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Transgenic almond (*Prunus dulcis* Mill.) plants obtained by *Agrobacterium*-mediated transformation of leaf explants

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Abstract Almond (*Prunus dulcis* Mill.) leaves were transformed with the marker genes *gus*A (β-glucuronidase) and *npt*II (neomycin phosphotransferase II) via *Agrobacterium*-mediated transformation. Bacterial strains and preculture of explants affected efficiency of gene transfer evaluated by transient expression assays. Following transformation, shoots were induced from primary explants on medium without kanamycin and exposed to selection 20 days after cocultivation. From 1419 original leaves, four shoots (A, B, C and D) were obtained that showed amplification of the predicted DNA fragments by polymerase chain reaction (PCR). After micropropagation of these shoots, only those cloned from shoot D gave consistently positive results in histochemical GUS detection and PCR amplification. Southern blot hybridisation confirmed stable transgene integration in clone D, which was also negative in PCR amplification of an *Agrobacterium* gene. Additional molecular analysis suggested that the remaining three shoots (A, B and C) were chimeric.

Key words Almond · *Prunus* · Transformation · *Agrobacterium* · Adventitious regeneration

Abbreviations *BA* N^6-Benzyladenine · *IAA* Indole-3-acetic acid · *IBA* Indole-3-butyric acid · *LB* Luria broth · *MS medium* Murashige and Skoog medium · *GUS* β-Glucuronidase

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Introduction

Genetic improvement of fruit tree species by classical breeding methods is a slow and difficult process due to the long generation time of these plants. This makes them ideal targets for gene transfer technologies which can provide a direct route for the introduction of a specific genetic change within a short period of time. One of the prerequisites for successful plant transformation is the availability of a regeneration protocol that is compatible with the gene transfer method of the target species. However, woody plants such as fruit trees are recalcitrant with respect to these processes. Transgenic plants from cultivars of species such as apple (James et al. 1989; De Bondt et al. 1996) pear (Mourgues et al. 1996), apricot (Machado et al. 1992) and chestnut (Seabra and Pais 1998) have already been obtained. In these cases, confirmation by Southern blot of transgene integration has only been reported for apple and pear. Many additional economically important fruit species have as yet not been transformed.

In almond (*Prunus dulcis* Mill.), a major nut crop worldwide, regeneration of adventitious shoots either from juvenile or from adult tissues has been demonstrated (Mehra and Mehra 1974; Miguel et al. 1996). Gene transfer through *Agrobacterium*-mediated transformation of leaf pieces with production of transformed calli has also been described (Archilletti et al. 1995). However, regeneration of transgenic almond plants has not yet been reported.

In this paper, we present a study on transformation of almond leaf explants by *Agrobacterium tumefaciens* for the first successful recovery of transgenic almond plants.

Materials and methods

Plant material

Leaf explants used in the experiments were taken from shoots derived from germinated almond seeds collected from cultivar Boa Casta, and propagated in vitro at 23±20 °C, 16 h photoperiod, on

Murashige and Skoog (1962) medium (MS) containing 0.3 mg/l N^6-benzyladenine (BA) and 0.01 mg/l indole-3-butyric acid (IBA) as previously described by Miguel et al. (1996).

Bacterial strains and vectors

Agrobacterium strains LBA4404 (Hoekema et al. 1983) carrying the plasmid p35SGUSINT (Vancanneyt et al. 1990) and EHA105 (Hood et al. 1993) carrying p35SGUSINT or the plasmid pFAJ3003 (De Bondt et al. 1996) were used as vector systems for transformation. Plasmid pFAJ3003 contains the *npt*II gene under the mannopine synthase (*mas*) promoter, and the *gus*A gene driven by the nopaline synthase (*nos*) promoter and terminator sequences located near the left border. Plasmid p35SGUSINT contains the *npt*II gene under regulatory control of the *nos* promoter and terminator, and the *gus*A coding region, containing a plant intron, linked to the cauliflower mosaic virus 35 S (*CaMV35S*) promoter, located near the left border. Both *Agrobacterium* strains were grown on Luria broth (LB) medium with appropriate antibiotics (50 mg/l kanamycin and 50 mg/l rifampicin for LBA4404 and EHA105 carrying p35SGUSINT, and 300 mg/l streptomycin, 100 mg/l spectinomycin and 50 mg/l rifampicin for EHA105/pFAJ3003). For cocultivation, isolated colonies of bacteria were picked from selection plates and grown overnight in 10 ml of LB liquid medium at 28 °C until an optical density of 0.6–0.8 at 600 nm was reached. Prior to plant inoculation, 20 μM of acetosyringone was added to the bacterial suspension.

Plant tissue culture and transformation

Explants used for transformation were the four youngest fully expanded leaves of 3-week-old micropropagated shoots. The leaves were wounded by making cuts perpendicular to the midrib, not reaching the leaf edges, with a scalpel previously dipped in the bacterial suspension. Prior to wounding, some of the leaves were precultured on induction medium for a period of 3–4 days. Cocultivation was carried out for 3 days in darkness with the adaxial side of the leaves in contact with an induction medium consisting of MS salts and vitamins supplemented with 1.5 mg/l thidiazuron, 0.5 mg/l indole-3-acetic acid (IAA), 0.01 mg/l 2,4-dichlorophenoxyacetic acid and solidified with 2 g/l gelrite (Miguel et al. 1996). After cocultivation, the leaves were transferred to the same medium supplemented with cefotaxime (300 mg/l), or cefotaxime (300 mg/l) and kanamycin (10 or 15 mg/l) and maintained in the dark at 23 ± 20 °C. After 20 days, all leaf explants were transferred to a shoot elongation medium containing 1 mg/l BA (Miguel et al. 1996), 200 mg/l cefotaxime and 50 mg/l kanamycin solidified with gelrite (2 g/l), under a 16-h photoperiod. Shoots surviving after 3 weeks on shoot elongation medium were excised from the explants and cultured on micropropagation medium containing 30 mg/l kanamycin, solidified with 7 g/l micro agar (Duchefa) for at least two subcultures. Shoots that were still green after this selection period were then subcultured every 3 weeks on medium without kanamycin, and propagated. The sensitivity of uninoculated almond leaves to kanamycin was previously tested by adding 0, 10, 15, 20 and 50 mg/l kanamycin to the induction medium. Control explants were treated as described above except that the cuts were made with a sterile scalpel. At least 20 control explants were tested per kanamycin concentration.

Histochemical β-glucuronidase (GUS) assay

Leaves were assayed for expression of the *gus*A gene following the histochemical staining procedure described by Jefferson (1987) with some modifications. The leaves were incubated overnight at 37 °C in X-Gluc dissolved in a small volume of dimethyl sulphoxide and diluted to 0.4 mg/l in 100 mM K_2HPO_4 (pH 7.2) containing 0.5% (vol/vol) Triton X-100. After the overnight staining (12–14 h) chlorophyll was extracted by soaking the tissues for several hours in 70% EtOH. β-Glucuronidase expression was measured immediately after cocultivation and 4 or 7 days after cocultivation. Quantification of the GUS-expressing units was made by counting the number of blue spots on the white leaf surfaces, using a stereomicroscope. Before and after passing through a multiplication phase, putatively transgenic shoots were also assayed for GUS expression using the same procedure. Control explants were also treated as described.

PCR and Southern blot analysis

DNA extraction

For PCR analysis, DNA was isolated from two to three young leaves of kanamycin-resistant shoots and wild-type control shoots using a CTAB procedure (Doyle and Doyle 1987). For Southern blot hybridisation, total genomic DNA was isolated from young shoots of putative transformants and controls, following the method of Doyle and Doyle (1987) as modified by Weising et al. (1995) with further modifications. One percent (wt/vol) polyvinylpyrrolidone was added to the extraction buffer and RNase A was added after precipitation and resuspension of the pellet in TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 8.0). After RNase treatment, the mixture was extracted once with phenol/chloroform (1 : 1) and once in chloroform. DNA was precipitated by the addition of 1/10 vol of 3 M sodium acetate and 2 vol 96% EtOH, washed and finally resuspended in TE buffer.

PCR analysis

PCR was performed in a Biometra (Uno-Thermoblock) thermocycler. The primers used for amplification of a 700-bp fragment of the *npt*II gene were 5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATACCGTA-3′ and those used for amplification of a 366-bp fragment of the *gus*A gene were 5′-CCCGGCAA-TAACATACGGCGT-3′ and 5′-CCTGTAGAAACCCCAACCCGT-3′. For each PCR, the amount of DNA used was 15–20 ng of the appropriate plasmid as positive control and 75–100 ng of plant DNA. The reaction mixture contained 0.4 mM NTPs, 2.5 mM $MgCl_2$, 0.4 μM of each primer, Taq polymerase buffer and 1 unit of Taq polymerase. After heating the samples to 94 °C for 5 min, Taq polymerase was added and the reaction proceeded with 30 cycles of 94 °C for 1 min, 65 °C (*npt*II primers) or 60 °C (*gus*A primers) for 1 min and 72 °C for 1 min. A final elongation step was carried out at 72 °C for 5 min. PCR products were separated by electrophoresis on 1% (wt/vol) agarose-ethidium bromide gels. To assure that amplification of DNA fragments obtained with the above primers would not be false positives produced by contaminating agrobacteria, an additional PCR was performed using primers for the amplification of a bacterial kanamycin resistance gene (*npt*I) which is located outside the T-DNA borders. The primers were 5′-ATCGGCTCCGTCGATAC-TAT-3′ and 5′-CGTTCCACATCATAGGTGGT-3′ and the reaction was carried out using identical conditions except that the annealing temperature of the primers was 56 °C.

Southern blot analysis

DNA (7–9 μg) from putative transformants and control plants was digested overnight at 37 °C, using 10 units/μg DNA of *Hind*III to generate an internal fragment corresponding to the *gus*A gene, or 5 units/μg DNA of *Eco*RI to generate a border T-DNA fragment. Five micrograms of undigested DNA from transformants along with digested genomic DNA samples and *Eco*RI and *Hind*III digests of p35SGUSINT (0.8 ng) were electrophoresed on a 0.8% (wt/vol) agarose gel and blotted onto a nylon membrane (Hybond-N+, Amersham) following standard procedures (Sambrook et al. 1989). A 366-bp fragment from the *gus*A gene as probe was generated through a PCR reaction using p35SGUSINT as template and the previously described primers and parameters. The amplified DNA fragment was separated by electrophoresis in a 1% (wt/vol) agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen). The fragment (50 ng) was fluorescein-labelled according to the instructions of Gene Images random prime labelling module (RPN 3540, Amers-

Table 1 Expression of the *gus*A gene in leaves cocultivated with LBA4404 (p35SGUSINT) or with EHA105 (p35SGUSINT/pFAJ3003) determined 0, 4 or 7 days after cocultivation. At least ten leaves were tested for each assay. (*NT* not tested)

Days after cocultivation	LBA4404/p35SGUSINT		EHA105/p35SGUSINT		EHA105/pFAJ3003	
	GUS+ leaves (%)	Mean number of blue spots/ leaf	GUS+ leaves (%)	Mean number of blue spots/ leaf	GUS+ leaves (%)	Mean number of blue spots/ leaf
0	40	3.0	86	23.3	83	8.5
4	40	2.5	81	11.7	83	8.0
7	30	2.3	81	9.5	NT	–

ham). Hybridisation of filters was carried out using the Gene Images CDP-Star detection module (RPN 3510, Amersham) and hybridisation signals detected by a 40 min exposure to Hyperfilm-MP (Amersham).

Rooting and acclimatisation

Three-week-old transgenic almond shoots, with 2–3 cm in length, were cultured overnight in darkness at 22 °C on a 170 mg/l IAA solution, pH 5.8, solidified with 2 g/l gelrite. Shoots were then transferred to MS half-strength salts and MS vitamins, supplemented with 20 g/l sucrose, pH 5.8, solidified with 2 g/l gelrite, under a 16-h photoperiod. One week later, shoots were again transferred to the same basal medium except that it was solidified with 7 g/l micro agar (Duchefa). When roots were at least 2 cm long, plants were transferred to a mixture of soil:vermiculite (3 : 1) in pots and humidity was gradually decreased to environmental conditions.

Results and discussion

Effect of bacterial strain, plasmid and preculture period

In species such as almond, where low transformation efficiencies are expected, the study of the effect of several factors, by comparing percentages of recovered transformed plants, may prove unsuccessful because limited numbers of transformants are produced. Therefore, as a first approximation to evaluate the influence of diverse factors on the efficiency of T-DNA transfer, experiments were conducted to observe the levels of transient GUS expression in inoculated leaves. It is known that results of transient GUS expression, originating mainly from non-integrated T-DNA copies, may not necessarily correlate with stable transformation events. However, these studies can be used as a guide, and only major differences between tested parameters were taken into consideration for the establishment of a transformation protocol in almond.

Cocultivation with the *Agrobacterium* strain EHA105 resulted in a much higher percentage of GUS-positive explants and GUS-expressing units when compared to the LBA4404 strain carrying the same plasmid, p35SGUSINT, and the percentage of GUS-positive explants remained similar from day 0 to day 7 after coculture (Table 1). With both strains, most of the blue spots were located near the cut edges, often associated with vascular bundles (Fig. 1 a). LBA4404/p35SGUSINT was tested in our experiments because it had been successfully used for the regeneration of transgenic plants in related *Prunus* species (Machado et al. 1992). This strain has also been used in almond to obtain transgenic calli from transformation of leaf pieces (Archilletti et al. 1995) and the levels of GUS expression 3 days after cocultivation were genotype dependent but considered satisfactory. In our work, we found EHA105 to be the most effective strain and it was therefore used in all subsequent experiments. The effectiveness of EHA105, or EHA101 from which it derives (Hood et al. 1993), has been observed for other fruit tree species (De Bondt et al. 1994; Peña et al. 1995; Mourgues et al. 1996). The supervirulence of A281 which is the parent oncogenic strain, is correlated with *vir*G and 3-*vir*B loci (Jin et al. 1987) and may result from enhanced transcription of the *vir* genes, leading to a more efficient transport of the T-DNA through the bacterial cell wall (Van Wordragen and Dons 1992).

Table 2 Influence of preculture of explants on transient GUS expression assayed immediately following cocultivation of leaves with *Agrobacterium tumefaciens* EHA105 carrying p35SGUSINT. At least 14 leaves were tested for each assay

Days of preculture	GUS + leaves (%)	Mean number of blue spots/leaf
0	13	12.0
3	86	23.3

The introduction of a preculture period of leaves prior to *Agrobacterium* infection led to an almost seven fold increase in the percentage of GUS-positive explants (Table 2). The number of gene transfer events also increased. The effect of preculture of explants on transformation efficiency is not clear. It has been proposed that extended preculture may be deleterious for transformation in some species (Janssen and Gardner 1993; De Bondt et al. 1994). However, we decided to introduce a preculture period in all subsequent experiments because it attenuated the drastic decrease in regeneration capacity after cocultivation, which in some cases dropped to zero even in the absence of kanamycin (data not shown). Here, the preculture period is applied without further wounding the excised leaves.

The results concerning gene transfer efficiencies when using EHA105 carrying two different plasmids, p35SGUSINT or pFAJ3003, were not significantly different (Table 1); both plasmids, therefore, continued to be used in further experiments. It should be noted that although the *gus*A gene in pFAJ3003 does not contain a plant intron as in p35SGUSINT, the *nos* promotor driving the gene does not direct detectable *gus*A expression in *A. tumefaciens* (De Bondt et al. 1996).

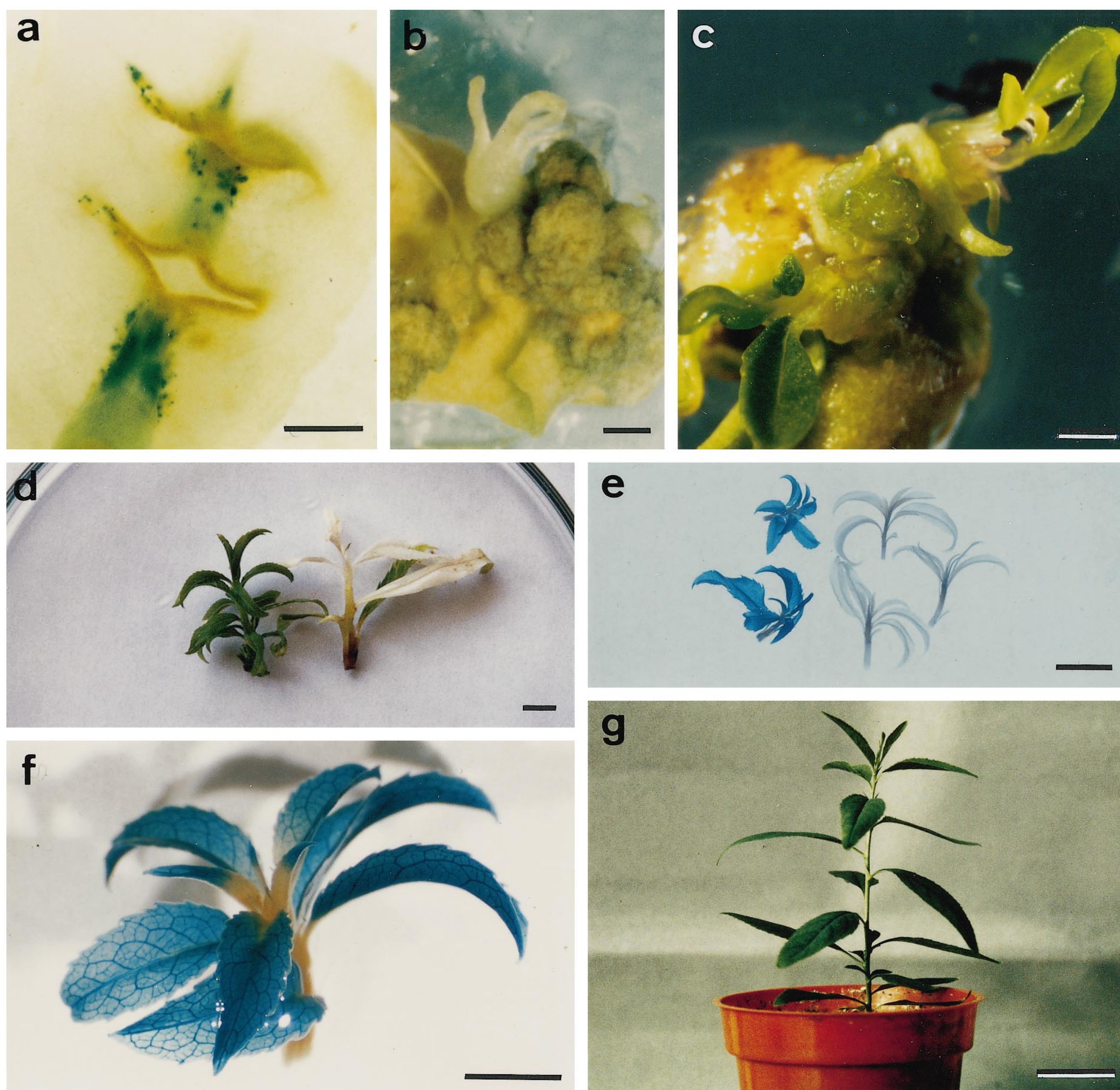

Fig. 1 a–g *Agrobacterium*-mediated transformation and plant regeneration in almond. **a** Staining pattern after histochemical GUS assay on precultured leaf following cocultivation with EHA105 strain (*bar* 0.8 mm). **b** Transformed leaf with white adventitious shoot on shoot elongation medium (50 mg/l kanamycin) following induction on medium containing 10 mg/l kanamycin (*bar* 1 mm). **c** Adventitious shoots obtained in identical conditions except that induction was performed on medium lacking kanamycin (*bar* 1 mm). **d** Kanamycin-resistant (*left*) and sensitive (*right*) shoots after two subcultures on selective micropropagation medium (30 mg/l kanamycin) (*bar* 3 mm). **e** GUS expression in transgenic shoots (*blue*) and in wild-type controls (*white*) (*bar* 9 mm). **f** Detail of transgenic shoot showing strong GUS expression (*bar* 3 mm). **g** Transgenic almond plant transferred to soil (*bar* 3.6 cm)

Selection strategy

When compared to medium lacking kanamycin, where 75% of control leaves (untransformed explants) regenerated shoots, all treatments we tested containing this antibiotic in increasing concentrations had dramatic effects on adventitious shoot regeneration. On medium with 50 mg/l kanamycin, all the explants showed severe necrosis and died without any callus formation. At 20 mg/l kanamycin, some callus formed initially but the explants gradually turned brown, and no regenerated buds developed. On media containing 10 and 15 mg/l kanamycin, a few buds could be regenerated (less than 15% of regenerating leaves) but these remained small and white and did not develop further.

Table 3 Percentage regeneration obtained at different levels of selection applied during shoot induction, following transformation with *Agrobacterium* strain EHA105, and number of surviving shoots after one or at least two subcultures on micropropagation medium with 30 mg/l kanamycin. Regenerated shoots, from independent sites, were recorded for 7 weeks after inoculation

Kanamycin (mg/l)	Regenerating leaves/ total leaf number	Total regenerated shoots	Surviving shoots after one subculture	Surviving shoots after two subcultures
p35SGUSINT				
0	546/1019	551	151	13
10	25/134	25	0	–
15	4/166	7	0	–
pFAJ303				
0	179/400	183	43	8
15	13/160	13	0	–

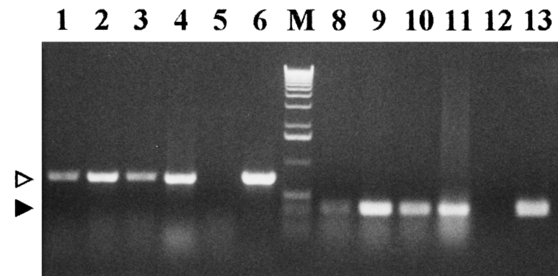

Fig. 2 Electrophoretic analysis of the PCR products of four putative transgenic almond shoots with primers for *npt*II (*lanes 1–6*) and *gus*A (*lanes 8–13*) genes. [*M* size marker 1-kb DNA ladder; *lanes 1*, *8* shoot A; *lanes 2*, *9* shoot B; *lanes 3*, *10* shoot C; *lanes 4*, *11* shoot D; *lanes 5*, *12* wild-type control plant; *lanes 6*, *13* positive control p35SGUSINT plasmid. *White and black arrowheads* point to amplification of the 700-bp and 366-bp fragments of the *npt*II and *gus*A genes, respectively

Due to the extreme sensitivity of almond control explants to kanamycin, we decided not to apply selection pressure until after 20 days post-cocultivation with *Agrobacterium* or to do so at low concentrations (10 and 15 mg/l) immediately after cocultivation. Shoots surviving this treatment were then subjected to higher levels of selection on shoot elongation and micropropagation media (50 and 30 mg/l, respectively).

Transformation

As shown in Table 3, the percentage of regenerating leaves, when kanamycin was present in the induction medium, was very low. Moreover, all shoots induced under these conditions and exposed for 3 weeks to 50 mg/l kanamycin on shoot elongation medium (Fig. 1b) were not able to survive on micropropagation medium with 30 mg/l kanamycin. Therefore, many explants were selected for the experiments in which a kanamycin-free induction medium was used. In these experiments, using either p35SGUSINT or pFAJ3003 as plasmid vectors, a higher regeneration rate was obtained, as expected, but the number of shoots rarely exceeded one per explant and drastically decreased after successive transfers through selection media (Fig. 1c, d). We conclude that many of the initial shoots were indeed escapes; however, 6 weeks after applying selection pressure, we were able to eliminate 73 or 77% of the total shoots that had been recovered using p35SGUSINT or pFAJ3003, respectively. After an additional 3-week period under selection, a reduced number of buds (21) survived for analysis.

In species showing high kanamycin sensitivity, alternative selection schemes may be an easier way of recovering transgenic shoots. Delayed selection as one of these alternative strategies, has proven successful in apple (Yepes and Aldwinckle 1994; Yao et al. 1995) and in apricot (Machado 1992). This strategy allows cell division to occur and also formation of transformed cell clusters or shoot initials, depending on the delay period. In many cases, the exposure of the few transformed cells to the toxic products resulting from necrosis of adjacent non-transformed cells may prove lethal.

PCR analysis and GUS expression

Due to the minimal amount of plant tissue needed for PCR analysis, all the shoots obtained in the transformation experiments that exhibited some level of resistance to kanamycin were analysed. Of the 21 surviving shoots, only 4 showed amplification of the predicted 700-bp internal fragment for the *npt*II gene and the 366-bp fragment of the *gus*A gene (Fig. 2). No amplification was observed in wild-type controls. Three of the PCR-positive shoots were derived from transformation with p35SGUSINT (B–D; Fig. 2) with the fourth (A; Fig. 2) resulting from transformation with pFAJ3003. For samples originating from transformation with p35SGUSINT, an additional PCR control was performed using primers for the bacterial kanamycin resistance gene located outside the T-DNA borders. In this reaction, only the positive control sample (p35SGUSINT) was amplified (data not shown), ruling out the possibility of bacterial contamination giving false-positive results.

Histochemical GUS assays on small leaf portions to detect expression of the *gus*A gene in these shoots, were also performed. One of the shoots always gave consistent GUS expression while the others did not consistently show the characteristic blue staining in repeated assays. The four putatively transformed almond shoots were then propagated to provide enough plant material for DNA isolation and Southern blot analysis. After a multiplication phase, small shoots were randomly picked and again assayed for GUS expression. All shoots from one of the clones (D) showed strong GUS expression, while shoots from the other clones and wild-type controls did not show any blue staining (Fig. 1e). GUS expression in GUS-positive shoots was

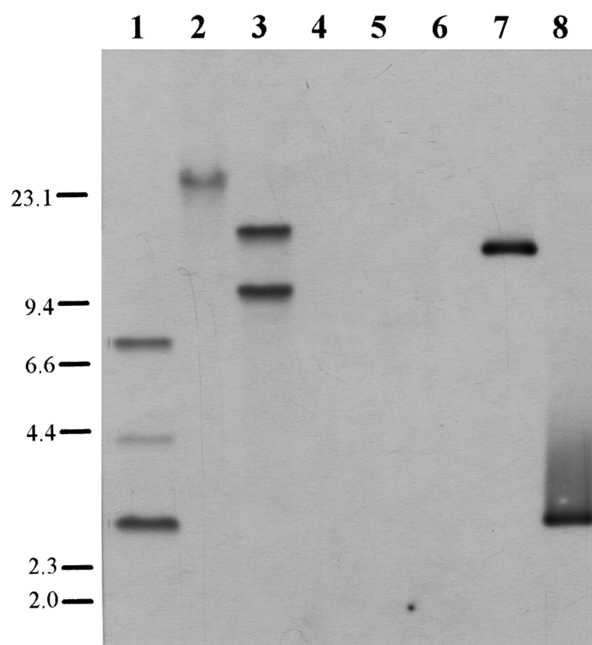

Fig. 3 Southern blot analysis of an almond clone transformed with EHA105 (p35SGUSINT). A 366-bp fragment of the *gus*A gene was used as probe. Total genomic DNA (5–9 μg) from transformed clone digested with *Hin*dIII (*lane 1*), undigested (*lane 2*) and digested with *Eco*RI (*lane 3*) and from a non-transformed control digested with *Eco*RI (*lane 4*). Lambda cut with *Hin*dIII as size marker (*lane 5*), empty (*lane 6*) and *Eco*RI and *Hin*dIII digests of DNA of 0.8 ng of p35SGUSINT (*lanes 7* and *8* respectively). Size markers in kbp

clearly visible in the leaves (Fig. 1 f), but the stems only showed the characteristic blue staining if cut in sections, due to easier penetration of substrate into the tissues (data not shown).

Southern blot analysis

When using a fragment of the *gus*A gene as probe, only one of the four propagated lines, the GUS-positive clone D, gave hybridisation bands in undigested and *Hind*III- or *Eco*RI-digested DNA samples (Fig. 3). As the T-DNA of p35SGUSINT contains only one recognition site for *Eco*RI, digestion of plant DNA with this enzyme should yield hybridising fragments that are composed of both T-DNA and flanking almond DNA sequences. Since the minimum number of T-DNA inserts is expected to equal the number of bands detected in this manner, the transgenic almond clone appears to have at least two inserts. In the digestion with *Hind*III that should generate an internal T-DNA fragment of 2.8 kb corresponding to the *gus*A gene, two larger bands were obtained in addition to the expected 2.8 kb fragment. This pattern can be explained in a number of different ways including integration of multiple copies of the gene or generation of new borders during the integration process.

Evidence collected from PCR, GUS expression assays and Southern blot analysis provided proof of integrative transformation of almond plants via *Agrobacterium*. These plants were derived from the propagation of a single transgenic shoot and have remained stable for 18 months. Additional shoots that showed amplification by PCR using *gus*A and *npt*II primers were probably chimeric and may have lost the foreign gene through the multiplication process. This chimeric status is supported by the fact that there was no contamination by endogenous bacteria, as shown by a PCR control, lack of sustained GUS expression and the absence of hybridisation in Southern analysis. The difficulty in regenerating transgenic almond shoots is probably related to the regeneration system currently available. Histology studies to elucidate the origin and timing of events leading to plant regeneration and the accessibility of transforming bacteria to the regeneration-competent cells is being investigated. This is the first report on the recovery of transgenic almond plants and the methods described here should serve as a useful experimental basis for the genetic engineering of almond for various agronomic traits, including virus resistance.

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