PROGRESS TOWARD THE DEVELOPMENT OF TRANSGENIC COTTON CULTIVARS ADAPTED TO GEORGIA ENVIRONMENTS

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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 a 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 marker in transgenic plants studies. The GFP visual selection system seems to hold the most promise for crops in which tissue culture/transformation system are inefficient or do not exist (Stewart, 2001), such as the case in cotton. The purpose of the present work was to investigate regenerability in elite Georgia cottons and the suitability of GFP as a visual selection system in particle bombardment transformation of these cotton lines.

Material and Methods

Fifteen cultivars/germplasm lines were tested; eight elite Georgia germplasm cultivar/lines (GA 161, GA 94894, GA 96199, GA 96211, GA 9654, GA 98015, GA 98033, and GA 98084, developed by Shelby Baker or Lloyd May with the University of Georgia Cotton Breeding Program) and seven Pee Dee lines (PD 97006, PD 97019, PD 97021, PD 97047, PD 97072, PD 97100, and PD 97101 developed by Lloyd May while with the USDA/ARS in Florence, SC). Coker 312, the standard cultivar for somatic embryogenesis (seeds were obtained from Dr. K. Rajasekaran, USDA/ARS, New Orleans, LA) was included 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 grown in a callus induction medium (CIM). Four weeks later, friable calli were transferred into 125 ml jars containing embryo induction medium (liquid), 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) (Sakhanokho et al., 2004). Mature embryos and embryogenic calli formed in EDM after about one month culture. Vigorously growing, friable, loose and light yellow embryogenic calli in EDM were transferred to medium and pre-cultured for two days before transformation by bombardment.

Plasmid construct p524EGFP.1 (Fleming et al., 2000), 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 provided by Dr J.W. Grosser, University of Florida. 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. Calli 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 calli were selected under the fluorescent microscope on the basis of fluorescence and transferred to fresh CIM medium every five days following bombardment until homogenously fluorescing calluses were obtained, GFP positive calli was transferred to EDM to induce somatic embryos. Plantlets were rooted, acclimatized and transferred to greenhouse. Transgenic confirmation was based on visual GFP expression/selection under ultraviolet 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 from the GFP coding sequence with a 548-bp fragment expected to be amplified from the open reading frame.

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 (Figure 1). 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 were been selected (Figure 2). Regenerated plants were grown in a greenhouse to produce seeds for next generation.

Highly embryogenic cell lines from the above study were used as explants for GFP gene transfer via bombardment. GFP 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. The second selection began 5 days after first selection to allow a desired, substantial 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-day 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 EDM for somatic embryo formation.

Fluorescent somatic embryos were regenerated from the selected fluorescent calli in EDM after 30 days. Different development stages of embryos emitted different GFP intensity. During the early stage, GFP transgenic cells exhibit green color. During the callus stage there is little difference in fluorescence in selected putatively transformed callus. When embryogenic cell developed to globe-stage, the embryos show a stronger fluorescence than nearby calli, while those from non-transformed calluses exhibit a red color. The transformed calli expressed a stronger fluorescence than the germinated somatic embryos did. As the regenerated plantlets grew over the next 2 months, they were potted in soil in the greenhouse. PCR amplification of selected GFP positive plants confirmed the presence 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 (Table 1).

Future work will focus on continuing to increase the regeneration efficiency of GA98033 and transferring agronomically/economically important genes to GA98033.

Acknowledgements

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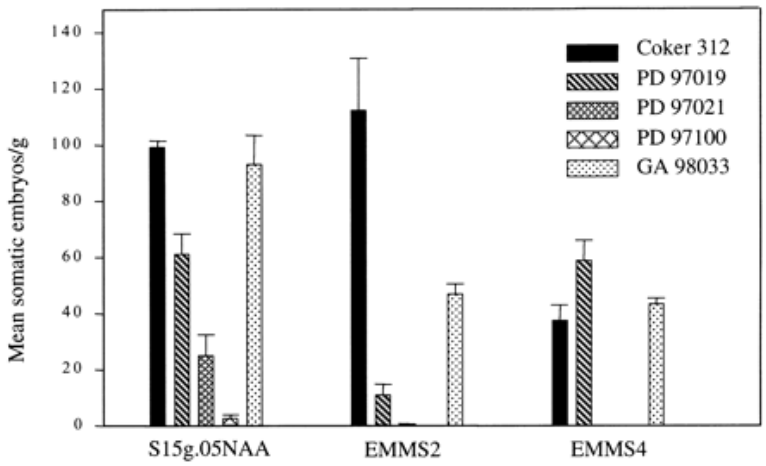

Figure1. Number of somatic embryos induced from calli of five cotton genotypes .

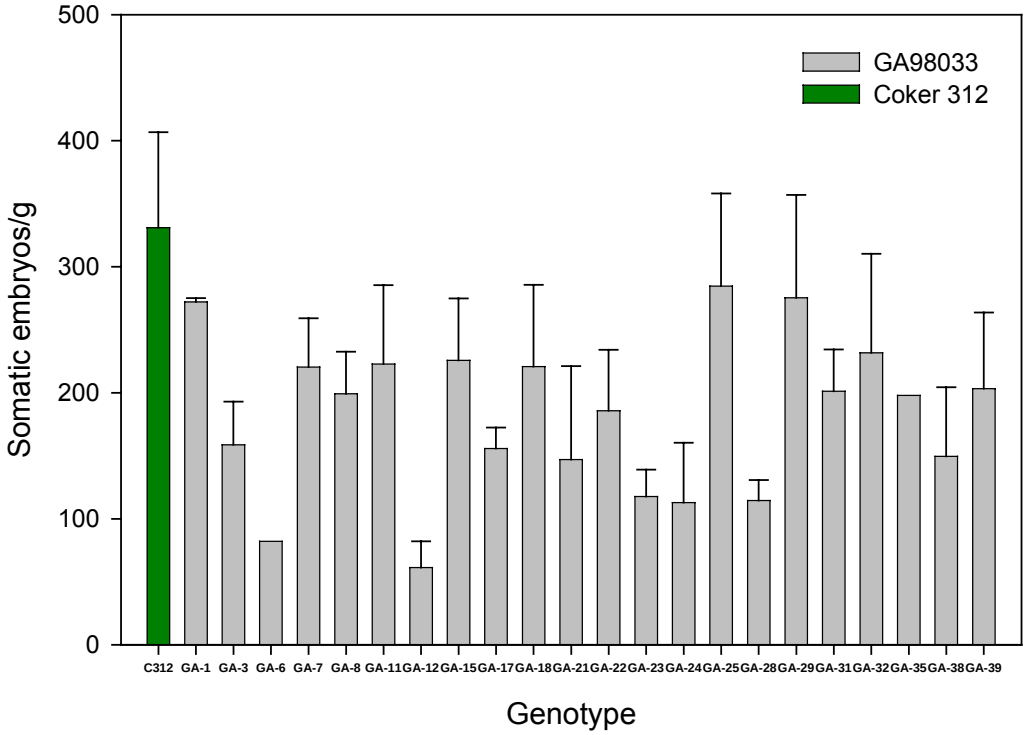

Figure 2. Improvement of somatic embryogenesis in GA98033

Table 1. Transformed cotton lines obtained from GFP visual selection

Experiment No.	No. of plates bombarded	GFP transient expression (2 days)	GFP stable expression (7 days)	Transformed lines obtained
1	3	433.3±30.55	136±14.11	2.67±0.58
2	3	502.3±26.76	165.7±66.71	4±0
3	3	490±26.46	137±11.14	3.33±0.58