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ELECTROPORATION OF EMBRYOGENIC PROTOPLASTS OF SWEET ORANGE (*CITRUS SINENSIS* (L.) OSBECK) AND REGENERATION OF TRANSFORMED PLANTS

RANDALL P. NIEDZ*, W. L. MCKENDREE, AND R. G. SHATTERS JR.

Agricultural Research Service, US Horticultural Research Laboratory, 2001 South Rock Road, Ft. Pierce, FL 34945-3030

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SUMMARY

Electroporation conditions were optimized for the transfection of protoplasts isolated from an embryogenic cell line of sweet orange [*Citrus sinensis* (L.) Osbeck cv. Hamlin]. Electric field strength (375–450 V cm^{-1}), vector DNA concentration (100 µg ml^{-1}), carrier DNA concentration (100 µg ml^{-1}), electroporation buffer (pH 8), and pre-electroporation heat shock of protoplasts (5 min at 45°C) were optimized. The plasmid vector pBI221 containing the β-glucuronidase (GUS) coding sequence under the control of the CaMV 35S promoter was used and GUS activity was measured 24 h after electroporation. All variables significantly affected transfection efficiency and when optimal conditions for each were combined, GUS activity was 7714 pmol 4-methylumbelliferone (MU) mg^{-1} (protein) min^{-1}. Protoplasts were then electroporated in the presence of green fluorescent protein (GFP) expression vectors pARS101 or pARS108. Green fluorescent embryos were selected, plants regenerated, and integration of the transgene was confirmed by Southern blot analysis. Both plasmids were constructed using EGFP, a GFP variant 35 times brighter than wtGFP, having a single, red-shifted excitation peak, and optimized for human codon-usage. pARS101 was constructed by placing EGFP under the control of a 35S–35S promoter containing 33 bp of the untranslated leader sequence from alfalfa mosaic virus. pARS108 was constructed similarly except sequences were added for transport and retention of EGFP in the lumen of the endoplasmic reticulum.

Key words: citrus; transformation; transient gene expression; GUS; transfection; endoplasmic reticulum; somatic embryogenesis.

INTRODUCTION

Electroporation is widely used to produce stable genetic transformants in both prokaryotic and eukaryotic organisms (Paszkowski et al., 1984). It is used extensively as an efficient method for loading cells with a wide array of exogenous macromolecules for studying cellular physiology (Abadía-Molina et al., 1998), transient gene expression (Hauptmann et al., 1987; Jones et al., 1989), and virus replication (Hibi et al., 1986; Nishiguchi et al., 1986). In plants, the major limitation of recovering stable transformants by protoplast electroporation is the requirement for an efficient protoplast-to-plant regeneration scheme. Plants can be regenerated from protoplasts of embryogenic callus of sweet orange (Vardi, 1977; Kobayashi et al., 1985). Numerous inter- and intrageneric somatic hybrids have been produced between *Citrus* and *Citrus* relatives, illustrating the amenability of *Citrus* embryogenic callus to cellular and genetic manipulation (Ohgawara et al., 1985; Grosser and Gmitter, 1990; Grosser et al., 2000).

*Author to whom correspondence should be addressed: Email rniedz@ushrl.ars.usda.gov

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Transgenic *Citrus* plants have been obtained by *Agrobacterium*-mediated gene transfer methods using embryogenic cell suspension cultures or stem explants (Hidaka et al., 1990; Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995a, b, 1997, 2001; Gutierrez et al., 1997; Bond and Roose, 1998; Cervera et al., 1998; Dominguez et al., 2000, 2002; Mendes et al., 2002; Yu et al., 2002) {Hidaka, Omura, et al. 1990 ID: 84}. Polyethylene glycol- (PEG) mediated DNA uptake methods were used by Vardi et al. (1990) and Fleming et al. (2000) to recover transgenic citrus from embryogenic callus-derived protoplasts. Hidaka and Omura (1993) reported the effects of capacitor size, electric field strength, heat shock, and $CaCl_2$ and vector DNA concentration on transient gene expression of electroporated mandarin protoplasts. Though no plants were regenerated, some GUS-positive, 2-mo.-old, protoplast-derived colonies were detected. Niedz et al. (1995) electroporated embryogenic protoplasts of sweet orange with p35S-GFP, a construct containing the wild-type green fluorescent protein (wtGFP) under the control of the 35S promoter. The protoplasts emitted an intense green fluorescence 24 h after electroporation; however, the fluorescence disappeared after 5 d, presumably because the wtGFP sequence contains a cryptic intron (Haseloff et al., 1997), and no transgenic plants were regenerated.

In this study we optimized electroporation efficiency of protoplasts derived from an embryogenic cell line of sweet orange (*C. sinensis*), electroporated these protoplasts in the presence of GFP constructs designed to produce transformed fluorescent plants,

identified and selected GFP-positive green fluorescence, and subsequently regenerated these selected protoplast-derived calluses into plants.

Materials and Methods

Plant material. Optimization of electroporation conditions and transformation experiments were conducted using a 2-yr-old embryogenic callus line derived from *Citrus sinensis* (L.) Osbeck cultivar Hamlin initiated as described by Kobayashi et al. (1985). The line was maintained on Murashige and Tucker's (MT) basal medium (Murashige and Tucker, 1969) at 27°C, 15–20 $\mu E\,m^{-2}\,s^{-1}$, 4-h photoperiod provided by coolwhite fluorescent lamps, and a 28-d subculture cycle. The protocol developed by Niedz (1993) to isolate, purify, embed in Ca-alginate beads, and measure the viability and plating efficiency (PE) of protoplasts isolated from embryogenic citrus callus was used.

Optimization of electroporation variables and transformation. Electroporation buffer, capacitor size, and incubation time proceding electroporation to assay for GUS activity were determined in preliminary experiments. Field strength, vector and carrier DNA concentration, electroporation buffer pH, and heat shock and their effect on DNA uptake into protoplasts were determined by GUS activity 24 h after electroporation. Carrier DNA was prepared by dissolving high molecular weight salmon sperm DNA in dH_2O (10 mg ml^{-1}) and sonicating on ice for 5 min with a Model 450 sonifier (Branson Ultrasonics Corp., Danbury, CT) fitted with a micro tip. Fifty watts of ultrasonic energy were delivered in pulse mode with a 1 s pulse duration of 50% (i.e., 0.5 s). The sonicated DNA was then rapidly drawn into and expelled from a syringe fitted with a 22-gauge needle. Vector and carrier DNAs were sterilized by ethanol precipitation (Sambrook et al., 1989). When testing the effect of pH on transfection efficiency, 'cell protoplast wash' (CPW) salts of Frearson et al. (1973) supplemented with 0.7 *M* mannitol and the pH adjusted with 10 m*M* 2-(4-morpholino)ethanesulfonic acid (MES) for pH 5 and 6, or 10 m*M* HEPES for pH 7 and 8 were used. When heat-shocked, the protoplasts were treated at 30, 35, 40, 45, or 50°C for 5 or 10 min, the DNA was added, and the protoplast/DNA mixture was electroporated.

Protoplasts were resuspended in electroporation buffer at a density of 1×10^6 protoplasts per ml. Plasmid DNA pBI221 (Jefferson, 1987) was purified by ultracentrifugation in a CsCl/ethidium bromide gradient (Sambrook et al., 1989) and was added to the protoplast suspension to a final concentration of 10 $\mu g\,ml^{-1}$. Protoplasts were electroporated within 15 min after the addition of DNA. Electroporation of the protoplast/DNA suspension was performed in 800 µl Gene Pulser electroporation cuvettes (Bio-Rad, Richmond, CA) with a 0.4 cm gap between the electrodes. A Gene Pulser apparatus (Bio-Rad) was used to deliver single or multiple exponential decay waveform pulses from a 960 µF capacitor. Immediately after electroporation, the protoplast/DNA mixture was removed from the cuvette, added to 800 µl of citrus protoplast medium CPM1 (Niedz, 1993) in 60 × 15 mm polystyrene culture dishes, and cultured for 24 h in a growth cabinet (27°C, 15–20 $\mu E\,m^{-2}\,s^{-1}$, 4 h photoperiod).

Each treatment combination included at least three plates and each experiment was conducted at least three times. Data were plotted, a graph fitted, and coefficient of determination, r^2, calculated for each experiment.

GUS assay. GUS activity was determined by the method of Jefferson (1987). Protoplasts from each 60 × 15 mm polystyrene culture dish were collected into 1.5-ml microcentrifuge tubes and gently pelleted by centrifugation (RCF = 2940 for 5 min). The supernatant was removed and replaced with 200 µl of extraction buffer (50 m*M* NaH_2PO_4, pH 7, 10 m*M* β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100) and vortexed for 15 s to rupture the cells. One hundred microliters of this extract were then added to 100 µl of GUS assay buffer (2 m*M* 4-methylumbelliferyl-β-D-glucuronide (MUG) in the extraction buffer) and the mixture incubated in a 37°C water bath for 1 h. The reaction was stopped by the addition of 800 µl of carbonate buffer (0.2 *M* Na_2CO_3). One hundred microliters of the stopped reaction solution were added to a cuvette containing 1.9 ml of carbonate stop buffer and the fluorescence of the liberated methylumbelliferone (MU) was measured in a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA) equipped with a mercury vapor lamp and a filtered detector that includes a 365 nm excitation filter and a 460 nm emission filter. Protein determinations were done according to the method of Bradford (1976) using bovine serum albumin as the standard.

Transformation vector construction. Two plant expression vectors were constructed to express EGFP (GenBank accession #U55761) nonspecifically in the nucleus and cytoplasm or to target EGFP expression to the endoplasmic reticulum (ER) using signal and ER-retention sequences. These EGFP variants were cloned into pBI524 downstream from a CaMV duplicated enhancer 35S promoter, and an untranslated leader sequence from alfalfa mosaic virus RNA4. This promoter–leader sequence combination was previously shown to provide a 20-fold increase in GUS activity in comparison to a standard CaMV 35S promoter (Datla et al., 1993).

EGFP was cloned into pBI524 by PCR amplification of EGFP from EGFP-1 (Clonetech Laboratories, Inc., Palo Alto, CA) with primers that produced a DNA fragment containing a *Nco* I and a *Bam* HI site 5′ and 3′ to the EGFP coding region, respectively (5′ primer: 5′-GAGATTCTAGACT-ACCATGGTGAGCAAGGGC-3′ – note that additional sequence added to the EGFP coding region includes the *Nco* I site, and an *Xba* I site added for other sequence cloning projects; 3′ primer: 5′-CTCAAGATCCGAGCTCT-TACTTGTACGGCTCGTCC-3′). After digestions with *Nco* I and *Bam* HI, the amplified EGFP was ligated into similarly digested pBI524. The resulting plasmid, pARS101, placed the EGFP start ATG within an optimized translation initiation consensus sequence (Kozak, 1986).

An EGFP variant encoding an N-terminal ER-targeting signal sequence and a C-terminal HDEL-ER-retention signal (Napier et al., 1992; Haseloff et al., 1997) was also inserted into pBI524 to produce pARS108 (Fig. 1). The DNA coding for the signal sequence was added as a synthetic double-stranded oligonucleotide copy of the *Arabidopsis thaliana* basic chitinase

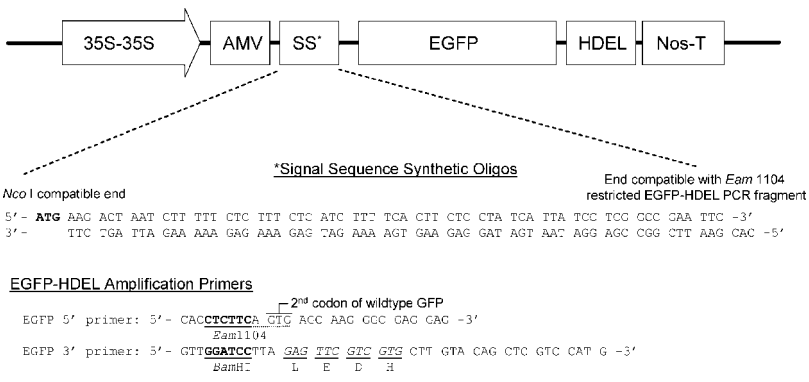


FIG. 1. Schematic of pARS108 ER-targeting and retention gene expression cassette used to transform sweet orange protoplasts. pARS108 contains the EGFP coding sequence placed under the control of the double 35S promoter with the AMV untranslated leader sequence and the *nos* terminator. An *Arabidopsis* signal sequence (SS) is included to target the protein to the ER, and the HDEL sequence was included for retention of the protein in the lumen of the ER. The SS-EGFP-HDEL sequence was cloned into pBI524 as a *Nco* I/*Bam* HI cassette, placing it under the control of the double 35S promoter with the AMV untranslated leader sequence and the *nos* terminator. The SS synthetic oligos and EGFP–HDEL primers used to construct pARS108 are shown.

signal sequence that was used for efficient expression of GFP in *A. thaliana* (Haseloff et al., 1997). Upon annealing, the single-stranded synthetic oligonucleotides produced a 63 base-pair double-stranded fragment with a 5′ *Nco* I compatible cohesive end encompassing the translational start ATG, and a 5′-CAC overhand immediately distal to the signal sequence coding region. This was ligated to an *Eam*1104 and *Bam* HI restricted PCR fragment produced by amplifying EGFP with primers encoding an *Eam*1104 restriction site 5′ to the EGFP sequence and an HDEL extension followed by a *Bam* HI site 3′ to the EGFP sequence. The full construct was inserted into *Nco* I/*Bam* HI restricted pBI524 by including this restricted plasmid in the ligation reaction.

Transformation. Transformation of protoplasts with GFP constructs was performed using optimized electroporation parameters. Briefly, purified protoplasts were resuspended at a density of 1×10^6 protoplasts per ml in electroporation buffer [CPW salts (Frearson et al., 1973) + 0.7 *M* mannitol in 10 m*M* HEPES, pH 8], heat shocked for 5 min at 45°C, sonicated salmon sperm DNA and expression vector pARS101 or pARS108 DNA were added at 100 μg ml^{-1} each, and a single exponential pulse from a 960 μF capacitor was then delivered to the mixture. The appropriate field strength was determined by running test samples between 300 and 500 V cm^{-1} at 25 V cm^{-1} intervals, and identifying the voltage that resulted in the lysis of 50% of the protoplasts with the remaining protoplasts appearing morphologically normal. Electroporated protoplasts were cultured and shoots regenerated as previously described (Niedz, 1993). Shoot-tip graft recovery of transgenic shoots was performed essentially as described by Peña et al. (1995b). Green fluorescent colonies were detected using an inverted microscope equipped with a FITC filter block (exciter 450–490 nm band-pass, 520 nm dichroic, and emission 515 nm long-pass), and subcultured individually once their diameter exceeded 400 μm (Niedz, 1993). Green fluorescent plantlets were observed and selected with a stereoscope equipped with a GFP filter block (exciter 470/40, dichroic 495 nm long-pass, and emission 500–550 nm band-pass). A 100 W high-pressure mercury vapor lamp with a heat-absorbing filter provided illumination.

Transformation frequency was defined as the percentage of transformants/survivors and was calculated from a total of 3.8×10^6 protoplasts electroporated and cultured, 50% viability, and 80% plating efficiency.

Presence of the introduced EGFP sequence in putative transgenic plants was verified by Southern blot analysis of genomic DNA isolated according to Dellaporta et al. (1983). Electrophoresis and capillary transfer for Southern analysis were performed by standard protocols (Southern, 1975) using 10 μg per lane of *Hin*dIII-digested DNA. A 1.38 kb EGFP *Hin*dIII/*Sac* I fragment from pARS101 or a 0.97 kb EGFP *Nco* I/*Eco* RI fragment were labeled with α-^{32}P using the Prime-a Gene® Labeling System (Promega, Madison, WI). Blots were exposed to X-ray film at −80°C.

GFP fluorescence analysis. Twenty fluorescent protoplast-derived calluses were selected from a total of 53 transformed cell lines, 10 each from pARS101- and pARS108-transformed fluorescent calluses. Approximately 200 mg of callus tissue was collected per sample with a spatula, homogenized in an ice-cold mortar with 1 ml buffer as described by Van der Geest and Petolino (1998) (10 m*M* Tris, pH 8, 100 m*M* $MgCl_2$, 10 m*M* dithiothreitol), and the homogenate centrifuged at 16 000 × ***g*** for 2 min. Three samples per plate were collected for a total of 60 samples. To measure fluorescence, 100 μl of supernatant were collected from each sample, loaded into a microtiter plate, and the fluorescence measured in a Bio-Tek FL500 Fluorescence Plate Reader (Bio-Tek Instruments, Winooski, VT) equipped with a filter setup for EGFP detection (excitation 485/20 BP, emission 530/25 BP). Protein determinations were done according to the method of Bradford (1976) using bovine serum albumin as the standard. Fluorescence measurements were normalized to the protein content of the sample.

Results and Discussion

Preliminary experiments indicated that the 'cell protoplast wash' (CPW) salts of Frearson et al. (1973) supplemented with 0.7 *M* mannitol and the pH adjusted to 5.8 would be a suitable base electroporation buffer (CPW). Sweet orange embryogenic protoplasts are stable in this medium (Niedz, 1993) and the resistance of CPW is sufficiently high (386 ohms) that a wide range of capacitor sizes could be used. The resistance–capacitance time constants, τ (the time for the pulse to decay 1/e or ~37% of the initial voltage), were then derived using eight capacitor sizes (0.25–960 μF) over four voltage settings (100–400 V) and ranged from <1 to 78 ms, verifying that a wide range of time constants could be derived from CPW.

Three capacitors (250, 500, and 960 μF) were tested in conjunction with a 500 V cm^{-1} field strength and GUS expression measured 24, 48, and 72 h after electroporation. The 960 μF capacitor was used in all subsequent optimization experiments, as it resulted in the highest GUS expression. Though we did not test a range of capacitors over a range of field strengths, the results of Joersbo et al. (1990) indicate that comparable electropermeabilization between capacitors can be achieved by optimizing field strength for any particular capacitor. GUS activity was highest at 72 h, though all three incubation times had GUS activity significantly higher than that of the control. For convenience, a 24 h incubation time was chosen for all further optimization experiments.

Field strength. To determine the optimum field strength to use in conjunction with the 960 μF capacitor, upper and lower field strength limits were defined first, followed by electroporation with field strength increments of 25 V cm^{-1} to define the optimum. To define the upper field strength limit, protoplasts were pulsed until a voltage was reached that visibly disrupted the spherical integrity of >50% of the protoplasts. Such treatment resulted in no viable protoplasts 24 h after electroporation based on morphology, fluorescein diacetate staining (Widholm, 1972), or Evan's blue dye exclusion (Kanai and Edwards, 1973). To define the lower field strength limit, protoplasts were pulsed until a voltage was reached that resulted in no disruption of their spherical integrity, and no decrease in 24 h viability as determined for the upper limit, in addition to no decrease in plating efficiency (PE) as compared to the nonelectroporated control. Optimum field strength was determined to range from 375 to 450 V cm^{-1} (Fig. 2), with a resulting time constant of 70–80 ms. Because this is a narrow range and the

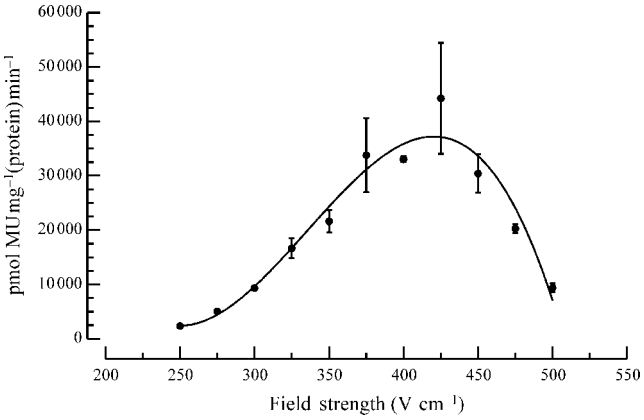


Fig. 2. Effect of field strength on GUS activity. H89 protoplasts were electroporated with 10 μg ml^{-1} pBI221 with a single pulse from a 960 μF capacitor at field strengths from 250 V cm^{-1} to 500 V cm^{-1} in 25 V cm^{-1} increments. Fluorescence measurements were taken 24 h after electroporation, adjusted for sample protein levels, and compared to nonelectroporated controls. Each *data point* represents the mean ± SE from three experiments and was fitted to a 3rd-order polynomial [pmol MU mg^{-1} (protein) min^{-1} = 450046.05 − 4463.96X + 14.23X^2 − 0.014X^3] where r^2 = 0.94.

various treatments may change the field strength limits, to achieve sufficient electropermeabilization of the protoplast membranes, we determined the optimum field strength for each protoplast preparation. A few test pulses were sufficient to determine the field strength that visibly disrupted >50% of the protoplasts. This field strength was then reduced by 100 V cm^{-1} to place the pulse in the effective range for electropermeabilization. Hidaka and Omura (1993) reported that *Citrus reticulata* embryogenic protoplasts had the highest transient GUS activity when electroporated with a field strength of 1200 V cm^{-1} generated with a 5.5 μF capacitor, with total cell lysis occurring at 1400 V cm^{-1}. Though they did not report a time constant, it was presumably less than the 90 ms that we observed. This is consistent with the observation of Abdul-Baki et al. (1990) of an inverse relationship between field strength and pulse duration over the effective poration range. A narrow effective field strength has been observed in other plant systems (Dhir et al., 1991) and may be characteristic of an exponential decay waveform.

Vector DNA. Vector DNA (pBI221) was added to the protoplast suspension to a final concentration ranging from 5 to 100 μg ml^{-1}. GUS expression increased with increasing amounts of vector DNA to 100 μg ml^{-1} (Fig. 3) and was significantly higher than the control, even at 5 μg ml^{-1}. Hidaka and Omura (1993) observed that maximal transient GUS activity was achieved with 100 μg ml^{-1} of vector DNA and that activity decreased when vector DNA was increased to 200 μg ml^{-1}.

Carrier DNA. Carrier DNA is often added to the protoplast/-vector DNA mixture (Bellini et al., 1989; Chang and Loescher, 1991) as it may protect the vector DNA from cellular nucleases by competitive inhibition. Sonicated salmon sperm DNA was used to test the effect of carrier DNA on GUS expression. Carrier DNA had no effect at 10 μg ml^{-1} but was effective at 50 μg ml^{-1} (Fig. 4), with little increase in GUS activity when concentrations greater than 100 μg ml^{-1} were used. The efficacy of carrier DNA in previous studies is mixed. Tagu et al. (1988) found that omitting carrier DNA reduced transformation frequency by three-fold. Conversely, carrier DNA at 150 μg ml^{-1} reduced chloramphenicol acetyl transferase (CAT) activity in electroporated larch protoplasts, but had no effect

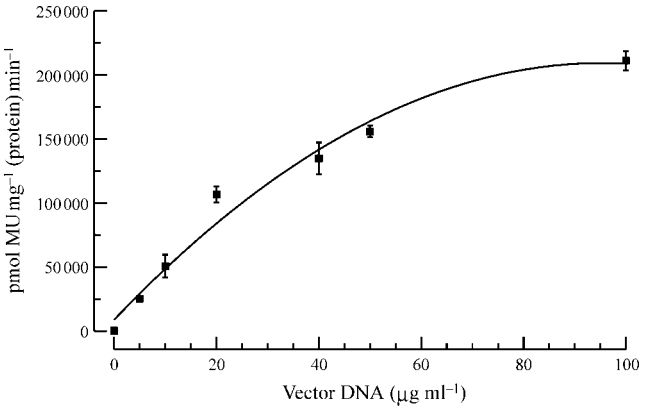


FIG. 3. Effect of vector DNA (pBI221) concentration on relative GUS activity. H89 protoplasts were electroporated with 10 μg ml^{-1} pBI221 with a single pulse from a 960 μF capacitor at field strength of 400 V cm^{-1}. Fluorescence measurements were taken 24 h after electroporation, adjusted for sample protein levels, and compared to nonelectroporated controls. Each *data point* represents the mean ± SE from three experiments and was fitted to a 2nd-order polynomial [pmol MU mg^{-1} (protein) $min^{-1} = 8707.46 + 4204.91X - 22.03X^2$] where $r^2 = 0.98$.

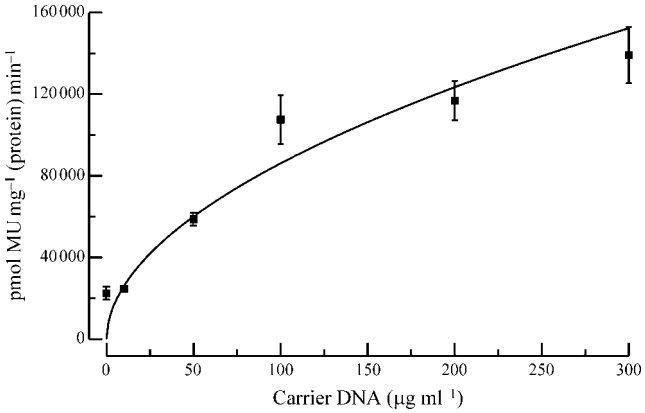


FIG. 4. Effect of carrier DNA (fish sperm DNA) concentration on relative GUS activity. H89 protoplasts were electroporated with 0–300 μg ml^{-1} carrier DNA and 10 μg ml^{-1} pBI221 DNA with a single pulse from a 960 μF capacitor at a field strength of 375 V cm^{-1}. Fluorescence measurements were taken 24 h after electroporation, adjusted for sample protein levels, and compared to nonelectroporated controls. Each *data point* represents the mean ± SE from three experiments and was fitted using a power function [pmol MU mg^{-1} (protein) $min^{-1} = 7872.92(X^{0.519})$] where $r^2 = 0.97$.

at lower concentrations (Charest et al., 1991). Negrutiu et al. (1990) reported that carrier DNA had little effect on increasing CAT activity in electroporated tobacco protoplasts.

Electroporation buffer pH. A pH range from 5 to 8 was tested. GUS expression was significantly higher at a pH of 8 [101 538 pmol MU mg^{-1} (protein) min^{-1}] than a pH of 5, 6, or 7 (34 210, 45 928, and 53 067 pmol MU mg^{-1} (protein) min^{-1}, respectively]. Ca^{2+} ions at high pH will induce fusion of plant protoplasts (Keller and Melchers, 1973) and the combination presumably facilitates the association of protoplasts and DNA, both of which are negatively charged. Rathus and Birch (1992) also observed high GUS activity when protoplasts were electroporated in a HEPES buffer of pH 8.0, but the effect was inconsistent.

Heat shock. Protoplasts were heat shocked at 30, 35, 40, 45, or 50°C for 5 or 10 min immediately prior to electroporation. All heat shock pretreatments increased GUS expression over non-heat shock-treated protoplasts, but 5 min at 45°C treatment was the most effective [26 911 vs. 6816 pmol MU mg^{-1} (protein) min^{-1}]. A heat shock treatment is often used to prepare protoplasts for electroporation (Guerche et al., 1987; Tagu et al., 1988), but the reasons why a heat shock treatment is sometimes effective are unclear.

Optimized protocol. The optimized protocol for protoplast electroporation utilized CPW buffered with 10 m*M* HEPES at pH 8, 100 μg ml^{-1} carrier DNA, 100 μg ml^{-1} vector DNA, a 5 min heat shock pretreatment at 45°C, and a single exponential wave pulse delivered from a 960 μF capacitor at a field strength of 375–450 V cm^{-1}. Protoplasts treated with the optimized protocol remained viable and exhibited a high plating efficiency when cultured in alginate beads. The optimized protocol resulted in an 886-fold increase of GUS activity over the control. GUS expression averaged 7714 pmol MU mg^{-1} (protein) min^{-1} compared to 8.7 pmol MU mg^{-1} (protein) min^{-1} for the control. There was significant variation between electroporation experiments that indicated additional unidentified variables important in consistent electroporation. Isolations of the highest 'quality' protoplasts, as defined by high yield, spherical morphology, and low background

debris, seemed to result in the most reproducible experiments from which meaningful data could be compiled.

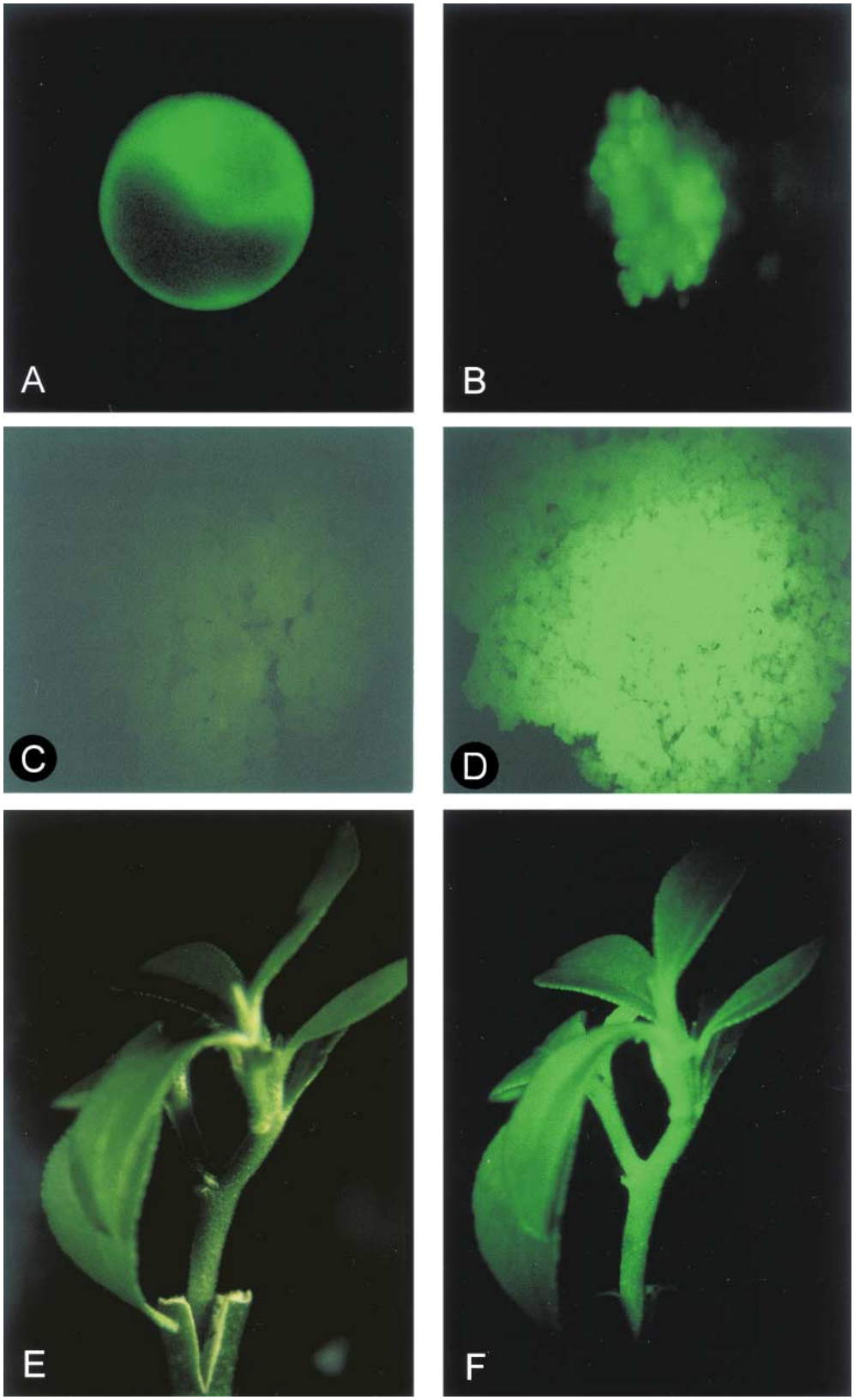
Transformation with optimized protocol. A highly embryogenic cell line of the primary early season Florida juice orange 'Hamlin' was used as the tissue source for protoplast isolation. Only quality protoplasts are suitable for electroporation as it is a damaging treatment only tolerated by highly viable protoplasts. The movement of DNA into a protoplast by electroporation occurs by electrophoresis through pores formed in the membrane by the electrical breakdown of the cell's membrane (Sowers, 1992). With a highly viable protoplast, the membrane breakdown is reversible. A good indicator to assess the suitability of a particular citrus protoplast preparation for electroporation is yield (Niedz, unpublished) and aroma (Niedz et al., 1997). Only isolations where the protoplast yield exceeded 1×10^7 per gram fresh weight of suspension-cultured cells were used. Aroma was not used as an isolation screen; however, high yield isolations were usually associated with a sweet aromatic aroma. The subculturing protocol of Niedz (1993) resulted in consistent production of protoplast populations suitable for electroporation. Protoplasts derived from lower-yielding isolations would generally die within 24 h. Electroporation of protoplasts was conducted using the optimized protocol. The appropriate field strength was determined for each protoplast population isolated. At the optimum field strength approximately 50% of the protoplasts were destroyed by lysis while the remaining protoplasts appeared morphologically normal and initiated cell division within 3–5 d. This loss is unavoidable when using any type of direct current (DC) pulse since the membrane potential induced is proportional to the diameter of the cell (Chang, 1992). The critical voltage will vary with the population since a typical protoplast population is heterogeneous for cell diameter. This results in a narrow window of voltages that will effectively electroporate a cell population; an electric field below the critical voltage will not induce pore formation while an electric field above the critical voltage will result in cell lysis.

To develop GFP constructs suitable for the identification of citrus transformants by visualization of green fluorescence, we tested a number of construct features that enhance various aspects of expression and protein fluorescent characteristics. First, we utilized the double 35S-AMV promoter sequence reported by Datla et al. (1993) to increase expression up to 20-fold relative to the 35S promoter. The 35S–35S-AMV promoter is suitable for the visual identification and recovery of GUS-positive citrus transformants (Niedz and McKendree, 1998). Second, we utilized an EGFP variant GFPmut1 that contains Phe-64 to Leu and Ser-65 to Thr mutations (Cormack et al., 1996). These mutations shift the maximal excitation peak to 488 nm resulting in a much brighter signal with our FITC filter set. EGFP also contains over 190 silent base changes corresponding to human codon usage and resulting in the removal of the cryptic intron that interferes with proper expression in plants (Haseloff et al., 1997). Third, we incorporated ER targeting and retention sequences for improved fluorescence (Haseloff et al., 1997).

Transient expression of GFP was visible 6 h after electroporation in both pARS101- and pARS108-treated protoplasts. Transient expression of green fluorescence was intense 16–24 h after electroporation (Fig. 5*A*) and was observed in >80% of the viable protoplasts. Visually there was no difference in fluorescence intensity between pARS101- and pARS108-treated protoplasts. The difference between the two constructs became particularly evident by day 5 when the cells had started dividing. Nuclear fluorescence was clearly evident in pARS101 but absent in the pARS108-treated cells, an observation consistent with other reports (Haseloff et al., 1997; Elliott et al., 1999). Fluorescent colonies were selected at 40 d (Fig. 5*B*). There was little visual difference in fluorescence at this stage between pARS101 and pARS108. Visual selection of transgenic citrus is particularly effective at this stage as the colonies are small enough (250–500 μm) so that a single plate containing thousands of colonies can be rapidly screened, but large enough to be easily rescued and recultured individually, as demonstrated by Niedz (1993). A single 15 × 100 mm Petri dish can be screened in less than 5 min.

Using a plasmid construct that placed wild-type GFP under the control of the 35S promoter, Niedz et al. (1995) first demonstrated in citrus that plant cells are capable of expressing functional GFP. However, fluorescence disappeared after 6 d and no GFP-positive calluses could be selected. Plants transgenic for GFP have been previously produced using conventional antibiotic or herbicide selection (Chiu et al., 1996; Pang et al., 1996; Haseloff et al., 1997; Maximova et al., 1998; Elliott et al., 1999; Ghorbel et al., 1999). Elliott et al. (1998, 1999) tested the efficiency of visual selection by GFP with no additional selection and concluded that without an additional selective agent preferential growth of GFP-positive tissue is difficult to maintain. However, when GFP-positive tissue can be identified, selectively cultured, and plants regenerated, GFP has been successfully used as a visual screenable marker (Niedz et al., 1999; Fleming et al., 2000; Kaeppler et al., 2001; Winfield et al., 2001). Protoplasts form colonies or embryoids directly from single cells, making the selection and regeneration of transgenic individuals an efficient process limited only by the efficiency of the particular protoplast system. A 6-mo.-old nontransformed control callus cell line is shown in Fig. 5*C*; fluorescent protoplast-derived colonies were regenerated into plants and maintained as cell lines (Fig. 5*D*). Nontransformed green fluorescent colonies were not observed in any of the control plates. Using a crude extract to assess the fluorescence levels in transformed cell lines, a significant difference in fluorescence intensity was observed between the pARS101- and pARS108-derived callus tissue. Tissue derived from pARS108-treated protoplasts was substantially brighter than callus derived from pARS101-treated protoplasts as determined by a *t*-test statistic ($P = 0.0067$) and visually.

FIG. 5. Selection and regeneration of sweet orange plants transgenic for *gfp* and expressing GFP. All fluorescent photographs were viewed under blue light illumination. *A*, Protoplast electroporated with pARS101 and viewed 24 h later. *B*, Protoplast-derived callus (pARS108; 40 d old). *C*, Nontransformed control callus (6 mo. old). *D*, Transformed callus (pARS108; 6 mo. old). *E*, Transformed (pARS108) sweet orange plant (4 mo. old) micrografted onto nontransformed grapefruit rootstock and viewed under bright-field illumination. *F*, Transformed (pARS108) sweet orange plant (4 mo. old) micrografted onto nontransformed grapefruit rootstock viewed under light emitted from a mercury vapor lamp and filtered (exciter: 480 nm ± 20; dichroic: 495 nm long-pass; emission: 500 ± 40 band-pass).


A
B
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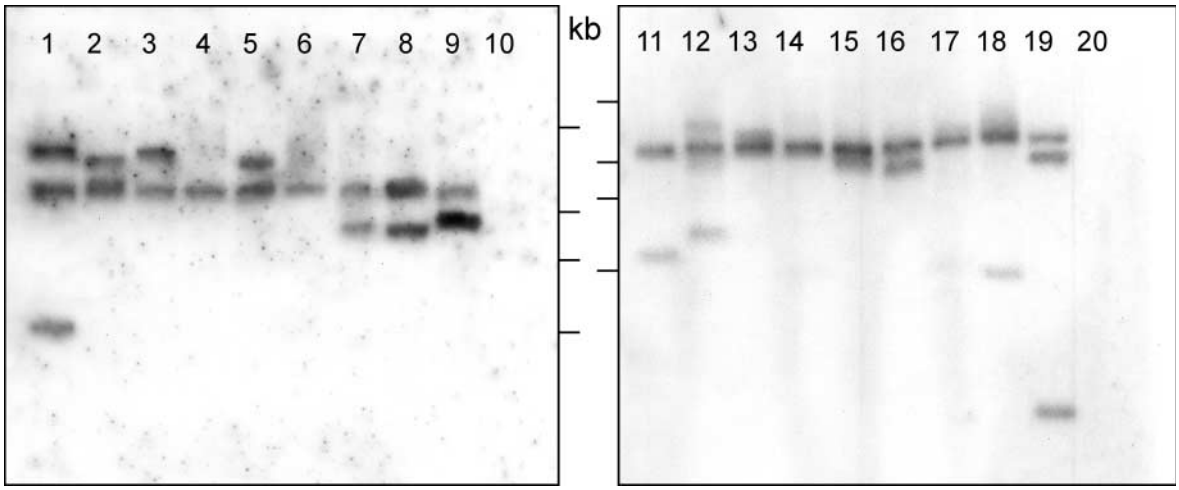

FIG. 6. Southern hybridization analysis of DNA from GFP-positive individuals transformed with pARS101 or ER-targeted GFP pARS108. Nine of 32 GFP-positive plants derived from protoplasts electroporated with pARS101 (*lanes 1–9*). Nine of 21 GFP-positive plants derived from protoplasts electroporated with pARS108 (*lanes 11–19*). No plasmid DNA electroporated controls (*lanes 10* and *20*). DNA (10 μg) digested with *Hin*dIII, blotted, and hybridized with the ^{32}P-labeled probe. *Marks* placed at 2, 3, 4, and 12 kb.

Regenerated shoots were grafted onto seedling rootstocks. Shoot-tip grafting has been used successfully to recover transgenic shoots regenerated from citrus epicotyls treated with *Agrobacterium* (Peña et al., 1995a, b, 1997; Bond and Roose, 1998; Cervera et al., 1998). Grafting of small shoots (< 1 cm) as they arise overcomes rooting difficulties that can occur (Moore et al., 1992). Rooting protoplast-derived sweet orange plants can take up to 3 mo. before roots emerge. Shoot-tip grafting greatly accelerates the recovery of plants (Fig. 5*E*). The double grafting technique reported by Peña et al. (1995a, b) further accelerates the development of the transgenic shoot. Of the 53 transgenic plants obtained from the two protoplast electroporation experiments, all were single-grafted and 26 have been double-grafted. The resulting transformation frequency was 1.38×10^{-3} and was calculated from $100 \times 53/(9\,600\,000$ total protoplasts electroporated $\times$ 0.5 viability $\times$ 0.8 PE).

There were distinct fluorescent differences between shoots transformed with the two constructs. Shoots transgenic for the ER-targeted EGFP (pARS108) were generally brighter than those derived from the nontargeted construct, and exhibited a uniform green fluorescence with minimal red fluorescence from chlorophyll (Fig. 5*F*). Haseloff et al. (1997) reported that they could consistently regenerate intensely fluorescent *Arabidopsis* plants when GFP was targeted to the ER. They also reported difficulty in regenerating plants from the brightest nontargeted transformants, something we did not observe in our citrus experiments. Other than the differences discussed above, development and regeneration responses appeared similar between plants transgenic for either pARS101 or pARS108.

Southern analysis confirmed the presence of the EGFP coding sequence and provided some information about its integration pattern. Eighteen green fluorescent cell lines derived from protoplasts treated with pARS101 or pARS108 were analyzed. Genomic DNA was digested with *Hin*dIII that cuts in a single site within the integrated plasmids (pARS101 and pARS108), therefore bands hybridizing to the probe result from one site within the vector and one site within the flanking genomic DNA. A relatively simple integration pattern of less than three copies of the gene in each of the transgenic lines was observed (Fig. 6).

The constructs used in these experiments utilized a strong constitutive promoter, and an altered codon usage to correct for aberrant splicing. The constructs also included targeting and retention sequences for cellular compartmentalization. Fluorescent plants were readily regenerated using both the nontargeted and targeted forms of EGFP. Though ER-targeting resulted in brighter fluorescence it was not required for selection. Features minimally required for use as a screenable marker cannot be answered from these results. For example, would a targeted EGFP under the control of a weaker promoter such as *nos* be suitable for selection? Other questions not addressed in this study included a determination of the correlation of fluorescence in the cell lines to fluorescence in the whole plants, the correlation between gene expression and fluorescence intensity, a comparison between pARS101- and pARS108-derived cells on the proportion of EGFP in the total protein complement, and further verification and elucidation of the cellular location of the nontargeted and targeted EGFP.

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