In Vitro Cell. Dev. Biol.—Plant 37:648–653, September–October 2001
DOI:10.1079/IVP2001208

1054-5476/01 $10.00+0.00

TRANSFORMATION OF SWEET POTATO TISSUES WITH GREEN-FLUORESCENT PROTEIN GENE

STACY WINFIELD[1†], RODRICK LAWTON[1‡], HENRY DANIELL[2], AND SARWAN K. DHIR[1]*

[1]*Plant Science, 141 Stallworth Research Bldg., Fort Valley State University, Fort Valley, Georgia 31030-1017*
[2]*Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, Florida 32816-2360*

(Received 3 February 2000; accepted 16 March 2001; editor T. A. Thorpe)

Summary

The expression of the green-fluorescent protein (GFP) gene from *Aequorea victoria* (jellyfish) was analyzed by transient and stable expression in sweet potato *Ipomoea batatas* L. (Lam.) cv. Beauregard tissues by electroporation and particle bombardment. Leaf and petiole segments from *in vitro*-raised young plantlets were used for protoplast isolation and electroporation. Embryogenic callus was also produced from leaf segments for particle bombardment experiments. A buffer solution containing 1×10^6 protoplasts ml^{-1} was mixed with plasmid DNA containing the GFP gene, and electroporated at 375 V cm^{-1}. Approximately 25–30% of electroporated mesophyll cell protoplasts subsequently cultured in KM8P medium regenerated cell walls after 48 h. Of these, 3% emitted bright green fluorescence when exposed to UV-blue light at 395 nm. Transformed cells continued to grow after embedding in KM8P medium solidified with 1.2% SeaPlaque agarose. Stable expression of GFP was observed after 4 wk of culture in approximately 1.0% of the initial GFP positive cells (27.5 GFP positive micro calluses out of 3024 cells which transiently expressed GFP 48 h after electroporation). In a separate experiment, 600–700 bright green spots were observed per plate 48 h after bombarding leaf segments or embryogenic callus. In bombarded cultures, several stable GFP-expressing sectors were observed in leaf-derived embryogenic callus grown without selection for 4 wk. These results show that GFP gene expression can occur in various sweet potato tissues, and that it may be a useful screenable marker to improve transformation efficiency and obtain transgenic sweet potato plants.

Key words: sweet potato; *Ipomoea batatas* L. (Lam.); green-fluorescent protein; electroporation; particle bombardment; protoplasts; intact cells; stable expression.

Introduction

Reporter genes have been used as convenient markers to visualize gene expression and protein localization *in vivo* in a wide spectrum of prokaryotes and eukaryotes (Jefferson et al., 1987). Commonly used reporters include genes encoding chloramphenicol acetyl transferase (CAT), β-glucuronidase (GUS), neomycin phosphotransferase (NPT-II), luciferase (LUC), and proteins involved in the regulation of anthocyanin biosynthesis. Luciferase and GUS are of special interest since their assays do not involve any radioactivity (Suter-Crazzolara et al., 1995). However, GUS is limited by destructive assays, endogenous activity, substrate uptake, leaching of reaction products (microbially-derived signals) and methylation (Reichel et al., 1996; Youvan and Larrick, 1996). Differential substrate uptake, cost of substract, and the requirement for sensitive low-light photographic or quantification equipment limit the utility of luciferase (Sheen et al., 1995). The maize *R*, *C1* and *B-Peru* transcription factors (Ludwig et al., 1990; Bowen, 1993) which initiate cell autonomous production of anthocyanin pigment have also been utilized for identification of transgenic cells. However, expression of these transcription factors or products of anthocyanin biosynthesis pathway can prove toxic during regeneration (Bower et al., 1996) or may depend on tissue-specific anthocyanin promoters or tissue pigmentation.

Green-fluorescent protein (GFP) genes from the jellyfish *Aequorea victoria* have recently been shown to have the characteristics of intrinsic signals (bioluminescent protein). This gene has been used as a screenable marker (Prasher et al., 1992; Chalfie et al., 1994; Haseloff et al., 1997). These are clear advantages over formerly used reporter genes such as GUS (Jefferson et al., 1987; Chilfie et al., 1994). Detection of GFP is non-invasive, non-destructive, and cell autonomous. The GFP absorbs UV or blue light maximally at 395 nm and emits bright green fluorescence light (peak emissions at 509 nm with a shoulder at 540 nm). Chromophore formation and light emission are intrinsic properties of this marker protein and it does not require any additional substrates for gene products from *A. victoria* (Prasher et al., 1992).

Expression of GFP has been reported in a variety of heterologous systems, including bacteria (Chalfie et al., 1994), yeast (Heim et al., 1995), *Drosophila* spp. (Wang and Hazelrigg 1994), and mammalian cells (Pines, 1995; Plautz et al., 1996). The GFP gene has also been extensively expressed in many plant species, both dicot and

*Author to whom correspondence should be addressed: Email dhirsO@mail.fvsu.edu

Present addresses: †Department of Biochemistry, Purdue University, West Lafayette, IN 47907; ‡Medical College of Georgia, 1120 15th Street, Augusta, GA 30912.

monocot (Pang et al., 1996; Reichel et al., 1996). Successful transient expression of GFP was observed in electroporated protoplasts of sweet orange (Neidz et al., 1995), maize (Galbraith, 1994; Sheen et al., 1995) and *Arabidopsis* (Sheen et al., 1995), and in bombarded intact tissues of *Arabidopsis* (Sheen et al., 1995) and conifer (Tian et al., 1997). Its expression in stably transformed plants has recently been reported in wheat and tobacco (Pang et al., 1996), corn (Pang et al., 1996; Geest and Petolino, 1998), and sugarcane (Elliott et al., 1999). Insertion of GFP genes into plant viruses has allowed the direct observation of viral movement through host plants during infection (Casper and Holt, 1996). Using GFP as a marker, visualization of nuclear and mitochondrial proteins in targeting studies has been reported recently (Grebenok et al., 1997; Kohler et al., 1997). In spite of initial success with GFP in plants, the general use of this gene as a reporter marker is not popular (Geest and Petolino, 1998).

Protoplasts provide a powerful tool to evaluate marker gene expression in plants (Neidz et al., 1995; Reichel et al., 1996). The investigation of GFP expression transiently in a variety of tissues provides valuable information on its potential applications (Sheen et al., 1995). Successful delivery of genes at a low frequency in sweet potato callus was achieved using microprojectile bombardment (Prakash and Varadarajan, 1992), but transformed plants were not obtained. Only recently, investigators have reported recovery of transgenic plants using *Agrobacterium*-mediated transformation at low efficiency (Newell et al., 1995; Gama et al., 1996). For this purpose, the potential of the GFP gene in sweet potato transformation was studied to determine whether it would maintain its fluorescent properties among the different tissues commonly used as target material in transformation experiments. Obtaining an early, strong signal is clearly advantageous in the identification and rapid isolation of transgenic cells with a subsequent reduction in tissue culture load for the production of transgenic plants. We also showed the stable expression of GFP in electroporated protoplast-derived callus and microprojectile-bombarded leaf tissue.

Materials and Methods

Protoplast isolation. For protoplast isolation, young petioles (2.5–5 cm) and leaves harvested from 2-wk-old *in vitro*-raised micropropagated sweet potato *Ipomoea batatas* L. (Lam.) cv. Beauregard were chopped into small pieces (1 mm) and incubated with enzyme solution as described previously (Dhir et al., 1998). Purified protoplasts were resuspended at 1×10^6 protoplast ml^{-1} in electroporation buffer (EPR) containing 10% glucose, 4 m*M* $CaCl_2$, 10 m*M* *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (HEPES), and 0.2 m*M* spermidine. Samples were stored on ice for 1 h prior to electroporation (Mitchell et al., 1998).

Embryogenic callus establishment. Leaf segments (5 × 5 mm) from 2-wk-old *in vitro*-raised plants were cultured adaxial side down on modified MS (Murashige and Skoog, 1962) medium supplemented with 11.3 μ*M* of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.2 μ*M* benzyladenine (BA) solidified with 0.3% gelrite (pH 5.7) for somatic embryo induction. After 2 wk of culture in the dark, proliferation of yellowish embryogenic callus tissue was observed. The leaf segments and embryogenic calluses (15–20 pieces) were placed in the center of a 150 × 20 mm Petri dish for the bombardment experiment.

Transformation vector. A modified mGFp4 sequence (Haseloff and Amos, 1995) was used as a source of exogenous DNA. The sequence contains a mutated coding region between the *Nde*I and *Aci*I sites in the gene which modifies the codon usage (not the amino acid sequence) and was designed to alter the 5′ splice site and to lower the A:T content around the cryptic intron. The mGFP4 expression cassette was taken out of the pBIN35SmGFP4 vector (Dr. J. Haseloff, personal communication) as a *Hin*dIII-*Eco*RI fragment, in which the sequence is driven by a 35S promoter and flanked by a *Nos* terminator at the 3′ end of the gene and cloned into pUC19 backbone. This has the *bla* gene for resistance to ampicillin and to allow for higher copy-number. All the plasmids were propagated in XL-1 blue cells. Bacterial cultures were grown in LB medium and plasmid DNA was prepared by alkaline lysis, followed by an equilibrium centrifugation in CsCl/ethidium bromide gradient (Sambrook et al., 1989).

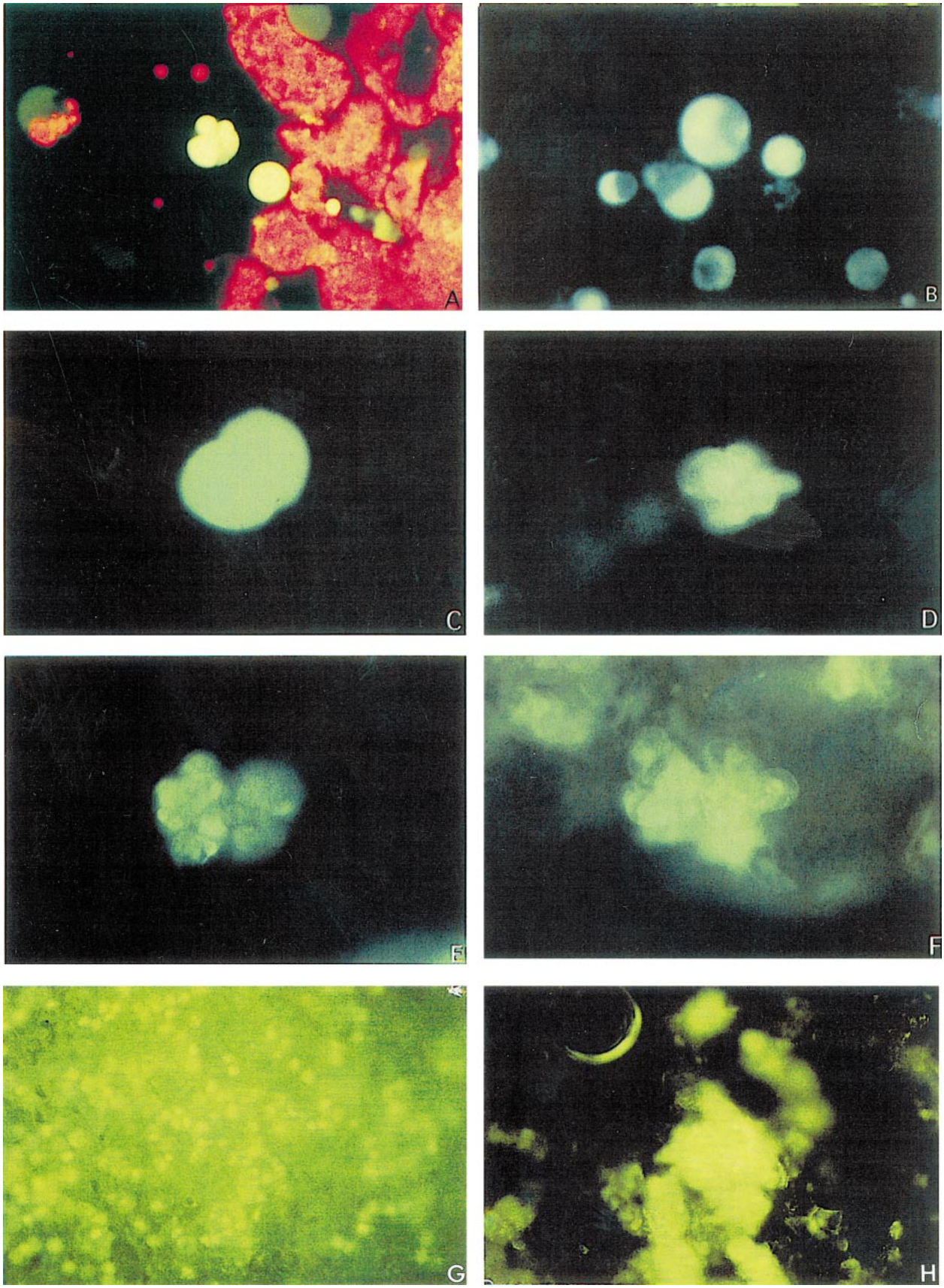
Electroporation. Sweet potato protoplasts isolated from mesophyll and petiole tissues of the *in vitro*-grown plants were electroporated in the presence of plasmid DNA. Electroporation conditions were used as previously described (Mitchell et al., 1998). One ml of protoplast suspension was mixed with 20 μg of the plasmid DNA to which was added 200 μl of 15% (w/v) PEG 4000. Electroporation was performed by delivering an electric pulse at 25 μF from the capacitor charged at 150 V (350 V cm^{-1}) as described previously (Dhir et al., 1991; Mitchell et al., 1998). Electroporated protoplasts were diluted immediately after electroporation with KM8P (Kao and Michayluk, 1975) medium to a density of 10^5 ml^{-1}, and incubated in the dark for 1 h before embedding in 1.2% SeaPlaque (FMC) agarose. Agarose plates were bathed with 2 ml KM8P medium. The osmolarity of the medium was progressively reduced by replacing 1 ml of the original liquid medium with fresh MS medium at 7, 14, 21, and 28 d, respectively (Dhir et al., 1998). All culture dishes were sealed tightly with 3M micropore tape and cultured at 26 ± 2°C in the dark. Cultures were observed periodically with an inverted microscope. The efficiency of cell division and number of colonies regenerated were calculated after 2, 4, 7, 14, 21, and 28 d of culture.

Particle bombardment. Microprojectiles were coated with DNA using a $CaCl_2$/spermidine precipitation technique (Dhir et al., 1994). Five μl of plasmid DNA (1 μg μl^{-1} in TE buffer), 50 μl of $CaCl_2$ (2.5 *M*), 20 μl spermidine (0.1 *M* free base) and 50 μl of sterile gold particles (60 mg ml^{-1} with 1.0 μm diameter; Bio-Rad, Hercules, CA, USA) were mixed with continuous agitation in 1.5 ml Eppendorf tubes. This mixture was vortexed continuously for 5 min followed by centrifugation (500 × ***g***, 10 min). The supernatant was removed and the pellet was resuspended in 250 μl of absolute ethanol. The centrifugation was repeated and the supernatant removed. Coated particles were resuspended in 75 μl of absolute ethanol and vortexed. Ten μl of the mixture was pipetted immediately into the center of each macroprojectile. The helium-driven Biolistics PDS-1000/He Device (Bio-Rad) was used for all bombardments. The conditions of bombardment were helium pressure of 1100 pounds per inch, a vacuum of 26 mmHg, and a 6 cm target distance. The target tissue, leaf segments and embryogenic callus, were placed in the center of a Petri dish and were bombarded twice. Bombarded tissue was sealed and incubated on the culture medium (Chee et al., 1990) in the dark at 26 ± 2°C for 48 h. After a further 48 h, tissues used for stable transformation were subcultured on the medium of Chee et al. (1990) supplemented with 11.3 μ*M* 2,4-D and 2.2 μ*M* BA solidified with 0.3% Gelrite (pH 5.7) for somatic embryo induction. Each experiment was repeated three times.

Detection of GFP gene expression. GFP expression following gene transfer was observed periodically using an Olympus IX 70/PM 30 epi-fluorescence automatic exposure photomicrographic inverted microscope. For fluorescence studies, filter blocks U-MWIB blue light excitation filter, exciter BP 460–490; beam-splitter mirror DM 500 nm; emitter 13A; 515 IF were used. The filter set: U-MNIBA (excitation filter BP 470–480; beam splitter 1 DM 500; emitter BA-515/550) was used to eliminate chlorophyll autofluorescence in mesophyll cells/protoplasts. This was integrated into the microscope setup through a slide module, which allowed visualization with or without the filter. The light source was provided by an HBO 50-W high-pressure mercury vapor lamp with a heat-absorbing filter. Photographs were taken using Kodachrome 200 ASA film. Results were expressed as the number of fluorescent protoplast/colonies or fluorescent cell units per bombardment.

Results and Discussion

GFP expression in protoplasts by electroporation. We have tested the application of the highly fluorescent, modified GFP vector for identifying transgenic sweet potato cells in a primary screen after transformation and without the need for fixation or removal from culture. Of 100 000 protoplasts electroporated,


A
B
C
D
E
F
G
H

approximately 25% had regenerated a cell wall after 48 h. Green fluorescence was observed only in viable protoplasts, not in damaged ones after electroporation. The expression of the GFP gene in protoplasts from both explants (as indicated by green fluorescence) was detected as early as 12 h after electroporation, but increased with longer incubation periods (Table 1). The highest level of expression was detected in mesophyll protoplasts under a fluorescence microscope 48 h after electroporation (Fig. 1A). The green fluorescence was clearly visible under UV light in about 3% of transfected mesophyll protoplasts. Similarly, Galbraith (1994) reported about 2–10% GFP expression in tobacco protoplasts using polyethylene glycol or electroporation. In the same population of sweet potato mesophyll protoplasts, bright yellow-orange protoplasts were also observed when the red autofluorescence of chlorophyll and green fluorescence of GFP overlapped after illumination with blue light (Fig. 1A). Elevated green fluorescence in the nuclei of the fluorescent cells was consistent with the accumulation pattern of GFP in other species, in the cytoplasm and at a higher level in plant nuclei (Haseloff and Amos, 1995; Kohler et al., 1997). The untransfected mesophyll protoplasts showed only red chlorophyll autofluorescence (Fig. 1A). To reduce/eliminate the chlorophyll autofluorescence problem, we subsequently used the filters (U-MNIBA Olympus Microscope) designed for GFP detection. These filters block red light without affecting the green fluorescence derived from GFP expression (Fig. 1B). Similarly, the filter set 41014 (exciter HQ 450/50: beam splitter Q 480 LP; Chroma Technology) was used to reduce the red autofluorescence in tobacco mesophyll protoplasts (Reichel et al., 1996). When petiole-derived protoplasts were electroporated in a similar manner, GFP gene expression was observed in approximately 2175 protoplasts after 48 h (Table 1). The intensity of the fluorescence patterns was variable among protoplasts and ranged from light green to very dark green. The higher number of fluorescent protoplasts from mesophyll versus petiole cells may be due to the size of the protoplasts and the exponential wave used for electroporating the protoplasts, as reported by Saunders et al. (1996).

Approximately 3% of mesophyll-derived protoplasts expressed GFP 48 h after electroporation. The proportion of green fluorescent cells was variable between experiments but averaged 3024 ± 330 per one million electroporated protoplasts. Similar variation was reported in sweet orange embryogenic suspension cells (Neidz et al., 1995) and tobacco mesophyll protoplasts (Galbraith, 1994).

To determine the stability of GFP in protoplast-derived cells, protoplasts were embedded in 1.2% agarose and GFP expression was monitored periodically in the dividing cells. Preliminary experiments provided evidence for stable incorporation of electroporated DNA into the genome of sweet potato protoplasts. The frequency of stable GFP-expressing cells observed 14 d after mesophyll protoplast electroporation was about 3.4% of the frequency of transiently expressing cells (101 out of 3024) observed 2 d after electroporation (Fig. 1C, D). Similarly, in petiole-derived protoplasts, the expression frequency was about 1.1% (25.3 out of 2175; Table 1). GFP fluorescence in cells was a good indicator of transformation with further evidence provided by the increased levels of green fluorescence in the nuclei of green-fluorescent cells (Fig. 1E, F). Also, continued growth and division of the fluorescent cells further support their transformed nature. The dividing cells grew continuously and after 4 wk the frequency of transient to stable (2–28 d) expressing cells was about 1% for mesophyll (27.5 out of 3024) and 0.7% for petiole protoplasts (15.3 out of 2175) (Table 1; Fig. 1F). This suggests continuous accumulation of GFP protein and/or relative stability of the introduced protein. The detection of strong green fluorescence in sweet potato cells was not impeded by endogenous fluorescence, and was always confined to cells with no leakage, whereas diffusion of the blue staining product produced during histochemical GUS staining is often observed (Hunold et al., 1994). GFP product activity in the dividing cells was quite resistant to photo-bleaching and strong GFP expression was still detected after the cells were exposed to blue light for 30 min. The estimated rates of conversion from transient expression to stable incorporation of DNA in cells of other species were in the range from less than 1.0% (Finer and McMullen, 1990) to about 4% (Frank and Birch, 1991).

GFP expression in leaf tissue using microprojectile bombardment. Although the expression of GFP could be detected in sweet potato mesophyll and petiole protoplasts, it was not known if it could be detected in other intact tissues. We used microprojectile bombardment to deliver DNA into leaves and leaf-derived embryogenic callus of sweet potato. The expression of GFP was evident in leaf tissue 48 h after bombardment (Fig. 1G). GFP was detected in individual cells or in groups of 600–700 cells, often

TABLE 1

EXPRESSION OF GFP IN SWEET POTATO MESOPHYLL- AND PETIOLE-DERIVED PROTOPLASTS AT DIFFERENT TIMES AFTER ELECTROPORATION

Time (d)	Mesophyll protoplasts/cells	Petiole protoplasts/cells
0.5	15.3 (± 11.2)*	17.8 (± 7.4)*
1.0	17.3 (± 11.9)	27.8 (± 18.6)
2.0	3024.0 (± 330.1)	2175.0 (± 172.9)
4.0	765.0 (± 118.5)	608.9 (± 174.9)
7.0	164.0 (± 16.4)	78.8 (± 14.9)
14.0	101.0 (± 15.6)	25.3 (± 10.9)
21.0	54.0 (± 7.5)	17.0 (± 11.0)
28.0	27.5 (± 15.9)	15.3 (± 6.4)

* The values represent the mean and the standard error of the mean. The means were obtained from three independent experiments, each with four replicates.

FIG. 1. Transient and stable expression of GFP in sweet potato mesophyll and petiole protoplasts using electroporation. *A*, The yellow-green fluorescence of GFP is visible in transfected mesophyll protoplasts and red autofluorescence from chlorophyll (×250). *B*, GFP expression in mesophyll protoplasts (protoplasts were viewed with a U-MNIBA filter set to eliminate the autofluorescence of chlorophyll, ×400). *C*, First division in a protoplast showing expression of GFP gene after 4 d in mesophyll protoplasts (×400). *D*, Six to eight-celled mesophyll protoplast-derived individual colonies expressing GFP gene. *E*, Twelve to sixteen micro-colonies expressing GFP after 2 wk of culture. *F*, Micro-calluses expressing GFP gene after 4 wk of culture. *G*, Each *green patch* represents an individual transient expression event 48 h after bombardment of leaf tissues using a U-MNIBA filter to eliminate the autofluorescence of chlorophyll (×250). *H*, Several stable events showing the expression of GFP in leaf-derived callus.

appearing as a yellow-orange units. This probably resulted from a combination of the red autofluorescence from the chloroplast and green fluorescence from GFP, each representing individual transient gene expression events. Forty-eight hours after bombardment, leaf tissues cultured onto embryo induction MS medium also continuously exhibited green fluorescence and after 2 wk several stable calluses (10–12) were observed. After 4 wk of culture, a total of six or seven stable GFP-expressing events of 6–10 mm size calluses were observed in the bombarded leaf as well as in the embryogenic callus tissues (Fig. 1H).

This study demonstrated that the GFP gene from *A. victoria* can be expressed transiently and stably in various tissues of sweet potato using electroporation and microprojectile bombardment. The ability to monitor constitutively expressed GFP in plants has many potential uses, such as developing and optimizing transformation methods by continuously monitoring transformation events at different stages, and non-destructive identification of transformed cells or cell lines. This also enables the removal of all untransformed material (usually the large majority of tissue) at an early stage and facilitates the prediction of the number of likely transformants. This methodology has not been possible using GUS histochemical assays which are toxic, or luciferase assays which frequently result in the reversion of embryogenic sugarcane callus to non-regenerable forms (Bower et al., 1996). The GFP gene may also be a useful reporter/marker in monitoring *in vivo* gene expression spatially and temporally at the subcellular and whole plant levels. Studies of these types have proven difficult in sweet potato using markers such as GUS. Combinations of any selection marker genes with GFP in detecting transgenic cells should lead to more widespread use in developing plant transformation in sweet potato.

Acknowledgments

We are very grateful to Dr. Marceline Egnin for providing the sweet potato genotypes used in this study, Dr. Upendra Sainju for performing the statistical analysis, and Ms. Cassandra L. Brown for excellent technical assistance. This research was supported by grants from USDA/CSREES Capacity Building, and Agricultural Research Station.

References

Bowen, B. A. Markers for plant gene transfer. In: Kung, S. D.; Wee, R., eds. Transgenic plants: engineering and utilization, vol. I. San Diego: Academic Press; 1993:89–121.

Bower, R.; Elliott, A. R.; Potier, B. A. M.; Birch, R. G. High-efficiency microprojectile-mediated co-transformation of sugarcane using visible or selectable markers. Mol. Breeding 2:239–249; 1996.

Casper, S. J.; Holt, C. A. Expression of the green fluorescent protein-encoding gene from tobacco mosaic virus-based vector. Gene 173:69–73; 1996.

Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C. Green fluorescent protein as a marker for gene expression. Science 263:802–805; 1994.

Chee, R. P.; Schultheis, J. R.; Cantliffe, D. J. Plant recovery from sweet potato embryos. Hortscience 25:795–797; 1990.

Dhir, S. K.; Dhir, S.; Hepburn, A.; Widholm, J. M. Factors affecting transient gene expression in electroporated *Glycine max* protoplasts. Plant Cell Rep. 10:106–110; 1991.

Dhir, S. K.; Oglesby, J.; Bhagsari, A. S. Plant regeneration via somatic embryogenesis, and transient gene expression in sweet potato protoplasts. Plant Cell Rep. 17:665–669; 1998.

Dhir, S. K.; Pajeau, M. E.; Fromm, M. E.; Fry, J. E. Anthocyanin genes as visual markers for wheat transformation. In: Henry, R. J.; Ronalds, J. A., eds. Improvement of cereal quality by genetic engineering. New York: Plenum Press; 1994:71–75.

Elliott, A. R.; Campbell, J. A.; Dougdale, B.; Brettell, R. I. S.; Grof, C. P. L. Green fluorescent protein facilitates rapid in vivo detection of genetically transformed plant cells. Plant Cell Rep. 18:707–714; 1999.

Finer, J. J.; McMullen, M. D. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep. 8:586–589; 1990.

Frank, T.; Birch, R. G. Gene transfer into intact sugarcane cells using microprojectile bombardment. Aust. J. Plant Physiol. 18:471–480; 1991.

Galbraith, D. W. Flow cytometric and sorting of plant protoplasts and cells. Methods Cell Biol. 42:539–561; 1994.

Gama, I. C. S.; Leite, R. P., Jr.; Cordeiro, A. R.; Cantliffe, D. J. Transgenic sweetpotato plants obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tiss. Organ Cult. 46:237–244; 1996.

Geest, A. H. M.; Petolino, J. F. Expression of a modified green fluorescent protein gene in transgenic maize plants and progeny. Plant Cell Rep. 17:760–764; 1998.

Grebenok, R. J.; Pierson, E.; Lambert, G. M.; Gong, F. C.; Afonso, C. L.; Haldeman-Cahill, R.; Carrington, J. C.; Galbraith, D. W. Green-fluorescent protein fusions for efficient characterization of nuclear targeting. Plant J. 11:573–586; 1997.

Haseloff, J.; Amos, B. GFP in plants. Trends Genet. 11:328–329; 1995.

Haseloff, J.; Siemering, K. R.; Prasher, D. C. Removal of cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl Acad. Sci. USA 94:2122–2127; 1997.

Heim, R.; Cubitt, A. B.; Tsien, R. Y. Improved green fluorescence. Nature 373:663–664; 1995.

Hunold, R.; Bronner, R.; Hahne, G. C. Early events in microprojectile bombardment: cell viability and particle location. Plant J. 5:593–604; 1994.

Jefferson, R. A.; Kavangh, T. A.; Bevan, M. W. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907; 1987.

Kao, K. N.; Michayluk, M. R. Nutritional requirements for growth of *Vicia hajastana* cell and protoplasts at very low population density in liquid media. Planta 126:105–110; 1975.

Kohler, R. H.; Zipfel, W. R.; Webb, W. W.; Hanson, M. R. The green fluorescent protein marker to visualize plant mitochondria *in vivo*. Plant J. 11:613–621; 1997.

Ludwig, S. R.; Bowen, B.; Beach, L.; Wessler, S. R. A regulatory gene as a novel visible marker for maize transformation. Science 247:449–450; 1990.

Mitchell, T.; Bhagsari, A. S.; Ozias-Akins, P.; Dhir, S. K. Electroporation-mediated transient gene expression in intact cells of sweet potato. In Vitro Cell. Dev. Biol. Plant 34:319–324; 1998.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–479; 1962.

Neidz, R. P.; Sussman, M. R.; Satterlee, J. S. Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Rep. 14:403–406; 1995.

Newell, C. A.; Lowe, J. M.; Merryweather, A.; Rooke, L. M.; Hamilton, W. D. O. Transformation of sweetpotato (*Ipomoea batatas* (L.) Lam.) with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. Plant Sci. 107:215–227; 1995.

Pang, S.; DeBoer, D. L.; Wan, Y.; Ye, G.; Layton, J. G.; Neher, M. K.; Armstrong, C. L.; Fry, J. C.; Hinchee, M. A. W.; Fromm, M. E. An improved green fluorescent protein gene as a vital marker in plants. Plant Physiol. 112:893–900; 1996.

Pines, J. GFP in mammalian cells. Trends Genet. 11:326–327; 1995.

Plautz, J. D.; Richard, N. D.; Dailey, G. N.; Welsh, S. B.; Hall, J. C.; Halpain, S.; Kay, S. A. Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells. Gene 173:83–87; 1996.

Prakash, C. S.; Varadarajan, U. Genetic transformation of sweet potato by particle bombardment. Plant Cell Rep. 11:53–57; 1992.

Prasher, D. C.; Eckenrode, V. K.; Ward, W. W.; Prendergast, F. G.; Cormier, M. J. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229–233; 1992.

Reichel, C.; Mathur, J.; Eckes, P.; Langenkemper, K.; Koncz, C.; Schell, J.; Reiss, B.; Mass, C. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cell. Proc. Natl Acad. Sci. USA 93:5888–5893; 1996.

Sambrook, K. J.; Fritsch, E. F.; Maniatis, T. C. Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

Saunders, J. A.; Matthews, B. F.; Miller, P. D. Plant gene transfer using electrofusion and electroporation. In: Neumann, E.; Sowers, A.; Jordan, C., eds. Electroporation and electrofusion in cell biology. New York: Plenum Press; 1996:343–354.

Sheen, J.; Hwang, S.; Niwa, Y.; Kobyashi, H.; Galbraith, D. W. Green-fluorescent protein as a new vital marker in plant cells. Plant J. 8:777–784; 1995.

Suter-Crazzolara, C.; Klemm, M.; Reiss, B. In: Galbraith, D. W., ed. Methods in cell biology: methods in plant cell biology, part B. San Diego: Academic Press; 1995:425–438.

Tian, L.; Sequin, A.; Charest, P. J. Expression of the green fluorescent protein gene in conifer tissues. Plant Cell Rep. 16:267–271; 1997.

Wang, S.; Hazelrigg, T. Implication for *bcd* mRNA localization from spatial distribution of *exu* protein in *Drosophila* oogenesis. Nature 369:400–403; 1994.

Youvan, D. C.; Larrick, J. W. Special issue on GFP. Gene 173:1–117; 1996.