GREEN FLUORESCENT PROTEIN AS A VISUAL SELECTION MARKER FOR COTTON TRANSFORMATION

KeDong Da[1], Peng W. Chee[1], and Peggy Ozias-Akins[2]
[1]Crop and Soil Sciences, University of Georgia, Tifton, GA
[2]Horticulture, University of Georgia, Tifton, GA

Introduction

Transgenic cotton plants have been produced by vacuum infiltration, particle bombardment, and *Agrobacterium*-mediated transformation. Most transgenic plants have co-introduced antibiotic or herbicide resistant genes which serve no useful purpose after the transgenic plants are produced. The presence of antibiotic and herbicide resistance marker genes in transgenic plants has raised public concern about ecological and food safety perspective of these genes (Puchta 2003). Generating a transformation system without antibiotic resistant and herbicide resistant genes would certainly contribute to public acceptance of transgenic cotton. The green fluorescent protein (GFP) gene has become a very effective marker gene for use in plant genetic transformation. Recent reports on GFP showed that apart from being used as a visual reporter gene instead of β-Glucuronidase (GUS) (Jordan 2000), it could also be used as a visual selection marker gene instead of chemical based antibiotic selection marker genes. Because of it, for the first time in plant transformation, researchers have at their disposal a universal, in vivo, and real-time transgenic visible marker (Stewart 2001). The GFP visual selection efficiency is much higher than antibiotic selection and can greatly reduce the time involved in transgenic plant production. GFP visual selection made transformation possible without antibiotic resistant marker genes without reducing transformation efficiency since there is no harmful effect on plants with the GFP gene. Establishment of GFP gene visual selection transformation system in cotton will provide a new transformation protocol in cotton.

Here we report a time efficient bombardment transformation of cotton using GFP visual selection that is free of selection via antibiotics and the GFP gene expression in different parts of cotton transgenic plant.

Materials and Methods

The seeds of cotton cultivar Coker 312 were kindly provided by Dr. K. Rajasekaran, USDA/ARS, New Orleans, LA. Plant embryogenic callus induction follows the method of Sakhanokho et al. (2004). 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). Plasmids were coated onto 1.0-µm gold particles (Bio-Rad) using a modified procedure of Sanford et al. (1991). 10 mg gold particles were first washed with 500 µl sterile distilled water and then resuspend in 200 µl sterile distilled water, a 5–µl aliquot of DNA (at 1 µg/µl) was added to particle suspension, a 35 µl aliquot of a solution consisting of a 5:2 ratio of 2.5M $CaCl_2$ and 0.1M spermidine was added and the tube was immediately finger-vortexed. The tube was incubated in room temperature for 20 min followed by a brief

centrifugation, the supernatant was then discarded, and the DNA coated particles were washed with 500 µl 100% ethanol, finger-vortexed, centrifuged, and resuspended in 200 µl 100% ethanol after removal of the supernatant. 10 µl of the suspension were loaded onto a macrocarrier for bombardment. Embryogenic calluses were bombarded with PDS-1000He Particle Delivery System (Bio-Rad) using 1,100 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. Three days following bombardment, the calluses were selected on the basis of florescence (Fig. 1) and transferred to fresh CIM medium. GFP gene expression (visual selection) was detected in callus/somatic embryos illuminated blue light using a Zeiss SV11 stereo microscope equipped for epi-fluorescence with a GFP filter system that allowed an excitation wavelength of 480 ± 30nm and viewing with a barrier filter cutoff wavelength of 515 nm. Images were recorded with a Zeiss AxioCam digital camera coupled to the microscope. The software used to capture and compile the image was Zeiss AxioVision 3.0.6 software and Paintshop 7.0. Fluorescing calluses were separated from non-fluorescing calluses five days later and transfer to EDM medium, selection cycle is five days until homogenously fluorescing calluses/embryos were obtained. The selection experiment was conducted three times; ten dishes were bombarded for each experiment. The results were summarized as the mean number of plants recovered per plate. Plantlet rooting and acclimatization follow the method of Sakhanokho et al. (2004).

Ten separately selected GFP positive plants were subjected to PCR analysis. Genomic DNA was isolated according to the method of Csaikl (1998). PCR primers was designed according to GFP gene sequence: 5′-AAG GGC GAG GAG CTG TTC AC-3′ and 5′-TTC TGC TGG TAG TGG TCG GC-3′. The PCR products were separated on a 0.8% agarose gel with the image recorded.

Transgenic plants grown in green house were subjected to GFP gene expression evaluation with nontransgenic Coker 312 plants as negative control. Different parts of the plant, including roots, stems, leaves, flower buds, and shoot meristems of both plants were sampled and subjected to GFP gene expression evaluation. Expression of GFP gene was measured by Axio software that came with the Zeiss SV11 stereo fluorescent microscope.

Results

Calluses with high embryogenic potential showed light yellow color and red color under a microscope with white light and green fluorescent light respectively. Calluses that expressed transient bright-green fluorescence dots of GFP gene were observed under fluorescent microscope 3 days after bombardment. Stable GFP expressions were observed 7 days after bombardment. Three different developmental pathways were observed on these fluorescent cells, some of the cells will divide and become homologous GFP transgenic callus, some will develop but can not match the division of non-transformed cells and be embedded by fast growing non-transformed cells, the third kind will gradually lose fluorescence and go back to normal non-transformed embryogenic cells. Early visual selection of GFP positive cells could help the transformed cells of the second and third groups to develop into transgenic cell mass with little disturbance by no-transformed surrounding cells. The first selection was performed 3 days after bombardment by transferring calluses with green GFP dots to

fresh medium. Selection for stably transformed cells began 5 days after first selection when there is a considerable increase in the mass of the transformed callus. At this stage, it is relatively easy to excise green-fluorescing cells from the larger callus mass and transfer these onto fresh medium. Growth of the fluorescing calluses/embryos was rapid. Each round of selection produced a larger, more homogeneous mass of rapidly growing, fluorescing cells. Calluses that exhibited homogeneous green fluorescence were obtained after approximately 20 days of repeated selection. The homogeneous fluorescing calluses were continually transferred to EIM for somatic embryo formation. A transformation efficiency of 13 GFP positive cell clones per petri-dish of bombarded embryogenic callus was obtained (Table 1).

Somatic embryos regenerated from the larger masses of fluorescing calluses in EIM medium within 25 days. The time used for transgenic callus selection and fluorescing embryo regeneration is same as the process in normal tissue culture somatic embryo regeneration because there is no side effect of the antibiotic selector found in the selection and regeneration medium. The germinated embryos rooted in root induction medium in 20-30 days; young plants were potted to soil in the growth chamber. It takes about 3 months for transformed cell to develop young plants that are suitable for potting from beginning of callus bombardment. In general, it takes about 6-8 months for regeneration of transgenic young plant.

A GFP gene PCR amplification was performed to confirm integration of the *GFP* gene in those plants that were regenerated from calluses transformed with the p254EGFP construct and selected by GFP fluorescing. All ten lines that showed green fluorescence were positive for GFP PCR amplification with the non-transformed lines showing no amplification.

Greenhouse grown, GFP transgenic, and non-transformed control plants were compared for GFP expression. Light images showed that organs from both transgenic and negative control plant were healthy and showed similar greenish color. Green fluorescent images showed that all organs from transgenic plants that were identified by PCR analysis emitted strong green fluorescence; organs from the negative control plant showed red fluorescence, no green fluorescence was observed in negative controls. Detailed measurement showed that all organs from transgenic plants showed a dramatic and significant increase in the level of green fluorescence. Compared with our average green fluorescence value of 6.8 in non-transformed negative control plants, the transgenic plant showed value as high as 171, a 25X increase. GFP expression in different organs is variable, strong expression was observed in fast growing, meristematic tissues (root tip and shoot tip) (Figure 1).

Discussion

Our results showed that GFP visual selection based antibiotic-free transformation system is possible in cotton genetic transformation. The cells that expressed transient and stable GFP were sufficiently bright and it is easy to isolate the transformed green fluorescing cells from the red non-transgenic fluorescing callus. Compared to stable transformation frequency (4%) by β-glucoronidase (GUS) histochemical detection, GFP visual gene selection could produce stable transformation frequency as high as 29%. A mean number of 13 transformed cell lines per plate were obtained; the brightness of fluorescence was maintained at full intensity during the subculture, growth, and development of somatic embryos from the cultures.

High embryogenic callus exhibited red fluorescence; we don't know from where the fluorescence comes, but the red fluorescence in mature plants is certainly from chlorophyll auto-fluorescence. During the embryo developmental stage, transgenic and non-transgenic embryos showed yellow-green fluorescent and red fluorescent separately, the yellow-green fluorescence came from interaction between green

fluorescence from GFP and red fluorescence from chlorophyll. In mature plantlets, transgenics showed yellow fluorescence in stem and leaf parts and green fluorescence in root parts, this confirmed that the yellow\yellow-green color is from chlorophyll and GFP interaction because there is no chlorophyll in root. The same phenomenon showed in mature plant organs; green fluorescence observed in meristematic organs: roots and shoots; yellow\yellow-green images showed in more mature organs like stems and leaves. The use of an appropriate yellow or orange filter to block the emitted red fluorescence showed the transformed plants expressing green fluorescence (Zhu et al. 2004).

In conclusion, an efficient antibiotic free transformation system is established in cotton, this system is based on high embryogenic callus bombardment transformation and GFP visual selection.

References

Csaikl U.M., Bastian H., Brettschneider R., Gauch S., Meir A., Schauerte M., Scholz F., Sperisen C., Vornam B. and Ziegenhagen B. (1998) Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. Plant Mol Biol Rep 16:69-86

Fleming G.H., Olivares-Fuster O., Fatta Del-Bosco S., and Grosser J.W. (2000) An alternative method for the genetic transformation of sweet orange. In Vitro Cell. Dev. Biol.-Plant. 36:450-455

Jordan M.C. (2000) Green fluorescent protein as a visual marker for wheat transformation. Plant Cell Rep 19:1069-1075

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Puchta H. (2003) Marker-free transgenic plants. Plant Cell, Tissue and Organ Culture. 74:123-134.

Sakhanokho H.F., Ozias-Akins P., May O.L. and Chee P.W. (2004) Induction of somatic embryogenesis and plant regeneration in select Georgia and Pee Dee cotton (*Gossypium hirsutum* L.) lines. Crop Science 44:2199-2205

Stewart Jr. C.N. (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376-382

Wilkins T.A., Mishra R., and Trolinder N.L. (2004) *Agrobacterium*-mediated transformation and regeneration of cotton. Food, Agriculture & Environment 2(1): 179-187

Zhu Y.J., Agbayani R., and Moore P.H. (2004) Green fluorescent protein as a visual selection marker for papaya (*Carica papaya* L.) transformation. Plant Cell Rep 22: 660-667

Table 1. Transgenic cotton obtained from GFP visual selection

Experiment number	Mean of transient GFP expression dot (two days)	Mean of stable GFP expression dot (seven days)	Mean of GFP and PCR positive lines (callus or somatic embryo)
I	847.7 ± 67.3963	265.0 ± 45.9086	11.0 ± 2.6077
II	1003.2 ± 125.3211	285.5 ± 37.8669	13.3 ± 2.5033
III	1312.3 ± 311.0618	367.3 ± 37.8876	14.5 ± 1.8708

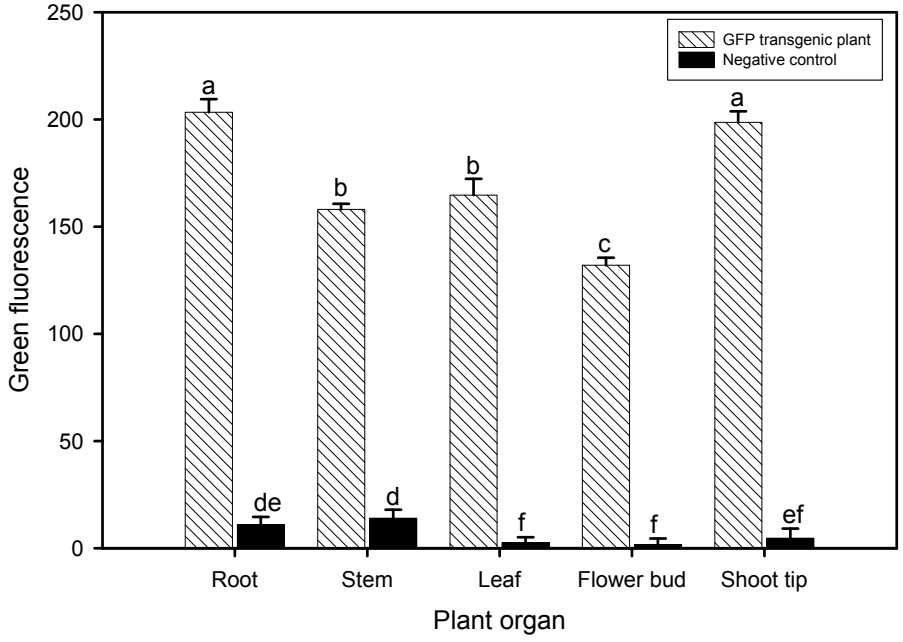

Figure 1. GFP expression in different organs of transgenic plant. GFP expression is significantly high in transgenic plants in all organ compared to expression in negative control plants