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Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology xxx (2006) xxx–xxx
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Long-term stability of marker gene expression in *Prunus subhirtella*: A model fruit tree species

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Received 11 April 2006; received in revised form 14 June 2006; accepted 23 June 2006

Abstract

Transgenic trees currently are being produced by *Agrobacterium*-mediated transformation and biolistics. Since trees are particularly suited for long-term evaluations of the impact of the technology, *Prunus subhirtella* autumno rosa (PAR) was chosen as model fruit tree species and transformed with a reporter gene (*uid*A) under the control of the 35S promoter. Using Southern and GUS fluorometric techniques, we compared transgene copy numbers and observed stability of transgene expression levels in 34 different transgenic plants, grown under *in vitro*, greenhouse and screenhouse conditions, over a period of 9 years. An influence of grafting on gene expression was not observed. No silenced transgenic plant was detected. Overall, these results suggest that transgene expression in perennial species, such as fruit trees, remains stable in time and space, over extended periods and in different organs, confirming the value of PAR as model species to study season-dependent regulation in mature stone fruit tissues.

While the *Agrobacterium*-derived *Prunus* transformants contained one to two copies of the transgenes, 91% of the transgenic events also contained various lengths of the bacterial plasmid backbone, indicating that the *Agrobacterium*-mediated transformation is not as precise as previously perceived. The implications for public acceptance and future applications are discussed.

Keywords: Transgenic woody plant; Step-by-step procedure; Long-term evaluation; GUS; *uid*A; *npt*II

1. Introduction

Genetic transformation technology has facilitated studies of gene regulation in several plant species including trees (Newton et al., 1992; Aronen et al., 1995; Deikman et al., 1998; Krasnyanski et al., 2001; Hawkins et al., 2003). Some of the most problematic barriers to genetic improvement of trees, such as their

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doi:10.1016/j.jbiotec.2006.06.016

large size and long breeding cycles, can be circumvented by the application of these techniques. Because trees have a long lifespan, knowledge of the genetic regulation of mature tissues is of major importance.

The successful introduction of transgenic trees depends on asserting and improving the agricultural achievement of the modified plants and on the stable expression of the transgene (McElroy, 1999). There is no need to consider the inheritance pattern to successive generations in trees, since grafting is the normal method of propagating fruit trees, providing unlimited numbers of selected transgenic lines. The study of transgene expression is of vital importance, whenever transgenic plants are produced. Transgene expression levels are influenced by many factors, in particular the site of integration of the transgene within the plant genome, gene silencing, and the promoter attached (Ainley and Kumpatla, 2004).

Since transgene silencing was reported (Finnegan and McElroy, 1994), the concern about the stability of transgene expression has been increasing. Inactivation of transgene expression has often been observed in annual herbaceous species; relatively little information is available about its occurrence in long-lived perennial species, such as trees. The phenomenon known as gene silencing, can occur immediately following the integration of a transgene or over several generations. Gene silencing in plants has been divided into transcriptional gene silencing (TGS, no mRNA is transcribed) and post-transcriptional gene silencing (PTGS, mRNA is transcribed, but then degraded). This is particularly important for trees, which undergo numerous dormancy cycles and are often exposed to extreme environmental changes during their life. Unstable expression or gene silencing is influenced by many factors, such as the presence of inverted repeats in the complex integration patterns, the nature of the insertion site, the AT/CG composition of the transgene, environmental factors and sexual reproduction (Brandle et al., 1995; Ellis et al., 1996; Metz et al., 1997; Stam et al., 1997; Matzke and Matzke, 1998).

Inactivation of expression in plants with single copies of the transgene can occur (Tingay et al., 1997), but it is not as frequent as the silencing observed in plants with multiple inserted copies. Therefore it is important to produce transgenic plants containing single or low and minimally rearranged transgene insertions. These approaches alone will not always overcome problems with instability of transgene expression. Transgene silencing has also been observed in events with single copy insertion associated with, e.g. position of the insert, GC content of the region and presence of vector DNA (Ainley and Kumpatla, 2004).

Although gene silencing was in the beginning perceived as an impediment to the use of genetic engineering for plant improvement, several studies discovered that such phenomena are involved in a number of important plant processes including plant development (Habu et al., 2001), plant defense mechanism against pathogens (Ainley and Kumpatla, 2004), and transposable elements (Kooter et al., 1999). Recent research focussed on resistance to viruses and bacterial DNA (Laimer et al., 2005), regulation of fruit ripening (Deikman et al., 1998), tolerance to biotic and abiotic stress (Cervera et al., 2000) and modification of fruit quality (Dandekar et al., 2004). Nevertheless, gene silencing remains a potential drawback for genetic engineering approaches.

Particularly in Europe potential adverse long-term effects are attracting major attention; among them the potential instability has been repeatedly addressed as potential hazard. Therefore the need exists for a model fruit tree to address questions of behaviour and stability of expression of transgenes in woody crop species over a longer observation period. It is extremely important that the transgene is expressed stably in a controlled way. The modifications targeted to all of the plant tissues must continue to be expressed until culmination age, even under stress conditions. In other words, transgene expression should remain stable in time and space throughout the lifetime of the plant.

Prunus subhirtella ($2n = 16$, diploid; Bennett and Leitch, 1995), family Rosaceae, genus *Prunus* L., section *Cerasus*, is an ornamental cherry, most often encountered as a horticultural selection (or botanical variety). Originating from Transcaucasia, Asia Minor and Persia, it is used as rootstock for cherries due to its cold hardiness and has some potential for the genetic improvement of cherry. In this study we address long-term transgene stability in *P. subhirtella* (PAR) as model species for molecular studies in stone fruit plants. Using Southern blot and GUS fluorometry techniques, we compared transgene copy number and expression level of transgenic plants generated by *Agrobacterium*-mediated transformation. The stability of transgenic expression was studied in 34 different

transgenic plants grown under *in vitro*, greenhouse and screenhouse conditions, over a period of 9 years in different organs.

2. Materials and methods

2.1. Transformation of P. subhirtella

Embryogenic lines of the cherry rootstock *P. subhirtella* autumno rosa (PAR) were established and transformed using the method described previously (da Câmara Machado et al., 1995). *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al., 1983) containing pBinGUSint (Vancanneyt et al., 1990) carrying the GUS gene with an intron and the *npt*II gene for selection under the control of the 35S promoter, was used for transformation. Transgenic shoots were selected and rooted on DKV-media (Driver and Kuniyuki, 1984) containing 100 mg/l kanamycin (Puschmann, 1993). Plants were acclimatised to greenhouse conditions at different time points, and used as own-rooted plants and for grafting experiments.

Selected lines were chosen for a prolonged period of observation from the originally regenerated 34 lines, according to the available data on molecular analyses and levels of GUS expression *in vitro*, in the greenhouse and under screenhouse conditions. Work was carried out according to the Austrian Gene Technology Law in the frame of the project "Characterisation of transgenic fruit trees and analyses of direct and indirect biological interactions", approved by the Austrian Ministries of Science and Agriculture in 2000 (http://www.boku.ac.at/sicherheitsforschung, Laimer, 2003) to create public perception and acceptance for the new technology. To precisely quantify the observations of phenological development stages of the young trees the scale of Meier et al. (1994) was used, where different stages corresponding to bud break (stages 0–9), leaf development (stages 10–19) and shoot development (stages 31–39) were marked.

2.2. Polymerase chain reaction and Southern blot hybridization

Total genomic DNA was extracted from 1 g of leaf tissue DNeasy® Plant Maxi Kit (QIAGEN) following the supplier's instructions. Gel electrophoresis and spectrophotometry determined the DNA quality and concentration, respectively.

The following pairs of primers were used for the detection of the integrated transgenes: GUS (5′-ATGTTACGTCCTGTAGAAACCC-3′ and 5′-TCATTGTTTGCCTCCCTGCTG-3′), amplifying a 1997 bp fragment of the *uid*A gene (Vancanneyt et al., 1990), *npt*II (5′-AGAGGCTATTCGGCTATGAC-3′ and 5′-ACTCGTCAAGAAGGCGATAG-3′) amplifying a 738 bp fragment of the kanamycin resistance gene (GenBank accession no. U09365), *npt*III (5′-ATCGGCTCCGTCGATACTAT-3′ and 5′-CGTTCCACATCATAGGTGGT-3′) amplifying a 349 bp fragment of the enterobacterium kanamycin resistance gene (GenBank accession no. U09365), backbone 1 (5′-CGTCCATTTGTATGTGCATG-3′ and 5′-GGCCCCAACAGTGAAGTAGC-3′) amplify a 830 bp fragment of backbone (GenBank accession no. U09365), and backbone 2 (5′-TGGCGGTCCTGGGGGCTATT-3′ and 5′-TCGGCACAAAATCACCACTC-3′) amplify a 856 bp fragment of backbone (GenBank accession no. U09365).

PCR amplifications were conducted in a total volume of 25 μl using 1× PCR buffer (QIAGEN), 2 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of each primer, 0.6 units HotStarTaq Polymerase (QIAGEN HotStarTaq™ PCR), 20–30 ng of total genomic DNA or 0.1–0.2 ng of plasmid-DNA. PCR-cycling conditions (Biometra) consisted of an initial denaturation step of 95 °C for 15 min followed by 35 cycles of 60 s at 95 °C, 60 s at annealing temperature (60 °C for GUS, 58 °C for *npt*II and III, 65 °C for backbone 1 and 70 °C for backbone 2), and 2 min at 72 °C. A final step of 10 min at 72 °C ended the cycle.

For Southern analyses 3 μg of genomic DNA was digested with 40 unit of the restriction endonucleases *Nco*I, *Hin*dIII, *Eco*RV (Roche). The digested DNA samples were separated on 1% (w/v) ultrapure agarose gel (GibcoBRL, Gaithersburg, MD), followed by depurination with 0.25 M HCl for 10 min, denaturation with 1.5 M NaCl and 0.5 M NaOH and neutralization with 10× SSPE (3 M NaCl, 0.2 M NaH_2PO_4 and 0.02 M EDTA) at room temperature. The gel was blotted onto Hybond-N^+ nylon membranes in 10× SSPE with the VacuGene XL Vacuum blotting system (Amersham) for 1 h at 50–60 mbar followed by baking at 80 °C for 2 h. Two PCR fragments amplified with the primers set (GUS and *npt*II) were used as

hybridisation probes for *uid*A and kanamycin genes, respectively. The probes were labelled with DIG-dUTP (Roche) and used for hybridisation overnight at 42 °C in DIG Easy Hyb containing 25 ng/ml of DIG labelled probe. Following hybridization, the membranes were detected with CSPD as described (Roche) and exposed to Hyperfilm MP X-ray films (Amersham).

2.3. Plant material to prove stability of transgene expression

To investigate the stability of transgene expression in PAR, three different groups of plants (grown under *in vitro*, greenhouse and screenhouse conditions) were studied. From group 1, 17 samples out of 34 *in vitro* plantlets were harvested at intervals and prepared for the histological and histochemical enzyme assays to gain a comparison of high and low expressing lines.

From group 2, in 1999–2000, different tissues of 34 lines growing in the greenhouse, including closed and developing buds in early spring, developing apical leaves from position one to four and stems were collected.

From group 3, in 2000–2003, samples of nine selected lines growing in the screenhouse were collected in order to have better insight into the effect of different physiological stages on gene expression. To verify the impact of horticultural practices, e.g. grafting on different rootstocks versus plants on own roots, the following types of plants were investigated: original transformants (A), t-buddings on the rootstock F12/1 (O) and self-rooted cuttings (S).

2.4. Fluorimetric GUS assay

For quantitative GUS analysis plant material of different organs and at various developmental stages, indicated in each experiment, was used. Samples were homogenised in GUS extraction buffer in a Retsch mill (MM 2000) and GUS activity in crude plant extracts was determined as described (Jefferson et al., 1987).

Fluorescence of the final product, 4-methylumbelliferone (4-MU), was measured after 24 h in a Hitachi F-2000 fluorimeter and quantified by using a standard curve. GUS activity is expressed as μmol 4-MU per mg protein per hour. Negative controls were leaves from untransformed *in vitro* shoots of PAR. The protein determination of the plant extracts was carried out with the Bio-Rad protein assay.

Data analyses for GUS expression involving statistical comparisons among treatments were made by analysis of variance (ANOVA) and means were compared by *t*-test using software Microsoft Excel 2000.

3. Results

3.1. Copy number determination using Southern analysis

Southern blot assays were performed on a subset of transgenic events generated *via A. tumefaciens*-mediated transformation to estimate the transgene copy number.

Thirty-four individuals of putatively transgenic PAR transformed with the binary vector pBinGUSint (Vancanneyt et al., 1990) were analyzed by PCR for the presence of sequences from the *uid*A gene and the *npt*II gene, respectively. All samples produced detectable transgene products of the expected size for both genes.

The number of integrated T-DNA copies in each plant was determined by Southern analyses with three restriction enzymes, *Hin*dIII, *Nco*I and *Eco*RV, using the *uid*A and *npt*II genes as probes. The restriction enzymes *Hin*dIII and *Nco*I produced variable fragments, which did not allow a clear estimation of the copy number. However the use of *Eco*RV allowed visualizing the presence of the *uid*A and *npt*II gene and yielded fragments that differed between independently transformed plants (Fig. 1). In the *Eco*RV digest, two constant (876 and 231 bp) and one variable fragment hybridizing to the *uid*A probe were shown per plant (Table 1 and Fig. 2b). The variable band represents the left border fragment (Fig. 1). After *Eco*RV digestion, in each plant, one to two variable fragments were found with *npt*II probe hybridization (Table 1 and Fig. 2a). The variable band represents the right border fragment (Fig. 1).

All individuals carried two copies of the *uid*A gene. However, only 15% of the transgenic lines carry a complete double T-DNA insert (Table 1). In fact 85% of transgene carry only one copy of the *npt*II gene.

In summary all samples carried 1–2 copy number of transgenes, which is in agreement with the

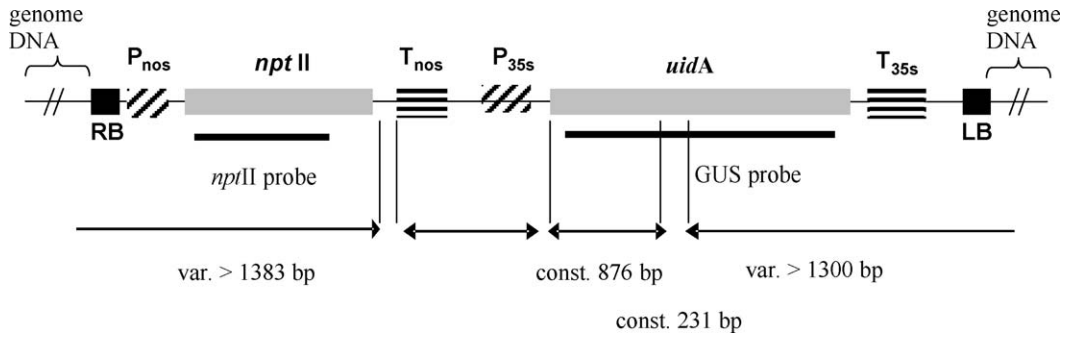

Fig. 1. Map of the T-DNA constructs for transformation, considering enzyme *Eco*RV. RB, T-DNA right border; LB, T-DNA left border; *npt*II, coding region of the neomycin phosphotransferase gene; *uid*A (GUS-intron), coding region of the β-glucuronidase gene; P_{nos}, promoter of the nopalin synthase gene; T_{nos}, terminator of the nopalin synthase; P_{35S}, promoter of the CaMV; T_{35S}, terminator of the CaMV.

reports indicating low copy number integration *via A. tumefaciens*-mediated transformation.

3.2. Integration of T-DNA binary vector backbone sequences

Since a binary vector was used for transformation, transgenic plants might also harbor an additional sequences inserted from the vector backbone, particularly the antibiotic resistance gene [e.g. *npt*III resistance gene (aminoglycoside phosphotransferase type III)]. Three primer pairs covering regions outside the T-DNA borders (see Section 2; Table 1) were used in PCR analysis to determine, whether vector backbone sequences were also transferred to plant cells by *Agrobacterium* during transformation (data not shown). Particularly primers backbone 1 and 2, covering regions near the right and left border, respectively, allow to determine only partial insertions. Results indicated, that 27 of the 34 individuals (79%) were PCR positive for all the screened regions, suggesting that the entire backbone region was transferred during transformation (Table 1). In four individuals (12%; PAR 6, 17, 30, 36), PCR analysis were negative for backbone 1 indicating that the backbone DNA may have been transferred by incomplete cleavage of the left border of the T-DNA. Three transgenic plants (9%; PAR 23, 24 and 65) were free of backbone DNA (Table 1).

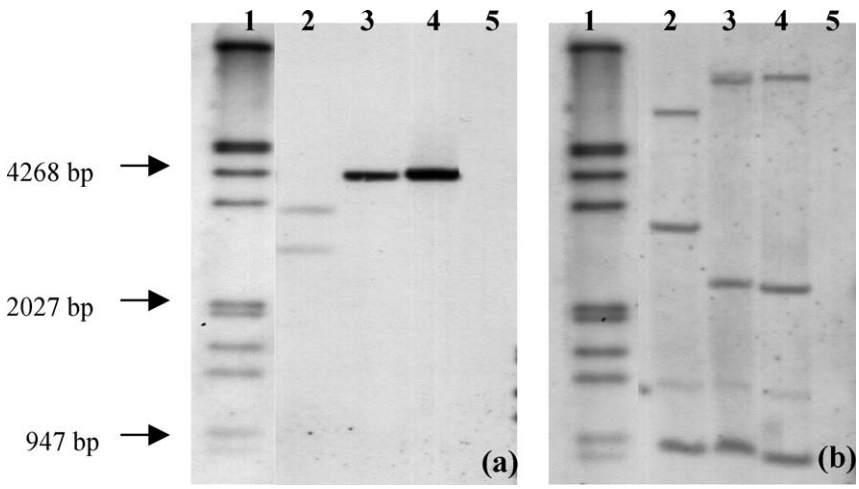

Fig. 2. Southern analyses of DNA from selected transgenic PAR plants digested with *Eco*RV: (a) DNA was probed with *npt*II and (b) DNA was probed with *uid*A. Lane 1: DIG-labeled DNA molecular weight marker III (Roche); lane 2: PAR 65; lane 3: PAR 66; lane 4: PAR 68; lane 5: control DNA from an untransformed plant.

Table 1
Transgene copy number estimation by Southern blot

No.	Transformed plants	PCR		Copy number (*Eco*RV)		PCR (backbone)		
		GUS	*npt*II	GUS	*npt*II	Backbone 1	*npt*III	Backbone 2
1	PAR 6	+	+	2	2	−	+	+
2	PAR 9	+	+	2	1	+	+	+
3	PAR 11	+	+	2	1	+	+	+
4	PAR 13	+	+	2	1	+	+	+
5	PAR 14	+	+	2	1	+	+	+
6	PAR 16	+	+	2	1	+	+	+
7	PAR 17	+	+	2	1	−	+	+
8	PAR 18	+	+	2	1	+	+	+
9	PAR 19	+	+	2	1	+	+	+
10	PAR 20	+	+	2	1	+	+	+
11	PAR 23	+	+	2	2	−	−	−
12	PAR 24	+	+	2	2	−	−	−
13	PAR 25	+	+	2	1	+	+	+
14	PAR 26	+	+	2	1	+	+	+
15	PAR 33	+	+	2	1	+	+	+
16	PAR 30	+	+	2	1	−	+	+
17	PAR 34	+	+	2	1	+	+	+
18	PAR 35	+	+	2	1	+	+	+
19	PAR 36	+	+	2	1	−	+	+
20	PAR 38	+	+	2	1	+	+	+
21	PAR 40	+	+	2	1	+	+	+
22	PAR 41	+	+	2	1	+	+	+
23	PAR 42	+	+	2	1	+	+	+
24	PAR 43	+	+	2	2	+	+	+
25	PAR 45	+	+	2	1	+	+	+
26	PAR 46	+	+	2	1	+	+	+
27	PAR 52	+	+	2	1	+	+	+
28	PAR 56	+	+	2	1	+	+	+
29	PAR 58	+	+	2	1	+	+	+
30	PAR 59	+	+	2	1	+	+	+
31	PAR 62	+	+	2	1	+	+	+
32	PAR 66	+	+	2	1	+	+	+
33	PAR 65	+	+	2	2	−	−	−
34	PAR 68	+	+	2	1	+	+	+
35	nt (non-transgenic)	−	−	0	0	−	−	−

Genomic DNA from 34 transgenic plants was digested by *Eco*RV and hybridized with a DIG-labelled *uid*A (GUS) and *npt*II probes. Integration of vector backbone sequences for *Agrobacterium* with three different primer pairs.

3.3. Transgene expression levels

Quantitative measurements of 35S-*uid*A transgene expression levels were studied in three plant groups by fluorometry based on Jefferson et al. (1987). After *Agrobacterium*-mediated transformation, 34 PAR transgenic plants were obtained. The GUS activity in leaves of 17 *in vitro* grown plants (group 1) of approximately 5–6 cm height ranged from 16.861 to 39.572 μmol/μg protein per hour (Fig. 3a).

GUS activity was assayed in 34 greenhouse grown plants (group 2) 3 years after transfer to soil, when plants were ca. 40–60 cm tall. GUS activity in examined lines was highest in young leaves (1–4 from the top), followed by open buds and stems. Closed buds displayed the lowest activity (Fig. 3b and e). The expression in leaves varied according to age and development in all plants. GUS activity in most transgenic plants was highest in immature leaves and consistently decreased as leaves matured (Fig. 3b). Analyses of variance

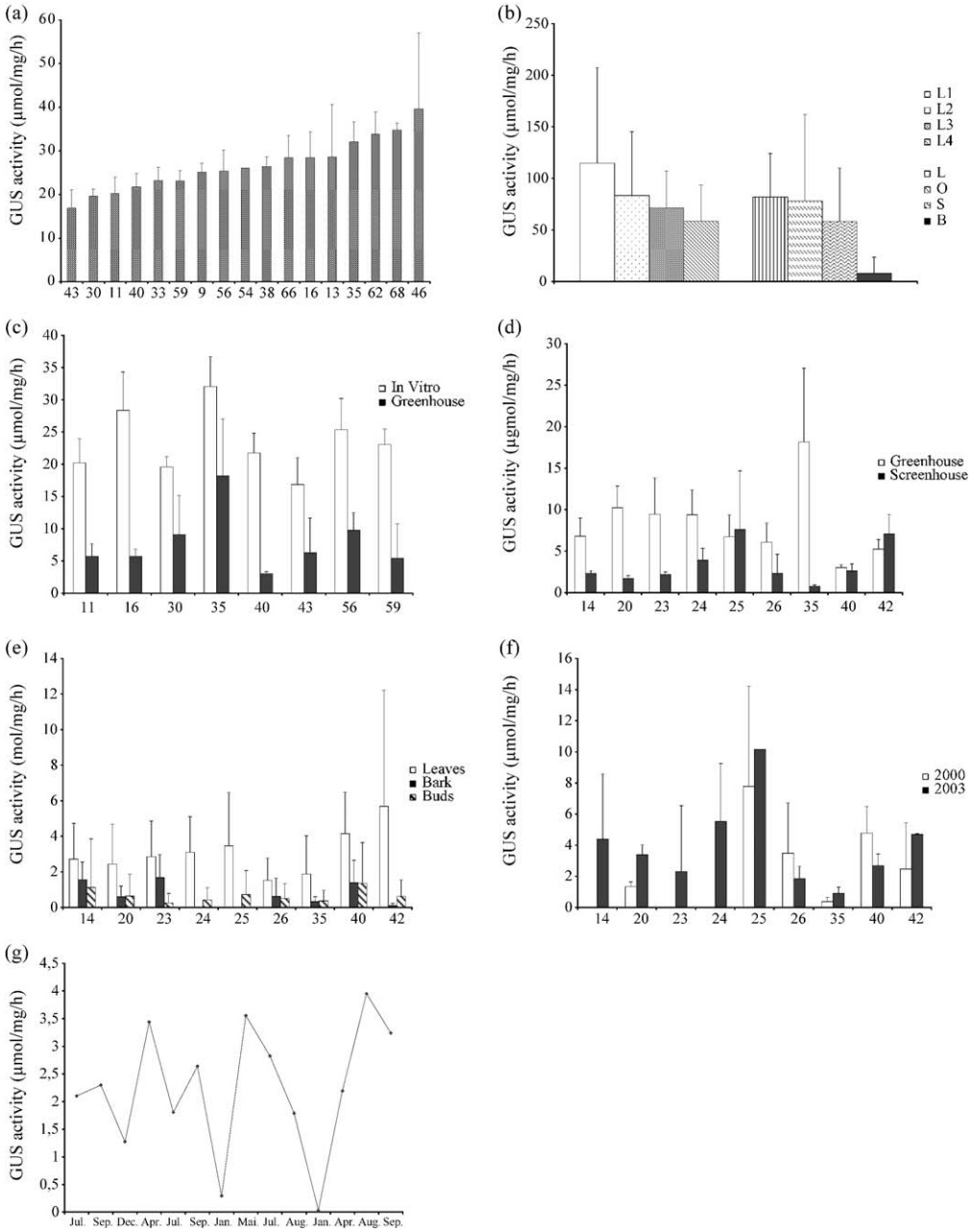

Fig. 3. (a) Transgene expression levels in 17 different individuals of group 1 (*in vitro*) plants. Error bars represent average values of three replications, (b) GUS activities in different tissues of 34 individuals of group 2 (greenhouse). Comparison of activity in leaves at different positions from the top (L 1–4) and different organs (L, leaves; O, open buds; S, stems; B, buds), (c) comparison of transgene activity in leaves of, *in vitro* (group 1) and greenhouse (group 2) plants, (d) comparison of transgene activity in leaves of greenhouse (group 2) and screenhouse (group 3) plants, (e) comparison of GUS activity in three different organs (barks, buds and leaves) of nine individuals of group 3 (screenhouse), (f) comparison of GUS activity levels in leaves of nine individuals of group 3 (screenhouse) harvested in summer 2000 and 2003, and (g) seasonal changes in transient GUS expression in the screenhouse plants from 2000 to 2003.

performed on samples collected from the greenhouse showed a significant difference in the transgene activity between different transgenic plants (data not shown).

In all plants of the nine lines studied from the screenhouse (group 3), GUS activity was found highest in young leaves, followed by bark and buds (data not shown), similar as in group 2. Comparison of the 35S-*uid*A transgene expression levels, as determined by fluorometry, between stems, leaves, buds and barks from plants under glasshouse or screenhouse condition (Fig. 3e) revealed the differences in activity levels between different transformants. However, no significant differences could be detected between leaves-stems and leaves-open buds ($P = 0.51$ and 0.82, respectively), barks and old leaves ($P = 0.07$). Significant differences were detected between young and old leaves ($P = 4.5 \times 10^{-5}$), leaves-buds and leaves-barks ($P = 3.8 \times 10^{-11}$ and 6.9×10^{-6}, respectively). The comparison of transgene activity levels in leaves of different transgenic plants from summer 2000 to 2003 (Fig. 3f) indicates, that the trees continue to express the transgene for three consecutive years in the screenhouse. The transgene expression levels of 2003 are not significantly different ($P = 0.55$) from those observed in 2000, a further evidence for the stability of transgene expression.

Transgenic GUS expression was found in all PAR plants collected from 2000 to 2003 grown under screenhouse conditions (data not shown). The highest amount of expression was observed between April and August and the lowest amount from December to February (Fig. 3g).

Comparison of the GUS activity by fluorometry in leaves of plants grown under *in vitro*, greenhouse and screenhouse conditions (Fig. 3c and d) showed, that in all examined plants significant differences ($P < 0.05$) in activity could be detected according to the growing conditions. For all transgenic lines, transgene activity was significantly higher in *in vitro* plants.

There was no correlation between transgene activity and the estimated number of 35S-*uid*A transgene loci under any kind of growing condition.

4. Discussion

Transgenic fruit trees have already been obtained in several laboratories (for review see: Laimer et al., 2005; Laimer, 2006). In most woody crop species, transformation and regeneration of commercial cultivars are not routine, generally being limited to a few examples with relatively low numbers of transgenic lines (Fladung et al., 1997; Dandekar et al., 2004; Petri and Burgos, 2005). Genetic engineering offers an exciting opportunity for improving woody crop species, since it allows the introduction of new desired characteristics into selected best genotype (Merkle and Dean, 2000). However, the future use of transgenic trees on a commercial basis will depend upon a thorough evaluation of the potential risk of modified plants and transgene stability over prolonged time periods (Ellis et al., 2001). In this paper we have addressed the stability aspect of transgenic PAR plants ($2n = 16$, self-sterile) used as cold tolerant rootstock. These plants are particularly useful because the activity of the transgene could be monitored over the years in different plant organs by qualitative and quantitative assays (Jefferson et al., 1987).

4.1. Southern blot

Thirty-four transgenic PAR lines carrying the *uid*A and *npt*II genes were analysed by PCR, Southern blotting and real time PCR (data not shown). All plants reacting positive in PCR analyses were also positive in Southern analyses when detected with the *uid*A and the *npt*II probes. This confirms the transgeneity of all transgenic lines, produced by the chosen procedure, and underlines the value of the prolonged selection procedure (Puschmann, 1993).

Southern blots further indicated that the transgenic PAR carry an average of 1.6 T-DNA copies. In fact 15% of the transgenic plants carry two copies, while the remainder contain one copy of the *npt*II gene and two copies of the *uid*A gene, rendering these low copy insertion lines interesting for further analyses and breeding purposes. The low value of T-DNA insertion should also facilitate the characterisation of flanking sequences of the T-DNA. In several lines (85%) the number of inserted copies of the *uid*A and *npt*II genes was not linearly corresponding, probably due to T-DNA re-arrangement or an incomplete copy insertion during the transformation process (Ohba et al., 1995; Spielmann et al., 2000).

To overcome limitations in copy number determination occurring with traditional Southern blotting, which

is costly in terms of reagents, labour, time and requires relatively large amounts of plant material, real time PCR (TaqMan) was used, as supposedly speedy, high throughput method with quantitative accuracy. For this study, 10 transgenic plants were chosen for real time quantitative PCR (data not shown). Results were consistent in 70% of analysed transgenic plants obtained using the traditional Southern analysis, which is in agreement with Weng et al. (2004), who described an agreement of 70% for Southern blot and real time PCR. However, in some studies, Mason et al. (2002) and Song et al. (2002) could not distinguish one copy transformants from two copy transformants, while transformants with copy numbers higher than five could be detected. Bubner et al. (2004) reported only 46% agreement in real time PCR and Southern copy number determination. Since the real time PCR (TaqMan) assay is specific to a small gene sequence, only rearrangements, which disrupt the primer binding site, may influence the truth of the result (Mason et al., 2002). Therefore, in this study it was preferred to report the Southern data only.

4.2. Vector backbone

Although *Agrobacterium* was believed to deliver only the T-DNA sequence between the right and left border sequences, integration of non-T-DNA binary vector backbone sequences into the genome of transgenic plant occurs frequently (Kononov et al., 1997; Wenck et al., 1997). It is known that the 25 bp sequence at the right and the left border regions delineate the transferred T-DNA from right to left border (Gelvin, 2000), and their flanking sequences enhance (on the right border) or attenuate (on the left border) their activity (Wang et al., 1987). Due to the fact, that the flanking sequence of the left border is not strong enough to attenuate the transfer of vector backbone sequences during transformation, backbone DNA contamination may exist commonly in *Agrobacterium*-derived transgenic plant and may not be due to the use of binary vectors.

Kononov et al. (1997) showed that about 75% of transgenic tobacco plants contained various lengths of *gus*A gene fragment placed outside of T-DNA region. Wenck et al. (1997) demonstrated, that the frequency of vector backbone insertion range from 30% to 60% depending on plant species, *Agrobacterium* strain and transformation method. Shou et al. (2004) reported, that 75% of transgenic maize events contained backbone DNA sequences of the binary vector. In the case of PAR (this paper) and grapevines (Maghuly et al., 2006), 91% and 15% of transgenic plants contained the backbone sequence of the binary vector, respectively. These sequences could result from one large extended T-DNA segment, that initiated from the right border or from two-separated T-DNA segments, initiated from the right and the left border sequences, respectively (Shou et al., 2004).

In addition, as many as 91% of the transgenic PAR plants also carry the antibiotic resistance marker gene *npt*III, which might be interesting from a scientific point of view to determine the capacity of protection of the different constructs. However, under the current legal situation these are to be excluded from further application for commercial release from the year 2008 onwards (EC, 2001), because *npt*III has been classified by EFSA (2004) as a non-appropriate marker gene.

4.3. GUS activity

We have addressed the stability aspect by following transgene expression in three groups of PAR plant species considered as model fruit tree grown under *in vitro*, greenhouse and screenhouse conditions. The analyses of transgene expression in a rather large number of different transgenic plants under different condition enabled us to evaluate conclusively the stability of expression.

Initially in group 1 a range of *in vitro* shoots were tested to confirm the success of the genetic transformation, to determine the expression level, and to attempt a first classification of low, medium and high expressing transgenic plants. From selected lines several plantlets were repeatedly tested to gain insight in the variation within plantlets due to the physiological stage of the plants. Since five-fold repetitions of 17 *in vitro* plants at five sampling times indicated high fluctuations among the measured values, we concluded that it was not possible to classify different lines as high or low expressors, due to the fact that they were growing *in vitro*, i.e. under altered physiological conditions.

Detailed assessments of group 2 in the greenhouse by GUS fluorometry indicated differences in expression levels between different organs, and between different leaf position from top (different stages of

maturity). It has been suggested, that the observed differences most probably reflect differences in general metabolic levels (Willmitzer, personal communication). However, such observations also emphasise the innate sampling difficulty, when working with trees compared to *in vitro* plants or herbaceous plants, growing under controlled conditions. It is known from studies with forest tree species (Ellis et al., 1996), that the activity of GUS under the 35S promoter is higher under *in vitro* than under *in vivo* conditions, and that we should expect seasonal variations, correlated most probably with the physiological activity of the plant material under study.

Differences in expression levels were detected between the leaves and stems of group 2 plants (greenhouse). Such differences in tissue specificity are perhaps associated with the process of lignification, which is more pronounced in woody tissues, or might be attributed to post-transcriptional regulation of GUS expression, as reported by Krasnyanski et al. (2001). Serres et al. (1997) reported, that the highest GUS activity was usually observed in young rather than in mature leaves, which might be correlated with the accumulation of polyphenol compounds, which inhibit GUS activity in mature leaves.

Nevertheless, it is interesting to note that most of the transgenic plants showed a reduction in transgene expression levels following transfer to greenhouse and screenhouse conditions. Such results are in agreement with other studies (Brandle et al., 1995; Pinçon et al., 2001; Hawkins et al., 2003), which reported that stress and other changes in environmental conditions, as well as the developmental stage of the plant could affect the expression level of transgenes, and consequently, the characteristics targeted. Although the level of transgene expression was reduced, the transgenes continued to be expressed without any case of gene silencing. In addition, phenological observations of the cherry plants during the vegetative growth phase over three consecutive years showed a normal development without morphological differences between the transgenic and the non-transgenic control plants.

Changes in transgene expression levels often accompanied by changes in DNA methylation levels have been associated with changes in environmental conditions and stress (Matzke and Matzke, 1995), a situation which could be experienced upon transfer to the greenhouse or the screenhouse.

Our results suggest, that transgene expression in woody plants, such as stone fruits, appears to be stable under *in vitro* and *in vivo* conditions, both in the greenhouse and in the screenhouse. Fladung et al. (1997) suggested, that gene silencing is relatively rare in woody trees, and indeed our results indicate that transgene expression is stable over long period under screenhouse conditions, which approximately reflect field conditions. Nevertheless, it is important to note, that gene silencing events in annual plants are often related with plants of the second generation, which are homozygous, whereas the plants analyzed in this study were the R0 generation, and hence heterozygous for the transgene.

Season had a pronounced effect on transgene expression in PAR plants. A marked seasonal variation in the overall gene expressions in Scot pine (Nuotio et al., 1990) and in other conifer species was reported by Ziegler and Kandler (1980). Moreover, during the growing season, GUS activity increased parallelly to growth initiation in early spring and to new flush development. In our study, the highest GUS expression was measured from April to August (summer) and the lowest from December to February (winter), which, according to Aronen et al. (1995), represents the transition phase between reduced wintertime activity and renewed metabolic activity. Typically, both in summer and winter periods, the values of GUS activity also varied between different individual plants. It is interesting to know, that during the transition phase in March several functions at the cellular level are activated, e.g. the size of nucleoli, and the amount of ribosomes increase (Häggman, 1987; Kupila-Ahvenniemi et al., 1987; Aronen et al., 1995). In addition, the *in vitro* translation capacity of ribosomes and the regenerative response of buds are increased (Hohtola, 1988). These changes, indicating a high cytoplasmic activity, co-occur with the period of maximum GUS expression.

Another reason for the highest activity in summer and the low values in winter could result from a general inability of bud cells to react to cytokinin at the end of the winter period. Likewise, Qamaruddin et al. (1990) observed, that cytokinin activity in pine buds decreased after a maximum in late March or early April. Newton et al. (1992) also reported that growth regulators are important for transient GUS expression in conifer species. Enhanced expression resulting from growth regulator pre-treatment may be due to a stimulation of

cell division (Aronen et al., 1995). These results confirm the value of PAR as model system to study season-dependent regulation in mature stone fruit tissues.

However, a final evaluation of a trial under open field conditions is necessary to confirm the genetic stability of the introduced genes and their interaction with other target and non-target organisms.

Acknowledgements

This work has been supported by the Austrian Federal Ministries of Science (BMBWK) and Agriculture (BMLFUW) in the frame of the project "Characterisation of transgenic fruit trees and analyses of direct and indirect biological interactions".

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