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Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants

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Abstract The green fluorescent protein (GFP) from *Aequorea victoria* has been introduced into three different citrus genotypes [*Citrus aurantium* L., *C. aurantifolia* (Christm.) Swing. and *C. sinensis* L. Osbeck × *Poncirus trifoliata* (L.) Raf.] which are considered recalcitrant to transformation, mainly due to low transformation frequencies and to the regeneration of escape shoots at high frequencies from the *Agrobacterium*-inoculated explants. High-level GFP expression was detected in transgenic cells, tissues and plants. Using GFP as a vital marker has allowed us to localize the sites of transgene expression in specific cells, always occurring in callus tissue formed from the cambium of the cut ends of explants. Whereas green fluorescent shoots regenerated in all cases from this callus, most escapes regenerated directly from explants with almost no callus formation. Thus, development of callus from cambium is a prerequisite for citrus transformation. Furthermore, in vivo monitoring of GFP expression permitted a rapid and easy discrimination of transgenic and escape shoots. The selection of transgenic shoots could be easily favored by eliminating the escapes and/or by performing shoot-tip grafting of the transgenic buds soon after their origin. GFP-expressing shoots have also been observed in citrus explants cocultivated with *Agrobacterium* but cultured in a medium without the selective agent kanamycin. This opens the possibility to rescue the transgenic sectors and to regenerate transgenic plants without using selectable marker genes conferring antibiotic or herbicide resistance, which is currently a topic of much discussion for the commercialization of transgenic plants.

Key words Citrus · Genetic transformation · Green fluorescent protein · Woody fruit plants

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Introduction

The selection of genetic transformants requires the use of marker genes that function as reporters of gene expression and so permit the recovery of transformed plants. Since the first demonstration of the green fluorescent protein gene (*gfp*) from the jellyfish *Aequorea victoria* as a vital marker gene in both bacteria and *Caenorhabditis elegans* (Chalfie et al. 1994), it has attracted increasing interest and is considered to have several advantages over other visual marker genes. The detection of commonly used reporters, such as β-glucuronidase (GUS) (Jefferson 1987), β-galactosidase (LacZ) (Helmer et al. 1984) and luciferase (LUC) (Millar et al. 1992), requires either exogenous substrates or co-factors. Their applications are limited by problems in substrate penetration, and the assays are generally destructive. In contrast, the fluorescence emission of GFP only requires the excitation of living cells by UV or blue light, which results from an internal p-hydroxybenzylideneimidazolinone fluorophore generated by an autocatalytic cyclisation and oxidation of a Ser-Tyr-Gly sequence at amino-acid residues 65–67 (Cody et al. 1993; Heim et al. 1994).

The use of the GFP marker protein has been described in various organisms, including bacteria, yeast, worm, fly and mammals (Chalfie et al. 1994, Heim et al. 1994, 1995; Wang and Hazelrigg 1994; Delagrave et al. 1995; Rosario et al. 1995; Takada et al. 1997). In plants, GFP has also been expressed in several species, both dicots and monocots. Transient expression was observed in electroporated protoplasts of maize (Hu and

Cheng 1995; Sheen et al. 1995), sweet orange (Niedz et al. 1995), and tobacco (Reichel et al. 1996), as well as in bombarded intact tissue of *Arabidopsis* (Sheen et al. 1995) and conifers (Tian et al. 1997). GFP was also detected following virus-mediated gene expression in tobacco mosaic virus-GFP and potato virus X-GFP-infected tobacco leaves (Baulcombe et al. 1995; Heinlein et al. 1995).

In spite of the successful transient expression of the wild-type GFP in plants, its expression in stably transformed plants has typically yielded faint or no green fluorescence. To improve expression, modifications of the *gfp* gene have been described. A mutation which results in an exchange of the amino-acid serine at position 65 to threonine dramatically increased the intensity of green fluorescence (Heim et al. 1995). Haseloff and Amos (1995) have demonstrated that the expression of the *gfp* cDNA is curtailed by aberrant mRNA splicing in *Arabidopsis*. Alteration of the codon usage of *gfp* to avoid recognition of the cryptic intron has resulted in efficient screening of stably transformed rice plants (Vain et al. 1998). Targetting of this GFP protein to the endoplasmic reticulum has improved its expression in stably transformed *Arabidopsis* plants (Haseloff et al. 1997). A re-engineered *gfp* gene sequence (s*gfp*) with the favored codons of highly expressed human proteins, designed to encode a peptide sequence identical to wild-type protein, combined with replacement of the serine at position 65 with a threonine, has resulted in high GFP expression in maize, tobacco, onion and *Arabidopsis* cells as well as in stably transformed tobacco plants (Chiu et al. 1996). Other synthetic *gfp* gene versions have improved the green fluorescence of the protein in transgenic maize, wheat, tobacco and *Arabidopsis* plants (Pang et al. 1996; Rouwendal et al. 1997; Van der Geest and Mandolino 1998). Recently, more soluble versions of the codon-modified *gfp*, including red-shifted and blue-fluorescent spectral variants, have been synthesized (Davis and Vierstra 1998).

Genetic transformation of woody fruit plants is a promising tool for their genetic improvement, since their breeding has limitations imposed in general by their high heterozygosity, long juvenile periods and auto-incompatibility. However, in procedures for the production of transgenic woody fruit plants, using *npt*II as a selectable marker gene and in most cases also *uidA* as a reporter marker gene, a high-frequency regeneration of escapes (from about 40% to more than 90%) has been reported for apple (James et al. 1989), pear (Mourgues et al. 1996), banana (May et al. 1995) and grapevine (Perl et al. 1996), as well as for citrange (Moore et al. 1992; Peña et al. 1995 a), sweet orange (Peña et al. 1995 b; Cervera et al. 1998 a) and lime (Peña et al. 1997) among citrus. To select the transgenic shoots or embryos, all the regenerated ones had to be analyzed, which is a very laborious, time- and space-consuming process. The goal of the present study is to investigate the value of *gfp* as a reporter marker gene for localizing the transgenic events and for improving their selection and thus the recovery of woody fruit transgenic plants. Furthermore, the effect of GFP in stable transformed woody species is unknown. We have chosen citrus plants for this study because of their economic importance since they are the most extensively grown fruit crop worldwide (FAO 1997), and because of their recalcitrance to transformation (Moore et al. 1992; Peña et al. 1995 a,b, 1997; Cervera et al. 1998 a).

Materials and methods

Plant material and culture media

Three different citrus genotypes were employed: Carrizo citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], Mexican lime [*Citrus aurantifolia* (Christm.) Swing.] and sour orange (*C. aurantium* L.).

Twelve-month-old greenhouse-grown Mexican lime and sour orange seedlings and 5-week-old Carrizo citrange seedlings, germinated and grown in vitro as described in Peña et al. (1995 a), were used as a source of tissue for transformation. Stem pieces (20-cm long) of Mexican lime and sour orange were stripped of leaves and thorns, disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile water.

Co-cultivation medium (CM) consisted of the plant cell-culture salt solution (MS) of Murashige and Skoog (1962), together with 0.2 mg/l thiamin hydrochloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid and 3% (w/v) sucrose. Shoot regeneration medium (SRM) consisted of CM plus 3 mg/l of benzylaminopurine for Carrizo citrange and 1 mg/l of benzylaminopurine for Mexican lime and sour orange, and was also supplemented with 10 g/l of agar and 100 mg/l of kanamycin for the selection of transgenic shoots and 250 mg/l of vancomycin and 500 mg/l of cefotaxime to control bacterial growth.

Tomato feeder plates were prepared by pipetting 2 ml of a 6–7-day old tomato cell suspension on the surface of 25 ml of tomato cell-suspension (TCS) medium, with sterile Whatman 5 filter paper on top, in 10 × 1.5 cm (diameter × height) plates. TCS medium consisted of MS salts, 1 mg/l of thiamine hydrochloride, 1 mg/l of pyridoxine hydrochloride, 1 mg/l of nicotinic acid, 3% (w/v) sucrose, 2 mg/l of indole-3-acetic acid, 1 mg/l of 2-isopentenyl-adenine, 2 mg/l of 2,4-dichlorophenoxyacetic acid, and 8 g/l of agar (Peña et al. 1997). Tomato cell suspensions were maintained in TCS liquid medium in a shaker at 100 rpm and 25°C and were subcultured to fresh medium every 15 days. In all media the pH was set at 5.7.

Bacterial strain and vector

Agrobacterium tumefaciens EHA105 (Hood et al. 1993) containing the binary plasmid pBin 19-s*gfp* was used as the vector system for transformation. Its T-DNA contains the **Nos**pro-*npt*II-**Nos**ter cassette as a selectable marker and the 35Spro-s*gfp*-35Ster cassette. This plasmid was kindly provided by Dr. Jen Sheen (Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, USA). The creation of this s*gfp* gene version is reported in Chiu et al. (1996). *A. tumefaciens* EHA105 is a disarmed derivative of *A. tumefaciens* A281 (Hood et al. 1993), which is super-virulent in citrus (Cervera et al. 1998 b). Bacteria were cultured overnight in an orbital shaker at

28°C and 200 rpm in LB medium (Sambrook et al. 1989) containing 25 mg/l of kanamycin and 25 mg/l of nalidixic acid. Bacterial cells were pelleted at 3500 rpm for 10 min, re-suspended and diluted to 4×10^7 cells/ml in CM.

Transformation and regeneration

Mexican lime and sour orange internodal stem segments and Carrizo citrange epicotyl segments (about 1-cm long in all cases) were cut transversely and incubated for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension in CM with gentle shaking. The infected explants were blotted dry on sterile filter paper and placed horizontally on tomato feeder plates for a 3-day co-cultivation period. After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to SRM. The cultures were maintained in the dark for 30 days at 26°C and then transferred to a 16-h photoperiod, with 45 $\mu Em^{-2}s^{-1}$ illumination, and 60% RH at 26°C. The explants were subcultured to fresh medium every 4 weeks.

The regeneration process of citrus transgenic plants involved several steps: (1) putative transgenic regenerated shoots from 0.1 to 0.8 cm in height were harvested from the cut ends of the explants; (2) they then were shoot-tip grafted in vitro on Troyer citrange seedlings (Navarro 1992), as previously described (Peña et al. 1995 a, b); (3) 2–4 weeks after shoot-tip grafting, scions had developed several leaves and one of them was PCR-analyzed; (4) putative transgenic plantlets were again grafted in the greenhouse on 5-month-old seedlings of Rough lemon (*C. jambhiri* Lush.).

PCR and Southern analyses

Standard PCR techniques were employed to detect the presence of the s*gfp* gene in leaf samples from the regenerated putative transgenic plantlets. The s*gfp* primers used were 5′-ATGGTGAG-CCAAGGGCGAGGA-3′ (nucleotides 1–20) and 5′-GGACCAT-GTGATCGCGCTTC-3′ (nucleotides 668–688). Reactions were performed in 50 µl containing 1 µl of DNA, 200 µM dNTPs, 0.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 0.25 µM of each primer and 2 u of *Taq* DNA polymerase. Reactions were subjected to 35 cycles of 0.5 min at 95°C, 0.5 min at 60°C and 1 min at 72°C. Amplified DNA was detected by ultraviolet light after electrophoresis of the amplification reactions for each plant sample on 2% (w/v) agarose-ethidium bromide gels.

Southern analyses were performed to confirm the stable integration of the s*gfp* gene in the transgenic plants. DNA was isolated from leaves according to Dellaporta et al. (1983). DNA samples (20 µg) were digested with *Eco*RI and *Hin*dIII or with *Bam*HI, separated on 1% (w/v) agarose gels and blotted to nylon membranes (Hybond-N^+, Amersham). Filters were probed with a digoxigenine (Boehringer-Mannheim)-labelled fragment of the coding region of the s*gfp* gene prepared by PCR following the supplier's instructions.

Detection of GFP by fluorescence stereomicroscopy

Once transferred to SRM, explants were examined periodically under a fluorescence stereomicroscope equipped with a Leica Fluorescence Module comprising a 480/40 nm exciter filter, a 505 nm LP dichromatic beam splitter and a 510 nm LP barrier filter. The light source was provided by a HBO 50 W high-pressure mercury bulb. The red autofluorescence from chlorophyll was not blocked with any interference filter. Photographs were taken using Kodak Ektachrome 400 film. The optimal exposure time was 2–4 min.

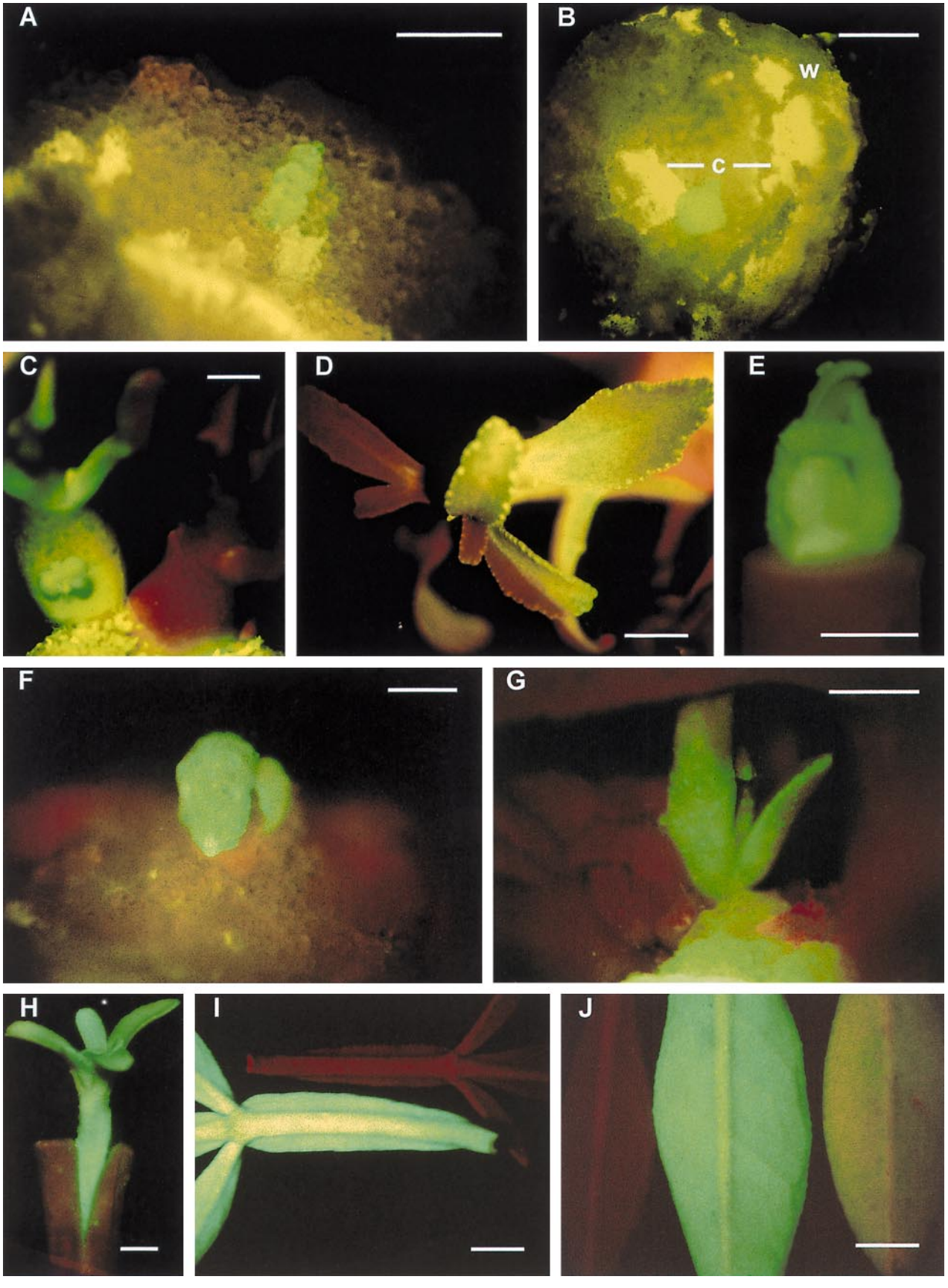
Results and discussion

GFP as a vital marker to localize the sites of transgene expression at early stages after transformation

Epicotyl segments from Carrizo citrange and internodal stem segments from lime and sour orange were co-cultivated with *A. tumefaciens* EHA 105 carrying the binary vector pBin 19-s*gfp* and transferred to shoot-induction and selective medium. After 1month in darkness, the explants developed a callus at the cut ends, which was formed from the cambium tissue. When this callus was examined under a stereomicroscope with 480 nm-excited blue light, it exhibited yellow autofluorescence, probably emitted by the cell walls. In some callus tissue, red autofluorescent cell clusters were also evident. Since chlorophyll fluoresces red at the wavelength used for GFP excitation, these clusters could be considered non-transformed cells initiating shoot regeneration. Green fluorescent sectors were also observed that were clearly distinguishable from the red and yellow ones (Fig. 1 A). Green fluorescent cell clusters corresponded to transgenic events. As each GFP molecule represents one fluorophore, high-level expression was detected in these cells. Furthermore, continuous monitoring of transformation events led us to observe that they always occurred in callus tissue formed from the cambium in all three citrus genotypes analyzed (Fig. 1 B).

Sour orange is a particularly recalcitrant-to-transformation species among citrus genotypes. The production of transgenic plants is rare and circumstantial. Only one or two transformants can be regenerated from thousands of explants (Gutiérrez et al. 1997; our unpublished results). In many recalcitrant plants, cells competent for transformation are restricted to specific

Fig. 1A–J Green fluorescent protein (GFP) expression in transgenic citrus cell clusters, buds, shoots and leaves observed under an stereomicroscope with 480 nm-excited blue light. **(A)** Development of a callus at the cut end of an explant. The green fluorescent cell cluster corresponds to a transgenic event and the red autofluorescent cell cluster is considered to be non-transformed cells initiating shoot regeneration. **(B)** Upper view of an explant showing a green fluorescent transgenic event at the callus formed from the cambium (*c*). No transformation is observed in the wound-response callus (*w*). **(C)** Shoots regenerating from an explant. A transgenic shoot shows bright green fluorescence whereas an escape clearly appears red. **(D)** Sectorial expression of GFP in leaves from a chimeric regenerated shoot. **(E)** Shoot-tip grafting of a transgenic scion (*green*) on a non-transformed decapitated seedling rootstock (*red*). **(F)** Visualization of a regenerating 0.1-cm-long transgenic bud. **(G)** A transgenic regenerated bud (*green*) and several escape shoots and buds (*red*) in a transformed explant cultured in a medium without kanamycin. **(H)** Production of a transgenic plantlet after successful shoot-tip grafting. **(I)** Leaves from a transgenic plant (*green*) and from a non-transformed control plant (*red*). **(J)** Different levels of GFP expression in central leaflets from two independent transgenic lines (*center and right*) and a non-transformed control line (*left*). Bar = 1 mm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tissues (Potrykus 1991). To develop transformation procedures in these cases, it is important to localize the sites of transgene expression in order to favor the regeneration of whole plants from such competent cells. When *uidA* is used as a reporter gene, the diffusion of the GUS indigo dye reaction, the possibility of the presence of bacteria expressing the *uidA* gene, the possible contact among different transformation events and the destructive character of GUS assays all contribute to the imprecise localization of cells or tissues competent for transformation. GFP provides the possibility to perform in vivo monitoring of *Agrobacterium*-inoculated plant tissues and thus allowed us to localize competent cells for transformation in de-differentiated callus from the cambium tissue of citrus explants. Therefore, treatments favoring the development of such callus tissue would be necessary to increase transformation frequencies and to be able to more efficiently recover citrus transgenic plants. These type of experiments are underway in our laboratory.

GFP as a vital marker to control the regeneration of escapes

Lime and citrange shoots started to regenerate from the explants after 1–4 weeks in the light. When they were excited at 480-nm blue light, transgenic shoots showed a bright green fluorescence whereas escapes and non-transformed control shoots appeared red, again because of the autofluorescence of the chlorophyll (Fig. 1 C).

It has been important in many plants to use reporter genes, mainly *uidA*, to test the influence of factors affecting both transformation and the regeneration of transformants and escapes. However, since assays to test reporter gene activity are destructive, such factors can not be evaluated continuously and simultaneously. Moreover, the destructive character of GUS assays precludes the recovery of the GUS+ putative transgenic regenerated shoots. To select transgenic shoots or embryos from systems in which escapes regenerate at high frequencies, as in many woody fruit species, all the regenerated ones have to be analyzed, which is a very laborious process. Here, we have shown that GFP expression permitted a rapid and easy discrimination of transgenic and escape shoots in citrus. Competition for growth between transformed and non-transformed shoots could be avoided by eliminating the escapes soon after their origin.

Green fluorescent shoots regenerated, in all cases, from callus from the cambium, whereas most escapes regenerated directly from the explants and with almost no callus formation. Regeneration of escapes is a common phenomenon widely documented from the original report of the co-cultivation of explants with *Agrobacterium* (Horsch et al. 1985). It is generally attributed to inefficient selection due to the protection of the non-transformed cells from the selective agent: (1) by the surrounding transformed ones, (2) by cells showing endogenous non-specific resistance to the selective agent, or (3) by persisting *Agrobacterium* contamination. In our experiments, no shoots were regenerated from non-transformed control explants exposed to kanamycin, so the regeneration of escapes was not directly due to resistance of the citrus cells to kanamycin. Probably the protection of non-transformed cells from kanamycin by the surrounding transformed cells, and/or the remaining *Agrobacterium* in the explants, could be the cause of the regeneration of escapes.

GFP as a vital marker for the easy discrimination of chimeric shoots

In some cases, lime and citrange regenerated shoots showed sectorial expression of GFP (Fig. 1 D). These plantlets were derived from transgenic and non-transgenic cells originating one shoot. The regeneration of chimeric transgenic plants, using GUS as a reporter, has been shown in many plants, mostly associated with transformation procedures such as particle acceleration and/or the use of meristems as source of tissue for transformation (Christou et al. 1989; Christou 1990; Lowe et al. 1995; Bean et al. 1997). Regeneration of chimeric transformants has also been shown in reports on the *Agrobacterium*-mediated transformation of explants (Schmülling and Schell 1993). In citrus, we earlier reported the regeneration of periclinal chimeras from citrange explants (Peña et al. 1995 a). However, periodical GUS assays of different tissues from such chimeric plants produced controversial results due to the irregularity of substrate penetration and the fact that small cells with dense cytoplasm appeared to be more intensely stained than large vacuolated ones (unpublished results). We show here that transgenic chimeras can be recognized at a very early stage of development and that the tissue pattern expression can be followed during the whole life of the plant. This could be extremely useful in transformation procedures generating high frequencies of chimeras, since transgenic sectors would be easily isolated to produce fully transgenic plants. Furthermore, such a simple discrimination of transgenic and non-transgenic sectors from chimeras would be very valuable for cell-lineage analyses using transgenic plants.

Shoot-tip grafting of green fluorescent transgenic shoots and buds soon after formation

The GFP-positive and GFP-chimeric regenerated shoots were successfully grafted on de-capitated Troyer citrange seedlings (Fig. 1 E). As shown in Fig. 1 F, transgenic buds of about 0.1–0.3 cm in height were

Table 1 Transformation efficiency from lime explants cultured in shoot regeneration medium with and without kanamycin selection

Culture	Total number of *Agrobacterium*-inoculated explants per experiment[a]	Frequency of explants with transformation events (%)[b]	Average number of regenerated shoots per experiment ± SE	Average number of regenerated GFP-positive shoots per experiment ± SE	Frequency of GFP-positive shoots (%)[c]
Without kanamycin selection	30	60	47.5 ± 5.5	2 ± 0	4.2
With kanamycin selection	170	62	52.5 ± 7.5	28 ± 3	43.3

[a] Data from two experiments
[b] Number of explants with transformation events per total number of *Agrobacterium* inoculated explants × 100
[c] Number of GFP-positive shoots per total number of regenerated shoots × 100

visualized under blue light soon after arising from the callus tissue. Buds of this size could be efficiently excised and grafted, thus avoiding the competition between transformed and escape shoots, and enhancing the rapid growth of the transgenic ones. This method could be applied to the recovery of transgenic plants from other woody plants, in which shoots are difficult to elongate and/or root, as in the case of plum (Mante et al. 1991), and where escapes regenerate at high frequencies competing with the transgenic shoots, as in the case of apple (James et al. 1989). Moreover, shoot-tip grafting has been successfully performed for these plants (Navarro 1988).

Localization of green fluorescent cell clusters in transformed explants cultured in a medium without kanamycin

Selectable markers, such as NPT II, have been widely used to produce transgenic plants (Croy 1993). However, the presence of these markers may restrict the release of transgenic products due to adverse influences on consumer perceptions. In fact, the expression of selectable markers coding for antibiotic or herbicide resistance has been a topic of much discussion for the commercialization of certain transgenic crops in Europe (Wadman 1996; Abbott 1997). Efficient monitoring by using GFP for the early identification and rescue of transgenic buds, as shown here, would circumvent the use of antibiotic or herbicide marker genes to produce transgenic plants. Interestingly, green fluorescent shoots were regenerated from control explants inoculated with *Agrobacterium* but cultured in a medium without kanamycin (Fig. 1 G; Table 1). These results open up the possibility of producing transgenic plants without using selective agents. Possibly, non-transformed buds would be periodically eliminated to avoid competition with the transgenic ones in order to favor the proper development of shoots only from the transgenic events, thus increasing the frequency of GFP-positive shoots regenerating in a medium without kanamycin selection. This type of experiment is currently under way in our laboratory.

High levels of GFP expression did not affect to the processes of transgenic cell division and shoot regeneration

As shown in Fig. 1, high-level GFP expression was usually detected in transgenic cells, buds and shoots, which did not seem to interfere with the transformation and regeneration of plants. Difficulties in obtaining whole plants from the brightest transformants using different improved *gfp* gene versions have been previously reported (Haseloff and Amos 1995; Chiu et al. 1996; Haseloff et al. 1997; Rouwental et al. 1997). It was suggested that high levels of constitutively expressed GFP were toxic, or else interfered with the regeneration of transgenic shoots. Since we have used the same *gfp* gene synthetic version as Chiu et al. (1996) and under the strong constitutive 35S promoter, such a toxic effect can not be attributed to the *gfp* gene version itself. It is possible that citrus cells are less sensitive to GFP than the *Arabidopsis* or tobacco cells, used by Chiu et al. (1996), or that the 35S promoter yields lower GFP expression in citrus than in other plants. These observations are supported by the results of Rouwental et al. (1997) who found that potato transformation was very inefficient and that most of the resulting transformants grew poorly whereas tobacco transformants regenerated normally using the same *gfp* construct. Maintenance of the citrus explants in darkness during the first month of culture in regeneration and selection medium could also preclude GFP-mediated phototoxicity (Haseloff et al. 1997), at least for the first steps of cell de-differentiation, transformation and re-differentiation. More recently, Davis and Vierstra (1998) have attributed this toxicity to the accumulation of GFP aggregates in plant cells.

Molecular analyses of the GFP-expressing plants

When scions grafted on Troyer citrange seedlings had developed several leaves (Fig. 1 H), one of them was removed from each plantlet, excited with blue light to confirm GFP expression (Fig. 1 I) and PCR-analyzed

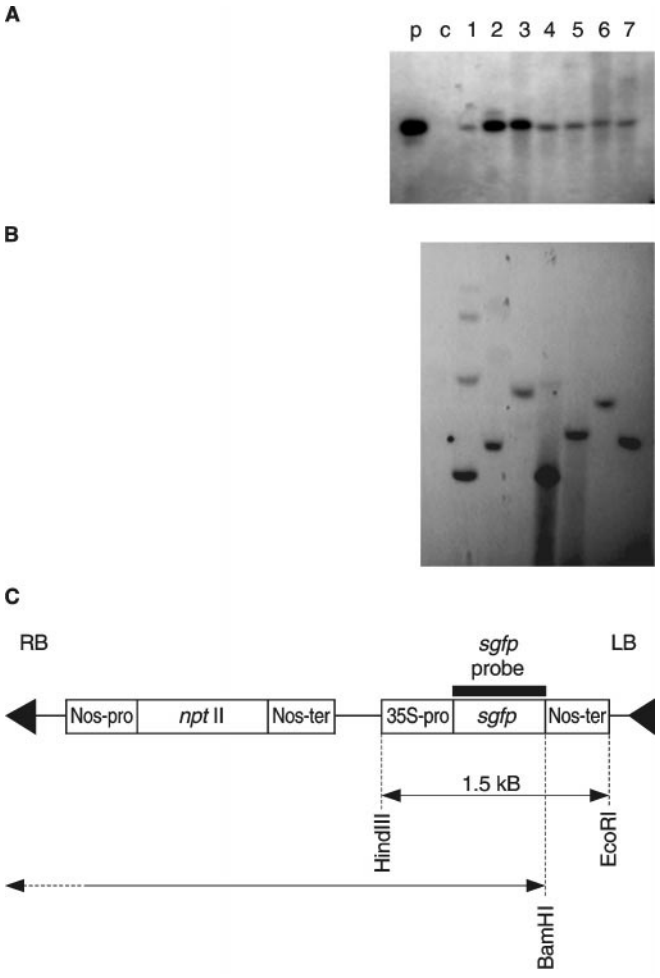

Fig. 2A–C Southern-blot analysis of the s*gfp* gene in leaves from green fluorescent protein (GFP)-expressing plants. *Lane p* pBin 19-s*gfp*; *lane c* a non-transformed control plant. *Lanes 1–7* GFP-expressing plants. **(A)** DNA digested with *Eco*RI + *Hin*dIII. **(B)** DNA digested with *Bam*HI. **(C)** Schematic representation of the T-DNA from pBin 19-s*gfp*. Restriction sites for *Eco*RI, *Hin*dIII and the *Bam*HI, and the position of the s*gfp* probe are indicated

to test for the presence of the s*gfp* gene in their genome. A predicted internal fragment of about 690 nucleotides was amplified in all DNA samples from green fluorescent leaves (data not shown). No amplification was detected in DNA samples from non-transgenic regenerated control plants, which emitted red autofluorescence under blue light (Fig. 1 I).

Then, plantlets were again grafted on vigorous seedlings of Rough lemon in the greenhouse. When plants measured 20–30 cm in height, Southern analyses were performed to confirm the stable integration of the s*gfp* gene cassette in the plant genome (Fig. 2). Digestion with *Eco*RI and *Hin*dIII produced a 1.5-kb fragment corresponding to the s*gfp* cassette (Fig. 2 A, lanes 1–7; Fig. 2 C). Digestion with *Bam*HI, which has a unique restriction site between the 35S promoter and s*gfp* gene (Fig. 2 C), revealed different integration patterns in the transgenic plants, with from one copy (Fig. 2 B, lanes 2, 3, 5, 6 and 7) to several copies (Fig. 2 B, lane 1 and 4) of the transgene. No hybridization signals were detected in non-transformed control plants (Figs. 2 A and 2 B, lane c). Therefore, the visual identification of transgenic plantlets based on GFP expression proved reliable, and was much faster and simpler than PCR and Southern analyses.

Differential patterns of GFP expression in plants

All cells or tissues from leaves of the transgenic plants showed green fluorescence. However, the fluorescence intensity varied among different parts of the plant, being higher in new growing leaves than in old ones. In old tissues, lower metabolic activity and chlorophyll accumulation partially masked the green fluorescence provided by GFP. Furthermore, differences in green fluorescence intensity could be observed associated with different GFP expression levels in apical leaves from independent transgenic lines (Fig. 1 J). This suggests that GFP can be used as a reporter of gene expression for the early non-destructive identification and selection of transgenic buds or shoots expressing the highest levels of protein.

Moreover, in vivo fluorescence could be an easily scorable marker for the ecological monitoring of transgene dispersion through the pollen and to investigate transgene persistence and stability in plants such as woody perennials which are clonally propagated and are grown over long periods of time in the field.

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