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A broad exploration of a transgenic population of citrus: stability of gene expression and phenotype

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Abstracts A collection of 70 transgenic citrus plants for the *uidA* and *nptII* genes have been maintained under screenhouse conditions over a period of 4–5 years. A detailed scanning of the plants allowed us to detect four phenotypic off-type plants and a large variation of transgene integration and expression patterns among the population. Off-type plants were analysed and characterised as nucellar tetraploids, probably originating from tetraploid starting tissues rather than from somaclonal variation events. Transgene integration and expression analyses revealed that: (1) a significant negative correlation was found between copy number and GUS activity; (2) rearrangements of the T-DNA inserts did not imply low expression levels; and (3) stability of integration and expression of the transgenes was confirmed for all the transformants grown under natural environmental conditions. These combined features validate transformation as a tool for the genetic improvement of citrus.

Key words Stability · *uidA* · *nptII* · Phenotype · Carrizo citrange

Introduction

In vegetatively propagated and long-lived perennial fruit crops, a major requisite for evaluating the validity of genetic-transformation technology in improvement programs is the stability of the modified genome and the stability of transgene integration and expression over long periods of time. However, little is known about these features in woody fruit crops.

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Carrizo citrange (*Citrus sinensis* L. Osbeck×*Poncirus trifoliata* L. Raf.) is currently one of the most extensively used citrus rootstocks and major efforts have been made to develop transformation procedures for this genotype (Moore et al. 1992; Peña et al. 1995; Gutiérrez et al. 1997; Cervera et al. 1998). We have established highly efficient procedures for the regeneration of transgenic citrange plants (Peña et al. 1995; Cervera et al. 1998). Nucellar embryony of citrus, an apomictic process in which adventitious embryos initiate directly from maternal nucellar cells (Koltunow et al. 1995), allows one to recover plants identical to the maternal line. Transgenic citrange plants regenerated from nucellar material would exhibit transgene expression differences related only to different T-DNA integration patterns.

Somaclonal variation has been extensively reported in both greenhouse experiments and field trials of transgenic plants: in the R_0 and progeny of cereals (Phan et al. 1996; Bregitzer et al. 1998), in herbaceous plants (Gonsalves et al. 1994; Singh et al. 1998) and in vegetatively propagated plants like potato (Imai et al. 1993; Kawchuk et al. 1997), and in trees such as poplar (Wang et al. 1996). Somaclonal variation affects plant phenotype producing abnormal morphology and has been correlated with genomic changes such as chromosomal deletions and alterations in ploidy.

In vivo expression of transgenes can be affected by numerous causes, including transgene-locus number (Hobbs et al. 1990; Matzke et al. 1994; Elmayan and Vaucheret 1996; Jorgensen et al. 1996), the genomic context of the integrated loci or position effects (Peach and Velten 1991; Iglesias et al. 1997), abnormal configurations of the integrated T-DNA (Hobbs et al. 1993; Stam et al. 1997) or even environmental conditions (Meyer et al. 1992; Neumann et al. 1997), all of which may contribute to differences in expression between plants with unlike integration patterns.

For a period of 4–5 years we have maintained a collection of 70 transgenic citrange plants in a screenhouse in order to investigate, for the first time in a woody fruit plant: (1) the origin of morphological variants in a trans-

genic population, (2) how factors related to T-DNA integration, the regeneration process and the expression of transgenes may be influenced by transformation conditions, (3) whether transgenes are stably integrated and expressed over long time periods in citrus plants grown under natural environmental conditions, and (4) whether correlations between integration patterns and transgene expression could be established in these plants.

Materials and methods

Plant material, bacterial strain and vector

Carrizo citrange seeds coming from the same tree stock were used for transformation experiments. Seedlings were prepared as described in Peña et al. (1995).

Agrobacterium tumefaciens EHA105 (Hood et al. 1993) containing the binary plasmid p35SGUSINT (Vancanneyt et al. 1990) was used as vector system for transformation. Two gene cassettes in the T-DNA, 35S-*uidA*(GUSINT)-35 S and NOS-*nptII*-NOS, served respectively as reporter and selectable marker genes. Bacterial suspensions were prepared as described in Peña et al. (1995).

Transformation and regeneration

A total of 70 transgenic Carrizo citrange plants were randomly selected from the experiments described in Peña et al. (1995) and Cervera et al. (1998), differing basically in the co-cultivation method. Briefly, 1-cm-long epicotyl segments were cut transversely from 5-week-old Carrizo citrange seedlings (8–10 cm in height). Explants were incubated for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension by gentle shaking. The infected explants were blotted dry on sterile filter paper and transferred to the co-cultivation medium. In Peña et al. (1995), co-cultivation was performed on a medium consisting of MS (Murashige and Skoog 1962) salts, 0.2 mg/l of thiamine hydrochloride, 1 mg/l of pyridoxine hydrochloride, 1 mg/l of nicotinic acid, 3% (w/v) sucrose, 1 mg/l of benzylaminopurine (BAP), pH 5.7, for 2 days. In Cervera et al. (1998), co-cultivation was maintained for 3 days in a co-cultivation medium consisting of the same basal medium but containing 1 mg/l of 2-isopentenyladenine, 2 mg/l of indole-3-acetic acid and 2 mg/l of 2,4-dichlorophenoxyacetic acid, instead of BAP. Acetosyringone at 100 µM was also added to this medium. In both cases, co-cultivation was performed under low irradiance (20 $\mu Em^{-2}s^{-1}$), a daily cycle of 16-h light/8-h dark, 60% RH and 26°C. After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to shoot regeneration medium, consisting of the same basal medium but with 3 mg/l of BAP and supplemented with 100 mg/l of kanamycin for selection and 250 mg/l of cefotaxime and 250 mg/l of vancomycin in order to control bacterial growth. The plates were maintained in the dark for 4 weeks at 60% RH and 26°C, and then transferred to a 16-h photoperiod, 45 $\mu Em^{-2}s^{-1}$ illumination, 60% RH and 26°C. The explants were subcultured to fresh medium every 4 weeks.

Regenerated shoots were harvested from the explants and excised into two pieces. The shoot basal ends were assayed for GUS activity and the remaining portions were shoot-tip grafted on to Troyer citrange seedlings growing in vitro (Peña et al. 1995). Three to five weeks after shoot-tip grafting, the plantlets were analysed by PCR-amplification of the *uidA* and *nptII* genes, and grafted again in the greenhouse on to 5-month-old seedlings of Rough lemon (*Citrus jambhiri* Lush). Putatively transformed Carrizo citrange plants were maintained in screenhouses for 4–5 years, periodically surveying their morphology and GUS expression. Enzymatic and DNA analyses were performed on leaf samples of these plants. Plants regenerated from the experiments of Peña et al. (1995) and Cervera et al. (1998) will be referred to as type 1 and type 2, respectively.

Isozyme analyses

Leaf tissue was used to obtain crude extracts for electrophoresis. The production of crude extracts, electrophoresis and gel-staining methods were carried out as described by Asíns et al. (1995). The enzymatic systems examined were: phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), malic acid dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), isocitric acid dehydrogenase (IDH), and leucine aminopeptidase (LAP). Leaves from the Carrizo citrange maternal line were used as a control for every electrophoresis.

PCR, ploidy and Southern analyses

PCR amplifications of the *uidA* and *nptII* genes were performed as described previously (Cervera et al. 1998). Ploidy determination of Carrizo citrange plants was performed from leaf pieces. Plant tissue was processed using a high-resolution DNA kit (PARTEC) and analysed by flow cytometry using a ploidy analyser (PARTEC PA). Genomic DNA was isolated from leaves according to Dellaporta et al. (1983). Southern analyses were performed with 20 µg of *Eco*RI-, *Dra*I-, *Hin*dIII- or *Pst*I-digested samples, which were separated on 1% (w/v) agarose gels and blotted onto nylon membranes (Hybond-N^+, Amersham). Filters were probed with a digoxigenine (Boehringer-Mannheim)-labelled fragment of the coding region of the *uidA* or the *nptII* genes prepared by PCR following the supplier's instructions.

Histochemical and fluorimetric GUS and ELISA NPTII assays

Histochemical GUS activity of the transgenic plants was analysed as described in Peña et al. (1995). GUS activity in leaf samples was quantitated by measuring the fluorescence emitted at 445 nm in the hydrolysis of 4-MUG to 4-MU (Jefferson 1987). NPTII activity in leaf samples was quantitated by ELISA, using a commercial kit (5 Prime→3 Prime, Inc.). Quantitative GUS and NPTII analyses were performed with samples from the upper fully expanded leaves of the last flushes from each plant.

Data analysis

To test the relationship between GUS activity and the number of *uidA* copies, a one-way analysis of variance using log-transformed data was carried out on two sets of data: the complete data and that excluding the class of six copies (only one observation). Additionally, data were re-coded for classes with a number of copies larger than two. Given that the natural-log transformation data did not achieve clear normality, data were re-analysed using a non-parametric approach (Nap et al. 1993). Plants of type 1 and type 2, obtained using different transformation procedures, were analysed in the same way.

Results

Isozymic analysis of transgenic citrus plants

Seven isozyme systems were analysed for ascertaining the nucellar or zygotic origin of transgenic plants. The segregation of genes in the sexual embryogenesis of Carrizo citrange (heterozygous for almost 50% of their enzymatic loci), and other genomic changes provided by tissue culture and/or transformation conditions, could induce

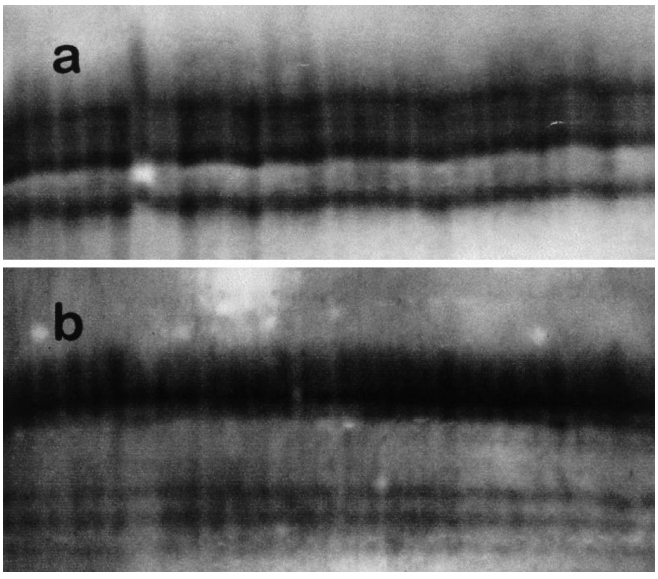


Fig.1 a GOT and **b** MDH isozymic analysis of 20 transgenic Carrizo citrange plants. In each gel, the *first lane* corresponds to a sample from leaves of the Carrizo citrange maternal line

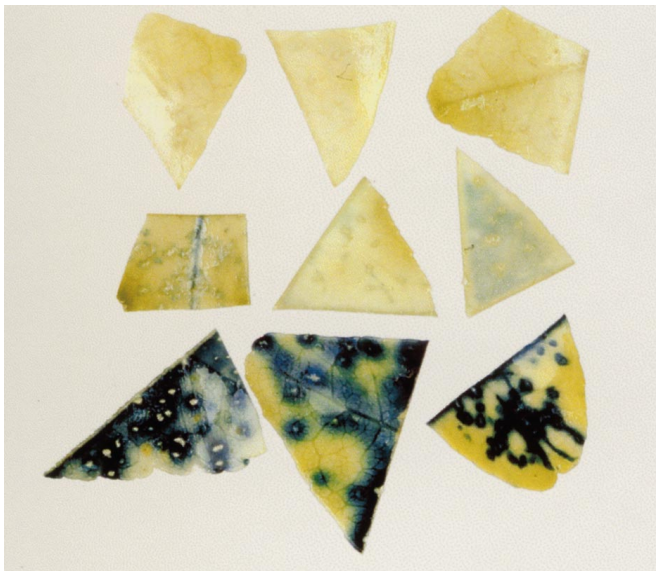


Fig. 2 Comparison among staining patterns exhibited by transgenic citrange plants with low and high GUS expression. *Upper row* control leaf pieces showing no coloration; *middle row* transgenic leaf pieces from some silenced plants showing a pale-blue staining; *lower row* transgenic leaf pieces from some non-silenced plants showing clear-blue staining. All leaves had been punched for easing substrate infiltration. After reaction, leaf pieces were discoloured by means of an ethanol series

variations in some of these enzyme-gene systems. However, no differences were observed in the zymograms (Fig. 1). The same band pattern was found for all the samples, which corresponded exactly to the pattern shown by the female parent from which the starting seeds were taken. These results indicated that no zygotic plant had been recovered from our transformation experiments and that, with the exception of differences in transgene integration loci, all the plants maintained the maternal genotype.

Phenotypical scrutiny of the transgenic citrange population

Four phenotypic variants (plants 40.21, 53.14, 54.2 and 54.20) were detected among the transgenic population. Several morphological features made these plants distinguishable from the maternal phenotype: they developed thicker and broader leaves, with a darker green colour, and usually with broader petiole wings, and showed a slower growth. Ploidy tests of the whole plant collection demonstrated that, except for four plants that were tetraploid (data not shown), all the plants in the population were diploid. Transgenic tetraploids could have originated either by the regeneration of transgenic shoots from tetraploid plant tissue or by a process of polyploidization during in vitro culture. Sixty seedlings coming from the in vitro germination of 40 Carrizo citrange seeds were tested for their ploidy. Seven seedlings (11.7%) proved to be tetraploid, which confirmed the natural occurrence of tetraploid seedlings among the source plant material, and thus the high probability that tetraploid transgenic plants came from initially tetraploid tissues.

No other phenotypic variations were observed among the 70 transgenic citrange plants.

Analysis of transgene expression

GUS analyses of leaf pieces from the transformed plants were performed periodically. All the transgenic samples showed blue staining in successive histochemical analyses during the 5 years of study, while no coloration was visible in the control samples. Obviously, the intensity of staining was different between plants depending on the level of expression of the transgene in each transgenic line. Some of the plants stood out in these assays because they exhibited a marked pale-blue staining in successive assays, only detectable when leaf pieces had been completely discoloured but distinguishable from control samples (Fig. 2). Transgenic plants conserved similar patterns of GUS expression during the whole period of study and no cases of a drastic decrease or increase in transgene activity were found. Among the transgenic citrange population we detected two plants showing a chimeric sectorial GUS expression pattern, in which only part of the plant exhibited GUS activity.

In order to quantitate *uidA* activities, fluorimetric GUS assays were performed on leaf samples from a set of 30 randomly selected GUS-expressing plants. Expression data varied from 0.1 to 154 pmol MU/min per µg of total protein. However, these values fluctuated drastically (nearly 40%) for the same transgenic line in different seasons depending on the physiological state of the plants. The measurements shown in Fig. 4 were carried out on homogeneous tissue collected from the same type of plant material and in the same month. Flu-

Table 1 Determination of the copy number for both transgenes *uidA* and *nptII* upon the hybridisation patterns shown by 30 plants of the transgenic citrus population

Plant	Copy number estimation with *Eco*RI[a]		Copy number estimation with *Dra*I[a]		Copy number determination		Total number of transgenes	plants showing *uidA* silencing[b]
	uidA	*nptII*	*uidA*	*nptII*	*uidA*	*nptII*	*uidA+nptII*	
2.10	2	2	nd	nd	2	2	4	
2.19	2	1	2	1	2	1	3	
3.12	4	4	3	2	4	3	7	S
33.12	2	2	1	1	2	2	4	S
33.14	1	1	1	1	1	1	2	
33.17	3	3	1	1	3	1	6	S
33.18	3	3	2	2	3	3	6	
33.22	2	2	2	nd	2	2	4	S
34.7	1	1	1	1	1	1	2	S
40.2	1	1	1	1	1	1	2	
40.18	2	2	2	2	2	2	4	
40.21	1	1	1	1	1	1	2	
40.24	1	1	1	1	1	1	2	
42.22	2	2	nd	nd	2	2	4	
43.3	2	2	1	1	2	2	4	
44.4	2	2	2	2	2	2	4	
44.11	4	4	4	nd	4	4	8	S
48.9	2	2	2	2	2	2	4	
51.17	4	5	3	4	4	5	9	S
51.22	4	4	4	nd	4	4	8	S
53.8	>6	>6	>4	>3	>6	>6	>12	S
53.13	nd	nd	1	1	1	1	2	
53.14	1	1	1	1	1	1	2	S
54.1	1	1	1	1	1	1	2	
54.2	2	2	1	1	2	2	4	
54.6	1	1	1	nd	1	1	2	
54.14	1	1	nd	nd	1	1	2	
54.16	3	4	2	3	3	4	7	
54.20	1	1	1	1	1	1	2	
54.21	2	2	2	2	2	2	4	

[a] nd: (non-determined value)
[b] S: only plants marked with S displayed *uidA* silencing

orimetric values for samples of 10 of these 30 plants (identified with an S in Table 1), which exhibited a pale-blue coloration in histochemical assays, approximated to zero, and then to values shown by control plants (less than 8 pg MU/min per µg of total protein). Probably the fluorimetric GUS assay is not sensitive enough in our case for differentiating values of GUS activity below a certain limit of detection or for quantitating differences of expression in independent transgenic lines over years. In one experiment, an NPTII ELISA assay was performed on these 30 plants, from samples that were simultaneously assayed for GUS activity. Expression of NPTII was very variable among the transgenic lines, ranging from 1.1 to 6.7 ng of NPTII protein/mg of total protein, but in all cases was significantly different from values obtained in the controls. No correlation was found between GUS and NPTII activities for the same line.

Analysis of transgene integration

Southern analyses were performed on the same 30 transgenic plants (Table 1). The T-DNA of p35SGUSINT has a unique restriction site for *Eco*RI and *Dra*I at the left and right borders of the sequence, respectively (Fig. 3a). DNA digestion with these enzymes generated fragments between T-DNA and plant DNA, and different number of insertions and integration patterns could be revealed after hybridisation with *uidA* or *nptII* probes (Fig. 3b, c, e and f). DNA digestion with *Hin*dIII or *Pst*I, both having two restriction sites at the beginning and at the end of *uidA* and *nptII* genes, respectively, liberates 2.8-kb and 1.6-kb fragments of each gene cassette (Fig. 3a). Hybridisation of *Hin*dIII-digested samples with the *uidA* probe and *Pst*I-digested samples with the *nptII* probe generally revealed fragments of the expected sizes, except for plants that had integrated truncated or rearranged inserts of the T-DNA fragment (Fig. 3d, g). All GUS- and NPTII-expressing plants showed a stable integration of both *uidA* and *nptII* genes, with different and distinguishable hybridisation patterns. The estimated transgene copy number was very variable among the plants analysed, ranging from one to six or more T-DNA copies (Table 1). A different number of inserts, inferred by cleavage with *Eco*RI and *Dra*I and hybridisation with the same *uidA* or *nptII* probe, might indicate the existence of a rearranged insert locus in the plant genome (Table 1). Data from these hybridizations led to the detection of T-DNA rearrangements in 11 of the 30 analysed transgenic lines, corresponding probably to contiguous inserts, in direct or inverted orientation. GUS- and NPTII-non-expressing samples and negative controls showed no T-DNA integration.

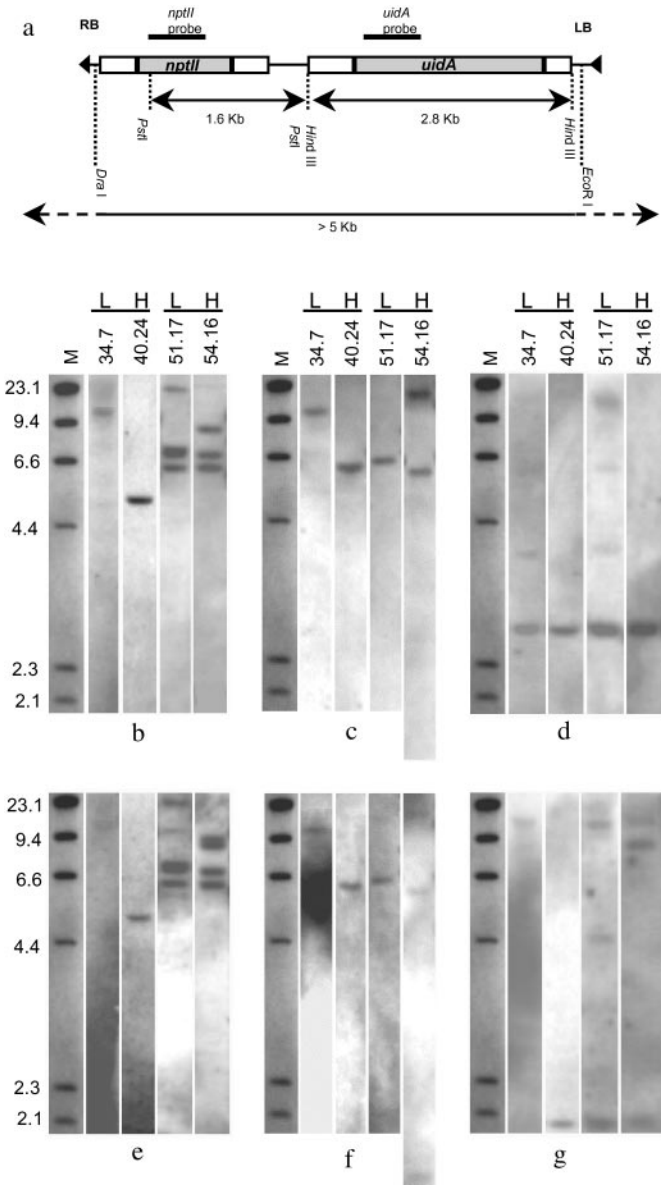
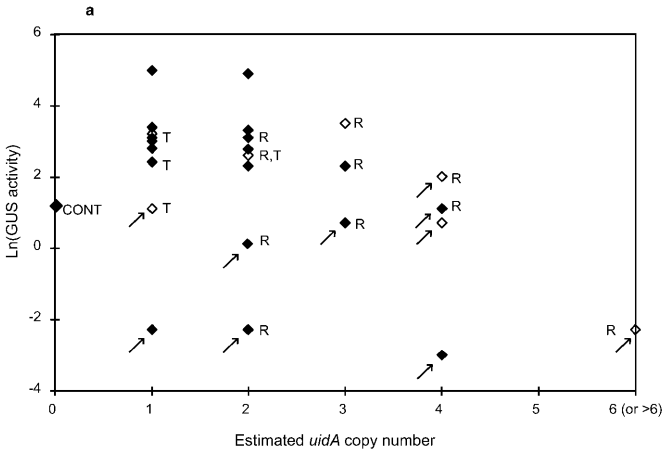


Fig. 3a–g Southern analysis of some transgenic citrange plants with similar integration patterns but showing different levels of *uidA* expression (i.e. plants 34.7 and 40.24, with only only *uidA* copy; plants 51.17 and 54.16, with possible rearrangements of foreign DNA; see also Table 1). **a** Representation of the p35SGUSINT T-DNA fragment, showing the restriction sites for the enzymes used, probes and sizes of hybridisation products. **b** and **e** *Eco*RI-digested samples; **c** and **f** *Dra*I-digested samples; **d** *Hin*dIII-digested samples; **g** *Pst*I-digested samples. Probes were PCR-amplified and digoxigenin-labelled fragments of the *uidA* gene were employed for blots **b**, **c** and **d**, and of the *nptII* gene for blots **e**, **f** and **g**. *L*, low-expressing plants; *H*, high-expressing plants. *M*, DIG-labelled Lambda/*Hin*dIII molecular-weight marker (in kb)



b

Level of *uidA* copies	Number of plants	Mean Ln (GUS act)	Standard deviation	Significance[a]
1	11	2.65	1.80	x
2	11	3.17	0.77	x
>2	8	1.24	1.89	y

[a] Different letters indicate significant differences between classes

Fig. 4 a Natural logarithm of GUS activity plotted against the number of *uidA* gene copies for 30 transgenic citrange plants. Values are the average of different measures taken during 1 month. *Closed symbols* correspond to plants of type 1 and *open symbols* to plants of type 2; plants marked with an *arrow* are those that displayed a pale-blue staining on histological GUS assays (marked with an *S* in Table 1); *R* are plants with possible rearrangements; *T* indicate tetraploid plants. **b** Analysis of variance using log-transformed data. Data are re-coded for classes with one, two and more than two *uidA* gene copies

Relationship between integration patterns and expression

The distribution of GUS activities among the transgenic population and the plotting of GUS activity against the number of transgene inserts (Fig. 4a) showed the high variability of transgene expression and integration patterns among the transgenic plants. Where T-DNA had been inserted at a single locus, transformants displayed either high (i.e. plant 40.24) or low (i.e. plant 34.7) GUS activity. A significant difference was found for GUS expression between plants carrying two, and more than two, *uidA* copies (Fig. 4b). Plants which had integrated more than three transgene copies showed a level of GUS activity lower than 8 pmol MU/min per µg of total protein in all cases and could be considered as plants with silenced GUS activity (or silenced plants). However, we did not find a strict correlation between plants with rearranged inserts and low expression levels (Fig. 4a). On the other hand, similar integration and expression patterns were found between plants of type 1 and type 2 (marked with different symbols in Fig. 4a), regenerated from remarkably different transformation experiments (see Peña et al. 1995 and Cervera et al. 1998, respectively).

Transgenic nature of the tetraploid citrange plants

Southern blot analyses confirmed the integration of the *uidA* and *nptII* genes in the genome of the four tetraploid plants. The levels of integration were similar to those

found for most of the transgenic diploid citrange plants. Transgenic tetraploid citrange plants were also tested for their GUS activity (Fig. 4). Only plant 53.14 showed *uidA* silencing, which represented a proportion similar to that found for diploid plants. Levels of transgene activity shown by the other tetraploid plants were also comparable to the values obtained from diploids.

Discussion

In this paper, we have not detected alterations from the mother genomic background in a population of 70 transgenic citrus plants, since no differences were observed among them in the isozyme and ploidy analyses. The lack of phenotypic variations in the transformants compared to control plants also supported this feature. Phenotypic variation could arise either from the sexual segregation of genes on zygotic seedlings or from events of somaclonal variation usually reported after in vitro culture and transformation processes (Imai et al. 1993; Phan et al. 1996; Wang et al. 1996; Bregitzer et al. 1998; Singh et al. 1998). Somaclonal variation may be even more common after expositing explants to auxins, which are used as a stimulus for cell de-differentiation, division and consequently callus induction. This de-differentiated state makes cells more likely to integrate foreign DNA (Villemont et al. 1997) but at the same time more predisposed to genomic alterations (Hammerschlag 1992). Nevertheless, we did not find phenotypic differences between plants of type 1, obtained after transformation on a culture medium without auxins, and plants of type 2, that were obtained after transformation on a culture medium rich in auxins and thus after regeneration through a prominent callus phase.

Observation of plant morphology led to the detection of four off-type citrange plants. After isozyme and ploidy analyses, they were identified as nucellar tetraploid plants. Some phenotypic characteristics, such as leaf shape and colour and growth habit, were coincident with those described for citrus tetraploids (Cameron and Frost 1968). Whether tetraploidy of these plants had occurred during the in vitro transformation process or was inherent to the source plant material cannot be determined. Nevertheless, the frequency of tetraploid plants within the transgenic population (4%), which was lower than that found for in vitro grown Carrizo citrange seedlings (11.7%), as well as the homogeneous genotype and phenotype of these four plants, led us to think that the most probable explanation for their tetraploidy was the tetraploidy of some of the starting-source seedlings. Regeneration of transgenic polyploids have also been reported for several plant species, such as potato (Imai et al. 1993), petunia (Tagu et al. 1990), muskmelon (Gonsalves et al. 1994) and *Arabidopsis* (Scheid et al. 1996). In all of these cases, it was suggested that the origin of tetraploidy could be attributed to the source plant material used for transformation. The known polysomaty of certain tissues, as in the case of tuber discs of potato or *Arabidopsis* tissues, with cells holding different amounts of nuclear DNA, or the use of protoplasts as the tissue for transformation, in which the formation of multinucleate cell aggregates during culture for regeneration has been usually reported, e.g. petunia, could explain the appearance of tetraploid transgenic plants. In some cases (Imai et al. 1993), the influence of in vitro culture and the genetic transformation process on the increased percentage of tetraploid regenerated plants has also been suggested. In citrus, in vivo occurrence of somatic tetraploids in nucellar seedling progeny of diploid parents is a rather common phenomenon that has been estimated from 0.8 to up 11.1% for Carrizo clones of diverse origin, depending on the cultivar and on environmental factors (Barrett and Hutchison 1978). No cases of citrus polyploids arising from diploid tissues after in vitro culture have ever been reported. In conclusion, although the processes of polyploidization cannot be discarded, it seems more probable that the citrus tetraploid plants originated from tetraploid starting tissues.

On the other hand, it would be interesting to transform tetraploid tissues of citrange with the aim of improving them as rootstocks. Lee et al. (1990) reported that citrus tetraploids, used as rootstocks, conferred a dwarf growth habit to the grafted variety. Dwarfed trees are certainly an interesting option for increasing planting densities, and therefore fruit yields, and for easing cultural practices.

The follow-up of the population of transgenic plants by means of histological and fluorimetric GUS analyses confirmed the stable expression of the *uidA* transgene in all plants over a period of 4–5 years. Patterns of expression were comparable for each line in successive histochemical analyses, although differences of 40% on measured values could be detected in fluorimetric assays, making the quantification of transgene activity over years very difficult. Similar fluctuations in time have also been reported in at least one other woody perennial, cranberry (Serres et al. 1997), and have been attributed to the developmental and physiological state of the plant, as seems also to be the case for citrus. Almost one-third of the population exhibited a typical pale-blue staining pattern, which recurred in the histochemical analyses performed over several years, and which in addition was correlated with very low values of expression as measured by fluorescence analysis. These plants seemed to be undergoing a process of transgene silencing. Depicker et al. (1996) attributed low, though not null, transgene expression levels in a population of transgenic tobacco plants to a process of post-transcriptional silencing, in which highly transcribed transgenes trigger a regulated mechanism of mRNA degradation that leads to low translation levels. Most usually these phenomena are related to partial methylation of the silenced gene sequence (Hobbs et al. 1990, 1993; Ingelbrecht et al. 1994; Matzke et al. 1994). Further analyses would be necessary to confirm this possibility in the silenced citrus plants. Silencing of the *uidA* gene did not correlate with silencing of the *nptII* gene, probably due to the fact that they

are regulated by different promoter and terminator sequences and to different integration features (Kohli et al. 1998). No completely silenced transgenic plants were detected among the population, which may be reasonable since the recovery of transgenic plants was performed after an histochemical GUS assay of regenerating shoots, and only those showing a detectable GUS blue-staining were shoot-tip grafted and allowed to progress into whole plants. This selection scheme possibly restricted the recovery of plants in which total silencing phenomena were occurring.

The most frequent number of T-DNA inserts was one and two, but plants having possibly more than six *uidA* copies were also found. According to data from the Southern analyses, it is suggested that at least a 35% of the analysed plants had integrated multiple T-DNA rearranged inserts at one locus. A significant tendency to low *uidA* expression levels was confirmed for transgenic citrus plants with more than two T-DNA copies, but levels of expression were highly variable for plants having less than two copies. Our data agree in general with the results described by other authors since, although it has been frequently reported that single copies of a transgene are generally more stably expressed than multiple or rearranged insertions, a clear-cut correlation has not been found (Hobbs et al. 1993; Elmayan and Vaucheret 1996; Stam et al. 1997; Kohli et al. 1998). Copy number, position effects and the organisation of a given insert could collectively account for the highly variable levels of expression displayed by the transgenic plants.

In conclusion, we have shown the stable integration and expression of foreign genes in a population of citrus transgenic plants over a period of 4–5 years under natural environmental conditions. This is essential in order to demonstrate the validity of genetic transformation technology for the improvement of perennial woody fruit plants, which are clonally propagated and grow in the field over several decades.

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