Agrobacterium-mediated genetic transformation of *Prunus salicina*

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Abstract We report *Agrobacterium tumefaciens*-mediated transformation of two *Prunus salicina* varieties, 'Angeleno' and 'Larry Anne', using a modification of the hypocotyl slice technique previously described for *P. domestica*. Regeneration rates on thidiazuron (TDZ) and indole-3-butyric acid (IBA) supplemented Murashige and Skoog (MS) media reached 11% for 'Angeleno' and 19% for 'Larry Anne' hypocotyl slices. Transformation using *Agrobacterium tumefaciens* GV3101 harboring a plasmid with the neomycin phosphotransferase II (*npt*II) and the green fluorescent protein (*gfp*) genes produced ten independent lines, six from 'Angeleno' and four from 'Larry Anne', representing transformation efficiencies of 0.8 and 0.3%, respectively, relative to the initial number of hypocotyl slices. Plants of six lines were found to produce the transgene encoded mRNAs. DNA blotting demonstrated the presence of transgene sequences in trees from five lines after 18 months of growth in the greenhouse.

Keywords *Prunus* · Rosaceae · *Prunus salicina* · Transformation · Regeneration · Genetic engineering

Abbreviations

PCR	Polymerase chain reaction
RT-PCR	Reverse transcription coupled to PCR
PGF	Plant growth factor
TDZ	Thidiazuron
IBA	Indole-3-butyric acid
BAP	6-Benzylamino purine
PPV	*Plum Pox Virus*
GFP	Green fluorescent protein
NPTII	Neomycin phosphotransferase II

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Introduction

In Chile "Japanese" (*Prunus salicina*) and "European" (*P. domestica*) plums together represent 6% of the total production of temperate fruits. *P. salicina* represents 70% of the commercial plum production (14,460 ha). Most of the production is of two varieties, 'Angeleno' and 'Larry Anne'. All the fruits from this production are used for internal and external fresh consumption. Plum production is affected by the typical phytosanitary and post-harvest problems that affect all Chilean stone fruit production, which include *Plum Pox Virus* (PPV) in the field (Reyes et al. 2003) and chilling injury during post-harvest storage and shipment to distant markets. In addition to traditional breeding approaches, new technologies including the use of molecular markers, gene function studies, and the

development of genetically engineered varieties offer approaches to control these problems. The latter technology has been demonstrated in the development of genetically engineered (GE) PPV resistant *P. domestica* (Malinowski et al. 2006; Scorza et al. 2007). Transformation of *P. salicina* would allow for improvement of this species through the development of GE varieties and additionally transformation of this diploid species would provide a platform for functional genomics studies that would be useful for all *Prunus* and Rosaceous species.

Multiple tissues have been used for plant regeneration in the *Prunus* genus, including leaves (Pérez-Tornero et al. 2000; Gentile et al. 2002; Yancheva et al. 2002; Burgos and Alburquerque 2003; Dolgov et al. 2005), cotyledons (Mante et al. 1989), embryos (Pérez-Clemente et al. 2004), and hypocotyls (Mante et al. 1991; Padilla et al. 2003). To date the more successful and reproducible system for plant regeneration in this genus is the use of hypocotyl segments of *P. domestica* described by Mante et al. (1991) and improved by Padilla et al. (2003). TDZ and IBA are used to regenerate shoots following *Agrobacterium*-mediated transformation of hypocotyl segments. In the case of *P. salicina*, previous reports have described in vitro micropopagation systems using local varieties (Rosati et al. 1980; Rogalski et al. 2003). Tian et al. (2007b) have reported the regeneration of *P. salicina* from hypocotyl segments as previously described in *P. domestica* using IBA combined with various levels of TDZ, benzylaminopurine (BAP), or kinetin. Shoots were induced from hypocotyl segments of the *P. salicina* cultivars 'Shiro', 'Early Golden', and 'Redheart' and regenerated plants were established in the greenhouse.

Preliminary experiments in our laboratory indicated the potential for regeneration and transformation of *P. salicina* (Prieto et al. 2005). The current study demonstrates regeneration and *Agrobacterium*-mediated transformation of this species. We utilized the IBA/TDZ-based hypocotyl segment regeneration protocol derived from the *P. domestica* model and demonstrate the production of transgenic shoots from hypocotyl segments of the *P. salicina* cultivars 'Angeleno' and 'Larry Anne', and the establishment of transgenic plants in the greenhouse, requiring a total period of time of about 10 months.

Materials and methods

Starting material

Fruits from *P. salicina* (cvs. 'Larry Anne' and 'Angeleno') were collected during the 2004 and 2005 seasons from the Central Valley in Chile 120 days after bloom. The flesh was removed from the stone (endocarp) which was then soaked in an antifungal solution of Benlate-Captan for 6 h, allowed to dry, and then stored in a dark chamber at 2–4°C for up to 3 months before use.

Explant preparation

Endocarps were removed under a clean air hood and the extracted seeds were soaked in a 25% (v/v) commercial bleach (6% NaClO)/water solution for 20 min. Seeds were rinsed three times with sterile water (supplemented with 2 drops of Tween-20/100 ml) for 20 min each time. Seeds were then split lengthwise and the hypocotyl was removed and cut into three segments, discarding the two terminal segments (radicle and epicotyl) (Mante et al. 1991). The central hypocotyl segment was sliced transversally generating explants measuring 0–1 mm in thickness.

Regeneration system

Basal media

Basal media (BM) consisted of three-fourth strength MS medium (Murashige and Skoog 1962) macrosalts and micronutrients and full strength MS vitamins. Sucrose (20 g/l) and agar (7 g/l; Merk KGaA, Darmstadt, Germany) were added and the pH was adjusted to 5.8.

Regeneration trials

Regeneration media (RM) were BM supplemented with TDZ at concentrations of 1, 3, 5 and 7 μM, together with IBA at concentrations of 0, 0.5, and 1 μM, in all possible combinations. *P. salicina* hypocotyl explants were cultured on RM (BM + variable TDZ + variable IBA) to the appearance of the first shoots after 15 days. Cultures were then moved onto fresh media of the same composition, with transfers to fresh media every 30 days. When individual shoots reached a length of approximately 5–10 mm, they were isolated and explanted onto multiplication medium (MM) (BM medium, supplemented with 4.4 μM BA and 0.4 μM IBA). Shoots were kept on MM for 30 days. After this period of time, shoot elongation was induced in double-phase cultures, which were established by adding 5 ml of liquid BM supplemented with 1 mg/l gibberellic acid (GA_3; Sigma Chemical Co., St Louis, USA) and 1 g/l activated charcoal into the vessels containing regenerated shoots in agar-solidified MM. Shoots were transferred between agar-solidified MM cultures and the double-phase cultures every 7 days. When elongated shoots reached approximately 3 cm in height, they were explanted onto rooting medium (RTM), which consisted of BM supplemented with 0.2 mg/l BAP, and 0.5 mg/l IBA.

Shoots were transferred to fresh RTM every 30 days. Whole plants were pre-conditioned by adding 20 ml of distilled water to solid RTM cultures that had 20 ml of solid medium in a container with a total volume of about 50 ml, and replacing foil closures with filter paper caps. After 15 days under these conditions plants were transferred to potting mix [soil:perlite (3:1)] in the greenhouse.

Statistical analyses

Regeneration percentages calculated as the number of hypocotyl slices producing shoots as a percentage of the total number of hypocotyl slices cultured, were obtained from at least three replicates during the seasons 2004–2005 and 2005–2006. Each replication consisted of 144 explants of each variety per treatment. Data were subjected to analyses of variance, and means were separated by an LSD test at the 5% level of significance using Statgraphics Plus 5.1 (Manugistics Inc., Rockville, MD, USA).

Transformation assays

Three *A. tumefaciens* strains, EHA105, GV3101, and LBA4404, were evaluated for their ability to transform *P. salicina* hypocotyl segments. All strains harbored the plasmid pCAMBIA2202 (*nptII-gfp*) (CAMBIA^{TM}, Canberra, Australia). Overnight *Agrobacterium* cultures grown in YEB (Sambrook et al. 1989), with appropriate antibiotics, were pelleted for 20 min. at 4,000 rpm at 4°C and then resuspended using either (a) water, (b) liquid MS (Murashige and Skoog 1962), or (c) Luria–Bertani broth, up to an OD_{600} between 0.1 and 1.0. Hypocotyl explants were infected in separated treatments with each one of these bacterial suspensions. The *Agrobacterium* infection procedures evaluated consisted of the following: (a) submersion for 20 min or submersion plus: (b) two pulses of vacuum (63.5 cm Hg by 15 s each), (c) 0.1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma), (d) 15 s of sonication at 25°C (8851 bath, Cole Palmer, Vernon Hills, Illinois) and,(e) 15 s of sonication and 0.1% Tween-20, or (f) 1 mM betain hydrocloride (Sigma–Aldrich, St Louis, MO, USA).

Explants were then blotted dry and co-cultivated in MM supplemented with 100 µM acetosyringone (Aldrich Chem. Co. Milwaukee, WI, USA) for 24 h and then washed for 10 min with liquid half-strength MS medium supplemented with 300 mg/l cefotaxime. They were then blotted dry and plated onto MM plus 300 mg/l cefotaxime. After 21 days, shoots started to appear and 30 days after infection, shoots were in the condition and size to be transferred into BM supplemented with 40 mg/l kanamycin which was increased up to 75 mg/l after 3 weeks. Selection in these conditions was kept by up to 50 days and the PCR screening was carried out. PCR-positive plants were propagated for additional 30 days in MM and then elongated by 14 days using the two-phase system previously described. Plants from each generated line were rooted by 15 days in RTM and then conditioned by 15 days as described.

Molecular characterization of transgenic events

DNA extraction

Preliminary analyses of DNA from regenerated plantlets were carried out using samples extracted from approximately 100 mg of leaf tissue from in vitro shoots, processed using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) following the protocol described by the manufacturer. DNA samples for blot analysis was isolated from leaves from 18-month-old trees kept in the greenhouse.

Polymerase chain reaction (PCR) analyses

Gene-specific primers for *npt*II and *gfp* were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by IDT (Coralville, San Diego, CA, USA). As a control for DNA quality the *18 S Ribosomal RNA* (*18S*) gene was amplified in separate reactions. Amplification of *npt*II was carried out using the primers: NPTII_F2 5′-TTCTTTTTGTCAAG ACCGACCTG-3′ and NPTII_R2 5′-CAGCAATATCAC GGGTAGCCAAC-3′, which amplify a fragment of 560 base pairs (bp). For *gfp* analyses, primers were GFP_F 5′-TTCTTCAAGAGCGCCATGCC-3′ and GFP_R 5′-TCC ATGCCATGTGTAATCCCAGC-3′, which amplify a fragment of 455 bp. For, *18S* amplifications primers were F18 s 5′-CCTCTGACTATGAAATACGAATG-3′ and R18 s 5′-GTGCCAGCAGCCGCGGTAATTC-3′, which amplify a fragment of 326 bp. PCR reactions (15 µl) were set up using 50 mM KCl, 10 mM Tris–HCl (pH 8.4), 1.25 mM $MgCl_2$ 50 mM, 250 µmol of each dNTP, 1 ρmol of each primer, 1 U of *Taq* DNA polymerase and 100 mg of DNA extract. Temperature profiles (for all the evaluated genes) consisted of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, which was applied for 35 cycles, and using an additional extension step at 72°C for 10 min. Amplified DNA samples were size separated by conventional electrophoresis using 1% agarose gels and ethidium-bromide staining (Sambrook et al. 1989).

DNA blotting

Genomic DNA (15 µg) was digested with *Hin*dIII enzymes (New England Biolabs, Ipswich, MA, USA), separated on a

1% (w/v) agarose gel, and then transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA). Filters were hybridized with a Digoxigein-11-dUTP alkali-labile (Roche) labeled probe coding for the NPT II gene. The probe was produced by PCR with the specific primers described above. Hybridizing bands were visualized with anti-DIG antibody-alkaline phosphatase and CDP-Star (Roche) on X-ray films.

RNA extraction

Total RNA was prepared from putative transgenic *P. salicina* leaves from trees 18 months after transfer to the greenhouse, using the Invisorb Spin RNA Mini Kit following protocols supplied by the manufacturer. After column purification, RNA's were treated with DNase I (RNase-Free) (New England Biolabs Inc., Ipswich, MA, USA) at 10 U/μg of total RNA following manufacturer instructions.

Reverse transcription: PCR (RT-PCR)

Reverse transcription reactions were run with 2 μg of RNA as template to synthesize single-strand cDNA's using MMVL-RT reverse transcriptase (Promega, Madison, WI, USA) and oligo dT primer (Invitrogen, Breda, The Netherlands) following the manufacturer's protocol. Ten percent of the final volumes generated in the single-strand cDNA synthesis reactions were used for specific PCR detection of *npt*II, *gfp*, and *18S*, following the same procedures and specific primers as described above, including cDNA's in the place of DNA in the reaction mixtures.

Results

Responses to regeneration in the selected varieties

TDZ at concentrations of 1, 3, 5 and 7 μM, without IBA, resulted in low levels of regeneration (Table 1; Fig. 1a, d). After 2 weeks in the TDZ-alone treatments few explants produced shoots. In the third week, surviving explants began to develop necrotic spots and by the fourth week, most of the explants were completely necrotic. When TDZ was combined with IBA at 0.5 (Fig. 1b, e) or 1.0 μM (Fig. 1c, f) shoot regeneration and survival dramatically increased. The highest average regeneration rates obtained were 11% for hypocotyl slices from 'Angeleno' with treatments 3 and 5 μM TDZ with 0.5, or 5 and 7 μM TDZ and 1.0 μM IBA; and 19% for 'Larry Anne' hypocotyl slices with 3 μM TDZ and 0.5 μM IBA or 7 μM TDZ and 1.0 μM IBA (Table 1). Regeneration was genotype-dependent and differences in regeneration response between hypocotyl slices from the two varieties were significant.

Transformation assays

Transient expression assays of *P. salicina* hypocotyl segments showed that *A. tumefaciens* strain LBA4404 caused significant browning of explants regardless the treatment

Table 1 Comparative shoot generation of hypocotyl slices in response to different TDZ/IBA ratios in two varieties of *P. salicina* ('Angeleno' and 'Larry Anne')

Plant growth regulator (μM) TDZ-IBA	Total explants treated	'Angeleno'		'Larry Anne'	
		% Regeneration	Multiple (M) or single (S) shooting pattern of explants	% Regeneration	Multiple (M) or single (S) shooting pattern of explants
1–0.0	144	0	–	2.8 a	S
1–0.5	144	8.3 b	M–S	16.7 d	M–S
1–1.0	144	2.8 a	M–S	13.9 c	M–S
3–0.0	144	3.2 a	S	6.0 ab	S
3–0.5	144	11.1 c	M–S	19.4 d	M–S
3–1.0	144	5.6 b	M–S	8.3 b	M–S
5–0.0	144	2.8 a	S	5.0 ab	S
5–0.5	144	11.3 c	M–S	11.0 c	M–S
5–1.0	144	11.4 c	M–S	13.9 c	M–S
7–0.0	144	3.0 a	M–S	2.8 a	S
7–0.5	144	8.3 b	M–S	8.3 b	M–S
7–1.0	144	11.1 c	M–S	19.4 d	M–S

Percentage of shooting refers to the number of explants showing single (S) and multiple (M) shoots after 4 weeks of treatment, compared to the total number of initial explants on each season. Two different seasons were evaluated. Data were subjected to analyses of variance, and means were separated by an LSD test at $P = 0.05$

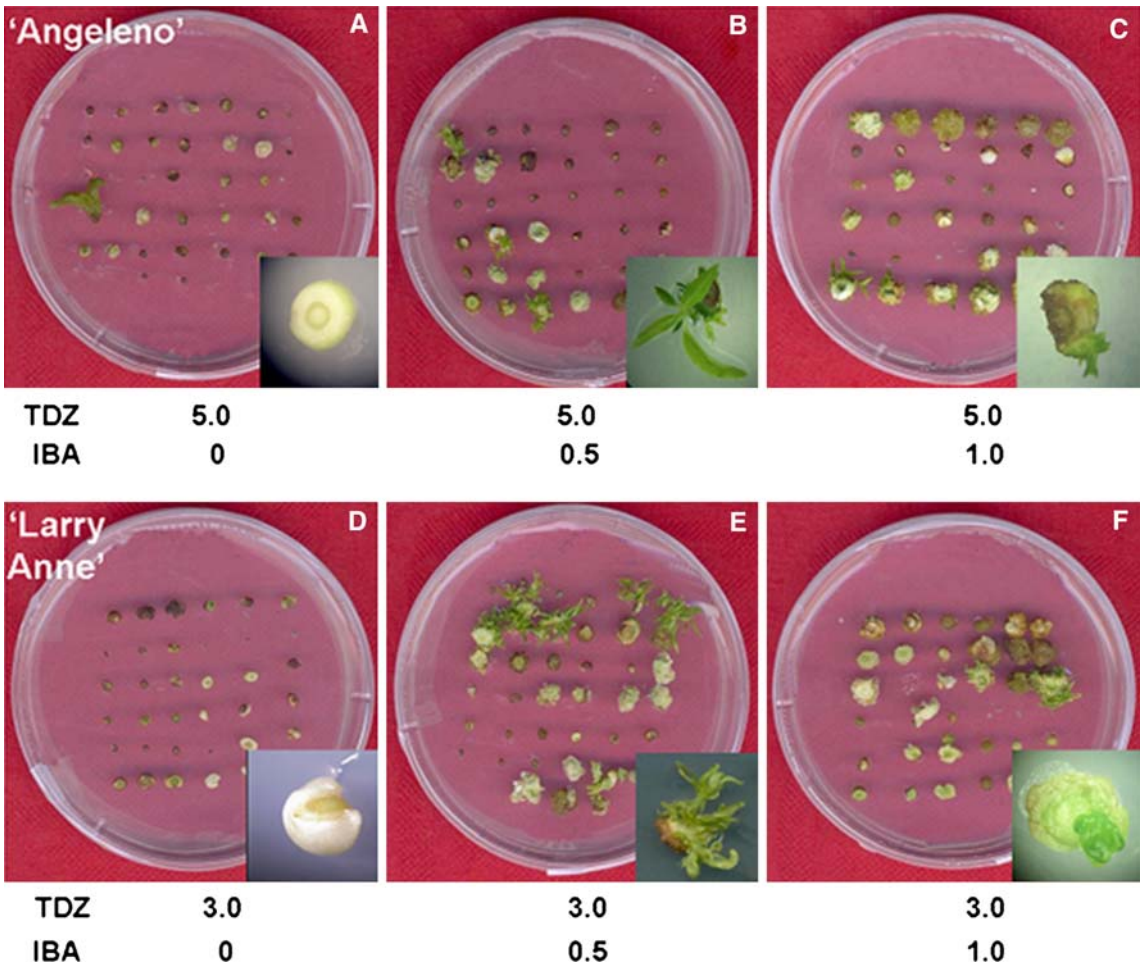

Fig. 1 Response of *P. salicina* in regeneration experiments based on the hypocotyls segment system described for *P. domestica.* Two representative sets of results, for regeneration with the best TDZ and indole-3-butyric acid (IBA) concentrations (μM) of 'Larry Anne' (**a**–**c**) and of 'Angeleno' (**d**–**f**) hypocotyl segments are shown 2 weeks after treatments. Detailed views are provided in the *lower right* of each frame

[(a) two pulses of vacuum, (b) 0.1% Tween-20, (c) 15 s of sonication, (d) 15 s of sonication and 0.1% Tween-20, or (e) 1 mM betain] and no GFP transient expression was observed up to 10 days post-infection of hypocotyl slices of either *P. salicina* variety. GV3101 and EHA105 generated strong GFP expression between 7 and 10 days post-infection in all infection treatments with more than 60% of the infected explants expressing GFP. We observed striking differences in the pattern of GFP expression in different infection treatments. Using 20 min of infection with 0.1% Tween-20 (treatment b) induced a peripheral pattern of GFP expression, while the use of vacuum pulses (treatment a) was also efficacious in producing transformed explants, the GFP expression was mostly detected in the central areas of the hypocotyl slices from both cultivars (not shown). This central area, in contrast to the peripheral areas of the hypocotyl slices, was observed have a low regenerative response. Finally, the effects of sonication and betain (treatments c, d, e), did not exhibit qualitative or quantitative (number of explants expressing GFP) differences in transient transformation when compared with Tween-20 (not shown). Based on these results, transformation experiments of *P. salicina* hypocotyl segments were carried out, assuming as optimal conditions by the use of MS-based suspensions of *A. tumefaciens* GV3101, with the addition of 0.1% Tween-20 and 20 min infections. Following infection and co-cultivation, explants were cultured on RM with the combinations of TDZ/IBA that were found to produce the highest rates of regeneration for the particular variety (Table 1) and with the addition of 40 mg/l kanamycin. Preliminary experiments with hypocotyl segments of both *P. salicina* varieties showed that this concentration of antibiotic was an effective dose for initial selection, which was applied 30 days after infection. This concentration severely reduced regeneration but permitted the survival of 65% of the non-transformed explants. After 3 weeks, the selection pressure was increased to 75 mg/l kanamycin.

Shoots that were able to grow on 75 mg/l kanamycin for 50 days were screened by PCR for the presence of *npt*II and *gfp*. From a total of 1,896 explants treated over two seasons, ten putative transgenic PCR positive lines were isolated (data not shown). Six of these transformed lines were derived from 'Angeleno' and four from 'Larry Anne' hypocotyl segments, representing efficiencies of 0.8 and 0.3%, respectively, relative to the initial number of hypocotyl slices.

Transgenic lines were propagated in vitro for an additional 30 days, during which four shoots were produced from each original explant. The shoots were elongated, rooted and conditioned as described in Materials and methods, requiring about 60 days. The total time from initiation of transformation was approximately 6 months. These plants were moved to the greenhouse and maintainted at approximately 25°C with 16 h light. Figure 2a shows the trees after 18 months in the greenhouse. DNA and total RNA samples were isolated from leaves from trees at this stage. Figure 2b shows results from PCR and RT-PCR analysis for three of the lines, in which the *gfp* and *npt*II transgenes and their transcripts are evident.

DNA samples were also cut with *Hin*dIII and analyzed by blotting for the *npt*II gene as described in Materials and methods. Figure 3 shows hybridization results from the evaluation of three 'Angeleno' (lanes 2–4) and three 'Larry Anne' (lanes 5–7) putative transgenic lines.

Discussion

The most robust transformation system in *Prunus* appears to be that of *P. domestica* (Mante et al. 1991; Padilla et al. 2003) and transformation success is likely related to the high level of regeneration from hypocotyls of this species (Tian et al. 2007a). Testing of a limited number of genotypes has shown that regeneration of *P. salicina* is lower than that of *P. domestica* (Tian et al. 2007b) and therefore transformation rates would be expected to be reduced in comparison. We show in this report that *P. salicina* is amenable to *Agrobacteriu*m-mediated transformation even though rates of regeneration are lower than reported for *P. domestica*.

We show the importance of the *A. tumefaciens* strain, with LBA4404 dramatically reducing explant survival in contrast to EHA105 and GV3101. The use of a surfactant (Tween-20) improved *A. tumefaciens* infection of hypocotyl slices, especially in the peripheral zone where shoot regeneration is generally obtained. In contrast, sonication improved transient transformation but in cells of the central region of the segments with low regeneration potential.

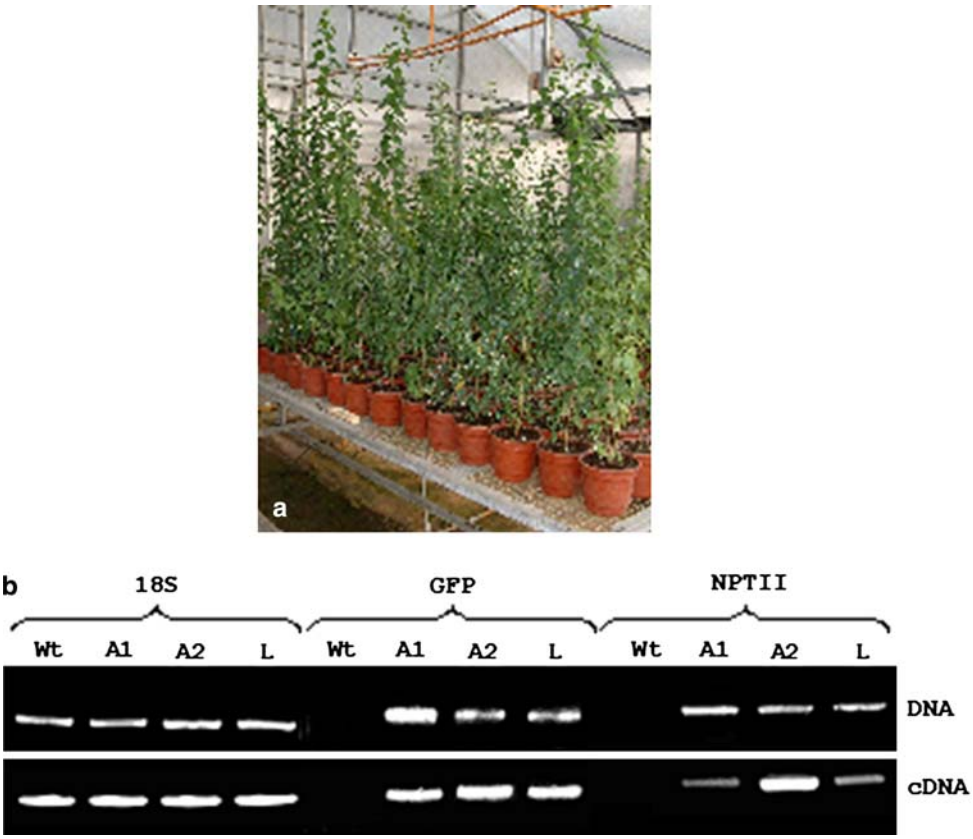

Fig. 2 Transgenic *P. salicina* and their molecular characterization. **a** Trees derived from the genetic transformation of *P salicina* one and a half years after transfer to greenhouse. **b** PCR results for amplifications of genomic DNAs (DNA) and reverse transcribed mRNAs (cDNA), isolated from leaves of greenhouse-grown tress of two 'Angeleno'- (A1 and A2) and one 'Larry Anne' (L)-derived line. *18S Ribosomal RNA*, *gfp,* and *npt*II genes and transcripts were amplified as described in Materials and methods

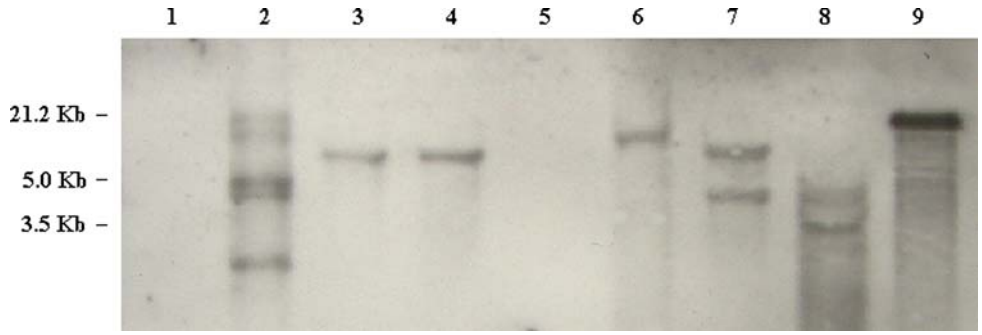

Fig. 3 Southern-blot analysis of putative *P. salicina* transgenic lines. Genomic DNA was digested with the restriction enzyme *Hind*III and hybridized with an *npt*II probe labeled with digoxigenin-11-dUTP. *Lane 1* Negative control, non-transformed regenerated plant. *Lanes 2–7* Candidate transgenic lines from 'Angeleno' (*lanes 2–4*) and 'Larry Anne' (*lanes 5–7*). *Lane 8* Transgenic *P. domestica* plum C-5 (Scorza et al. 1994). Lane 9: *p*CAMBIA2202 (*npt*II-*gfp*) (5 pg)

In spite of our attempts to optimize regeneration and transformation the efficiency of transgenic plant recovery was low, about 0.5%. Transformation of woody species depends upon a complex interplay of many factors including genotype, explant source, media nutrients, growth regulators, gelling agents, culture environment, *A. tumefaciens* strains, infection and co-cultivation methods, and selection scheme. The transgenic plants that we report were the product of a limited set of manipulations of some of these factors. We clearly show that *P. salicina* is amenable to transformation. Resulting efficiencies under the current experimentation are still low (Table 1) although similar to rates reported for other varieties (Tian et al. 2007a, b). Improvement of the system will require optimization of multiple factors. We suggest that among the most important is an improvement of regeneration (Negri et al. 2007). High levels of regeneration are critical for increasing transformation rates. Work with *P. domestica, P. salicina* and other woody species has demonstrated the importance of the genotype of the explant source (Damiano et al. 2007; Petri and Burgos 2005; Tian et al. 2007a, b). Therefore a wide range of *P. salicina* genotypes should be evaluated for regeneration. The timing and severity of kanamycin selection is another important factor for the optimization of transformation (Damiano et al. 2007; Petri and Burgos 2005). The reduction of kanamycin level in the second round of selection should be evaluated in order to decrease potential losses of transgenic shoots.

The present work demonstrates the first report, to our knowledge, of the transformation and regeneration of transgenic trees of *P. salicina* and paves the way for the genetic engineering of this species. Further optimization of the system will require continued investigation into the parameters evaluated in this report in addition to factors such as genotype and antibiotic selection strategies. Such work may also lead to systems for the transformation of the species from clonal explant sources such as leaves and internodes.

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