Development of an Efficient Regeneration and Transformation System for Commercially Grown South African Cotton Cultivars

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ABSTRACT

While establishing a system for genetic transformation of South African cotton cultivars, a method of regeneration and transformation of the shoot apical meristem of seedlings was developed. Shoot apices from young cotton seedlings (Gossypium hirsutum cvs Sabie, Jassid, Palala and 107/1) were placed onto Murashige and Skoog (MS) basal medium (pH 5.8) containing MS vitamins, 2% sucrose, 0.7% agar and 0.1 mg/L kinetin. Within two weeks of culture, the shoot meristems differentiated into multiple green shoots and spontaneous root regeneration occurred two months later. Plantlets were placed in soil and hardened off in the growth chamber. Four months later the plants had grown to maturity and cotton bolls had formed. This regeneration protocol demonstrated a successful technique for the recovery of cotton plants from tissue culture using the shoot apical meristem. Shoot apical meristems of seedlings were used for transformation and were co-cultivated with Agrobacterium tumefaciens containing the phosphinothricin acetyltransferase (pat) and the neomycin phosphotransferase (npt II) genes. After co-cultivation, selection was carried out on regeneration medium containing 100 mg/L kanamycin and 1 mg/L glufosinate ammonium (PPT). Control explants were completely inhibited from growing on the selection medium, whereas transformed explants gave rise to multiple shoots resistant to PPT and kanamycin after four weeks. The integration of the pat gene in the transformed plant DNA was confirmed by polymerase chain reaction.

Introduction

Cotton (*Gossypium hirsutum* L.) is an excellent natural source of textile fiber and is cultivated in many countries (Agrawal *et al.*, 1997). Cotton production is of high economic importance and considerable attention has been paid to improving cotton plants through genetic engineering. Regeneration of cotton plants has been notoriously difficult and genotype has been shown to play an important role in successful regeneration (Trolinder and Chen, 1989). Somatic embryogenesis and subsequent plant regeneration has been obtained in cotton (Davidonis and Hamilton, 1983; Rangan *et al.*, 1984; Gawel *et al.*, 1986; Umbeck *et al.*, 1987; Trolinder and Goodin, 1988). These protocols presently remain restricted to a few cultivars (Trolinder and Chen, 1989; Firoozabady and DeBoer, 1993). Aside from genotype limitations, the major impediments to somatic embryogenesis have been the low rate of regeneration from the explants, phenotypic abnormalities and cytogenetic changes (Stelly *et al.*, 1989; Firoozabady and DeBoer, 1993). To circumvent the problems of somatic embryogenesis, cotton plants have been regenerated from shoot meristems (Gould *et al.*, 1991; McCabe and Martinell, 1993; Gupta *et al.*, 1997; Saeed *et al.*, 1997; Hemphill *et al.*, 1998). *Agrobacterium*-mediated transfer systems (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987; Perlak *et al.*, 1990) and particle bombardment procedures (Finer and McMullen, 1990; McCabe and Martinell, 1993) have been used to deliver transgenes into cotton. However, the reports on regeneration and transformation of cotton pertain to either wild or Coker varieties of *G. hirsutum* which are not highly cultivated. Therefore, in view of the economic importance of cotton in South Africa and the potential to improve commercially grown cultivars by genetic transformation, an efficient regeneration and transformation protocol utilizing the shoot apical meristem was developed.

Materials and Methods

Seed material

Seeds of South African cotton cultivars Sabie, Jassid, Palala and 107/1 were obtained from the Tobacco and Cotton Research Institute, Rustenburg, South Africa. The seeds were washed briefly in soapy water and surface sterilised with 2.6% sodium hypochlorite solution containing 3 drops of Triton X-100 per 200 ml for 20 minutes followed by 70% ethanol for 2 minutes. The seeds were then rinsed 4 times in sterile distilled water and placed onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing MS vitamins, 3% sucrose and 0.8% purified agar (pH 5.8) for germination. The seeds were germinated at 25$_o$C with a 16h photoperiod. After five days, shoot apical meristems measuring 0.5-1 mm in length were aseptically isolated from the seedlings and used for regeneration and transformation trials.

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Plant regeneration

Shoot apical meristems were placed onto MS medium containing MS vitamins, 2% sucrose, 0.7% purified agar and 0.1 mg/L kinetin (pH 5.8) at a density of 5 explants/plate. All explants were maintained at 25$_o$C with a 16h photoperiod and subcultured to fresh medium biweekly. For rooting of elongated shoots, regenerated shoots were transferred to media containing the following combinations: MS + 0.1 mg/L kinetin, ½ MS with or without 0.05 mg/L α-naphthaleneacetic acid (NAA). Rooted shoots were transferred to sterile soil and hardened off in the growth chamber (16h photoperiod; 25$_o$C day temperature and 20$_o$C night temperature).

Transformation of shoot meristems by Agrobacterium tumefaciens

The *Agrobacterium* strain LBA4404, containing plasmid pBI101 with the phosphinothricin acetyltransferase (*pat*) and the neomycin phosphotransferase (*npt II*) genes, was used for transformation. Shoot apical meristems were injected with 10 µL of an overnight culture of *Agrobacterium* and then placed onto MS medium for two days co-cultivation with the *Agrobacterium*. After co-cultivation, the meristems were placed onto regeneration medium containing 50 µg/mL cefotaxime and 250 µg/mL carbenicillin for 1 week and then transferred to regeneration medium containing 1 mg/L glufosinate ammonium (PPT) and 100 mg/L kanamycin for selection of transformed shoots.

Plant DNA extraction and polymerase chain reaction (PCR) analysis

Total genomic DNA was isolated from control and transformed shoots according to Doyle and Doyle (1987) and tested for the presence of the *pat* gene by PCR. Two primers were used to amplify 558 bp of the *pat* gene,: left primer 5'GTCTCCGGAGA GGAGACCAG3' (synthesized by University of Cape Town, South Africa) and right primer 5'CCTAACTG GCCTTGGAGGAG3' (received from Johan Burger, CSIR, South Africa). PCR products were separated on a 0.8% agarose gel and visualized with ethidium bromide.

Results and Discussion

Plant regeneration

After two weeks on MS medium containing 0.1 mg/L kinetin, 90-100% of the shoot meristems differentiated into multiple green shoots which elongated to 2-3 cm a week later. Multiple shoot formation and rooting was observed when elongated shoots were transferred to MS medium containing 0.1 mg/L kinetin and maintained on this medium for 4 weeks. No multiple shoot formation and rooting was observed when the shoots were placed onto ½ MS with or without NAA. All regenerated shoots that were advanced to soil were phenotypically normal and all of the matured plants regenerated to date have initiated flowers and set viable R1 seeds under growth chamber conditions. These results showed that shoot meristems from all four South African cotton cultivars tested were responsive to tissue culture and that regeneration from the shoot meristem was simple and direct. Theoretically, the tissues of the apical meristem are best suited for regeneration because these tissues are programmed for shoot organogenesis and do not need to differentiate to a meristematic state. In practice, this method has few genotype limitations, and the incidence of somaclonal variation in regenerated plants is low (Murashige, 1974).

Transformation of shoot meristems by Agrobacterium tumefaciens

During the initial seven days on non-selective medium, all co-cultivated and control explant material retained a healthy green colour. After transfer to selective medium, uninoculated control explants turned brown and died within one week. In contrast, transformed shoot meristems placed onto selection medium produced healthy green shoots within 2 weeks. The presumptive transgenic plants were tested for the presence of the *pat* gene by PCR, and the presence of a 558 bp fragment in the transformed shoots indicated successful transformation of meristematic tissues. We are presently doing Southern blotting to determine copy number of the *pat* gene in the transformed shoots.

The results obtained in the present study are of enormous significance, since data on regeneration and transformation of South African cotton cultivars are not available. The development of a rapid, reliable and efficient genetic transformation and regeneration system will allow for the transfer of useful genes to South African cotton cultivars for crop improvement.

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References

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

Davidonis, G.H. and R.H. Hamilton. (1983): Plant regeneration from callus tissues of *Gossypium hirsutum* L. Plant Science Letters 32:89-93.

Doyle, J.J. and J.L. Doyle. (1987): A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11-15.

Finer, J.J. and M.D. McMullen. (1990): Transformation of cotton (*Gossypium hirsutum*) via particle bombardment. Plant Cell Reports 8:586-589.

Firoozabady, E. and D.L. DeBoer. (1993): Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev. Biol. 29P:166-173.

Firoozabady, E., D.L. DeBoer, D. Merlo, E. Halk, L. Amerson and K. Rashka. (1987): Transformation of cotton (*Gossypium hirsutum* (L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Molecular Biology 10:105-116.

Gawel, N.J., A.P. Rao and C.D. Robacker. (1986): Somatic embryogenesis from leaf and petiole callus cultures of *Gossypium hirsutum* L. Plant Cell Reports 5:457-459.

Gould, J., S. Banister, O. Hasegawa, M. Fahima and R. H. Smith. (1991): Regeneration of *Gossypium hirsutum* and *G. barbadense* from shoot apex tissues for transformation. Plant Cell Reports 10:12-16.

Gupta, S.K., A.K. Srivastava, P.K. Singh and R. Tuli. (1997): *In vitro* proliferation of shoots and regeneration of cotton. Plant Cell, Tissue and Organ Culture 51:149-152.

Hemphill, J.K., C.G. A. Maier and K.D. Chapman.

(1998): Rapid *in-vitro* plant regeneration of cotton (*Gossypium hirsutum* L.). Plant Cell Reports 17:273-278.

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

Murashige, T. (1974): Plant propagation through tissue cultures. Annual Review of Plant Physiology 25:135-166.

Murashige, T. and F. Skoog. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Perlak, F.J., R.W. Deaton, T.A. Armstromg, R.L. Fuchs, S.R. Sims, J.T. Greenplate and D.A. Fischhoff. (1990): Insect resistant cotton plants. Bio/Technology 8:939-943.

Rangan, T.S., T. Zvala and A. Ip. (1984): Somatic embryogenesis in *Gossypium hirsutum* L. In Vitro 20:256.

Saeed, N.A., Y. Zafar and K.A. Malik. (1997): A simple procedure of *Gossypium* meristem shoot tip culture. Plant Cell, Tissue and Organ Culture 51:201-207.

Stelly, D.M., D.W. Altman, R. J. Kohel, T. S. Rangan and E. Commiskey. (1989): Cytogenetic abnormalities of cotton somaclones from callus cultures. Genome 32:762-770.

Trolinder, N.L. and J.R. Goodin. (1988): Somatic embryogenesis in cotton (*Gossypium*) II. Requirements for embryo development and plant regeneration. Plant Cell, Tissue and Organ Culture 12:43-53.

Trolinder, N.L. and X.X. Chen. (1989): Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Reports 8:133-136.

Umbeck, P., G. Johnson, K. Barton and W. Swain. (1987): Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/Technology 5:263-266.