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Green fluorescent protein: an *in vivo* reporter of plant gene expression

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Summary. Protoplasts were isolated from H89, an embryogenic sweet orange (*Citrus sinensis* (L.) Osbeck cv. Hamlin) suspension culture, and electroporated with p35S-GFP, a plasmid carrying the gene for the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria*. p35S-GFP was constructed by replacing the GUS coding sequence of pBI221 with a functional GFP gene, thereby placing the GFP gene under the control of the CaMV 35S promoter. Protoplasts were viewed by incident-light fluorescence microscopy twenty-four h after electroporation. 20-60% of the protoplasts emitted an intense green light when illuminated with blue (450-490 nm) light.

Abbreviations: GUS, β-glucuronidase; LUC, luciferase; NPTII, neomycin phosphotransferase; CaMV, cauliflower mosaic virus; MUG, 4-methylumbelliferyl β-D-glucuronide

Introduction

Gene expression and the selection of genetic transformants requires the use of genes that respectively function as reporters of gene expression, and permit the recovery of transformed cells, tissues, or organs. There are a number of genes widely used in plant biology as 'reporters' and/or selectable markers. Each gene has specific, inherent characteristics that both define its limitations and the applications where it will be useful. For example, the gene for GUS has been extensively used as a reporter of gene expression in plants (Martin et al., 1992), but because the available substrates are membrane impermeable the assays are generally toxic and destructive (Naleway, 1992), and cannot be used to follow gene expression *in vivo*. The gene for firefly LUC is easily detected and can be monitored *in vivo* over time (Howell, 1989), but LUC expression has not been used to select for genetic transformants. This is probably because the emitted light is not readily visible but requires detection by X-ray film, luminometer, or video enhancement. The gene for NPTII has been widely used as a selectable marker. Plants transformed with NPTII are resistant to aminoglycoside antibiotics such as kanamycin, paromomycin, or Geneticin (GIBCO BRL, Gaithersburg, MD). NPTII has also been used as a reporter of gene expression (Töpfer et al., 1988), but like GUS the assays are destructive.

Our interest was to identify genes that would function as reporters of gene expression *in vivo* over time in a single individual, and as selectable markers that would readily identify transformants, and be suitable for both manual and automated selection. A recent report by Chalfie et al. (1994) indicated that GFP had the characteristics to function both as a sensitive reporter of *in vivo* gene expression and an efficient selectable marker.

The green bioluminescence of the jellyfish *Aequorea victoria* is due to a protein-protein energy transfer from a blue fluorescent protein, aequorin, to GFP in the presence of Ca^{2+} (Morise et al., 1974). The GFP chromophore appears to be derived from the primary amino acid sequence of the protein (Cody et al., 1993). Fluorescence of purified GFP requires no exogenous cofactors, only excitation with near UV or blue light, and

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is easily visible under normal room fluorescent lighting (Morise et al., 1974). GFP is stable and is only denatured under extreme conditions (Ward & Bokman, 1982). GFP maintains its fluorescence when expressed in heterologous systems such as *Escherichia coli* and *Caenorhabditis elegans* (Chalfie et al., 1994), and *Drosophila melanogaster* (Wang and Hazelrigg, 1994). These results suggested that it may also function in plant cells, and if so, might be useful as a sensitive reporter of *in vivo* gene expression, and an efficient selectable marker suitable for automated procedures. The purpose of this study was to determine whether GFP would maintain its fluorescent properties when expressed in a plant cell.

Materials and methods

Plant material. An embryogenic cell line (H89) was initiated and maintained from *Citrus sinensis* (L.) Osbeck cv. Hamlin as previously described (Kobayashi et al., 1985; Niedz, 1993). Protoplasts were isolated from log-phase H89 suspension cultures, purified, and cultured in Ca-alginate beads as previously described (Niedz, 1993).

Construction of expression vector. In order to obtain a plant GFP expression construct, the polymerase chain reaction was performed using custom primers to add convenient restriction enzyme sites. Oligonucleotides GFP1 [5'TTAGGATCCATAGATCTGA TAACAAAGATGAGTAAAGG3'] and GFP2 [5'AGAATTC GAGCTCTTATTTGTATAGTTCATCCAT3'] were synthesized and used to amplify gfp10 template DNA (Prasher, et al., 1992). This resulted in a 757 base pair PCR fragment containing unique 5' BamH1 and BglII sites, 10 bases of the 5' untranslated GFP leader, the GFP coding sequence, and a SacI and EcoR1 site immediately following the GFP stop codon. A BamH1/EcoR1 fragment was subcloned into the GST-KT vector (Hakes and Dixon, 1992) which resulted in a construct that allowed isopropyl-β-D-thiogalactopyranoside (IPTG) inducible expression of GFP as a glutathione S-transferase (GST) fusion protein in *E. coli*. This provided a biological assay for GFP function so that isolates containing mutations deleterious to GFP function could be avoided. The GST-GFP fusion protein was expressed in DH5α by growing cells to pre-log phase, followed by a 4 h induction with 0.5 mM IPTG in the dark at 37° C. Bacteria were observed using incident-light fluorescence microscopy with a filter cube for fluorescein isothiocyanate (FITC). A plant GFP expression construct, p35S-GFP, was constructed by replacing the GUS gene of pBI221 (Clontech, CA; pBI221.1 in Jefferson, 1987) with the BamH1/SacI fragment containing a functional GFP gene. This placed GFP under the control of the cauliflower mosaic virus 35S promoter, and positioned the nopaline synthase (*nos*) terminator at the 3' end of the gene. p35S-GFP was maintained in DH5α and purified for electroporation by ultracentrifugation in a CsCl/ethidium bromide gradient (Sambrook et al., 1989).

Electroporation of protoplasts. Purified protoplasts were resuspended at a density of 1 x 10^6 protoplasts/ml in electroporation buffer (CPW salts (Frearson et al., 1973) + 0.7 M mannitol in 10 mM Hepes, pH 8). The protoplasts were heat shocked for 5 min at 45° C. Immediately after the heat shock treatment, sonicated salmon sperm DNA and plasmid expression vector DNA (pBI221, p35S-GFP, or both) were added to the protoplast suspension at 100 μg/ml each. The experiment included five treatments. In the first treatment protoplasts were electroporated without carrier or vector DNA. In the second treatment protoplasts were mixed with carrier DNA and electroporated. In the third treatment protoplasts were electroporated with both carrier DNA and the GUS-containing plasmid pBI221. In the fourth treatment protoplasts were electroporated with the GFP-containing plasmid p35S-GFP and carrier DNA. In the fifth treatment protoplasts were electroporated with both vector plasmids and carrier DNA. Protoplasts were electroporated within 15 min after the addition of the DNA. Electroporation of the protoplast/DNA mixture was performed in 800 μl electroporation cuvettes (Bio-Rad, Richmond, CA, USA) with a 0.4 cm gap between the electrodes. A Gene Pulser apparatus (Bio-Rad) was used to deliver a single exponential 0.425 kV/cm pulse from a 960 μF capacitor to the mixture. Protoplasts were then embedded in Ca-alginate beads and incubated in a growth cabinet (27°C, 15-20 μmol·m^{-2}·s^{-1}, 4 h photoperiod) for 24 h. Protoplasts were observed with an inverted microscope using a filter block for FITC which consists of a 450-490 nm band-pass filter, a 520 nm reflection short-pass filter, and a 515 nm long-pass filter. The light source was a 100 W high pressure mercury vapor lamp with a heat- absorbing filter.

GUS assay. Protoplasts were released from the beads by depolymerizing the Ca-alginate matrix in citrate buffer medium (5% sucrose (w/v) in 0.1M citric acid, pH 5.8; Niedz, 1993). GUS activity was determined by the method of Jefferson et al. (1987). Protoplasts from each 60 x 15 mm polystyrene culture dish were collected into 1.5 ml microcentrifuge tubes and gently pelleted by centrifugation. The supernatant was removed and replaced with 200 μl of extraction buffer (50 mM $NaHPO_4$, pH 7, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) and vortexed to break up the cells. One hundred μl of this extract was then added to 100 μl of GUS assay buffer (2 mM MUG in the extraction buffer) and the mixture incubated in a 37°C water bath for 1 h. The reaction was stopped with the addition of 800 μl of carbonate buffer (0.2M Na_2CO_3). One hundred μl of the stopped reaction solution was added to a cuvette containing 1.9 ml of carbonate stop buffer and a reading taken in a fluorometer equipped with a mercury vapor lamp and filters to yield a peak emission spectrum of 365 nm and a peak emission spectrum of 460 nm. Protein determinations were done according to the method of Bradford (1976) using bovine serum albumin as the standard. GUS activity is expressed as pmoles MU·min^{-1}·mg protein and are reported as the mean values from three experiments, each with three samples and three replications.

Results and Discussion

Sweet orange protoplasts isolated from H89, an embryogenic cell line derived from nucellar tissue were electroporated in the presence of plasmids p35S-GFP or pBI221, coding for the genes for GFP and GUS, respectively. Both GFP and GUS coding regions were under the control of the CaMV 35S promoter and contained the *nos* polyadenylation region. GUS expression and protoplast fluorescence were determined for each treatment combination and are reported in Table 1.

Table 1. GUS activity and green fluorescence in sweet orange protoplasts 24 h after electroporation.

Treatment	GUS Activity[a]	Green Fluorescence
No DNA	48 ± 7.0	no
Carrier DNA	42 ± 3.5	no
pBI221 (GUS)	2958 ± 521.8	no
p35S-GFP (GFP)	41 ± 2.6	yes
pBI221 + p35S-GFP	3276 ± 637.6	yes

[a] mean pmoles MU·min^{-1}·mg protein^{-1} ± SE from three experiments each with three samples.

The only green fluorescent protoplasts observed were those electroporated with p35S-GFP containing the gene for GFP (Table 1). A very faint yellow fluorescence, not visible in Fig. 1, was observed in all treatments, and may come from autofluorescence of phenolic compounds produced by damaged cells. The intensity of fluorescence was variable among protoplasts and ranged from barely visible to an intense green that shrouded the protoplast in a green aura (Fig. 1). The greatest fluorescence appeared

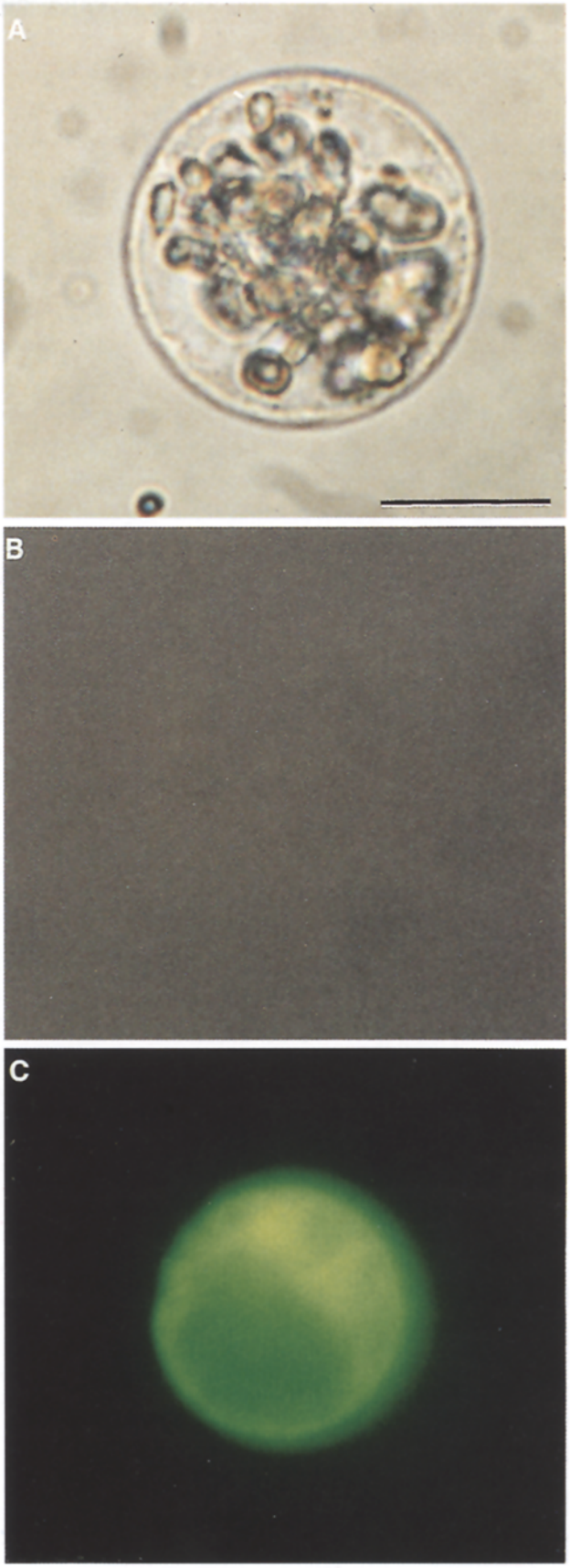

Fig. 1. H89 sweet orange protoplasts electroporated with or without GFP vector DNA, cultured in Ca-alginate beads, and viewed 24 h after isolation. All photographs taken with Fujicolor Super G 400 print film (Fuji Photo Film Co., Tokyo, Japan). Bar = 10 μm. A, H89 protoplast electroporated with carrier DNA and viewed by brightfield illumination. B, H89 protoplast pictured in A but illuminated with blue light (450-490 nm) and photographed with a 1 min 20 s exposure. Protoplasts electroporated with carrier DNA and pBI221 gave identical results. C, A green fluorescent H89 protoplast electroporated with carrier DNA + p35S-GFP, illuminated with blue light, and photographed with a 40 s exposure.

to originate from the cytoplasm; the larger darker region is presumably the nucleus or vacuole. GUS activity was observed only in protoplasts electroporated with pBI221, and confirmed that vector DNA had entered the cells.

The proportion of fluorescent protoplasts was highly variable between experiments and ranged from 20 to 60 percent. This is not unexpected since high levels of variability are often reported for exponential wave electroporation (Saunders et al., 1989).

To the best of our knowledge this is the first report that plant cells are capable of expressing functional GFP. Because GFP fluorescence is not the result of an enzymatic reaction, the signal is not amplified as it is with GUS or LUC. This may result in less sensitivity than GUS or LUC under certain conditions. However, GFP fluorescence should still be useful in monitoring *in vivo* gene expression, something now impossible with current reporter genes that require membrane impermeable substrates or cumbersome equipment. GFP could also be useful in protein targeting experiments, since GFP fusion proteins such as GST-GFP appear to fluoresce. GFP may also be useful for the identification and selection of genetic transformants using both manual and automated procedures, such as flow cytometry. The intense and stable fluorescence of GFP may also be usable in the horticulture industry. GFP fluorescence in plant cells containing chloroplasts should be readily detected since chlorophyll fluoresces red at the wavelength used for GFP excitation (Goedheer, 1972). Fluorescent plants transgenic for GFP would be novel specimens for terrariums and night gardens illuminated with 'black lights.'

This experiment demonstrates that GFP maintains its green fluorescent properties in plant cells, making GFP an attractive alternative to currently available reporter and selectable marker systems now used in plant molecular biology.

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