Transgenic Plant Journal ©2008 Global Science Books

Expression of Three Different Mutant Green Fluorescent Protein Genes in Transgenic Carrizo Citrange

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ABSTRACT

The expression of three different GFP mutants was studied in Carrizo citrange (*C. sinensis* [L.]) Osb. X *Poncirus trifoliata* [L.] Raf.) using strain AGL1 in *Agrobacterium tumefaciens*-mediated transformation. The localization of green fluorescent protein (GFP) expression in citrus tissue was compared with three different GFP mutants: *EGFP.1*, *GFPC3*, and *mGFP5-ER*. All three were driven by the CaMV35S promoter, but EGFP.1 and C3 gene cassettes have the double 35S promoter with an AMV enhancer sequence from the Alfalfa mosaic virus (AMV). Strong GFP expression was provided with the double 35S CaMV promoter and AMV enhancer in front of *EGFP* and *GFPC3* in transgenic citrus shoots. However, the brightest expression of GFP was observed in transgenic callus and shoots transformed by *GFPC3*. Most of the stable transgenic shoots survived when transformation was performed with *mGFP5-ER* for GFP expression. GFP expression in transgenic tissue was detected by stereo and confocal microscopy for three different GFP variants. The intensity of GFP fluorescence varied in transgenic plants regardless of the GFP variant. Sixty stable transgenic citrus shoots developed whole plants by *in vivo* shoot tip grafting. In the greenhouse, however, 50% of these transgenic plants were silenced for GFP expression. The presence of transgenes in both silenced and transgenic plants was verified by gene amplification and Southern analysis. GFP synthesis was also confirmed by Western blotting using GFP-PCA only in GFP-expressing shoots and plants.

Keywords: *Agrobacterium tumefaciens*, confocal microscopy, genetic transformation, GFP
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **AMV**, Alfalfa mosaic virus enhancer sequence; **BAP**, 6-benzylaminopurine; **CaMV 35S promoter**, Cauliflower mosaic virus 35S promoter; ***EGFP-1***, Encodes a red-shifted variant of wild-type green fluorescent; ***GFPC3***, modified GFP from DNA shuffling of the wild-type *GFP*; **GA_3**, gibberellic acid; ***mGFP5-ER***, modified GFP with an endoplasmic reticulum targeting sequence; medium; **MES**, Morpholinoetane sulfonic acid; **MS**, Murashige and Skoog (1962); **MSCC**, co-culture medium; **MSE**, shoot elongation medium; **MSI**, inoculation solution; **MSP-10M**, plasmolysis solution with 10% maltose; **MSP-8S**, plasmolysis solution with 8% sucrose; **MSR**, selection and regeneration medium; **NAA**, α-naphthalenacetic acid; ***NPT*II**, neomycin phosphotransferase II; **NOS-P**, nopaline synthase promoter; **NOS-T**, nopaline synthase terminator

INTRODUCTION

The green fluorescent protein (*GFP*) from a bioluminescent Jellyfish, *Aequorea victoria*, is an ideal visual marker gene when compared to the most widely used visual reporter marker, β-glucuronidase (*uid*A), which is destructive and needs substrate addition (Haseloff and Amos 1995). GFP has advantages over the *uid*A gene because it permits direct visual detection of transgene expression in the living plant with either a fluorescent microscope or a long wavelength hand-held UV lamp (Haseloff and Amos 1995). The expression of the *GFP* gene has been observed in a wide range of plant species including dicots and monocots, but most of the expression of GFP in the plants was transient until variant GFPs could be developed. The first transient expression was reported in sweet orange protoplasts (Niedz *et al.* 1995), followed by maize protoplasts (Hu and Cheng 1995), *Arabidopsis* tissue (Sheen *et al.* 1995), and tobacco (Reichel *et al.* 1996). However, stable expression of GFP was faint or not expressed at all.

Modifications of the wild type GFP sequence made it possible for stable transgenic plants to be produced. The cryptic intron has been altered in all variant GFPs, which will be used as a stable marker gene. Of those variants, Enhanced GFP (*EGFP*), a red-shifted GFP variant that contains the double-amino acid substitutions Phe-64 to Leu and Ser-65 to Thr, has been optimized for brighter fluorescence and higher expression in mammalian cells (Cormack 1996). Based on spectral analysis of equal amounts of soluble protein, *EGFP* fluoresces 35-fold more intensely than wt *GFP* when excited at 488 nm because of an increase in its extinction coefficient (Em) (Clontech). The coding sequence of the *EGFP* gene contains more than 190 silent base changes, which correspond to human codon-usage preferences (Yang *et al*. 1996). The cycle 3 GFP (*GFPC3*) is a modified GFP gene resulting from DNA shuffling of the native gene and selection for increased fluorescence in *E. coli* (Crameri *et al*. 1996). Compared with 238 amino acids in the wild type *GFP*, the *GFPC3* protein has 239, resulting from an insertion of an Ala at the N-terminus, in addition to two amino acid substitutions and 25 silent nt changes. The *mGFP5-ER* has an mutated nucleotides to improve folding of the apo-GFP during post-translational maturation (V163A, S175G), dual excitation peaks at 395 and 475 nm, and an emission peak at 509 nm (Siemering *et al*. 1996) to provide equalized UV and blue light excitation (I167T); peptide sequences were added to allow targeting of the protein to the lumen of the endoplasmic reticulum to reduce the toxicity of GFP in the cytoplasm of the plant cell (Haseloff *et al*. 1997). These modified *GFP* genes lead to express transient and stable bright green fluorescence in both monocot and dicot cells (Siemering *et al*. 1996; Yang *et al*. 1996; Haseloff *et al*. 1997).

As a visual marker, GFP currently has been used intensively in the genetic transformation of both monocot and dicotyledonous plant species such as wheat (*Triticum aesti-*

Received: 5 February, 2008. Accepted: 12 May, 2008.

Original Research Paper

vum L.), oat (*Avena sativa* L.), sugarcane (*Saccharum* L. hybrid) cv. 'Q117', maize (*Zea mays* L.), *Arabidopsis thaliana*, lettuce (*Lactuca sativa* L.), tobacco (*Nicotiana tabacum* L.) (Hu and Cheng 1995; Reichel *et al*. 1995; Sheen *et al*. 1995; Vander Geest and Petolino 1998; Elliott *et al.* 1999; Jordan 2000; Cho *et al.* 2003), and in a few trees such as *Citrus* spp. and papaya (*Carica papaya* L.) (Niedz *et al*. 1995; Ghorbel *et al*. 1999; Tian *et al*. 1999; Fleming *et al*. 2000; Zhu *et al*. 2006; Duan *et al*. 2007). The use of GFP in citrus transformation has more advantages than another most commonly used marker gene, GUS. Selection of GFP-expressing cell cluster, tissue or shoots is non-destructive because transformed selection is performed directly by visual observation under UV (390 nm) or microscope with equipped EGFP filter set (excitation 470/20 nm-emission 510/20 nm, dichromatic mirror 490 LP). However identification of GUS-expressing tissue needs fatal substrate application and this assay is time consuming and laborious since each cell cluster and shoot needs to be analyzed individually. GFP has the advantage in reducing the laborious and time-consuming analysis process in transgenic selection since there is a high frequency of regeneration of escapes in citrus (Ghorbel *et al*. 1999; Fleming *et al*. 2000; Kayim *et al*. 2004). Chimeric shoots regenerated from transformed epicotyl segments are also easily detected by GFP marker gene (Ghorbel *et al*. 1999). The intrinsic fluorescence of GFP allows monitoring to track the expression and location of proteins and other microstructures within organisms such as viruses, nematodes, and fungi (Baulcombe *et al*. 1995; Oparka *et al*. 1996; Plautz *et al*. 1996), and allows screening and monitoring somatic hybrids cells at an early developmental stage during somatic hybridization (Guo and Grosser 2005).

A limited number of transgenic citrus plants, expressing three different variant GFP genes, s*GFP* (Ghorbel *et al*. 1999), *EGFP.1* (Fleming *et al*. 2000; Omar and Grosser 2008) and mGFP5-ER (Duan *et al*. 2007; Omar and Grosser 2008) have been reported. The reason for the limitations of transgenic plant regeneration is either overexpression of GFP resulting in toxicity to cells (Haseloff and Amos 1995; Köhler 1998) or over expression of GFP interfering with regeneration of shoots (Haseloff and Siemering 1998). To prevent mild toxicity of high concentrations of GFP in the cytosol and nucleus of the cells, GFP was targeted to the ER (Haseloff and Siemering 1998). The expression of *EGFP-1* has been reported in "Hamlin" sweet orange (*Citrus sinensis* Osb. L.) protoplasts and protoplast-derived calli and embryo (Fleming *et al*. 2000), and the expression of *sGFP* has been studied in stem segments of Carrizo citrange (*C. sinensis* [L.]) Osb. X *Poncirus trifoliata* [L.] Raf.), Mexican lime (*Citrus aurantifolia* [Christm.] Swing.) and sour orange (*Citrus aurantium* L.) (Ghorbel *et al*. 1999). Recently, Duan *et al*. (2007) regenerated mgfp5-ER-expressing shoots from epicotyl segments of "Bing-tang" sweet orange (*C. sinensis* L. Osb.). However there is no detailed report on ER-localized GFP expression in citrus transgenic plant and no further information on GFP-expressing transgenic plants which have been kept for over a year in greenhouse conditions. In the present study, we report and compare the expression of three different GFP variants: *EGFP-1*, *GFPC3*, and *mGFP5-ER* in Carrizo citrange.

MATERIALS AND METHODS

Plant material and culture media

Carrizo citrange (*C. sinensis* [L.]) Osb. X *Poncirus trifoliata* [L.] Raf.) seeds were germinated *in vitro* and the cultures were maintained in darkness at 27 ± 1°C for 3-4 weeks prior to harvesting epicotyls, as described by Kayim *et al*. (2004). Seeds taken from fresh fruit were peeled to remove both seed coats. After the seed coats had been discarded, the seeds were surface sterilized for 10 min. each in 1% and 2% (v/v) sodium hypochlorite solutions containing 0.1% Tween-20, respectively, and rinsed 5 times with sterile distilled water. Two-to-three seeds were placed in 25 × 150 mm glass culture tubes containing 12 ml of MSG medium consisting of Murashige and Skoog (MS) inorganic salts and vitamins (Murashige and Skoog 1962), supplemented with 0.5 mg/l α-naphthaleneacetic acid (NAA), 500mg/l activated charcoal, 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma-Aldrich, Germany) (pH 5.7 ± 0.1). Protocols and media used for genetic transformation and regenerations were performed according to Kayim *et al*. (2004).

Two different plasmolysis solutions were used. One of them MSP-8S consisted of half-strength of MS salts and vitamins, 500 mg/l MES (morpholinoethane sulfonic acid), 8% (w/v) sucrose, pH 5.7. The second plasmolysis solution, MSP-10M consisted of MSP with 10% maltose instead of sucrose. Inoculation solution (MSI) consisted of half-strength of MS salts, MS vitamins, and 500 mg/l MES supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 μM acetosyringone and 3% (w/v) sucrose (pH 5.6). Co-culture medium (MSCC) consisted of MS medium containing 3.0 mg/l 6-benzylaminopurine (BAP), 0.5 mg/l NAA, and 0.9% Difco bacto agar (pH 5.6). Selection and regeneration medium (MSR) consisted of MSCC medium supplemented with 500 mg/l cefotaxime to inhibit further growth of bacteria and 50 mg/l kanamycin to select putative transgenic shoots. Shoot elongation medium (MSE) consisted of MS medium containing 1.0 mg/l BAP, 0.1 mg/l NAA, 0.3-mg/l gibberellic acid (GA_3), 300 mg/l cefotaxime, 50 mg/l kanamycin, 3% (w/v) sucrose, and 0.9 % Difco bacto agar (Becton Dickinson) (pH 5.7).

Plasmid vector constructions

We have constructed two different binary plasmids including GFP mutants. Only the binary plasmid, pBin-mGFP5-ER, was kindly provided by Dr. Ananthakrishnan (CREC, University of Florida) and was ready to use. Standard methods used for plasmid constructions and cloning were performed essentially as described by Sambrook and Russell (2001). A promotorless *EGFP* on the plasmid pEGFP-1 (Clontech, Palo Alto, CA, USA) cut with *Bam*HI and *Afl*II was ligated into the *Bam*HI/*Afl*II sites of pBI524 (kindly provided by Dr. Grosser, CREC, University of Florida) between the double 35S *Cauliflower mosaic virus* (35S-35S CaMV) promoter followed by the *Alfalfa mosaic virus* (AMV) enhancer sequence and NOS terminator. The ligated plasmid was designated pBI524EGFP-1. *EGFP-1* expression cassette was excised as an *Hind*III/*Eco*RI (Promega, Madison, USA) fragment and ligated into the same cuts in the binary plasmid pCAMBIA2200 (http:/www.cambia.org.au) vector. The ligated binary plasmid was referred as pC2200-524EGFP-1 (**Fig. 1A**). Two different GFPs (*EGFP-1*, *GFPC3*) were cloned into binary vector pC2200 for their delivery into Carrizo citrange.

GFPC3 (Crameri *et al*. 1996) was amplified as a 720 bp fragment from the plasmid p30B-GFPC3 (kindly provided by Dr. Shivprasad CREC, University of Florida in 2001) with designed primers including the *Bsp*HI/*Eco*RI sites. These primers were C3-BspHI-F (5′-CGATTCATGATGGCTAGCAAAGGAGAAG-3′) and C3-EcoRV-S (5′-CGATGATATCTTATTTGTAGAGCTCATC CAT-3′). The plasmid pBI524 was cut first with *Bam*HI, the 3′ terminus filled to make a blunt end, and then cut with *Nco*I. The amplified fragment of the GFPC3 was inserted between the double CaMV35S promoter with an enhancer, AMV, and NOS terminator at the blunt end and *Nco*I sites. The ligated plasmid was designated pBI524-GFPC3. The *GFPC3* gene cassette was removed by *Pst*I and *Eco*RI from pBI524-GFPC3 plasmid and inserted into the binary vector pCAMBIA2200 at the *Pst*I/*Eco*RI cut ends. This new binary plasmid was referred to as pC2200-524GFPC3 (**Fig. 1B**).

The *mGFP-ER* gene was under the control of a single CaMV 35S promoter with a NOS terminator. The Bin plasmid contained an antibiotic selectable marker gene (*nptII*, neomycine phosphotransferase II) as well as the pCambia2200 binary vectors. The binary vectors pC2200-524EGFP-1, pC2200-524GFPC3, and pBin-mGFP5-ER (**Fig. 1C**) were introduced into *Agrobacterium tumefaciens* AGL1 (Lazo *et al*. 1991) (kindly provided by Vicente Febres, University of Florida) by the $CaCI_2$ method according to Chen *et al*. (1994). Bacteria cultures were prepared at 2×10^7 cell/ml in MSI.

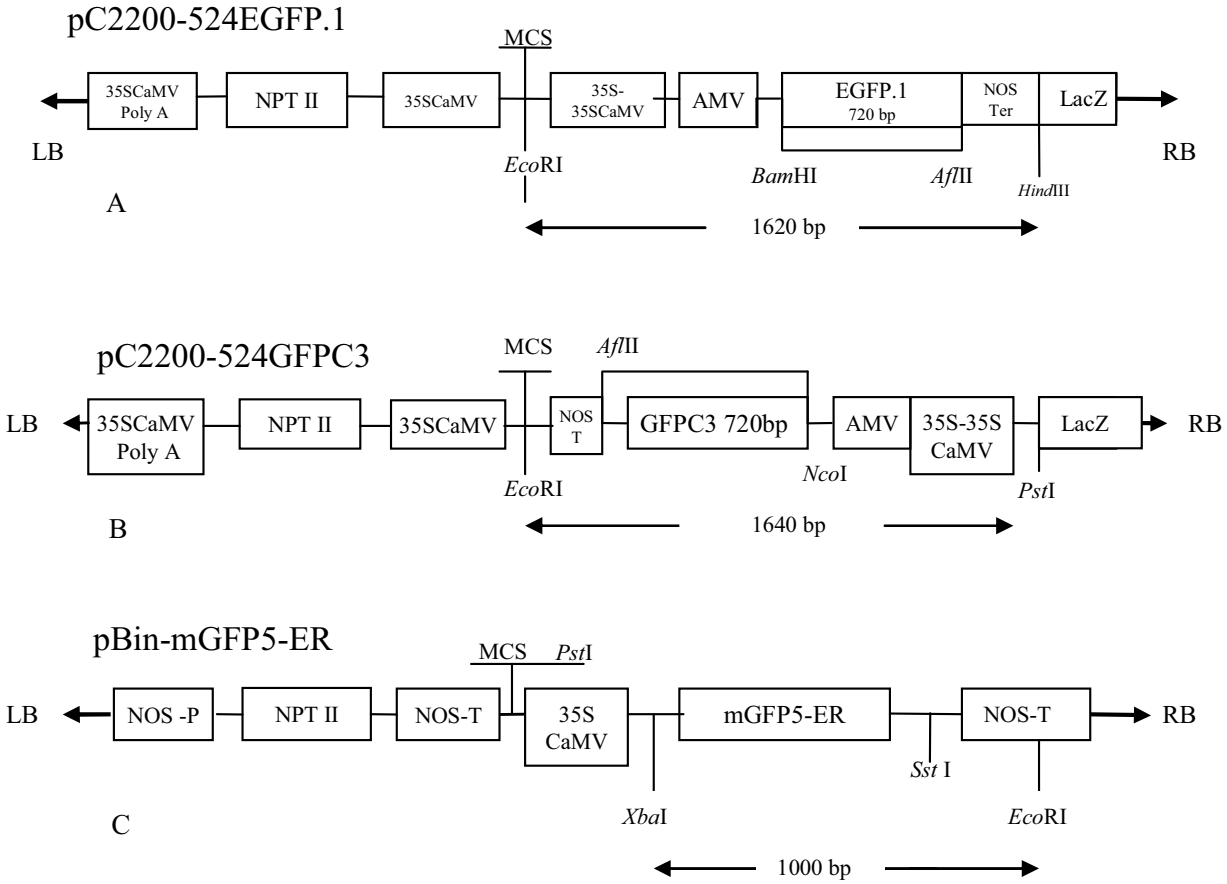

Fig. 1 Shematic diagram of mutant GFP expressing gene cassettes on the binary vectors. A promoterless EGFP cut with *Bam*HI/*Afl*II from pEGFP-1 (Clonthech) was ligated into the *Bam*HI/*Afl*II sites on the pBI524 between the double 35S *Cauliflower mosaic virus* (35S-35S CaMV) promoter followed by the *Alfalfa mosaic virus* (AMV) enhancer sequence and NOS terminator. EGFP-1 expression cassette was excised as a *Hind*III/*Eco*RI fragment and ligated into the same cuts on the binary plasmid pcambia2200 (**A**), GFPC3 was amplified as 720 bp fragment from the plasmid, p30B-GFPC3 with designed primers including *Bsp*HI/*Eco*RV sites. *Bam*HI-cut pBI524 was filled at the 3′ terminus and cut with *Nco*I. The amplified fragment of the GFPC3 was inserted into the cut pBI524 plasmid. The resulting GFPC3 gene cassette was removed by *Pst*I and *Eco*RI and inserted into the binary vector (**B**), and pBin-mGFP5-ER (**C**).

Transformation and plant regeneration

The transformation of longitudinally cut epicotyl segments and the selection and regeneration of transformed shoots were performed as described previously (Kayim *et al*. 2004; Kayim and Koc 2005). Briefly, epicotyl stems of Carrizo citrange were harvested from 3-weeks old *in vitro* grown seedlings and cut transversely into 1.5-2 cm length pieces; each piece then was cut into two halves longitudinally as two explants. To enhance the number of *Agrobacterium* cells enter the wounded tissue of epicotyl segments, the explants were dipped in 10 ml of plasmolysis solution, MSP-8S for 30 min before inoculation. The plasmolysed explants were dipped in MSI medium containing a cell suspension of *A. tumefaciens* for 10-15 min, blotted dry with sterile filter paper (309 grade, Watman Inc., Florham Park, NJ USA), and placed horizontally (cut surface upside) on solid MSCC medium for a 2 or 3-day co-cultivation period, depending on bacterial growth appearance around the explants. Following co-cultivation, explants were transferred to an MSR medium for selection. The cultures were maintained in darkness for 10 days at 27 ± 1°C until they started to regenerate shoots (Peña *et al*. 1997), followed by a continuous photoperiod. Two weeks later, epicotyl explants with shoots were transferred to MSE medium to elongate the shoots. Within 4-6 weeks, 0.6-1.3 cm long of transgenic shoots were regenerated from the cut surface of epicotyls. Regenerated shoots were harvested from epicotyl explants and shoot-tip grafted *in vivo* onto one-month-old Carrizo citrange seedlings (Kayim *et al*. 2004). The transformation frequency was evaluated as the total number of GFP^+ shoots per total number of *Agrobacterium*-inoculated explants × 100. The grafting of *in vivo* growing shoots on acclimated Carrizo citrange rootstocks allowed development of 4-6 expanded leaves of transgenic plants that could be analyzed within six months by PCR, Southern hybridization, and Western blotting.

Transgenic whole plant regeneration

Transgenic whole plants from Carrizo citrange were recovered by *in vivo* grafting of GFP^+ shoots. *In vivo* grafting of transgenic shoots was performed as described by Kayim *et al*. (2004). Three-four week-old *in vitro* grown Carrizo citrange plants were transferred to pots (15 × 15cm) including soil, turf and perlite mixture (1:1:1), one plant per pot and each pot covered with a plastic bag to prevent evaporation. One week later, acclimated seedlings were decapitated leaving 2-3 cm of the epicotyl, cut vertically 0.3-0.5 cm deep, and the cotyledons were removed. The stems of the transgenic shoots (apical portions ≥0.5) were cut into a V-shape, and then inserted into the vertical cut top of the decapitated Carrizo or sour orange seedlings and in contact with the vascular ring. Grafted plants were covered with plastic bags (one grafted plant/pot/plastic bag) and placed in the greenhouse (27 ± 2°C) until they started to grow. After approximately one month, the grafting plastic bags were removed, and the plants were irrigated by weekly and fertilized by liquid MS macro and micro elements (Murashige and Skoog 1962), and 21% superphosphate once a month. Transgenic plants were kept in the same conditions for another 2-3 months, and then moved to another greenhouse at 28-30°C. 5% Metadex bait was applied to each pot once a month for snails, but plants were not sprayed for flies since the greenhouse had an insect-proof screen.

GFP detection in shoots and whole plants

In order to detect GFP-expressing transgenic shoots, a Zeiss SV11 stereomicroscope equipped with a filter set (excitation filters BP 450-490nm; dichromatic mirror RKP 510; suppression filter LP 515) and computerized spot image was used. A confocal microscope (Leica TCS SL) was used to visualize and to detect the loca-

lization of expression of different mutant GFPs in the citrus cell and tissue used. A portable UV hand-lamp (365 nm) (UVP model B-100Ap 100W) and Nikon camera with an orange filter was also used to visualize the whole plants.

Molecular analyses of transgenic plants

PCR analysis

GFP^+ transgenic plants were screened for the presence of three different GFP marker genes (*EGFP.1*, *GFPC3*, *mGFP5-ER*) by PCR. Primers for the *EGFP.1*, *GFPC3*, and *mGFP5ER* genes were 5′-ATGGTGAGCAAGGGCGAGGA-3′ (GFP-F), 5′-TTACTTG TACAGCTCGTCC-3′ (GFP-R), 5′-cgattcatgATGGCTAGCAAA GGAGAAG-3′ (C3-*Bsp*HI-F), 5′-cgatgatatcTTATTTGTAGAGCT CATCCAT-3′ (C3-*Eco*RV-R), 5′-ATGAAGACTAATCTTTTTCT CT-3′ (mgfp5-F) and 5′-TTAAAGCTCATCATGTTTGTATA-3′ (mgfp5-R), respectively. DNA was extracted from leaves of transgenic and non-transgenic plants using a Qiagen DNeasy plant mini kit (Qiagen Val. CA. USA). A 720 bp of GFP DNA fragment was amplified by PCR (MJ Research PTC-100) using each primer set from all transgenic DNA templates. The template DNA was 50-100 ng, and the PCR cocktail consisted of primers (10 μM each; IDT, Inc. IA, USA), 3 mM Mg PCR buffer (Idaho Technology, Idaho Falls, ID), dNTPs (200 μM each; Idaho Technology) and *Taq* polymerase (1 U/0.2 μl; Promega, Madison, USA). The amplification program had one initial step of 94°C, 2 min., followed by 30 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 2 min; and one final 72°C elongation period of 5 min. PCR products were electrophorized on a 8% polyacrylamide gel (PAGE) and visualized with UV illumination (UXDT-47SL-15E, Fisher Scientific) after being stained with ethidium bromide (0.5 μg/ml).

Southern hybridization

Southern hybridization was performed for only transgenic plants expressing the mGFP-ER gene from Carrizo citrange. DNA was extracted from 1-2 g of leaves of transgenic and non-transgenic plants using DNAzol ES solution according to manufacturer's instructions (MRC, Inc. Cincinnati, Ohio) and purified using a DNeasy plant mini kit (Qiagen Val. CA. USA). Ten to fifteen micrograms of DNA was digested with *Bam*HI and *Afl*II for the *EGFP.1* transgene, *Bsp*HI and *Eco*RI for the *GFPC3* trangene, and *Xba*I *and Eco*RI for the mGFP5-ER transgene of transgenic plant DNAs based on GFP mutants on different the binary vectors (**Figs. 1A-C**). Gel electrophoresis, blotting to membrane, and hybridization using a DIG-labeled probe of the *EGFP.1*, *GFPC3* and *mGFP5-ER* genes were as described by Sambrook and Russell (2001).

Western blotting

Western blot analyses of proteins extracted from leaf tissues of transgenic plants, transgenic dead shoots, and silenced transgenic and non-transgenic plants were performed as described by Derrick *et al*. (1992) except for autoclaving of nitrocellulose membrane. Ten to twenty mg tissues from leaf of transgenic and non-transgenic shoots and plants were grinded with 100 μl phosphate-buffered saline (PBS) (Sambrook and Russell 2001) in 1.5 ml tubes using a plastic pestle. The remaining of the stages are the same protocol described by Derrick *et al*. (1992). GFP (FL) (Santa Cruz Biotechnology, Inc.), a rabbit polyclonal antibody was used at a 1:400 dilution.

RESULTS

We have tested the performance of GFP expression in transgenic citrus calli and shoots in the early stages of citrus transformation, and in plants under greenhouse conditions, using three different GFP mutants *EGFP.1*, *GFPC3*, and *mGFP5-ER*.

Comparison of GFP mutants at an early stage of stable transgenic calli and shoots and stability of GFP in whole plants

Longitudinally cut epicotyl explants of Carrizo citrange were co-cultivated with three different *A. tumefaciens*-plasmid combinations: AGL1 and pC2200-524EGFP-1, AGL1 and pC2200-p524C3, and AGL1 and pBin-mGFP5-ER (**Fig. 1A-C**). The explants were then transferred to shoot induction and selection medium to compare the stability of the fluorescent signal in transgenic calli and shoots. Two weeks in darkness after the transformation, calli initiated from the cambium tissue of longitudinally cut surface expressed the GFP signal under a stereo-microscope with 480 nm blue light. The intensity of GFP expression varied in calli and shoots depending on the used GFP variant. The *GFPC3* expressing callus on the cut surface of epicotyl segments was significantly brighter than the calli expressing *EGFP.1* and *mGFP5-ER* (**Fig. 1A-C**). At an early stage of transformation, all segments exhibited yellow autofluorescence in tissue on the cut surface, which is attributed to the cell wall emission (**Fig. 2D-E**) (Ghorbel *et al*. 1999) however no red autofluorescent cell clusters were observed because the transformed segments etiolated with no chlorophyll.

Within two months, fluorescent transgenic shoots from Carrizo were developed and selected from regenerated escapes under the fluorescent microscope (**Fig. 2D-F**). The *EGFP.1*, *GFPC3* expression in transgenic citrus cells, tissue, and young shoots was brighter than the expression of the *mGFP5-ER* gene in plant cell and tissue (**Fig. 2D-I**). Non-transgenic escapes appeared red because of autofluorescence of the chlorophyll (**Fig. 2F**: see red shoot on the right). Several chimeric transgenic shoots expressing GFP were also identified under the fluorescent microscope (**Fig. 2G-H**).

The localization of the three different GFPs expressed also was detected under the confocal microscope (**Fig. 2J-K**), and the localization of *mGFP5-ER* slightly differed from *EGFP.1* and *GFPC3*. The *mGFP5-ER* gene expression in the cell occurred within a line very close to the cell wall (**Fig. 2J**). This line is probably of the ER along the cell wall, but cytoplasmic-localized GFP expression was observed throughout the cytoplasm of the cell. *EGFP.1* such as cytoplasmic-localized GFP expression in root cells of Carrizo citrange was observed in whole cells as it diffused to cytoplasm (**Fig. 2K**), and other cell compartments such as vacuole, ER, and stoma in leaf tissue (**Fig. 3**). Since *EGFP.1* and *GFPC3* have a double 35S CaMV promoter with an AMV enhancer, shoots containing these genes were brighter at the early stages of shoot development (**Figs. 2A-B**) than whole plants (**Fig. 4**). Furthermore, in whole plants, GFP expression was brighter in the mid-vein of leaves, very young leaves, and in young roots more than in old leaves and other part of tissues (data not shown).

Regeneration of transgenic shoots and shoot-tip grafting

Transformation frequencies per explant were calculated as 1.81, 1.93, and 2.72 for Carrizo (**Table 1**) by scanning the transgenic shoots under a stereo-microscope with 480 nm blue light. In total, 211 transgenic shoots expressed the *EGFP.1*, *GFPC3* and *mGFP5-ER* out of 428 regenerated shoots. One hundred and thirty one transgenic shoots out of 180 that included the *mGFP5-ER* transgene, 50 transgenic shoots out of 120 that included the *E-GFP.1* transgene, and 30 transgenic shoots out of 128 that included the *GFPC3* transgene survived. Sixty transgenic shoots were selected from a total of 211 transgenic shoots to be grafted *in vivo* by shoot-tip and further analysis. Transgenic shoots were grafted onto Carrizo citrange rootstock. All grafted shoots developed into whole plants within 3 months. Molecular and serological analyses were performed on one year-old transgenic plants.

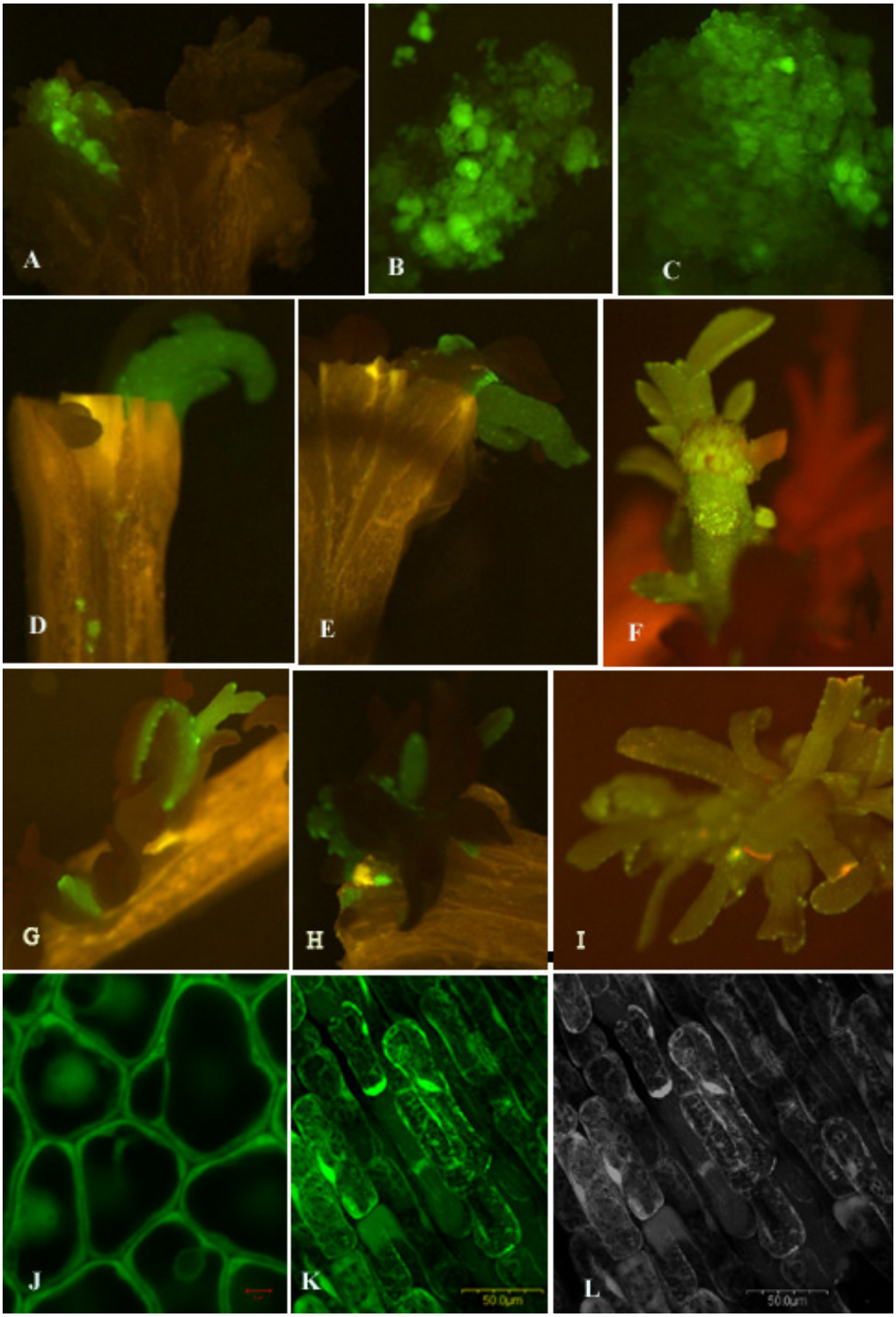

Fig. 2 The expression of three different gfp mutants in Carrizo citrange. GFPC3 expression in cell cluster of epicotyl segment (**A**), EGFP.1 expression in callus of epicotyl segment (**B**), mGFP5-ER expression in callus of epicotyl segment (**C**), GFPC3 expression in transgenic shoot from epicotyl segment (**D**), EGFP.1 expression in transgenic shoot from epicotyl segment (**E**), mGFP5-ER expression in transgenic shoot (**F**), GFPC3 expression in chimeric transgenic shoot (**G**), EGFP.1 expression in chimeric transgenic shoot (**H**), mGFP5-ER expression in transgenic shoot (**I**) under a Zeiss, SV11 stereomicroscope equipped with a filter set (excitation filters BP 450-490 nm; dichromatic mirror RKP 510; suppression filter 515) and spot image (red autoflorescence shows non transgenic shoots). Detection of localized mGFP5-ER expression in the cells of transgenic leaf of Carrizo citrange (**J**), localization of EGFP.1 expression in the cells of young root of Carrizo citrange (**K**) with a confocal microscope (TLC Leica) and root cells under normal light with a confocal microscope (**L**).

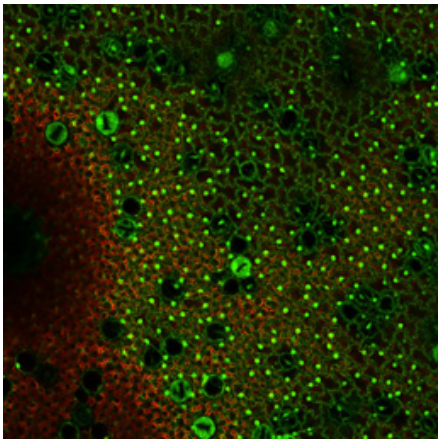

Fig. 3 Localization *of EGFP.1*-expression in the cells of young Carrizo citrange leaf with under the confocal microscope (TLC Leica). Photograph is digitized.

Fig. 4 Fluorescence emitted under UV illumination (366 nm) from transgenic Carrizo citrange expressing the *mGFP5ER* gene. Photograph is digitized.

Table 1 Effect of tree different GFP mutants on transformation efficiency of epicotyl segments of Carrizo citrange.

Plasmid/ *A. tumefaciens* combination	№ of GFP^+ explants / № of transformed explants	№ of GFP^+ buds / № of responding explants	Transformation frequency[a]	GFP^+ shoot regeneration
pCambia2200-EGFP.1/AGL1	49/66	120/49	1.81	50
pCambia2200-GFPC3/AGL1	45/66	128/45	1.93	30
pBin-mGFP5-ER/AGL1	60/66	180/60	2.72	131

a: Transformation frequency per explant was calculated as the total number of buds expressing gfp (GFP^+) / total number of explants evaluated

Stability of GFP expression in *in vitro* shoots and greenhouse plants

Three months after transformation, most of the transgenic shoots expressing high intensity of *EGFP.1* and *GFPC3* started to die *in vitro*. The number of dead transformed shoots increased when extending the period of sub-culture (4-6 months) of transgenic shoots expressing *EGFP.1* and *GFPC3* on the selection medium. But transgenic shoots expressing ER-localized mGFP5 grew faster and survived mostly *in vitro* on selection medium.

Sixty of transgenic plants adapted to soil in the greenhouse and continued to expression of GFP for 6 months (**Fig. 4**). Due to the GFP expression, none of the transgenic plants died; however, when plants reached 30-50 cm in height within one and half years, the ones with the GFPC3 and E.GFP.1 transgenes no longer showed GFP expression in the greenhouse when the plants were scanned by a hand-held UV lamp (365 nm, UVGL-58, UVP Upland, USA) and under a stereo-microscope with a 480 nm blue light filter and GFP was found to be silenced. Silenced plants grew as well as the transgenic plants expressing GFP. Approximately 50% of transgenic plants were silenced for GFP expression.

PCR and Southern analysis of transgenic plants

Before sampling the leaves for PCR and Southern analyses, whole plants were scanned by a hand-held UV lamp for fluorescent emission. Seven leaves from at least one year-old transgenic Carrizo plants were detached and analysed by PCR to test for the presence of the *EGFP.1*, *GFPC3*, and *mGFP5-ER* transgenes in their genome. All designated primers for each GFP mutants were the same size of the expected DNA fragment, i.e. 720 bp. This DNA fragment was amplified in all DNA samples from the green fluorescent leaves (**Fig. 5**). The DNA from the DNA samples of one year-old non-transgenic plant and regenerated escapes which emitted red autofluorescence under blue light was not amplified (**Fig. 5** Lane N).

Southern analysis was performed to confirm the stable integration of the *EGFP.1*, *GFPC3*, and *mGFP5-ER* gene cassettes in the genome of GFP-expressing and silenced transgenic plants. Digestion of DNA samples with *Bam*HI and *Afl*II, with *Bsp*HI and *Eco*RI, and with *Xba*I *and Eco*RI produced a 720 bp fragment corresponding to the *EGFP.1* and *GFPC3* (data not shown), and an approximately 1000 bp fragment corresponding *mGFP5-ER* (**Fig. 6**), respectively. In these plants, only the integration of transgenes was confirmed. The *mGFP5-ER* transgene integration into the plant genome was shown in one restriction pattern of DNA from *mGFP5-ER*-expressing transgenic plants. In order to demonstrate a certain size of the *mGFP5-ER* gene insertion into the transgenic plant genome, we digested DNA from six transgenic plants with *Xba*I and *Eco*RI (**Fig. 1C**). Southern hybridization of DNA from six transgenic plants released a 1000 bp DNA fragment, which included the *mGFP5-ER* gene with a NOS terminator. Lanes 1 and 4 included an additional 750 bp DNA fragment of lower molecular weight, suggesting that rearrangements may have occurred that affected the *mGFP5-ER* gene cassette. Lane 6 was DNA from a silenced transgenic plant, but released the expected size of DNA fragment as seen in DNA from non-silenced transgenic plants. As was expected, DNA from non-transgenic plant did not produce the expected size of DNA fragment by Southern hybridization.

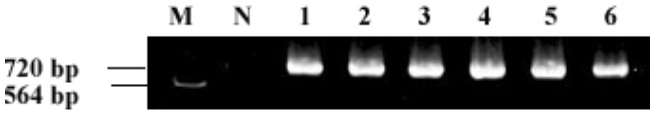

Fig. 5 PCR analysis of three different mutant GFP-expressing transgenic Carrizo citrange. N: Non-transgenic plant, 720 bp EGFP.1 (lanes 1-2), GFPC3 (lanes 3-4), and mGFP5ER (lanes 5-6) gene fragments in DNA samples of transgenic pants. M: Lambda DNA with *Hin*dIII. Photograph is digitized.

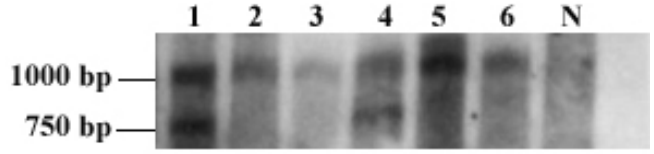

Fig. 6 Southern blot analysis of DNA isolated from transgenic Carrizo citrange plants transformed with pBin-mGFP5-ER. *Xba*I and *Eco*RI used for the digestion. The blot was hybridized with a DIG-labeled DNA probe for *mGFP5-ER*. N: Control DNA isolated from a non-transgenic plant; Lanes 1-6: transgenic plants expressing the *mGFP5-ER* gene. Photograph is digitized.

Western analysis of transgenic plants

Western blot analysis has proved the expression of GFP in all tested transgenic plants. Protein extracts were prepared from 1-year-old transgenic, non transgenic, silenced transgenic plants and dead transgenic shoots. GFP was detected in all tested eight individual transgenic plants tested, including three different GFP mutants using a polyclonal antibody of GFP. Control negative plant and dead transgenic shoot extracts did not show any protein expression. Intensity of green fluorescent emission varied among the individual transgenic plants depending on the version of the GFP expressed. However, the tissue expressing the brightest GFP did not show significant differences among the protein band expressed on the nitrocellulose membrane (**Fig. 7**). All living transgenic plant leaf tissues revealed strong immunoreactive bands, with an expected size of the protein, 28 kDa by Western blotting (**Fig. 7** Lanes 1-7, 12-14) except for silenced transgenic tissues and dead transgenic shoots (**Fig. 7** Lanes 8-11).

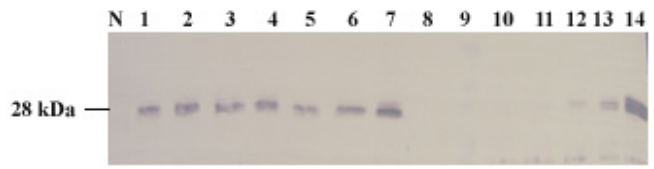

Fig. 7 Western immunoblot analysis of transgenic Carrizo citrange plant and shoot tissues with mutant GFP genes detected with GFP-specific polyclonal antibodies. N: Non-transgenic plant; Lanes 1-3: EGFP.1-expressing transgenic plants; Lanes 4-7: GFPC3-expressing transgenic plants; Lanes 8-9: Dead transgenic shoots; Lanes 10-11: Silenced transgenic plants; Lanes 12-14: mGFP5ER-expressing transgenic plants. Photograph is digitized.

DISCUSSION

The performance of GFP variants has not yet been compared in *Citrus* spp. so far. However some directed experiments have been performed with *EGFP* and (wild type) GFP (wt *GFP*) in citrus protoplasts and with *sGFP* in Carrizo, sour orange, and Mexican lime epicotyl segments (Hu and Cheng 1995; Fleming *et al*. 2000). In these experiments, green fluorescent protein was mainly under the control of the 35S CaMV promoter, the double 35S CaMV promoter sequence, the FMV promoter, and the double 35S CaMV promoter sequence, followed by the AMV-enhancer sequence (Fleming *et al*. 2000). It has been reported that all of wt *GFP* and *EGFP* constructs with the mentioned promoter sequences above gave strong transient expression of GFP in citrus protoplasts but none of them had stable expression at the colony or plant level. This lack of expression has been explained as the insufficient accumulation of protein for the detection in multicellular structures (Fleming *et al*. 2000). From the results obtained, it was suggested that strong GFP expression was critical to assure transformation identification at the calli and colony levels since citrus calli tissue sometimes autofluoresces yellow when illuminated with blue light. Therefore, Fleming *et al*. (2000) constructed an enhanced form of the protein *EGFP.1*, which is 35 times brighter than wild type GFP with the double 35S CaMV promoter plus AMV enhancer (pB524EGF.1), and introduced the EGFP.1 gene into protoplast of many sweet orange cultivars, including 'Itaborai', 'Succari', 'Early Gold', and 'Hamlin' as well as 'Murcott' mandarin hybrid and many citrus species (Data not reported). They have been successful in expressing the *EGFP.1* at the protoplast level with a transformation efficiency ranging from 5 to 15%, depending on the culture cycle stage and cultivar of the culture used as source of protoplast (Fleming *et al*. 2000). They evaluated the percentage of transient transformation frequency of GFP-expressing protoplast in 100 treated protoplasts. On the other hand Ghorbel *et al*. (1999) used the *SGFP* under the control of 35S CaMV promoter sequence to transform epicotyl segments of three different citrus species; Carrizo, sour orange, and Mexican lime. Ghorbel *et al*. (1999) gave the results from Mexican lime epicotyl segments with the successful stable expression of *SGFP* in shoots with 43% transformation efficiency. Their calculation was based on the number of GFP-positive shoots per total number of regenerated shoots × 100.

Here we compared the expression of three different GFP mutants mainly in Carrizo citrange. In these experiments, *EGFP.1* and *GFPC3* have been derived from the double 35S CaMV promoter plus AMV enhancer to enhance the GFP expression in transgenic cells, tissue, or shoots. But *mGFP5-ER* has not been modified by the double 35S CaMV promoter. It was derived by a single 35S CaMV promoter. This comparison is the first report in Carrizo citrange using three different GFPs to detect the most stable version of GFP in shoots and whole transgenic plants under greenhouse conditions. The pBI524-based GFP constructions with the double 35S CaMV promoter followed by AMV enhancer tested produced very bright GFP expression in cells and shoots of the epicotyl segments of three different citrus species. The high expression of GFP (*EGFP.1* and *GFPC3*) in the cell or calli stage was stable and most transgenic cells and calli remained alive for a long time by culturing of epicotyl explants on selection medium. The color of fluorescence of *EGFP.1* in the transgenic cells and shoots was a bright dark green. But a transgenic cell cluster expressing *GFPC3* was brighter than those transgenic expressing *EGFP.1* and *mGFP5-ER*. There was no difference in color or intensity of the fluorescence emission between *in vitro* transgenic shoots expressing *EGFP.1* and *GFPC3*. This is probably due to the double 35S promoter with AMV enhancer, which causes over-expression of GFP, since all tested GFP versions here had enhanced GFP expression. *GFPC3* is also an enhanced version of a protein expressed strongly when fused to the viral vector (Shivprasad *et al*. 1999). This version of *GFPC3* was first used in citrus transformation to produce transgenic Carrizo, sour orange, and Cleopatra mandarin plants after the infection of *Nicotiana benthamiana* leaves with TMV-based vector, 30B-*GFPC3*, to increase the level of transient GFP production (Shivprasad *et al*. 1999). The expression of GFP with the *GFPC3* in transgenic citrus calli and shoots was very bright green and strong as was reported in *N. benthamiana* by Shivprasad *et al*. (1999). Shivprasad *et al*. (1999) constructed several TMV hybrid vectors producing GFP to compare fluorescence of the wild type GFP and GFPC3 *in N. benthamiana* and inoculated plants with the viral vectors of 30B-GFP and 30B-GFPC3 separately. They found the 30B-GFPC3 infected plant was brighter green homogenously and had more soluble protein respectively than the plant infected with 30B-GFP by under UV illumination and western blot analysis. When some of these *GFPC3* expressing Carrizo cell clusters turned to embryo and shoot stages started to die or silenced for the GFP expression**.** A similar effect of GFP was previously observed in transgenic *Arabidopsis* cells by Haseloff and Amos (1995) who reported that the brightest transformants failed to regenerate fertile plants although the cells expressing highly fluorescent calli and masses of shoots survived after several months of culture. It was concluded that high levels of GFP expression can cause mild phytotoxicity, and that the regeneration of fertile plants is diminished in highly expressing GFP transformants of tobacco and *Arabidopsis.* Similarly, under natural conditions in jellyfish photocytes, where high levels of GFP are tolerated, the protein is found to be sequestered in microbody-like lumisomes. In contrast, the mature protein was found throughout the cytoplasm and nucleoplasm in transformed Arabidopsis by Haseloff and Amos (1995). Besides, Köhler (1998) reported that GFP alone did not penetrate membranes, but that GFP enters the nucleus by diffusion through the nuclear pore complex and is observable in the nucleus and cytosol of plant cells. Moreover GFP can be excluded from all compartments that are enclosed in membranes such as vacuoles and plastids without targeting signal peptides or mitochondria and ER when GFP was fused with targeting signal peptides (Köhler 1998).

Stewart (2001) thoroughly reviewed the utility of GFP variants in transgenic plants and pointed out that the evidence of GFP expression in plants was not cytotoxic. In our experiments, we found that dead transgenic shoots only occurred when they expressed high GFP by *EGFP.1* and *GFPC3* genes. A similar result has been reported by Haseloff and Amos in transgenic Arabidopsis cells (Haseloff and Amos 1995). We did not see any dead transgenic shoots expressing GFP directing the ER (*mGFP5-ER*) during *in vitro* selection culture. This may, however, be related to the amount of GFP protein expressed in the cell, which may cause a toxic effect, since GFP expression of *EGFP.1* and *GFPC3* gene cassettes in pBI524 was increased by adding an AMV enhancer and double 35S promoter. GFP expression in transgenic plants that included those genes was higher and brighter than the other transgenic shoots including *mGFP5-ER* (**Fig. 2B**). In shoots and plants where high GFP expression occurred, several tannins appeared under the confocal microscope (**Fig. 3**). The cause of death of shoots is probably due to of a high tannin contents that precipitates proteins. But transgenic shoots expressing *mGFP5-ER* was yellowish green. This is probably related to enveloping of protein accumulation by ER in the cell. On the other hand, one-year-old grafted transgenic whole plants did not show any difference in fluorescent color amongst the transgenic plants based in any of the GFP versions. All living and non-silenced transgenic plants including either *EGFP.1* and *GFPC3* or *mGFP5-ER* expressed GFP at normal levels based on fluorescence emission under a hand-held UV, stereo-and confocal microscopes. However, GFP expression of three GFP mutants in young shoots and mid-vein of leaves was stronger and brighter than the other part of transgenic plants. Parallel to this research, Harper and Stewart (2000) found that GFP synthesis was higher in vas-

cular tissue (mid-vein), younger leaves of transgenic tobacco plants than older leaves by Western blot analysis.

We succeeded in the production of 30 stable transgenic Carrizo plants expressing three different versions of GFP mutants under greenhouse conditions. The high number of regenerated transgenic shoots was not important to us since a given number of transgenic plants were enough for the evaluation of stability of GFP expression during the growth of plants in the greenhouse. Therefore, we grafted a total of 60 shoots onto rootstock for the evaluation of GFP stability in the plant. The overall transformation frequency per explants was 1, which corresponds to 100 % transformation frequency. We have also produced a few transgenic shoots expressing *EGFP.1* and *mGFP5-ER* from sour orange and Cleopatra mandarin, but with no further development of transgenic plants (data not shown). Sour orange has been reported as an intractable species for transformation by Ghorbel *et al*. (1999). But this is still related to optimization of regeneration and transformation since Carrizo has a good potential for regeneration on the medium used here. Chimeras which were observed in the three different GFP mutants used here, were also reported previously by Ghorbel *et al*. (1999) and reported by using GUS as a visual marker gene by Peña *et al*. (1997) and Kayim *et al*. (2004, 2005). We also found that the three GFP mutants were strong determinants for chimeras amongst the transformed shoots and escapees (**Fig. 1G-H**). Chimeric shoots included both GFP-expressing transgenic leaves (bright green) and non-transgenic leaves appeared in red in the same shoot. GFP might have a greater more advantage in detection of chimeras than GUS, since there is no false positive or negative reaction based on substrate penetration of tissue. Besides, when transgenic shoots were grafted onto rootstock and adapted to soil and greenhouse conditions none of the transgenic plants expressing any of *EGFP.1*, *GFPC3* or *mGFP5-ER* died. There was a contradiction between shoot and plants in the stability of GFP expression. While shoots with high expression of GFP died, whole plants that survived were mostly silenced based on visual observation of GFP under a fluorescent light and molecular analysis by PCR and Southern blot. The cause of silencing of the GFP gene has not been tested in detail. The amount of GFP content in shoots is probably of critical importance in remaining alive and whole plants expressing GFP may tolerate or silence the gene when toxic amounts of protein are expressed.

Consequently, the construct of a *GFPC3* binary vector may be useful for at the cell level of GFP expression, as it is expressing a strongly bright green. But the production of stable transgenic plants with GFPC3 may not be the best choice because of the high number of silenced plants that emerged. Consequently, we can conclude that *EGFP.1* and *GFPC3* with the single 35S CaMV promoter sequence without the AMV enhancer can be useful for the epicotyl explant transformation to produce stable transgenic plants as seen with the *mGFP5-ER* construct. However, we found that the mGFP5-ER is the most stable visual marker gene in transgenic Carrizo citrange plants under greenhouse conditions. Duan *et al*. (2007) were also successfully to regenerated twelve stable transgenic "Bingtang" sweet orange (*Citrus sinensis* L.) plants harboring the mGFP5-ER gene. The versions of GFP, we constructed here might be useful for protoplast transformation to increase the protein expression in cells and to maintain the stability of protein at the cell level in plants. But we found that high protein expression of GFP transgenes in shoots was somehow cytotoxic. However GFP constructs that have been built here that could be useful for protoplast transformation and protoplast fusion to select somatic hybrids (Guo and Grosser 2005) or a viral vector system.

ACKNOWLEDGEMENTS

We thank Drs. Grosser, Shivprasad and Ananthakrishnan for providing pBI524 and 30B-GFPC3 plasmids and pBin-mGFP5ER binary vector respectively and John L. Cook for editorial assistance. This project was supported in part by the Florida Citrus Production Research Advisory Council.

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