) Abstract</b><br><br><p>A method is disclosed for producing a transgenic cotton plant comprising the steps of (a) obtaining cottonfibrous root explants, (b) culturing the fibrous root explants to induce callus formation, (c) exposing root callus to a culture of <i>Agrobacterium tumefaciens</i> that harbors a vector comprising an exogenous gene and a selectable marker, the <i>Agrobacterium</i> being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the explant, (d) culturing the callus in the presence of the selection agent to which the selection agent resistance gene confers resistance so as to select for transformed cells, (e) inducing somatic embryo formation in the selected callus culture, and (f) regenerating the induced somatic embryos into whole transgenic cotton plants.</p> |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |

*Agrobacterium*-Mediated Transformation of  
Cotton With Novel Explants

Technical Field

5 The present invention relates to the general field of genetic engineering of plants, in particular to the introduction of exogenous genetic material into cotton by *Agrobacterium* transformation of novel explants followed by somatic embryo regeneration.

Background

10 Cotton is the most extensively used natural fiber in the textile industry. Its annual production worldwide is over 100 million bales, valued at US\$45 billion. Cotton lint or seed hair is a terminally differentiated single epidermal cell from 50 species of the genus *Gossypium* of the family Malvaceae. It is  
15 classified as a natural, cellulosic, monocellular and staple fiber. The cultivated cotton varieties, which have been cultivated for more than 5000 years, all come from two diploids ( $2n=2x=26$ ) (*G. herbaceum* and *G.*  
20 *arboreum*) and two allotetraploids ( $2n=4x=52$ ) (*G. hirsutum* L., Upland; and *G. barbadense* L., Sea Island). In 1997 the top five world cotton producers were the United States, China, India, Pakistan and Uzbekistan, producing about 63 million bales.

In the next century, most crops, including cereals, oil crops, fruits, vegetables and other economically important crops, will be genetically engineered with added or modified traits ranging from improvement of yield and quality, to herbicide resistance and pest resistance (Chappell, 1996; Fraley et al., 1986; Herrera-Estrella et al., 1983; Hoekema et al., 1983; Horsch et al., 1985; Jefferson, 1987; Ryals, 1996). In cotton, the new technology will be used to increase yield, improve fiber quality and create new varieties which are resistant to herbicides, pest insects, nematodes and diseases (John, 1996; John & Keller, 1996; John & Stewart, 1992; Murray et al., 1993; Rajasekaran et al., 1996; Schell, 1997; Stewart, 1992).

1. Tissue Culture of Cotton: In 1935, Skovsted reported the first embryo culture of cotton. Beasley (1971) reported callus formation in cotton as an outgrowth from the micropylar end of fertilized ovules on MS medium. Somatic embryogenesis was achieved from a suspension culture of *G. klotzschianum* (Prive & Smith, 1979). In 1983, Davidonis & Hamilton first succeeded in efficient and repeatable regeneration of cotton (*G. hirsutum* L.) plants from callus after two-year cultivation. Cotton plants were since regenerated through somatic embryogenesis from different explants (Zhang & Feng, 1992; Zhang, 1994) including cotyledon (Davidonis et al., 1987; Davidonis & Hamilton, 1983; Finer, 1988; Firoozabady et al., 1987), hypocotyl (Cousins et al., 1991; Rangan & Zavala, 1984; Rangan & Rajasekaran, 1996; Trolinder & Goodin, 1988; Umbeck et al., 1987, 1989), stem (Altman

et al., 1990; Finer & Smith, 1984), shoot apex (Bajaj et al., 1985; Gould et al., 1991; Turaev & Shamina, 1986), immature embryo (Beasley, 1971; Eid et al., 1973; Stewart & Hsu, 1977, 1978), petiole (Finer & Smith, 1984; Gawel et al., 1986; Gawel & Robacker, 1990), leaf (Finer & Smith, 1984; Gawel & Robacker, 1986), root (Chen & Xia, 1991; Kuo et al., 1989), callus (Finer & McMullen, 1986; Trolinder et al., 1991) and protoplast (Chen et al., 1989).

2. Cotton Transformation: Explants (such as hypocotyl, cotyledon, callus generated from hypocotyl and cotyledon, as well as immature embryos) have been used for *Agrobacterium*-mediated transformation and particle bombardment (de Framond et al., 1983; Finer & McMullen, 1990; Firoozabady et al., 1987; Perlak et al., 1990; Rangan & Rajasekaran, 1996; Rajasekaran et al., 1996; Trolinder et al., 1991; Umbeck et al., 1987, 1989, 1992). In addition, meristematic tissue of excised embryonic axes has also been used for cotton transformation by particle bombardment (Chlan et al., 1995; John, 1996; John & Keller, 1996; McCabe & Martinell, 1993). Zhou et al. (1983) transformed cotton by injecting DNA into the axile placenta one day after self-pollination. However, cotton transformation is highly dependent on genotype (Trolinder, 1985a, 1985b, 1986; Trolinder & Goodin, 1987, 1988a, 1988b). Apart from a few cultivars which are regeneratable and transformable, such as *Gossypium hirsutum* cv. Coker 312 and *G. hirsutum* Jin 7, most other important elite commercial cultivars, such as *G. hirsutum* cv. D&P 5415 and *G. hirsutum* cv. Zhongmian 12, are not regeneratable and transformable by these methods.

Based on previous reports and the inventor's own experimental data, high efficiency of callus induction (60%) can be achieved using the hypocotyl as an explant. However, the transformation rate was only 20% (Firoozabady et al., 1987; Umbeck et al., 1987). Several factors can lead to breakthrough of nontransformed calli, or to chimeric calli consisting of predominantly nontransformed cells: (1) low kanamycin levels (a high level of kanamycin is toxic to cotton explants and calli); (2) experience-dependent selection in later stages of callus proliferation; and (3) use of explants such as the hypocotyl which has only partial contact with the selective media (Firoozabady et al., 1987). When the cotyledon was used as an explant, although the transformation rate was higher than that with the hypocotyl, it was often difficult to eliminate *Agrobacterium* during subsequent culture (Jiao G.-L and Chen, Z.-X., personal communication; Umbeck et al., 1987, 1989). The transformation rate of meristemic tissue through particle bombardment was simply too low (0.02%-0.22%) compared to that of *Agrobacterium* mediated transformation.

There thus remains a need for methods of producing transgenic cotton plants that provide high rates of transformation along with high rates of transformants among regenerated somatic embryos.

Summary of the Invention

The present invention relates to a method for producing transgenic cotton plants, comprising the steps of (a) obtaining cotton fibrous root explants, (b) culturing the fibrous root explants in the presence of hormones to induce callus formation, (c) exposing root callus to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the explant, (d) culturing the callus in the presence of the selection agent to which the selection agent resistance gene confers resistance so as to select for transformed callus, (e) inducing somatic embryo formation in the selected callus culture, and (f) regenerating the induced somatic embryos into whole transgenic cotton plants.

The present method provides for an improved rate of transformation when compared to previous methods that employ hypocotyl and cotyledon tissue. The method is believed to have wide applicability to a variety of cotton varieties.

Brief description of the Figures

Figure 1 shows the plasmid pBK9, containing a luciferase gene used to detect positive transformants obtained by the methods of the present invention.

Figure 2 shows the plasmid pVIP96, the plasmid from which pBK9 was derived by insertion of the luciferase gene.

Detailed Description

In order to overcome the problems seen with prior art methods and increase the efficiency of transformation, fibrous root explants were used for *Agrobacterium*-mediated transformation of cotton. Although in *Arabidopsis* high efficiency of transformation was achieved in *Agrobacterium*-mediated transformation with fibrous root explants (Valvekens, et al., 1988), and the differentiation of young fibrous roots from cotton on MS medium containing 2.0 mg/L IAA, 0.02-0.04 mg/L IBA has been reported (Kuo, C.C., et al., 1989), there is no report in the literature about using fibrous roots as explants for cotton transformation.

Fibrous roots now have been successfully used as explants for *Agrobacterium*-mediated transformation and plant regeneration. In the process modified media for seedling culture, and regeneration and differentiation of embryogenic calli were used.

Media used to culture seedlings to obtain explant material was designed to minimize browning of the roots (browning adversely effects the ability of explants to grow in culture and form callus), and to promote overall vigorous root growth. Thus fibrous root explants were cultured in the presence of hormones to induce callus formation. In a preferred embodiment MET (multi-effect triazole, a chemical agent used in agriculture to promote root growth) and NAA ( $\alpha$  naphthalene acetic acid) are used together in the seedling culture medium to reduce the proportion of browned roots and increase callus initiation rate. MET is preferably used in concentrations ranging from about 0.05 mg/l to about 0.2 mg/l, most preferably about 0.1 mg/l. NAA is preferably used in concentrations ranging

from about 0.01 mg/l to about 0.2 mg/l, most preferably about 0.05 mg/l. MET and NAA are also preferably used in the medium used to root transgenic seedlings regenerated from callus, in amounts similar to those described for the seedling culture medium. In a preferred embodiment of the callus-forming medium vitamin B<sub>5</sub>, 2,4-D ((2,4-dichlorophenoxy)acetic acid, MgCl and glucose are used, preferably about 0.05 mg/l to about 0.15 mg/l 2,4-D, about 0.4 mg/l to about 1.2 mg/l MgCl, and about 1% to about 5% glucose, most preferably about 0.1 mg/L 2,4-D, 0.8 mg/L MgCl and 3% glucose. In an alternate preferred embodiment of the callus-forming medium myo-inositol, vitamin B<sub>1</sub>, and dimethylallyl(amino)purine are used, a, preferably about 50 mg/l to about 150 mg/l myo-inositol, about 1 mg/l to about 10 mg/l vitamin B<sub>1</sub>, and about 0.1 mg/l to about 7.5 mg/l dimethylallyl(amino)purine, most preferably about 100 mg/l myo-inositol, about 0.4 mg/l vitamin B<sub>1</sub> and about 5 mg/l dimethylallyl(amino)purine. The same media used for callus induction can also be used during selection with antibiotics -- for example with 300-400 mg/L cefotaxime or 15-30 mg/L kanamycin. The presence of high concentrations (preferably about 1900 mg/l to about 5700 mg/l, most preferably about 3800 mg/L) of nitrates (preferably NaNO<sub>3</sub>) was crucial for the observed effectiveness of the differentiation medium. With the fibrous roots as explants, although the rate of callus-induction was lower compared with hypocotyl and cotyledon, a higher rate of transformation was achieved.

Techniques for introducing exogenous genes into *Agrobacterium* such that they will be transferred stably

to a plant or plant tissue exposed to the *Agrobacterium* are well-known in the art and do not form part of the present invention. It is advantageous to use a so-called "disarmed" strain of *Agrobacterium* or Ti plasmid, that is, a strain or plasmid wherein the genes responsible for the formation of the tumor characteristic of the crown gall disease caused by wild-type *Agrobacterium* are removed or deactivated.

5 Numerous examples of disarmed *Agrobacterium* strains are found in the literature (e.g., pAL4404, pEHA101 and pEH 105 (Walkerpeach & Veltern, 1994)). It is further advantageous to use a so-called binary vector system, such as that described in U.S. Patent Nos. 4,940,838 and 5,464,763 (Schilperoort, et al.) and Hoekema et al., 1983. A binary vector system allows for manipulation in *E. coli* of the plasmid carrying the exogenous gene to be introduced into the plant, making the process of vector construction much easier to carry out.

20 Similarly, vector construction, including the construction of chimeric genes comprising the exogenous gene that one desires to introduce into the plant, can be carried out using techniques well-known in the art and does not form part of the present invention.

25 Chimeric genes should comprise promoters that have activity in the host in which expression is desired. For example, it is advantageous to have a series of selectable markers for selection of transformed cells at various stages in the transformation process. A 30 selectable marker (for example a gene conferring resistance to an antibiotic such as kanamycin, cefotaxime or streptomycin) linked to a promoter active

in bacteria would permit selection of bacteria containing the marker (i.e., transformants). Another selectable marker linked to a plant-active promoter, such as the CaMV 35S promoter or a T-DNA promoter such as the NPT II NOS promoter, would allow selection of transformed plant cells. The exogenous gene that is desired to be introduced into the plant cell should comprise a plant-active promoter in functional relation to the coding sequence, so that the promoter drives expression of the gene in the transformed plant. Again, plant-active promoters, such as the CaMV 35S, the NPT II NOS promoter or any of a number of tissue-specific promoters, are well-known in the art and selection of an appropriate promoter is well within the ordinary skill in the art.

The present method can be used to produce transgenic plants expressing any number of exogenous genes, and is not limited by the choice of such a gene. The selection of the desired exogenous gene depends on the goal of the researcher, and numerous examples of desirable genes that could be used with the present invention are known in the art (e.g., the family of *Bacillus thuringiensis* toxin genes, herbicide resistance genes such as shikimate synthase genes that confer glyphosate resistance, U.S. Patent No. 5,188,642, or a 2,4-D monooxygenase gene that confers 2,4-D resistance, Bayley et al., Theoretical and Applied Genetics, vol. 82, pp. 645-49, male sterility genes such as the antisense genes of U.S. Patent No. 5,741,684 (Fabijanski, et al.), or even the elaborate crop protection systems described in U.S. Patent No. 5,123,765 (Oliver, et al.)).

*Agrobacterium*-mediated cotton transformation is considered in the art to be heavily variety-dependant. The Coker series of cotton varieties have been shown to be relatively easy to transform. However, DP 5412, 5 Zhongmain 12 and many other varieties still have difficulties associated with transformation. The situation is the same for *G. barbadense* and other diploid species. Particle bombardment, DNA injection and infection of meristem tissue with *Agrobacterium* are 10 some alternative methods, which can be used to transform, in theory, all the cotton varieties. The problems associated with these methods are: low efficiency of transformation and unstable/unreliable results. It is believed that the present method has 15 broad applicability to transformation of cotton varieties, as it overcomes or minimizes several of the problems associated with previous work relating to cotton transformation (such as breakthrough of non-transformed callus, poor explant growth and low 20 transformation rate, poor somatic regeneration) through the use of fibrous root explants.

The following abbreviations are used to designate culture media useful in connection with the present invention:

- 25        LB medium (10g bacto-tryptone + 5g bacto-yeast extract + 10g NaCl);  
         B5 medium (Gamborg et al., 1968; Sigma, Cat. No. G-5768);  
         MS medium (Murashige et al., 1962; Sigma, Cat. No. 30 M-5524);  
         SH medium (Stewart & Hsu, 1977. Planta 137, 113-117);

- CB-1.1 ( $\frac{1}{2}$  MS +  $\frac{1}{2}$  B5 Vitamin + 0.1mg/L NAA);  
CB-1.2 ( $\frac{1}{2}$  B5 medium);  
CB-2.1 (MS macro + B5 micro + 0.05mg/L 2,4-D +  
 0.1mg/L kinetin + 3% glucose + 2g/L gellan gum  
 5 (PhytaGel™, Sigma) + 0.93mg/L  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH5.8);  
CB-2.2 (MS macro + 100mg/L myo-inositol + 0.4mg/L  
 vitamin B1 + 5mg/L 2iP (6 - (γγ-  
 dimethylallyl(amino)purine) + 0.2mg/L NAA + 3%  
 glucose + 2g/L gellan gum (PhytaGel™, Sigma) +  
 10 0.93mg/L  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH5.8);  
CB-3.1 (CB-2.1 + 500 mg/L cefotaxime + 50mg/L  
 kanamycin);  
CB-3.2 (CB-2.2 + 500mg/L cefotaxime + 50mg/L  
 kanamycin);  
 15 CB-4 (Modified CB-3.1 or CB-3.2 by adding double  
 amount of  $\text{KNO}_3$  and removing  $\text{NH}_4\text{NO}_3$  with 250 mg/L  
 cefotaxime and 20mg/L kanamycin);  
CB-5 (SH + 1.5% Sucrose + 2g/L gellan gum  
 (PhytaGel™, Sigma) + 0.93g/L  $\text{MgCl}_2$ , pH7.0).

20       The following Examples are intended to illustrate  
 the present invention, and not in any way to limit its  
 scope, which is solely defined by the claims.

EXAMPLE 1:       Regeneration of Cotton Plants from Root  
 Tissue Culture

25       Preparation of Root Explants: Cotton seeds were  
 sterilized in 70% ethanol for 10-15 min., and then  
 treated with 10%  $\text{H}_2\text{O}_2$  for 30-120 mins. Treated seeds  
 were rinsed in sterile water for 24 hrs at 28°C and  
 germinated on either CB-1.1 medium or CB-1.2 medium at  
 30 28°C - 30°C, 16h light ( $60-90 \mu\text{E m}^{-2} \text{ s}^{-1}$ ). Seven to ten

days sterile seedlings thus grown were used to prepare explants. It was found that plentiful healthy roots (longer and thicker) with white color were obtained using CB-1.1 medium, whereas shorter and thinner roots with grey to brown color were obtained using the CB-1.2 medium. Therefor, CB-1.1 was chosen for further work.

Induction of calli: Fibrous roots were cut from seedlings and cultured on CB-2.1 medium or CB-2.2 medium at 28°C - 30°C for three days, 16h light (60-90  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). The optimum size for root explants was 5-7 mm. A few small calli initiated on the cut sites of root segments in as little as 3 days. In general, transformed hypocotyl or cotyledon explants started to initiate callus on inducing medium after 3 days. However, previous to the present invention, transformed root explants were generally found to initiate callus only after 10 days of cultivation. The color of the root explants was white. One week later, small calli were also initiated from other parts of the root segments. The color of the root explants changed to grey or even brown. At the end of 2 weeks of cultivation, calli initiated from the whole root explants and grew well. Of the two inducing media, CB-2.2 was found to induce good callus formation, while CB-2.1 did not. On CB-2.2 medium, root explants grew well, and the microcallus initiated on the cut sites of the explant. About 10% of root explants initiated callus after only 3 days on the medium. On the other hand, while CB-2.1 medium supported the growth of the root explants well, there was no callus initiation on the cut sites. The efficiency of callus-induction with root explants on the CB-2.2 medium was 10%, which was

lower than that with hypocotyl or cotyledon explants (20-30%). A summary of results showing callus induction and transformation efficiency appears in Table 1, below.

5           Regeneration of root calli: After one month, the calli were transferred to new medium for subculture on either CB-2.1 medium or CB-2.2 medium. After 2 months of subculture, the mature calli were transferred to CB-4 (without antibiotic) for induction of somatic  
10           embryos. Glutamine and L-asparagine were added in amounts of 0.5 mg/L and 0.2 mg/L, respectively, to promote embryogenesis. Primary somatic embryos were formed on the embryogenic calli after 2 months of cultivation, with 2 subcultures in between on the same  
15           media. Primary somatic embryos were subcultured on the same media for another month before mature somatic embryos were formed. Some of the somatic embryos developed to plantlets. These small plantlets were transferred to CB-5 medium for root induction. When  
20           the plantlets had made roots on the CB-5 medium (4-6 weeks), they were transferred to soil and maintained in an incubator under high humidity for 3-4 weeks at 28°C, 16h light (60-90  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), and then transferred to large pots with soil in a green house.

25           EXAMPLE 2:       *Agrobacterium* Transformation and Culture

          The plasmid pBK9 (35S:LUC) (see Fig. 1) was generated by cloning the luc coding sequence from the BamHI/Stul fragment of plasmid pGEMluc into the blunt-ended Stul site of the plasmid pVIP96 (see Fig.  
30           2).

Prepared competent cells (400 microliter) in Eppendorf tube from -80°C were put on ice to thaw. Plasmid DNA was added in the cells. After gentle mixing, the mixture was incubated on ice for 45 minutes. The Eppendorf tube containing the mixture was put into liquid nitrogen for 1 minute and afterwards in a water bath (37°C) for 3 minutes. After the incubation, 800 microliter LB medium (without antibiotics) was added into the mixture and the tube with the mixture was incubated at 28°C for 3 hours. After a brief centrifugation at 12,000 rpm, 800 microliter supernatant was removed. The rest of the medium was mixed well with the cell pellet and the mixture was plated onto LB plates containing 100 mg/L kanamycin and 100 mg/L streptomycin. Successful transformed LBA4404 cells formed colonies on the plates in about 48 hours at 28°C. *Agrobacterium* strain LBA4404 harboring the plasmid pBK9 (35S:LUC) was initiated on LB plate with kanamycin (50 mg/L), streptomycin (50 mg/L) and rifamycin (50 mg/L). A single colony was inoculated into LB liquid medium without antibiotics and grown overnight for about 18 h at 28°C on a gyratory shaker. The optical density ( $A_{600}$ ) value was adjusted to 0.1 - 0.4 in liquid LB medium prior to use.

EXAMPLE 3: Transformation of Root Explant Tissue and Regeneration of Transgenic Cotton Plants

Root explants were obtained by cultivating sterile cotton seeds as described in Example 1, above, on CB-1.1 medium. Fibrous roots were cut from seedlings and

cultured on CB-2.2 for two days, 16h light ( $60-90 \mu\text{E m}^{-2}\text{s}^{-1}$ ). The fibrous roots were then cut into small segments (5-10 mm) and incubated with the cell suspension culture of *Agrobacterium tumefaciens* strain LBA4404 harboring the plasmid pBK9 (35S:LUC) ( $A_{600} = 0.1-0.6$ ) of Example 2 for 15 min. After drainage of the bacterial solution, the root explants were cultured at  $28^{\circ}\text{C}$ , 16h light ( $60-90 \mu\text{E m}^{-2}\text{s}^{-1}$ ) for an additional two days. The optimum concentration of the *Agrobacterium* strain LBA4404 for root explants was lower ( $A_{600}=0.1-0.4$ ) than that for hypocotyl and cotyledon explants ( $A_{600}=0.3-0.6$ ). Optimal bacterial concentrations did not affect the growth of the root explants and the subsequent callus induction.

Co-cultured explants were washed twice with sterile distilled water and transferred to CB-3.1 medium or CB-3.2 medium for cultivation at  $28^{\circ}\text{C}$ , 16h light ( $60-90 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

After four weeks, kanamycin-resistant calli were selected and subcultured on the same media for the second selection. At the same time, some of the calli were selected to detect the LUC expression with the luciferase luminescence image system (see Example 4, below). The process of inducing callus took about 2 months. The efficiency of callus induction from root explant was lower compared with that from hypocotyl and cotyledon explants.

Kanamycin-resistant calli were transferred to CB-4 medium to induce embryogenic calli and somatic embryos. After 4-6 weeks of cultivation, with one subculture, mature somatic embryos appeared on the calli. Plantlets developed afterwards from some of the

embryos. The green plantlets were then transferred to rooting medium (CB-5) for root induction. When plantlets had made roots, they were transferred to soil and maintained in an incubator under high humidity for 3-4 weeks at 28°C, 16h light ( $60-90 \mu\text{E m}^{-2} \text{s}^{-1}$ ), and then transferred to large pots with soil in a green house.

#### EXAMPLE 4: Detection of Luciferase Activity

Plant materials (such as callus, leaf and whole plantlet) were sprayed with a solution containing 0.5 mM potassium luciferin and 0.01% (w/v) polyoxyethylenesorbitan monolaurate (Tween-20) and left for 30 min. The luciferase luminescence from these plant materials was visualized using an image-intensifying camera and photon-counting image processors purchased from Princeton Instruments Inc., 3660 Quakerbridge Road, Trenton, NJ 08619. The exposure time was 6 min. The electronic images were converted to Microsoft Powerpoint TIFF files and printed out from a standard color printer.

Callus growing on selected medium for one month was selected to test LUC expression with the video image system. The positive transformed callus had white spots whereas untransformed callus did not. Out of the 139 pieces of kanamycin resistant calli, 49 pieces were positive with LUC activity. The successful transformation rate was therefor 35%, which was much higher than that seen using cotyledon or hypocotyl as explant (20%).

**Table 1.** Brief summary of root as explant for transformation

| Variety     | Construct | Conc. of Strain | Date of selected medium | No. of explant | No. of callus | No. of LUC test | No. of LUC+ |
|-------------|-----------|-----------------|-------------------------|----------------|---------------|-----------------|-------------|
| 5 Coker 312 | 35S-LUC   | 0.1-0.2         | 9/10/97                 | 2574           | 156           | 16              | 12          |
|             | 35S-LUC   | 0.43            | 23/10/97                | 1255           | 70            | 8               | 5           |
|             | 35S-LUC   | 0.438           | 23/10/97                | 564            | 46            | 28              | 19          |
|             | 35S-LUC   | 0.2-0.4         | 5/3/98                  | 520            | 33            | 15              | 11          |

References

- Altman, D. W. et al. 1990. Economic Botany 40, 106.
- Bajaj, Y. P. S. 1985. Theor. Appl. Genet. 70, 363.
- Beasley, C. A. 1971. In vitro culture of fertilized  
 10 cotton ovules. Biosci. 21, 906-7.
- Chappell, J. 1996. Plant biotechnology comes of age -  
 again. Mol. Breeding 2, 1-6.
- Chen, Z. X., Li, S. J., Yue, J. X., Jiao, G. L. and Liu  
 S. X. 1989. Plantlet regeneration from protoplasts  
 15 isolated from an embryogenic suspension culture of  
 cotton (*Gossypium hirsutum* L.). Acta Botanica  
 Sinica 31, 966-9.
- Chen, Z. X. and Xia Z. A. 1991. Acta Botanica Sinica  
 33, 98.
- 20 Chlan, C. A., Lin, J., Cary, J. W. and Cleveland, T. E.  
 1995. A procedure for biolistic transformation  
 and regeneration of transgenic cotton from  
 meristematic tissue. Plant Mol. Biol. Rep. 13,  
 31-7.
- 25 Cousins, Y.L., Lyon, B. R. and Llewellyn, D. J. 1991.

- Transformation of an Australian cotton cultivar:  
prospects for cotton improvement through genetic  
engineering. Aust. J. Plant Physiol. 18, 481-94.
- Davidonis, G. H., and Hamilton, R. H. 1983. Plant  
5 regeneration from callus tissue of *Gossypium*  
*hirsutum* L. Plant Sci. Lett. 32, 89-93.
- Davidonis, G. H., Mumma, R. O. and Hamilton, R. H.  
1987. Controlled regeneration of cotton plants  
from tissue culture. US Patent No. 4,672,035.
- 10 de Framond et al. 1983. Mini-Ti: a new vector strategy  
for plant genetic engineering. Bio/technology 1,  
262-9.
- Finer, J. J. and Smith, R. H. 1984. Initiation of  
callus and somatic embryos from explants of mature  
15 cotton (*Gossypium klotzschianum* Anderss). Plant  
Cell Reports 3, 41-43.
- Finer, J., 1988. Plant regeneration from somatic  
embryogenic suspension cultures of cotton  
(*Gossypium hirsutum* L.). Plant Cell Rep. 7,  
20 399-402.
- Finer, J. J. and McMullen, M. D. 1990. Transformation  
of cotton (*Gossypium hirsutum* L.) via particle  
bombardment. Plant Cell Reports 8, 586-9.
- 25 Firoozabady, E., Deboer, D., Merlo, D., Halk, E.,  
Amerson, L., Rashka, K. and Murray, E. 1987.  
Transformation of cotton (*Gossypium hirsutum* L.)  
by *Agrobacterium tumefaciens* and regeneration of  
transgenic plants. Plant Mol. Biol. 10, 105-16.
- Fraley, R. T., Rogers, S. G. and Horsch, R. B. 1986.  
30 Genetic transformation in higher plants. CRC Cri.  
Rev. Plant Sci. 4, 1-46.
- Gamborg, O., Miller, R. and Ojima, K. 1968. Exp. Cell

Res. 50, 148.

Gawel, N. J., Rao, A. P. and Robacker, C. 1986.

Somatic embryogenesis from leaf and petiole callus  
cultures of *Gossypium hirsutum* L. Plant Cell

5 Reports 5, 4579.

Gawel, N. J. and Robacker, C. 1986. Plant Cell Rep. 5,  
67.

Gawel, N. J. and Robacker, C. 1990. Genetic control of  
somatic embryogenesis in cotton petiole callus  
10 cultures. Euphytica 49, 249-53.

Gould, J., Banister, S., Hasegawa, O., Fahima, M. and  
Smith, R. H. 1991. Regeneration of *Gossypium*  
*hirsutum* and *G. barbadense* from shoot apex tissues  
for transformation. Plant Cell Reports 10, 12-6.

15 Herrera-Estrella, L., Depicker, A., Van Montagu, M. and  
Schell, J. 1983. Expression of chimeric genes  
transferred into plant cells using a  
Ti-plasmid-derived vector. Nature 303, 209-13.

Hoekema et al. 1983. A binary plant vector strategy  
20 based on separation of Vir- and T-Region of the  
*Agrobacterium tumefaciens* Ti-plasmid. Nature 303,  
179-80.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz,  
D., Rogers, S. G. and Fraley, R. T. 1985. A  
25 simple and general method for transferring genes  
into plants. Sci. 227, 1229-31.

Jefferson, R. A. 1987. Assaying chimeric genes in  
plants: the GUS gene fusion system. Plant Mol.  
Biol. Rep. 5, 387-405.

30 John, M. E. 1996. Structural characterization of genes

- corresponding to cotton fiber mRNA, E6: reduced E6 protein in transgenic plants by antisense gene. Plant Mol. Biol. 30, 297-306.
- 5 John, M. E. and Keller, G. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93, 12768-73.
- John, M. E., and Stewart, J. McD. 1992. Genes for jeans: biotechnological advances in cotton. Trends Biotechnol. 10, 165070.
- 10 Kuo, C. C. et al., 1989. Proc. Beltwide Cotton Prod. Res. Confs, 638.
- McCabe, D. E. and Martinell, B. J. 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. Bio/technol. 11, 596-8.
- 15 Murray, E. E., DeBoer, D. L. and Firoozabady, E. 1993. Transgenic cotton. In "Transgenic Cotton", Vol 2, 153-68.
- 20 Murashige et al., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-97.
- Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, T. A., Sims, S. R., Greenplate, J. T. and Fischhoff, D. A. 1990. Insect resistant cotton plants. Bio/technol. 8, 939-943.
- 25 Price, H. J. and Smith, R. H. 1979. Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anderss. Planta 145, 305-6.
- 30 Rajasekaran, K., Grula, J. W., Hudspeth, R. L., Pofelis, S. and Anderson, D. M. 1996. Herbicide-resistant Acala and Coker cottons

- transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. Mol. Breeding 2, 307-19.
- 5 Rangan, F. J. and Zavala, T. Ip. A. 1984. Somatic embryogenesis in tissue culture of *Gossypium hirsutum* L.). In Vitro 20, 256.
- Rangan, R. and Rajasekaran, K. 1996. Regeneration of cotton plant in cell suspension culture. US Patent No. 5,583,036 (continued from US Patent 10 5,244,802, 1993 and 122,200, 1987).
- Ryals, J. 1996. Agricultural Biotechnology '96. Molecular Breeding 2, 91-93.
- Schell, J. 1997. Cotton carrying the recombinant insect poison Bt toxin: no case to double the benefits of 15 plant biotechnology.
- Skovsted, A. 1935. Cytological studies in cotton. III. A hybrid between *Gossypium davidsonii* Kell, and *G. sturrtii* F. Muell. J. Genet. 30, 397405.
- 20 Stewart, J. McD. 1992. Biotechnology of cotton: achievements and perspectives. ICAC Review Articles on Cotton Production Research No. 3, 1-50.
- 25 Stewart, J. McD and Hsu, C. L. 1978. Hybridization of deploid and tetraploid cottons through in-ovulo embryo culture. J. Heridity 69, 404-8.
- Srewart, J. McD. and Hsu, C. L., 1977. In ovulo embryo culture and seedling development of cotton (*Gossypium hirsutum* L.). Planta 137, 113-7.
- 30 Thomas, J. C., Adams, D. G., Keppene, V. D., Wasmann, C. C., Brown, J. K., Kanost, M. R. and Bohnert, H. J. 1995. Protease inhibitors of *Manduca sexta*

- expressed in transgenic cotton. Plant Cell, Rep. 14, 758-62.
- 5 Trolinder, N. L. 1985a. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). A Dissertation in Biology (Dec., 1985).
- Trolinder, N. L. 1985b. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Reports 8, 133-6.
- 10 Trolinder, N. L. 1986. Somatic embryogenesis and plant regeneration in *Gossypium hirsutum* L. Dissertations Abstracts International 47, 2550B-2551B.
- 15 Trolinder, N. L. and Goodin, J. E. 1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Reports 6, 231-4.
- 20 Trolinder, N. L. and Goodin, J. E. 1988a. Somatic embryogenesis and regeneration in cotton. I. Effects of source of explant and hormone regime. Plant Cell Tissue Organ Culture 12, 31-42.
- 25 Trolinder, N. L. and Goodin, J. E. 1988b. Somatic embryogenesis and regeneration in cotton. II. Requirements for embryo development and plant regeneration. Plant Cell Tissue Organ Culture 12, 43-53.
- Trolinder, N. L., Quisenberry, J., Bayley, C., Ray, C., and Ow, D. 1991. 2,4-D resistant transgenic cotton. Proc. Beltwide Cotton Prod. Res. Conf., 840.
- 30 Turaev, A. M. and Shamina, Z. B. 1986. Soniet Plant Physiol. 33, 439.
- Umbeck, P. F., 1991. Genetic engineering of cotton

- plants and lines. US Patent No. 5,004,863.
- Umbeck, P. F. 1992. Genetic engineering of cotton  
plants and lines. US Patent No. 5,159,135  
(continued from US Patent 5,004,863, 1991).
- 5 Umbeck, P. F., Johnson, G., Barton, K. and Swain, W.  
1987. Genetically transformed cotton (*Gossypium*  
*hirsutum* L.) plants. *Bio/technol.* 5, 263-6.
- Umbeck, P. F., Swain, W. and Yang, N.-S., 1989.  
Inheritance and expression of genes for kanamycin  
10 and chloramphenicol resistance in transgenic  
cotton plants. *Crop. Sci.* 29, 196-201.
- Valvekens, D., Van Montagu, M., Van Lijsebettens, M.  
1988. *Agrobacterium tumefaciens*-mediated  
transformation of *Arabidopsis* root explants by  
15 using kanamycin selection. *Proc. Natl. Acad. Sci.*  
USA 85, 5536-40.
- Walkerpeach, C.R. and Veltern, J. 1994. *Agrobacterium*-  
mediated gene transfer to plant cells: cointegrate  
and binary vector systems. *Plant Mol. Biol.*  
20 *Mannuel B1*, 1-19.
- Zhang, H.-B. 1994. The tissue culture of cotton (II).  
*Plant Physiol. Commun.* 30, 386-91.
- Zhang, H.-B. and Feng, R. 1992. The tissue culture of  
cotton (I). *Plant Physiol. Commun.* 30, 386-91.
- 25 Zhou, G.-Y., Weng, J., Zeng, Y.-S., Huang, J.-G., Qian,  
S.-Y. and Liu, G.-L. 1983. Introduction of  
exogenous DNA into cotton embryos. *Methods in*  
*Enzymology* 101,433-81.

We claim:

1. A method for producing a transgenic cotton plant comprising the steps of:
  - (a) obtaining cotton fibrous root explants,
  - (b) culturing the fibrous root explants in the presence of hormones to induce callus formation,
  - 5 (c) exposing root callus to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the callus,
  - 10 (d) culturing the callus in the presence of the selection agent to which the selection agent resistance gene confers resistance so as to select for transformed callus,
  - 15 (e) inducing embryogenic callus and somatic embryo formation in the selected callus culture, and
  - 20 (f) regenerating the induced somatic embryos into whole transgenic cotton plants.
2. The method of claim 1 wherein the cotton fibrous root explants are obtained by growing cotton seedlings in the presence of multi-effect triazole.
- 25 3. The method of claim 2 wherein the multi-effect triazole is in a concentration of about 0.05 mg/l to about 0.2 mg/l.

4. The method of claim 3 wherein the multi-effect triazole is in a concentration of about 0.1 mg/l.
5. The method of claim 1 wherein the cotton seedlings are grown in the presence of  $\alpha$  naphthalene acetic acid.  
5
6. The method of claim 5 wherein the  $\alpha$  naphthalene acetic acid is in a concentration of about 0.01 mg/l to about 0.2 mg/l.
7. The method of claim 6 wherein the  $\alpha$  naphthalene acetic acid is in a concentration of about 0.05 mg/l.  
10
8. The method of claim 1 wherein the step of regenerating the somatic embryos is carried out in the presence of multi-effect triazole.
9. The method of claim 8 wherein the multi-effect triazole is in a concentration of about 0.05 mg/l to about 0.2 mg/l.  
15
10. The method of claim 9 wherein the multi-effect triazole is in a concentration of about 0.1 mg/l.
11. The method of claim 8 wherein the step of regenerating the somatic embryos is carried out in the additional presence of  $\alpha$  naphthalene acetic acid.  
20

12. The method of claim 11 wherein the  $\alpha$  naphthalene acetic acid is in a concentration of about 0.01 mg/l to about 0.2 mg/l.
13. The method of claim 12 wherein the  $\alpha$  naphthalene acetic acid is in a concentration of about 0.05 mg/l.
14. The method of claim 1 wherein the step of inducing callus formation is carried out in a callus inducing culture medium comprising myo-inositol, vitamin B<sub>1</sub> and a dimethylallyl(amino)purine.
15. The method of claim 1 wherein the step of inducing somatic embryo formation is carried out in a somatic embryo inducing culture medium comprising myo-inositol, vitamin B<sub>1</sub>, and a dimethylallyl(amino)purine.
16. The method of claim 14 wherein the callus inducing culture medium comprises myo-inositol in an amount from 50 mg/L to 150 mg/L, vitamin B<sub>1</sub> in an amount from 0.2 to 10 mg/L and a dimethylallyl(amino)purine in an amount from 0.1 to 7.5 mg/L.
17. The method of claim 16 wherein the callus inducing culture medium comprises 100 mg/L myo-inositol, 0.4 mg/L vitamin B<sub>1</sub> and 5 mg/L dimethylallyl(amino)purine.

18. The method of claim 15 wherein the somatic embryo inducing culture medium comprises myo-inositol in an amount from 50 to 100 mg/L, vitamin B<sub>1</sub> in an amount from 0.2 to 10 mg/L and  
5 dimethylallyl(amino)purine in an amount from 0.01 to 0.5 mg/L.
19. The method of claim 18 wherein the somatic embryo inducing medium comprises 100 mg/L myo-inositol, 0.4 mg/L vitamin B<sub>1</sub> and 5 mg/L dimethylallyl(amino)purine.
20. The method of claim 1 wherein the step of inducing callus formation is carried out in a callus inducing culture medium comprising vitamin B<sub>5</sub>, (2,4-dichlorophenoxy)acetic acid, MgCl and glucose.
21. The method of claim 1 wherein the step of inducing somatic embryo formation is carried out in a somatic embryo inducing culture medium comprising vitamin B<sub>5</sub>, (2,4-dichlorophenoxy)acetic acid, MgCl  
5 and glucose.
22. The method of claim 20 wherein the callus inducing culture medium comprises vitamin B<sub>5</sub> in an amount from 0.2 mg/L to 10 mg/L, (2,4-dichlorophenoxy)acetic acid in an amount from 0.05  
5 mg/L to 0.15 mg/L, MgCl in an amount from 0.4 mg/L to 1.2 mg/L and glucose in an amount from 1% to 5%.

23. The method of claim 22 wherein the callus inducing culture medium comprises 0.4 mg/L vitamin B<sub>5</sub>, 0.1 mg/L (2,4-dichlorophenoxy)acetic acid, 0.8 mg/L MgCl and 3% glucose.
24. The method of claim 21 wherein the somatic embryo inducing culture medium comprises vitamin B<sub>5</sub> in an amount from 0.2 mg/L to 10 mg/L, (2,4-dichlorophenoxy)acetic acid in an amount from 0.05  
5 mg/L to 0.15 mg/L, MgCl in an amount from 0.4 mg/L to 1.2 mg/L and glucose in an amount from 1% to 5%.
25. The method of claim 24 wherein the somatic embryo-inducing medium comprises 0.4 mg/L vitamin B<sub>5</sub>, 0.1 mg/L (2,4-dichlorophenoxy)acetic acid, 0.8 mg/L MgCl and 3% glucose.
26. A method according to any of claims 14-25, wherein the medium further comprises gellan gum.
27. A method according to claim 26 wherein the gellan gum is present in an amount from 1.0 g/L to 3.0 g/L.
28. The method of claim 1 wherein the step of inducing somatic embryo culture is carried out in a somatic embryo-inducing medium comprising a nitrate in an amount from 1900 mg/L to 5700 mg/L.
29. The method of claim 28 wherein the somatic embryo-inducing medium comprises 3800 mg/L nitrate.

30. A method according to either claim 28 or 29,  
wherein the nitrate is  $\text{NaNO}_3$ .