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Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting

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Abstract

To investigate early events of *Agrobacterium*-mediated transformation of apple cultivars, a synthetic green fluorescent protein gene (SGFP) was used as a highly sensitive, vital reporter gene. Leaf explants from four apple cultivars ('Delicious', 'Golden Delicious', 'Royal Gala' and 'Greensleeves') were infected with *Agrobacterium* EHA101 harboring plasmid pDM96.0501. Fluorescence microscopy indicated that SGFP expression was first detected 48 h after infection and quantitative analysis revealed a high T-DNA transfer rate. Plant cells with stably incorporated T-DNA exhibited cell division and developed transgenic calli, followed by formation of transgenic shoots at low frequencies. The detection of SGFP expression with an epifluorescence stereomicroscope confirmed the effectiveness of SGFP as a reporter gene for detection of very early transformation events and for screening of putative transformants. The efficiency of the transformation and regeneration process decreased ca. 10 000-fold from *Agrobacterium* infection to transgenic shoot regeneration, suggesting that factors other than *Agrobacterium* interaction and T-DNA transfer are rate-limiting steps in *Agrobacterium*-mediated transformation of apple.

Introduction

Genetic engineering offers an exciting opportunity for improvement of plants with long generation and breeding cycles such as apple. Transformation of apple plants expressing marker genes such as nopaline synthase (*nos*), β-glucuronidase (GUS), and neomycin phosphotransferase (*nptII*) have been achieved, but the transformation frequencies reported are relatively low [19, 20]. Regardless of the high shoot regeneration frequencies achieved for many apple cultivars with leaf explants cultured on non-selective media [1, 2, 11, 19–22, 26, 31, 36, 38, 39], the regeneration of transformed plants remains difficult and is genotype-dependent. Recent reports with *Agrobacterium*-mediated transformation of the commercial cultivars 'Delicious' [36] 'Royal Gala' [40], 'Golden Delicious' and 'Elastar' [32], indicated that the regeneration of GUS- and *nptII*-positive plants per total number of explants varied from 1.5 to 8.7%.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a novel genetic reporter system [24] and has become an important *in vivo* reporter in plants [13, 16, 29, 30, 35]. When expressed in either eukaryotic or prokaryotic cells and illuminated with blue light (395 nm), GFP yields bright green fluorescence. This fluorescence is stable, species-independent, and can be monitored non-destructively. Light-stimulated GFP fluorescence does not require any co-factors, substrates, or additional gene products. GFP has now been successfully expressed in a large number of organisms including *Escherichia coli*, yeast, the nematode *Caenorhabditis elegans* [3], *Arabidopsis thaliana* [13], and *Nicotiana tabacum* [30]. In these organisms, GFP accumulation did not appear to have a toxic effect.

The SGFP-TYG gene is a synthetic GFP gene with codon usage optimized for eukaryotes and the major restriction sites and the cryptic intron site found in wild type GFP removed [13]. The serine at position 65 was mutated to threonine, which has previously been shown to result in a single excitation peak by blue light with the emmision of green light [14]. The combination of all the modifications of the gene resulted in 120-fold brighter green fluorescence than the original jellyfish gene (Dr Jen Sheen, personal communication).

The purpose of this study was to investigate the early events in the transformation and regeneration of various apple cultivars in order to identify the rate-limiting steps which dramatically lower the regeneration of transgenic tissues. The GFP gene was used as a sensitive and non-invasive marker to visualize transformed cells. Our results demonstrate that individual cells in leaf explants from all apple cultivars tested can be efficiently transformed by *Agrobacterium* and that GFP expression can be detected in single cells within 48 h after agrobacterial infection. However, 11 days after agrobacterial infection; expression levels decreased, indicating that many of the GFP-expressing cells were not stably transformed. No correlation between transformation rates and recovery of transgenic plants was observed. Together, these results suggest that factors other than *Agrobacterium* interaction and T-DNA transfer are the rate-limiting steps in apple transformation.

Materials and methods

Bacterial strain and vectors

The synthetic SGFP-TYG gene used in this study was provided in plasmid pUC18-HBT-SGFP-*nos* plasmid by Dr Jen Sheen [35]. The SGFP-TYG gene was PCR-amplified from pUC18-HBT-SGFP-TYG-*nos* with the PCR primers 5′-CAGATCTTTACTTTGACAGCTCGTCCATGCCGT-3′ and 5′-GGGATCCATGGTGAG CAAGGGCGAGGAGCTGTT-3′. The primers contain 5′ *BgL*II and 3′ *Bam*HI restriction sites, to facilitate cloning. The PCR fragment was then purified and cloned into plasmid pCR II (Invitrogen, San Diego, CA) resulting in plasmid pDM96.0417, which was verified by DNA sequencing.

Binary plasmid pDM96.0501 was constructed by the ligation of SGFP-TYG as a *BgL*II-*Bam*HI fragment (excised from pDM96.0417) into the *Bam*HI cloning site of binary plant transformation vector pDU92.3103 [37] placing SGFP-TYG under the control of CaMV 35S promoter and terminator (Figure 1). Plasmid pDU92.3103 is a derivative of pCGN1559 [27] and contains CaMV35S5′-*nptII*-tml3′ expression cassette, GUS reporter gene with CaMV 35S promoter and terminator, and a gentamycin bacterial selection marker. The CaMV 35S promoter-GFP fusion junction was verified by DNA sequencing.

Introduction of pDM96.0501 in the disarmed *Agrobacterium* strain EHA101 was performed by electroporation [25]. Plasmid integrity in *Agrobacterium* was verified by Southern blot analysis (not shown).

Growth and virulence induction of Agrobacterium

Agrobacterium tumefaciens EHA101/pDM96.0501 was maintained on a selection plate with 50 mg/l kanamycin and 20 mg/l gentamycin at 4 °C. One full loop of bacteria was used to inoculate 10 ml of 523 bacterial media [6] in a 50 ml screw top centrifuge tube. The culture was incubated for 18–20 h at 25 °C in the dark with rotary agitation (200 rpm). The 10 ml overnight bacterial culture was centrifuged for 20 min at 1600 × g, resuspended in MS20 induction medium [23] to 0.5 OD 420 mm, and incubated for 5 h at 25 °C in the dark with shaking at 100 rpm as described by James *et al.* [23].

Apple shoot culture

Leaf explants were obtained from shoots of 'Delicious' (RD), 'Golden Delicious' (GD), 'Royal Gala' (RG), and 'Greensleeves' (GS) which had been *in vitro* propagated by the method of James and Dandekar [18]. Single, 1.5–2.0 cm long, axillary shoots were excised aseptically from shoot clusters. At the basal end of the stems, the shoots were cut in a V-shape at an angle of ca. 30° to the shoot axis. Three to five shoots were placed upright in disposable 20 ml blood dilution vials (Fisher Scientific) containing 5 ml of a simplified liquid root induction medium (RI, Table 1) with 1.5% sucrose and 15 μM IBA. The shoots were incubated in the dark for 2 days at 25 °C. After root induction, all shoots were transferred to an agar-solidified root elongation medium (RE, Table 1). Root elongation cultures were exposed to cool-white fluorescent lamps (110 μmol m^{-2} s^{-1} at a 16/8 h light/dark photoperiod and 25 °C for 10 to 20 days before the explants (leaf pieces) were removed.

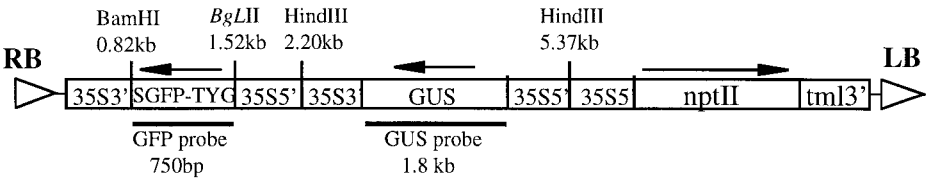

Figure 1. Schematic representation of the T-DNA region of the binary plant transformation vector pDM96.0501. Abbreviations: RB, right T-DNA border; 35S5′, 35S promoter of the cauliflower mosaic virus (CaMV), SGFP, coding region of the synthetic green fluorescent protein gene; 35S3′, termination signal of CaMV; GUS, coding region of the β-glucuronidase gene; *nptII*, coding region for the neomycin phosphotransferase gene; tml3′, tml termination signal [27].

Table 1. Tissue culture media formula.

	MBNZ511	BN505	RI	RE
MS basal medium g/l	4.4	4.4	–	2.2
BA (mg/l)	5	5	–	–
NAA (mg/l)	1	0.5	–	–
TDZ (mg/l)	1	–	–	–
IBA (mg/l)	–	–	3	–
Sucrose (g/l)	–	–	15	20
Sorbitol (g/l)	30	30	–	–
Agar Type A (g/l)	–	6	–	6
PhytaGel (g/l)	3	–	–	–
pH	5.5	5.5	5.2	5.5

Abbreviations: MBNZ511, modified BNZ511 medium [18]; RI, root induction medium; RE, root elongation medium; BA, 6-benzylaminopurine, NAA, α-naphthaleneacetic acid; TDZ, thidiazuron; IBA, indole-3-butyric acid. All chemicals were purchased from Sigma Chemical Company, St Louis, MO.

Plant transformation and regeneration

The top three fully unfolded leaves of rooted apple shoots were cut with a scalpel blade perpendicular to the midvein into 2 to 3 mm explants and infected with 5 ml solution of induced *Agrobacterium* for 10–20 min in a Petri dish. After infection, the leaf discs were blotted dry and placed on sterile Whatman #4 filter paper overlaying the co-cultivation modified BNZ511 medium (MBNZ511; Table 1). The explants were co-cultured for three days in the dark at 25 °C. After co-cultivation, the explants were transferred to MBNZ511 selection medium with 75 mg/l kanamycin and 400 mg/l cefotaxime.

Forty days after culture on MBNZ511 selection medium, the explants were transferred to secondary regeneration medium BN505 (Table 1). The explants were observed weekly by fluorescent microscopy (see below). Leaf explants with green fluorescing leaf primordia were transferred to light with a 16/8 h light/dark photoperiod under cool-white fluorescent light (110 μmol m^{-2} s^{-1}). The remaining explants were cultured in the dark until GFP-expressing primordia were developed. Every 20 days all explants were transferred to fresh selection media.

The numbers of GFP-expressing calli were counted 40 days after bacterial infection and the results were analyzed to determine the percent of explants with one or more GFP-expressing calli and the mean number of expressing calli per explant. Leaf explants with one or more GFP-expressing shoots are presented as a percentage of the total explants infected. Two different *Agrobacterium* infections were performed for each cultivar. Fifteen explants were cultured in individual Petri dishes as one replicate. The total numbers of infected explants were 257 for GD, 423 for RD, 300 for RG, and 159 for GS. The results from the two separate infections were combined. Variation among cultivars was analyzed with analysis of variance techniques and means were separated using Duncan New Multiple Range Test at $P < 0.05$. Control regeneration and selection efficiencies were obtained by culturing 30 uninfected leaf explants from each cultivar on MBNZ511 regeneration medium with and without selection. The leaf explants with one or more shoots were counted and the results are presented as percentages of explants with one or more shoots from the total number of explants and mean number of shoots per regenerating leaf explant.

Rooting and acclimatization of plants to greenhouse conditions

Putatively transformed shoots were transferred to propagation media with 50 mg/l kanamycin and 400 mg/l cefotaxime. After one or two subcultures, 2–3 cm long shoots were transferred to liquid root initiation medium (RI, Table 1) and incubated in the dark for 48 h at 25 °C. The shoots were then transferred to root elongation medium (RE, Table 1) and cultured in

a 16/8 h day/night photoperiod at 25 °C. After 10 to 15 days, plants with well developed root systems (3–4 roots, 3–5 cm long) were transferred to sterile soil in magenta boxes (Sigma, St. Louis, MO), and gradually exposed to the air in a growth chamber by gradually opening the lids for 5–7 days. The acclimatized plants were transferred to 20 cm pots with a soilless growing mix (Pro-mix BX, Premier Horticulture, Red Hill, PA) and transferred to a greenhouse.

Fluorescent microscopy

The fluorescent images were taken with a Nikon SMZ-4 dissecting microscope equipped with an epi-fluorescence attachment, a 100W mercury light source, and a Nikon N6006 35 mm camera. Two different emission filters were used separately: a 515 nm long pass emission filter (transmitting red and green light) and a 520–560 mm emission filter (transmitting only green light). A 450–490 nm excitation filter was used with each of the emission filters.

For measuring GFP fluorescence intensity of 'Delicious', ten random areas from different leaf explants were selected and measurements of the fluorescence values (expressed as integrated pixel values) were recorded every other day starting 3 days after the end of the co-cultivation period (6 days after the agrobacterial infection). Seven data sets were collected over a time period of 13 days. At each data collection point three images were recorded per area with different focal planes at a magnification of 75× and 30 s exposure time (a total of 30 measurements per data set) using a 3 CCD video camera system (Optronics Engineering, Goleta, CA). Fluorescence was quantified using NIH Image 1.6 image processing and analysis program for Macintosh. Analysis of variance and mean separations were performed with Duncan New Multiple Range; significance was determined at $P < 0.05$.

GUS histochemical assay

GUS histochemical assays were performed weekly for the first four weeks after co-cultivation. Random samples of 10 explants per cultivar were placed in microtiter plates with X-Gluc solution and tested for GUS activity. X-Gluc solution was prepared by dissolving 10.4 mg X-Gluc in 240 μl dimethyl formamide and adding 10 ml GUS incubation buffer to a final concentration of 2 mM [10, 12]. The explants were incubated overnight at 37 °C in the dark. Afterwards, the explants were washed with 70% ethanol to bleach chorophyll from the tissue.

Southern blot analysis

Apple genomic DNA was isolated from 'Royal Gala', 'Greensleeves', and 'Delicious' transgenic and non-transgenic *in vitro* propagated shoots as previously described [9]. One additional phenol:chloroform extraction and chloroform:isoamyl alcohol extraction were performed. Genomic DNA was dissolved in 100 μl TE buffer and digested with RNase A (50 μg/ml) at 37 °C for 1 h. Ten μg of DNA from each line were digested with *Hin*dIII restriction enzyme, fractionated on a 0.8% agarose gel, and transferred onto HybondN^+ (Amersham) membrane by the alkaline blotting method [33]. A 25 ng portion of each probe (700 bp *Bam*HI-GFP-*Psi*I DNA (pUC18-HBT-SGFP-TYG-*nos*) and 1.8 kb *Bam*HI-GUS-*Sac*I DNA (pBI121; Clontech Laboratories, Palo Alto, CA) was ^{32}P-labeled using the random primer DNA labeling kit (Boehringer Mannheim, Germany). The membrane was hybridized sequentially at 65 °C for 20 h in hybridization solution (7% SDS, 0.5 M sodium phosphate), washed 2 times under low-stringency conditions in 100 ml of 5% SEN (5% SDS, 400 mM sodium phosphate, 10 mM EDTA) for 20 minutes at 65 °C, followed by wash under high-stringency conditions in 100 ml of 1% SEN (1% SDS, 400 mM sodium phosphate, 10 mM EDTA) at 65 °C, and exposed to a phosphorus screen (Molecular Dynamics, Sunnyvale, CA) for 48 h. Between the hybridizations the probe was removed from the membrane by incubation of the blot at 45 °C for 30 min in 100 ml of 0.4 M NaOH, followed by transfer to 50 ml 0.1× SSC, 0.1% SDS, 0.2 M Tris-HCl pH 7.5 at the same temperature for 15 mins. The filter was then transferred to 100 ml boiled 0.5% SDS, cooled to room temperature for 30 mins and pre-hybridized. The images were scanned using PhosphorImager and Image Quant software (Molecular Dynamics).

Results

*Construction of a SGFP-TYG/GUS/*nptII *co expression vector*

The Ser65Thr mutated synthetic GFP (SGFP) is a reporter gene that potentially allows rapid *in vivo* detection of DNA transfer events in plant tissues. The binary vector pDM96.0501 (Figure 1) was constructed

to investigate early events of transformation using the SGFP gene. The parental binary vectors have high stability in *Agrobacterium*, and both high stability and copy number in *Escherichia coli* [27]. This is due to the presence of two different origins of replication, ColE1 from *E. coli*, and pRiHRI from *Agrobacterium*. The GUS reporter gene and the *nptII* selectable marker gene also present in this plasmid provide additional alternatives for detection of DNA transformation. The presence of the bacterial gentamycin resistance gene allows for efficient selection of this vector in the highly virulent and kanamycin-resistant *A. tumefaciens* strain EHA101 [15]. Unlike the GUS reporter gene without a plant intron, the SGFP gene under the control of 35S CaMV promoter reproducibly showed no background expression in *A. tumefaciens* (data not shown), thus eliminating the problem of false-positive reporter gene detection in plant tissue containing *Agrobacterium*.

Transient GFP expression reveals a high gene transfer rate

Leaf explants from *in vitro* propagated and rooted apple plants were inoculated with *Agrobacterium tumefaciens* EHA101 harboring plasmid pDM96.0501. After the initial infection, the explants were co-cultivated on MBNZ511 medium for three days and GFP expression was monitored daily with a fluorescence stereomicroscope. The first fluorescence was detected 48 h after agrobacterial infection. Fluorescence was first observed along the cut edges and wounded sites on the leaf surface (Figure 2 a,b,c,d). About 100–500 fluorescing cells were observed per leaf explant and high numbers of these cells were seen at cut vascular tissues (Figure 2d). Between 95 and 100% of the explants from all cultivars had a large number of infection sites, most of which lost their fluorescence within the following few weeks. Fluorescence was not observed at the cut edges and the injured sites of the control uninfected leaf explants at any time (not shown). Control co-cultivation experiments with pDU92.3103 [37] (the same Ti plasmid without the GFP gene) were also performed, and no fluorescence was observed after infection (not shown). This excludes the possibility that wounding or *Agrobacterium* infection alone induces the production of fluorescing compounds in apple leaf explants.

The quantitative analysis of the fluorescence of selected areas from *Agrobacterium*-infected leaf explants of 'Delicious' indicated that GFP fluorescence increased by 9 days after bacterial infection (DAI), followed by decrease and stabilization from 11 to 15 DAI and a sharp increase after 15 DAI (Figure 3). The bright fluorescence at DAI 9 is apparently due to transient expression of the GFP gene, as it declined in the following few days. The decline most likely is the result of DNA degradation of non-integrated T-DNA. Alternatively, it could be caused by silencing of integrated T-DNA copies. The sharp increase in GFP fluorescence at 15 DAI reflects the division of cells with stably incorporated T-DNA and the formation of brightly fluorescing microcalli.

GFP expression in apple calli

Only a few of the large number of cells that were fluorescing immediately after transformation continued to express GFP after 30 days and formed calli on selection media. The 'Golden Delicious' fluorescing calli displayed on Figure 2, panels m, n, q and r were grown on selection media for 34 days. The non-transformed tissue from the original explant still contained chlorophyll, but cell division was suppressed by the selective antibiotic (Figure 2e). Upon illumination with blue light only the GFP expressing calli emitted green light and were visible (Figure 2f). A 34-day old culture of *Agrobacterium*-infected 'Delicious' tissue is presented in Figure 2g, recorded with 450–490 nm excitation and 515 nm emission filters. This filter setting allowed imaging of the red fluorescence from the chlorophyll and some endogenous background fluorescence from the untransformed tissue. The formation of non-transgenic calli was closely associated with the GFP-expressing cells. After an extended period of culture on selection media, the development of the non-transgenic calli was suppressed.

Forty days after *Agrobacterium* infection, the numbers of developing calli expressing GFP were counted (Table 2). 'Delicious' and 'Golden Delicious' had the highest percentage of transformed calli and mean number of GFP expressing calli per leaf explant, followed by 'Royal Gala'. 'Greensleeves' produced the lowest percentage of transformed calli and mean number of GFP expressing calli.

Detection of GFP fluorescence in etiolated and green shoots

Sixty DAI some of the GFP-expressing calli developed leaf primordia (Figure 2h). Compared to nontransformed leaf primordia regenerated without selection which showed no fluorescence (Figure 2j and k),

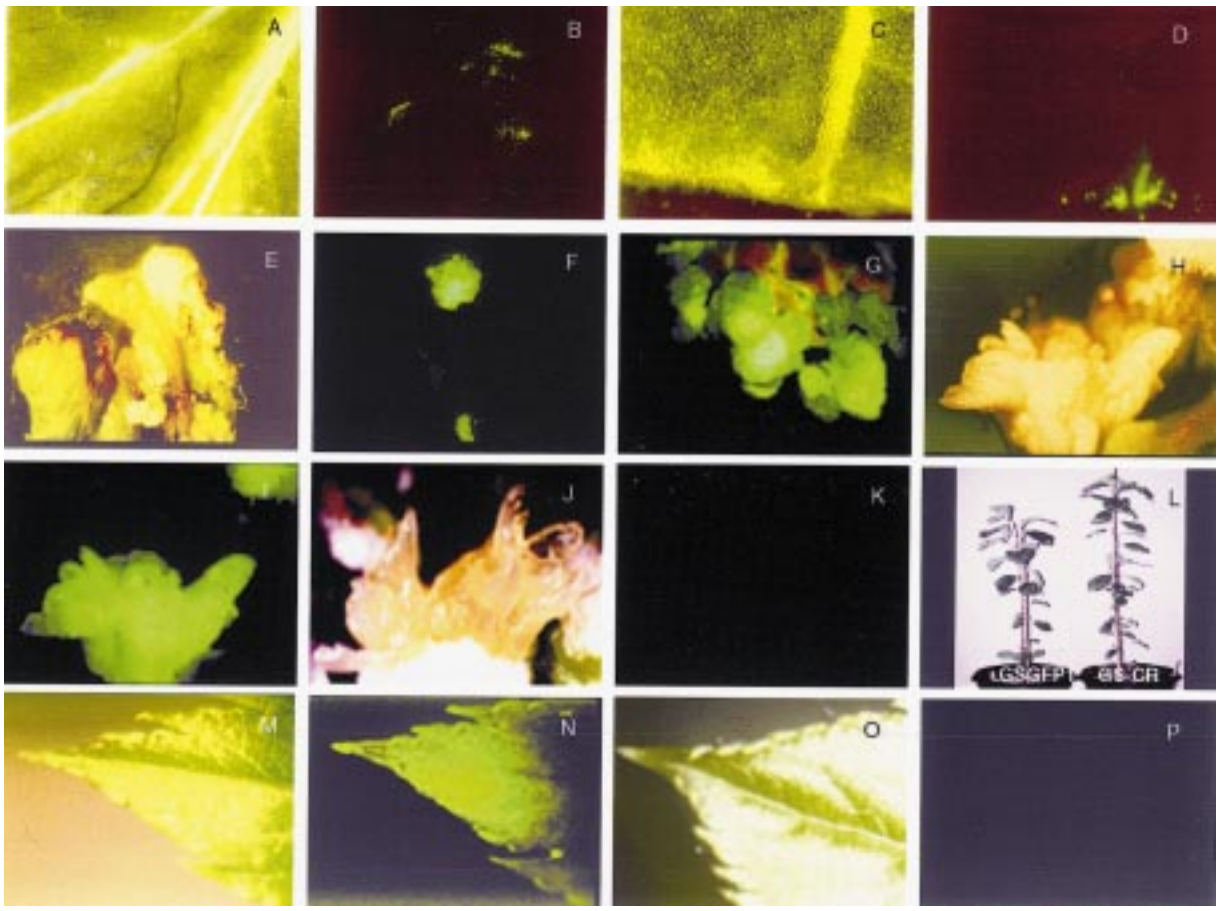

Figure 2. Green fluorescent protein expression in apple leaf explants and transgenic apple plants visualized by fluorescent microscopy. Panels A, C, E, H, J, M and O, light micrographs; panel G, micrographs taken with 515 nm emission filter and 450–490 nm excitation filter, panels B, D, F, I, K, N and P, micrographs taken with 520–560 nm emission filter and 450–490 nm excitation filter. A. Leaf explant 48 h after *Agrobacterium* infection. B. Fluorescent images of the explants from panel 'A'. C. The cut edge of leaf explant 48 h after *Agrobacterium* infection. D. Fluorescent images of the explants from panel 'C'. E. *Agrobacterium*-infected leaf explant from 'Golden Delicious' cultured on selection media, 30 days after infection. F. Fluorescent images of the leaf explants from panel 'E'. G. Fluorescent image of 'Delicious' leaf explant with developed calli, 30 days after bacterial infection. H. Leaf primordia formed on *Agrobacterium*-infected leaf explant after 60 days culturing on kanamycin medium. I. Fluorescent images of the shoot primordia from panel 'H'. J. Leaf primordia developed on non-infected leaf explant grown without selection for 30-days. K. Fluorescent image of shoot primordia form panel 'J'. L. Control and transgenic 'Greensleeves' plants grown in greenhouse conditions. M. Light image of a green leaf from transgenic 'Greensleeves' apple plant. N. Fluorescent image of the leaves from panel 'M'. O. Light image of a leaf from non-transgenic 'Greensleeves' plant. P. Fluorescent image of the leaf explant from panel 'O'.

the GFP-expressing leaf primordia fluoresced brightly (Figure 2i).

After the first few leaves on the newly regenerated shoots had formed, the explants were transferred to light. The shoots developed chlorophyll and were transferred to micropropagation media with selective antibiotics. The green leaves of the transformed plants also expressed GFP and the green fluorescence was easily detectable (Figure 2 m and n). However, the intensity of the fluorescence in green leaves was less than the intensity of etiolated shoots (Figure 2i), possibly due to absorption of the fluorescence from some of the newly developed leaf pigments, and/or the enlargement of the cell vacuole and dilution of the GFP protein in the cell.

All cultivars exhibited high shoot regeneration potential on non-selective media, but genotypic variation was observed under selection pressure (Table 2). The cultivar with the highest transformation percentage ('Golden Delicious', 96.7%) did not produce any transgenic shoots. Surprisingly, 'Greensleeves', which had the lowest transformation percentage, produced

Table 2. GFP expression in apple calli and regeneration of control and transgenic shoots.

Apple cultivar	No *Agrobacterium*		With *Agrobacterium* infection			
	explants regenerating 1 or more shoots[1] (%)	Mean number of shoots per explant[2]	GFP-expressing explants[3] (%)	Mean number of GFP calli per explant ± SE[4]	explants regenerating 1 or more shoots[5]	Total number of GFP plants regenerated
GD	71	5.6	95	4.21 ± 0.23[a]	0.0	0
RD	72	8.2	89	4.85 ± 0.59[a]	0.2	1
RG	87	4.5	63	2.37 ± 0.36[b]	3.0	9
GS	90	7.9	37	0.87 ± 0.13[c]	5.8	9

[1] Expressed as a percentage of the total number of explants; [2] Only explants with one or more shoots were considered; [3] GFP-expressing calli were counted 40 days after bacterial infection, percentage of explants with one or more GFP expressing calli were calculated; [4] Only explants with one or more expressing calli were considered, means of all replicates for the individual cultivars were averaged and were compared by Duncan's New Multiple Range test. Mean values followed by different letters (a, b, c) are significantly different at $P < 0.05$; [5] Number of leaf explants with one or more GFP expressing shoots as a percentage of the total number of explants infected. Abbreviations: GD, 'Golden Delicious'; 'Delicious'; 'Royal Gala'; GS, 'Greensleeves'; SE, Standard error.

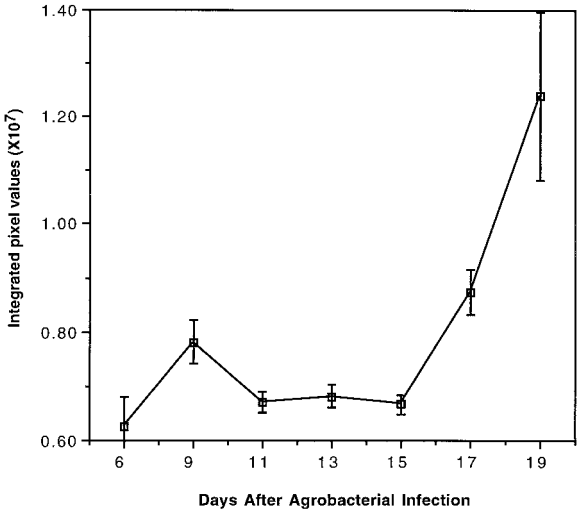

Figure 3. Quantitative measurement of the fluorescence intensity of randomly selected areas from *Agrobacterium*-infected leaf explants of 'Delicious'. Fluorescence was quantified using NIH Image 1.6 image processing and analysis program for Macintosh and fluorescence intensity was expressed as integrated pixel values ($\times 10^7$). Analysis of variance and mean separations were performed with Duncars' New Multiple Range statistics; significance was determined at $P < 0.05$.

the largest number of transgenic shoots. This finding is consistent with previous studies that showed a higher frequency of production of transgenic plants from 'Greensleeves' than from other apple cultivars [17, 20, 21]. In addition to stably transformed apple shoots, all of the infected cultivars regenerated a number of shoots on kanamycin medium that were usually associated with GFP-expressing calli but which did not exhibit green fluorescence. When transferred to light in the presence of kanamycin, these shoots did not develop chlorophyll and died. These shoots could represent escapes which survived selection perhaps due to cross feeding from transgenic cells, or alternatively, could result from gene silencing events.

Rooting and acclimatization of transgenic GFP apple plants to greenhouse conditions

Twenty-six plants from seven independent transgenic lines were cultured on RI and RE media (Table 1) with 50 mg/l kanamycin. Of these plants, 18 (69%) developed 2 to 5 roots and were transferred to soil (Table 3). The development of the transgenic plants was compared to control plants from the same cultivars regenerated without selection. All transgenic and control plants grew 10–15 cm within the first two weeks and developed 5–9 new leaves. As of the time of submission, the plants were 150 cm high after growth in soil for 10 months and show no visual abnormalities compared to control regenerated plants. No toxic or negative effects of the GFP protein on the transgenic plants were noticed (Figure 2l).

Southern blot analysis

To confirm the integration of T-DNA in transgenic apple plants, Southern blot analysis of three inde-

Table 3. Rooting frequency of control and transgenic apple shoots.

Transgenic line	Total number of shoots	Number of plants rooted
RG-CR	5	3
RGGFP2	5	3
GS-CR	5	3
GSGFP1	10	8
GSGFP4	1	1
GSGFP5	3	1
GSGFP6	1	1
GSGFP8	3	1
GSGFP9	3	3

Abbreviations: RGCR, 'Royal Gala', control regeneration; GSCR, 'Greesleeves', control regeneration; RGGFP2, 'Royal Gala'-GFP transgenic line 2; GSGFP1, 'Greensleeves'-GFP transgenic lines 1, 4, 5, 6, 8, and 9.

pendent transgenic clones and two untransformed clones was performed. Genomic DNA was isolated from untransformed 'Royal Cala' and 'Greensleeves' *in vitro* propagated plants (Figure 4, lanes 2 and 4) and transformed 'Delicious' clone RDGFP1 (lane 1), 'Royal Gala' clone RGGFP2 (lane 3), and 'Greensleeves' clone GSGFP1 (lane 5). DNA was digested with *Hin*dIII and hybridized sequentially with ^{32}P-labeled GFP (Figure 4a) and GUS probes (Figure 4b). As expected the GFP probe hybridized to fragments longer than 2.2 kb with DNA isolated from transgenic plants and did not hybridize to digested DNA from non-transformed plants (Figure 4a). The sizes of the bands (5.09, 3.05, and 3.50 kb) indicates the distance between the *Hin*dIII site at a position 2.20 kb in the T-DNA region and the nearest *Hin*dIII site in the genomic DNA flanking the insertion sites. The different fragment sizes for each line indicate that each plant arose from separate transformation events. When the intact Ti plasmid pDM96.0501 was cut with *Hin*dIII, a single band of 16.5 kb was produced (data not shown), indicating that the various bands in the apple DNA digests did not result from contamination from *Agrobacterium* DNA. The presence of single bands indicates one T-DNA insertion per apple genome. When hybridized with a GUS probe, DNA from all of the transgenic plants produced expected 3.17 kb bands, corresponding to the intact internal *Hin*dIII fragment of the transformation construct (Figure 4b).

Discussion

Four different apple cultivars were utilized in genetic transformation experiments to express the green fluorescent protein. A synthetic, codon-optimized GFP gene was demonstrated to be an efficient reporter gene in transgenic apple plants and to have a number of advantages over other commonly used reporter genes. Using a stereo-microscope with an epifluorescent attachment, the green fluorescence can be detected within 48 h after infection without the use of a lethal assay system as for GUS. This provides an opportunity for monitoring early events of transformation over time and rapidly testing the influence of different factors on the efficiency of DNA transfer.

The results from this study confirmed that the procedure used for inducing virulence in *Agrobacterium* for the infection of apple leaf tissue provided a large number of transformation events. The highest number of GFP expressing cells were associated with the cut vascular tissues. This could be explained by the higher cell density and the larger number of cells in the vascular tissue of the apple leaf, compared to the lower cell density (due to large air spaces) and cell number in the leaf blade. Another possibility is that the vascular cells have specific morphological or biochemical characteristics, perhaps wound induced, which enables them to become highly susceptible to *Agrobacterium*. The type and the metabolic state of cells competent for *Agrobacterium* transformation appear to vary among plant species and different tissue explants and to a certain extent, depend upon the phytohormone content in the culture media [4, 7, 8, 28, 34]. For example, when *Arabidopsis thaliana* cotyledon explants were

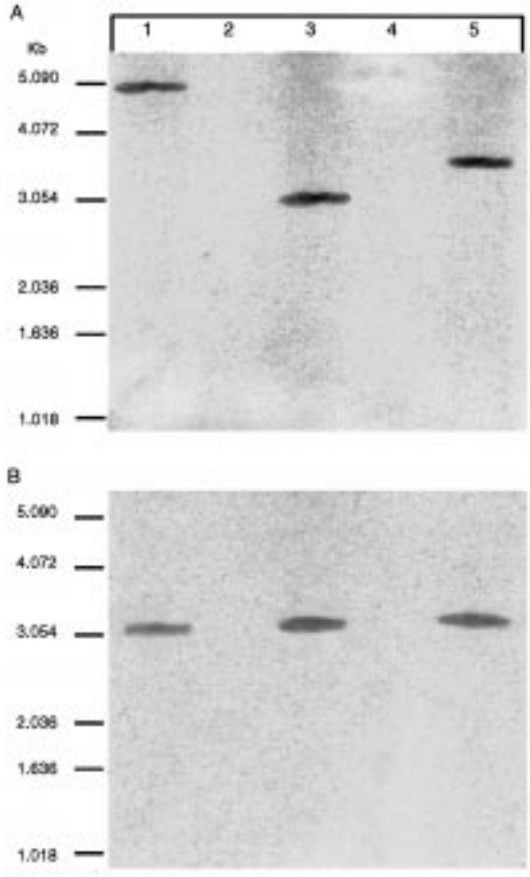

Figure 4. Southern blot analysis of genomic DNA from transgenic apple plants containing the T-region of pDM96.0501. Genomic DNA was isolated from untransformed 'Royal Gala' and 'Greensleeves' *in vitro* grown plants (lane 2 and 4) and transformed 'Delicious' clone RDGFP1 (lane 1), 'Royal Gala' clone RGGFP2 (lane 3), and 'Greensleeves' clone GSGFP1 (lane 5). 10 μg of genomic DNA digested with *Hin*dIII from each sample were electrophoresed on a 0.8% agarose gel, transferred onto Hybond N+ membrane and hybridized sequentially with ^{32}P-labeled GFP (panel A) and GUS probes (panel B).

cultured on callus-induction media and infected with *Agrobacterium*, competent cells were found among the mesophyll cells located both around vascular traces and beneath the epidermal layer in contact with the medium [34].

The localization of the GFP protein/fluorescence allows a precise identification of individually transformed cells shortly after the agrobacterial infection. GFP does not appear to move intercellularly or to be secreted out of the cell. A key feature of GFP as a reporter gene for these experiments was the absence of endogenous background fluorescence in intact apple tissue or in control tissues transformed without GFP gene. However, in some plant species, for example *Nicotiana tabacum*, the use of GFP as a genetic reporter gene could be restricted due to the high autofluorescence of some tissues, particularly roots (data not shown). The *Agrobacterium* strains examined did not have significant endogenous fluorescence at the wave lengths used for GFP detection. Additionally, when pDN96.0501 was introduced into EHA101, the GFP gene did not produce any bacterial expression.

The *in vivo* GFP assay permits monitoring the growth of individually transformed cells and their development into calli and/or shoots. The results from this experiment indicated that significant numbers of transformed cells underwent cell division and formed GFP-expressing calli (up to 4.58 calli per leaf disk), but only a few developed leaf primordia. This was independent of the regeneration potential of the leaf tissue or the frequency of the DNA transfer, and seemed to be due to the continuous selection pressure applied. Although all cultivars expressed very high regeneration potential on non-selective media, when infected with *Agrobacterium* and cultured on selection media, the percent regeneration decreased dramatically regardless of the high transformation efficiency in some cultivars. Surprisingly, the cultivars that produce highest percentage of transformed calli did not regenerate, or regenerated a very low number of transgenic shoots (Table 2).

The results also indicated that there were differences in the response to the selection pressure among cultivars. Two of the cultivars, 'Greensleeves' and 'Royal Gala', regenerated a number of transgenic shoots, which suggested a higher tolerance of the tissue to the antibiotic or perhaps a different mechanism of adventitious shoot regeneration. In all cultivars it was observed that most of the shoots formed on non-selection media occurred within 30–40 days after culture initiation and regenerated without an intermediate callus phase. Perhaps these shoots had a multicellular origin and arose from adjacent healthy, unwounded cells. Similar shoots that regenerated from infected leaves under antibiotic selection were non-transgenic escapes. On untransformed explants, a very small number of shoots, primarily associated with the cut edges, developed with an intermediate callus phase (data not shown). This mode of shoot regeneration was slower (60 days to 9 months) than direct shoot regeneration (30 days). A similar, slow shoot development was observed after *Agrobacterium* infection, where the cut edges of the explants were the main sites of DNA transfer. The regeneration process in these explants may have also been inhibited by the presence of the selective antibi-

otic in the media. The fact that transgenic cells from some of the cultivars cannot overcome the selection pressure, and failed to regenerate shoots, indicates that the original regeneration medium, although inducing a very high number of primordia with multicellular origins from leaf explants without selection, even over an extended period of time, did not effectively induce morphogenesis from single cells or unorganized callus cells. It is possible that the content of the regeneration medium needs to be adjusted for the individual cultivars considering the extended time of culture and the tissue requirement changes in relation to *Agrobacterium* infection.

One of the potential concerns about expressing GFP in plants under the control of a constitutive promoter has been its toxicity at the cellular level and on general plant development under direct light. Regardless of the very high fluorescence in etiolated tissues, the plants regenerated in this study developed a normal phenotype when transferred to light. Eighteen plants from different transgenic lines were rooted on selection medium and successfully acclimatized to greenhouse conditions. These plants continued to grow 10 months later, and do not display developmental abnormalities or differences from the control plants.

These observations raise a number of possibilities as to the rate limiting step in apple *Agrobacterium*-mediated transformation. Since large numbers of fluorescing cells per leaf explant were observed 48 hs after infection (100–500 per explant), *Agrobacterium* infection and DNA transfer are not the rate limiting steps. However, two weeks following infection only a few of these cells developed calli and showed stable expression (1–5 per explant), indicating that either the DNA was not efficiently integrated into the genome, or if integrated, gene silencing occurred at high frequencies. Additionally, regeneration of transgenic apple shoots appears to be a second low efficiency process, possibly because selection with kanamycin forces a single cell origin. In the case of regeneration in the absence of selection, which occurs at very high frequencies, regeneration is evident with no calli intermediate, perhaps from multiple cells in the leaf tissue [4, 5, 28]. In the case of regeneration with selection, the new shoots must arise from a single cell, which first forms a disorganized callus tissue. It is possible that positional information in the different cell layers involved is a factor in generation of new meristems with high efficiency. In the case of single cell origin, disorganized calli must organize into a meristematic structure. A similar process has been observed during shoot formation from 5-day old tomato cotyledon cultures, where groups of 2–4 initial cells differentiated within the callus and led to formation of zones of intensive meristematic activity [28]. Detailed histological analysis of apple tissue undergoing regeneration is necessary to resolve this question.

Together, the two steps of DNA transformation and shoot regeneration reduced the efficiency of the transformation and regeneration process about 10 000-fold, suggesting that factors other than *Agrobacterium* interaction and T-DNA transfer are rate limiting steps in *Agrobacterium*-mediated transformation of apple. The GFP reporter gene can be used as a powerful tool to optimize the transformation system for individual cultivars and to test for variables which could enhance the integration and regeneration steps.

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