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DEVELOPMENT OF PLANT REGENERATION AND TRANSFORMATION PROTOCOLS FOR ELITE GEORGIA COTTON LINES

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Introduction

In cotton, the ability to produce embryogenic cells is genotype dependent with only a few genotypes known to be capable of regenerating plants from cell culture. Because of this limitation, most transgenic cultivars are produced by inserting the transgene into the highly embryogenic but obsolete cultivar and then introduced into the desired cultivars through backcrossing (Wilkins et al. 2000). Efficient cotton regeneration/transformation, particularly of commercially important cultivars, remains a major obstacle to cotton cultivar improvement by genetic transformation. The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been proven to be a convenient and powerful vital marker in transgenic plants studies. GFP visual selection system seems to hold the most promise for commercially important genotypes (cultivars and breeding materials), which tissue culture/transformation system that are inefficient or no system exist.
The purpose of the present work was to investigate the regeneration ability in elite Georgia cottons and the suitability of GFP as a visual selecting system in particle bombardment transformation of these cotton lines.

Material and methods

The eight elite Georgia germplasm lines tested were those developed by S. Baker, retired Univ. of Georgia cotton breeder, and lines bred by O.L. May (GA 161, GA 94894, GA 96199, GA 96211, GA 9654, GA 98015, GA 98033, and GA 98084). The seven Pee Dee lines (PD 97006, PD 97019, PD 97021, PD 97047, PD 97072, PD 97100, and PD 97101) from the USDA/ARS, Florence, SC, were bred by O.L. May in the late 1990s. Seeds of Coker 312, the standard cultivar for somatic embryogenesis, were obtained from Dr. K. Rajasekaran, USDA/ARS, New Orleans, LA, and included in the study as a positive control.

Seeds were surface sterilized and germinated on MS0 solid medium. Hypocotyl explants 5~7 mm in length were excised from 7 to 10 day-old seedling and grow in a callus induction medium (CIM). Four weeks later, friable callus was transferred into 125 ml jars containing embryo induction medium (liquid) (EIML), and shaken at 130 rpm under a 16/8 h light/day cycle at 28C for a period of 4 to 6 weeks. After 4 to 6 weeks, cell suspension cultures containing white embryogenic cells were placed on embryo development medium (EDM). Mature embryos and embryogenic callus formed in EDM after about one month culture. Vigorously growing, friable, loose and light yellow embryogenic calluses in EDM were transferred to CIM medium and pre-culture for two days before bombardment transformation.

Plasmid construct p524EGFP.1 expressing visual selection marker gene EGFP from a double 35S cauliflower mosaic virus (35–35S CaMV) promoter with an alfalfa mosaic virus (AMV) enhancer sequence was kindly provided by Dr J. W. Grosser, University of Florida (Fleming et al., 2000). Plasmid DNA was coated onto 1.0-μm gold particles (Bio-Rad) using the procedure of Sanford et al. (1990). Ten microliters of the suspension was loaded onto a macrocarrier for bombardment. Calluses were bombarded with the PDS-1000He Particle Delivery System (Bio-Rad) using 1,100/1350 psi rupture disk, 28 in. of Hg vacuum, a gap distance of 0.32 cm and a target distance of 6 cm. Each target callus plate was bombarded two times. The calluses were selected under the GFP light on the basis of fluorescence and transferred to fresh CIM medium every five days following bombardment, until homogenously fluorescing calluses were obtained. Plantlets were rooted, acclimatized and transferred to green house. Transgenic confirmation was based on visual GFP expression/selection under GFP light and PCR based molecular biological confirmation. PCR primers 5′-AAG GGC GAG GAG CTG TTC AC-3′ and 5′-TTC TGC TGG TAG TGG TCG GC-3′were designed according to GFP coding sequence, a 548-bp fragment from the open reading frame was expected to be amplified.

Results and discussion

All genotypes tested produced callus on CIM medium within 2 to 4 weeks. The hypocotyl explants formed callus more readily than cotyledons. Not all seeds in an embryogenic line produce somatic embryos; specific individuals within a cultivar may be more embryogenic than others. In this experiment, Coker 312 showed a high frequency of embryogenesis and among the fifteen Georgia and Pee Dee lines, four of the genotypes PD 97019, PD 97021, PD 97100, and GA 98033 were found to be embryogenic (Sakhanokno et al. 2004). Seed-to-seed variability in embryogenic capability was observed and these could have originated during the cultivar development process where different F4 or F5 plants were bulked. The embryogenic cell lines from GA98033 were subcultured/selected for 6 months, and highly embryogenic cell lines have been selected. Regenerated plants were grown in green house to produce seeds for next generation.

Visual selection was performed 2 days after bombardment. Small pieces of callus with green fluorescence dots were selected and transferred to fresh CIM medium for callus proliferation. Early selection helps transformed cells to proliferate without disturbance by non-transformed surrounding cells. Second selection begins 5 days after first selection when there has been a considerable increase in the mass of the transformed callus. At this stage, it was relatively easy to excise green-fluorescing cells from the non-transformed calluses mass. Repeated selection to remove the green-fluorescing cells from the non-fluorescing ones was carried out at five-days intervals. Each round of selection produced a larger, more homogeneous mass of rapidly growing, fluorescing cells. Calli exhibiting homogeneous green fluorescence were obtained after approximately two months of repeated selection. The homogeneous fluorescing calli were transferred to embryo development medium (EDM) for somatic embryo formation.

Fluorescent somatic embryos were regenerated from the selected fluorescent calluses in EDM after 30 days. Different development stages of embryos emitted different GFP intensity. GFP transgenic cells exhibit green color at early developmental stage. During the callus stage there is little difference in fluorescence in selected putatively transformed callus. When developed to globe-stage, the embryos have more fluorescence than calli nearby, while those from non-transformed calluses exhibit red color. The transformed calli express strong fluorescence even though the fluorescence of the germinated somatic embryos was less. As the regenerated plantlets grew over the next 2 months, they were potted to soil in the greenhouse. PCR amplification of selected GFP positive plants confirmed integration of the *gfp* gene in those plants that were regenerated from calluses transformed with the p254EGFP construct and selected by GFP fluorescence. All plant lines that showed green fluorescence were positive for GFP but those of the non-transformed lines were negative. A mean of 3.3/plate transgenic cell lines was recovered by using present method.

References

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