Plant Cell Rep (2004) 22:832–838
DOI 10.1007/s00299-004-0766-2

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Regeneration of herbicide-tolerant black locust transgenic plants by SAAT

Received: 1 July 2003 / Revised: 7 January 2004 / Accepted: 8 January 2004 / Published online: 6 February 2004
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Abstract A protocol based on SAAT (sonication-assisted *Agrobacterium*-mediated transformation) has been developed to obtain herbicide-resistant transgenic black locust (*Robinia pseudoacacia* L.) plants. Cotyledon explants were co-cultivated with *Agrobacterium* AGL1 strain carrying the pTAB16 plasmid (*bar* and *gus*A genes). The effects of bacterial concentration (OD_{550} of 0.3, 0.6, 0.8) and method of infection (sonication vs immersion) on bacterial delivery were determined by assaying cotyledons for transient β-glucuronidase expression 3 days after infection. SAAT increases transient expression efficiency especially at an OD_{550} of 0.6. After determining bacterial concentration and infection method, other factors affecting transformation efficiency, such as explant preconditioning and period of time before applying selection, were tested. From these experiments, the preferred protocol for black locust cotyledon transformation should include sonication of preconditioned cotyledons in AGL1 suspension, coculture for 3 days with 100 μM acetosyringone and transfer to selection medium with 4 mg/l phosphinothricin and 150 mg/l timentin. Of the initial explants, 2% produced at least one transgenic shoot. Genetic transformation was confirmed by Southern hybridization, chlorophenol red assay and herbicide tolerance of the regenerated plants.

Keywords *Agrobacterium* · Cotyledon explants · Marker gene · Organogenesis · *Robinia pseudoacacia*

Abbreviations *AS:* Acetosyringone · *BA: N*-(Phenylmethyl)-1*H*-purin-6-amine (benzyladenine) · *CR:* Chlorophenol red · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GUS:* β-Glucuronidase · *IAA:* Indole-3-acetic acid · *IBA:* Indole-3-butyric acid · *PPT:* Phosphinothricin

Communicated by P. Ozias-Akins

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Introduction

Black locust (*Robinia pseudoacacia* L.) is a nitrogen-fixing leguminous tree valued for its agroforestry qualities (ornamental, timber, animal feed, and honey production). Moreover, its fast growth rate and potential for environmental restoration have drawn attention to the species as one of the leading candidates for woody biomass plantations (Mitchell 1988; Ranney et al. 1988). The amenability of black locust to be regenerated by tissue culture (Arrillaga and Merkle 1993) and its susceptibility to *Agrobacterium* infection (Han et al. 1999) could facilitate development of biotechnological programs for genetic improvement of this species. In spite of this, previous transgenic work produced phenotypically abnormal black locust plants after infection with *Agrobacterium rhizogenes* (Han et al. 1999). Arrillaga and Merkle (1994) reported transient β-glucuronidase (GUS) expression after bombardment of black locust proembryogenic masses but no production of transgenic somatic embryos. Recently, phenotypically normal transgenic plants were obtained after infection of black locust stem and leaves with *Agrobacterium tumefaciens* (Igasaki et al. 2000). In this work, we present a transformation protocol, based on SAAT (sonication-assisted *Agrobacterium*-mediated transformation, Trick and Finer 1997), to obtain herbicide-resistant transgenic black locust plants from cotyledon explants.

Materials and methods

Plant material

Mature black locust pods were collected in September, from several open-pollinated trees growing on the Campus of The University of Valencia, (Burjassot, Valencia, Spain). Seeds were removed from

pods, mixed randomly and stored at 4°C until used. Scarification and seed sterilization were as described in Merkle and Wiecko (1989). Seed coats were removed and embryos were used as a source of cotyledon explants.

Plant regeneration and acclimatisation

Two regeneration protocols were applied to cotyledon explants from freshly isolated black locust mature embryos. These protocols had been previously reported by Arrillaga and Merkle (1993) for cotyledon and leaf explants (hereafter referred to as protocols A and B, respectively) of a black locust genotype (BL698). Cotyledon sections (~4 mm long) were cultured in 90×15 mm Petri plates containing 25 ml culture medium. In protocol A, cotyledons were cultured for 45 days on a modified woody plant medium (WPM) containing major WPM salts (Lloyd and McCown 1980), MS minor salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% sucrose, 22.2 μ*M* benzyladenine (BA) and 0.4 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D). Subsequently, explants were transferred to the same medium without growth regulators. In protocol B, cotyledons were cultured on MS salts with B5 vitamins, 3% sucrose, 4.4 μ*M* BA and 24.6 μ*M* indole-3-butyric acid (IBA) (hereafter referred to as MSBIB medium) for 30 days and then transferred to the same medium supplemented with 4.4 μ*M* BA and 0.5 μ*M* IAA (MSBIA medium). In all cases the pH was adjusted to 5.7 and 0.8% agar (Difco-Bacto) was added before autoclaving for 20 min at 120°C. Cultures were incubated initially in darkness at 26±2°C. After 15 days, explants were placed under a 16-h photoperiod (100 μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps (GTE Gro-lux, F36W; Gro, Germany). Morphogenic responses (% of caulogenic explants and mean number of shoots) were recorded at the end of the culture period (90 or 60 days for protocols A and B, respectively). Five explants, adaxial surface down, were inoculated per plate and a total of five plates were used for each protocol. Shoots longer than 1 cm were isolated and rooted on hormone-free SH medium (Schenk and Hildebrandt 1972).

Regenerated plants were transplanted to 100 ml pots containing 1:1 perlite:peat moss and adapted to growth chamber conditions [75% relative humidity (RH), 25±1°C and a 16-h photoperiod of 80 μmol m^{-2} s^{-1} irradiance at culture level] with gradual exposure to reduced RH by progressively removing a transparent plastic cover over 2–3 weeks. Once acclimatisation was accomplished, plants were transferred to the greenhouse. Drip irrigation provided moisture for maintenance of vigorous growth.

Bacterial strain and plasmid

Agrobacterium tumefaciens strain AGL1 (Lazo et al. 1991), carrying the binary plasmid pTAB16 (Schröeder et al. 1993), was used in the experiments. Plasmid pTAB16 includes the *bar* gene, which confers tolerance to the herbicide phosphinotricin (PPT), and the *gus*A gene, both driven by the CaMV 35S promoter. The *gus*A gene is not expressed in *A. tumefaciens* due to insertion of a plant intron in the protein-coding region.

Bacteria were grown overnight on a horizontal gyratory shaker (200 rpm) at 28°C in LB with 0.4 g/l $MgSO_4$, 20 mg/l rifampicin and 5 mg/l tetracycline. Bacterial cells were collected by centrifugation (3,000 rpm for 20 min) in a 50 ml tube, resuspended to appropriate OD_{550} (see below) in liquid MS (pH 5.5) containing 100 μ*M* acetosyringone (AS), and used for cocultivation experiments.

Tolerance of black locust to herbicide

The herbicide sensitivity of black locust was tested by culturing 25 cotyledon explants on regeneration medium (MSBIB medium) containing varying levels of PPT (0, 5, 10, 15 mg/l). After 4 weeks, survival and regeneration of explants were scored.

Transient expression assays

Effect of bacterial dilution and sonication

Cotyledon explants were preconditioned for 3 days on solidified MSBIB medium with 100 μ*M* AS, soaked for 20 min in 10 ml of the bacterial suspension (OD 0.3, 0.6, 0.8), dry blotted with sterile filter paper and placed back on preconditioning medium at 25±1°C. Alternatively, SAAT was applied by sonicating cotyledons immersed in 20 ml bacteria in 50 ml Falcon tubes for 1 min at 60 W (Selecta ultrason, Abrera, Barcelona, Spain). After sonication, explants were maintained in the bacterial suspension for 20 min. To test the effectiveness of the protocols described, transient GUS expression was determined 3 days after infection (see below). Each treatment included five replications with ten cotyledon explants each. A control treatment with cotyledons immersed in MS liquid medium with and without sonication was also tested.

Regeneration of transgenic plants

After deciding upon the method of bacterial delivery, and dilution, a series of experiments was carried out to determine factors affecting transformation efficiency in black locust cotyledons. Factors tested included preconditioning of the explant (0, 3 or 4 days), and period of time before applying herbicide selection (0 or 3 days). For each experiment, between 125 and 350 cotyledon explants (either preconditioned on MSBIB with AS or not) were sonicated in a bacterial dilution (OD=0.6) as described above. After 3 days of coculture, explants were placed on selection medium, MSBIB with 150 mg/l timentin (a gift from SmithKline Beecham Pharmaceuticals, Madrid, Spain) and 4 mg/l PPT. When selection was delayed, cotyledons were transferred after coculture to MSBIB medium with timentin for 3 days. Culture medium was replaced every 15 days. After 30 days, regenerating explants were transferred to MSBIA medium with the appropriate selective agents as described above. Finally shoots were transferred to rooting medium with 4 mg/l PPT. Plants were acclimatised and transferred to the greenhouse. In each transformation experiment, 25 non-infected explants were cultured on the same medium with or without selection agents.

Histochemical GUS assay

Histochemical GUS assays were performed on cotyledons (transient expression assays) and on leaves isolated from putatively transgenic plants. In both cases, plant material was immersed in the reagent mix described by Jefferson et al. (1987) containing 2 m*M* 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), and incubated at 37°C for 16 h. Subsequently, the plant material was rinsed in 70% ethanol to extract pigments.

Molecular analysis

Molecular analysis was performed on putative transgenic and control plants by polymerase chain reaction (PCR) and Southern hybridisation. For PCR analysis, genomic DNA was isolated by the simplified CTAB (hexadecyl trimethyl ammonium bromide) method of Doyle and Doyle (1990). PCR was performed in a Perkin-Elmer GeneAmp PCR system 9600 thermocycler. The *bar* gene was amplified according to Toki et al. (1992) and the *gus*A gene according to Peña et al. (1995). Reaction mix (50 μl) contained 50 ng DNA, 0.2 m*M* of each dNTP, 0.25 μ*M* of each primer, 1× PCR buffer (1.5 m*M* $MgCl_2$) and 2 U *Taq* polymerase (Pharmacia). Amplified DNA was electrophoresed on 1% (w/v) agarose gels in TBE. Gels were stained with ethidium bromide and photographed under UV light.

For Southern blot analysis, about 30 μg DNA, from fresh or frozen leaf tissue, was digested with 320 U *Eco*RI for 16 h at 37°C,

and subsequently separated on a 1% agarose gel at 35 V for 16 h. *Eco*RI cuts at a single site in the plasmid outside of the *bar* gene. DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) by capillary blotting. DNA was bound to the membranes by UV crosslinking. Positively charged nylon membranes (Boehringer Mannheim) were prehybridised, as specified by the manufacturer, in a hybridisation oven (Ecogen SRL, Barcelona, Spain) at 60°C. The *bar* gene probe used in the hybridisation reactions was prepared and labeled with digoxigenin-11-dUTP by PCR (PCR DIG probe synthesis kit, Boehringer Mannheim) using plasmid pTAB16 as template. PCR conditions were as described above. Hybridisation and detection of the digoxigenin-hybridised fragments was carried out according to the manufacturer's recommendations.

Chlorophenol red assay

The accumulation of ammonium in non-transformed plants in the presence of PPT was determined by the chlorophenol red (CR) method as described by Kramer et al. (1993). Putatively transgenic shoots (4 cm length) and controls, previously micropropagated through axillary bud proliferation on SH medium, were cultured in test tubes containing 25 ml SH medium with 2 mg/l PPT and 50 mg/l CR solidified with 0.7% agar. The pH of the medium was adjusted to 6.0, a pH at which the medium is a deep red colour. The change of colour to yellow was evaluated after 3 days.

Herbicide application

Regenerated plants were acclimatised and tested for their response to herbicide by a leaf spray assay. Transgenic and control plants were sprayed with an aqueous solution containing 160 mg/l glufosinate ammonium and 0.1% Tween 20. Results were scored after 4 days.

Statistical analysis

Significance of the different treatment effects on organogenesis, transient GUS expression and transformation efficiency were determined using analysis of variance (ANOVA). Percentage data were subjected to arcsine transformation prior to statistical analysis. Variation among treatments means was analysed using the procedure of Tukey (1953).

Results and discussion

Organogenesis

The results obtained in the organogenesis experiments showed that the protocols described by Arrillaga and Merkle (1993) for genotype BL 698 can be applied successfully to regeneration of plants from cotyledons of our black locust material (Fig. 1). No significant effect of the treatment was observed on the percentage of explants producing shoots and on the mean number of shoots per explant. The percentage of cotyledons forming shoots ranged from 74.2%, in protocol A, to 82.9%, in protocol B ($P \geq 0.1$). In turn, the mean numbers of shoots per cultured explant were 3.8±2.5 and 9.1±4.2 for treatments A and B, respectively ($P \geq 0.1$). Based on these trends, protocol B (culture of explants on MSBIB medium for 30 days, and transfer to MSBIA medium) was used for transformation experiments. About 90% of shoots rooted on SH medium. Plantlets were maintained on SH medium until acclimatisation.

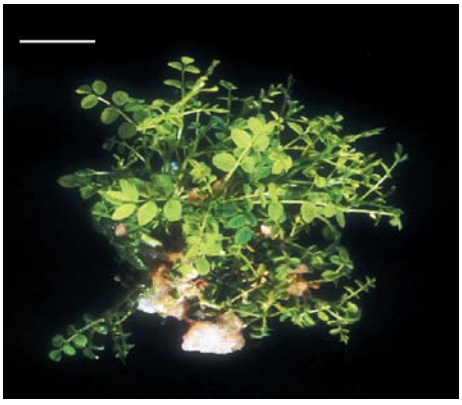

Fig. 1 Organogenesis in black locust cotyledon explants. *Bar* 0.7 cm

Tolerance of black locust to herbicide

The sensitivity of black locust explants to PPT was established prior to transformation experiments. After 30 days, 90% of cotyledons in control treatments differentiated adventitious shoots. The presence of 5 mg/l PPT inhibited organogenesis (data not shown). None of the timentin dosages tested affected organogenesis (data not shown). Therefore, for selection of putative transgenic black locust shoots, explants were subcultured on regeneration medium containing 4 mg/l PPT and 150 mg/l timentin.

Transient expression assays

The preliminary assays on survival and regeneration of black locust cotyledons following sonication indicated that 1 min sonication did not affect black locust regeneration (data not shown); this sonication period was therefore used in the experiments. Sonication significantly increased the percentage of cotyledons with transient GUS activity (75.3 vs 96.7, $P \leq 0.05$). In addition, no significant differences were observed among the three bacterial dilutions tested (Tables 1 and 2). However, GUS staining was always diffuse or weak in non-sonicated cotyledons (Fig. 2a), whereas when SAAT was used, transient expression was so high in areas of some tissues that individual foci could not be distinguished (Fig. 2b). Because of this, data on the percentage of explants that showed such a high expression (blue spots ≥ 1 mm^2) are also presented and analyzed. ANOVA demonstrated a significant interaction between bacterial concentration and delivery method (Table 2). Thus, maximal transient expression occurred when cotyledons were sonicated in a bacterial suspension at an OD of 0.6 (Table 1). This treatment was used to regenerate transgenic plants.

Table 1 Effect of bacterial dilution (OD) and sonication on β-glucuronidase (GUS) transient expression in cotyledon explants of *Robinia pseudoacacia*. Cotyledons were preconditioned for 3 days and sonicated or not with a suspension of *Agrobacterium tumefaciens* AGL1vir carrying the binary vector pTAB16. Data are means of five replications with ten cotyledons each

Bacterial dilution (OD)	Percentage of cotyledons with GUS activity		Cotyledons with blue foci ≥1 mm^2 (%)[e]	
	Not sonicated	Sonicated	Not sonicated	Sonicated
0.0[d]	0.0	0.0	0.0	0.0
0.3	70.0	92.0	2.0a	40.0b
0.6	70.0	98.0	2.0a	76.0c
0.8	86.0	100.0	8.0a	42.0b
Mean[f]	75.3a	96.7b		

[d] Control treatment, with cotyledons immersed in MS medium without bacteria, was omitted from the analysis
[e] Effect of interaction (OD × sonication) on the percentage of cotyledons with aggregate blue foci ≥1 mm^2
[f] Effect of sonication on the percentage of cotyledons with GUS activity
[e],[f] For each entry, data followed by the same lower case letter are not significantly different according to Tukey's test ($P \leq 0.05$)

Table 2 Analysis of variance for data in Table 1

		Mean square	
Source of variation	*df*	Percentage of cotyledons with GUS activity	Cotyledons with blue foci ≥1 mm^2 (%)
Bacterial dilution (A)	2	427.9 NS[a]	380.5*
Sonication (B)	1	2,863.4*	12,152.1**
A×B	2	57.6 NS	792.45*
Error	24	252.2	99.56

[a] Non significant
*, ** Significant at $P \leq 0.05$ or 0.01, respectively

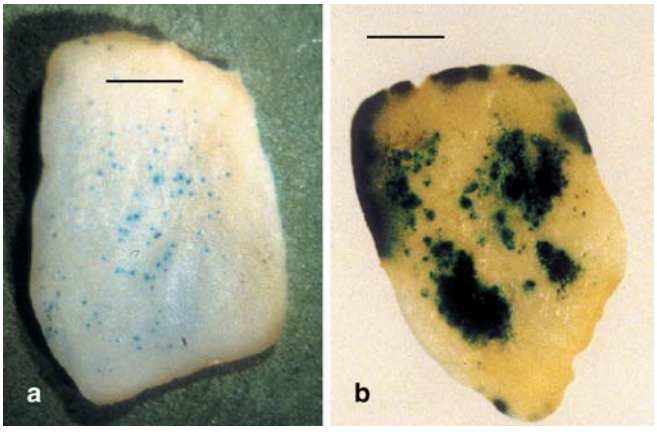

Fig. 2a,b Transient β-glucuronidase (GUS) expression on black locust cotyledons after infection with *Agrobacterium* strain AGL1. **a** Without sonication, **b** with sonication. *Bar* 0.2 cm

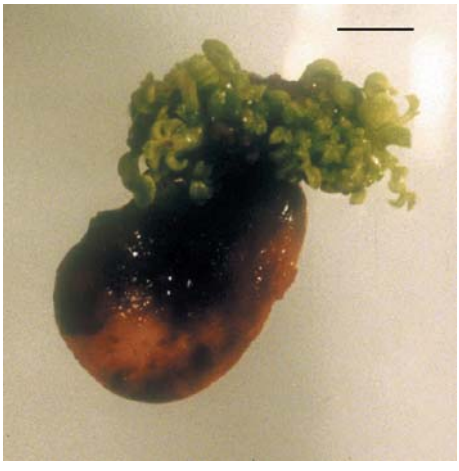

Fig. 3 Black locust cotyledon explant regenerating shoots after 30 days on selection medium. *Bar* 0.3 cm

Regeneration of transgenic plants

Plants were obtained when 3-day-preconditioned cotyledons were sonicated in an AGL1 bacterial suspension (OD=0.6). Under the same conditions, non-sonicated cotyledons failed to produce transgenic plants (control treatment). Within 3 weeks of culture, untransformed but sonicated control explants on MSBIB with the appropriate selection agents turned yellow and produced no callus or adventitious buds. By this time, about 8% of the black locust cotyledons sonicated and co-cultured on AGL1 had differentiated adventitious buds, either directly (Fig. 3) or via callus formation, at the wounded edges of the explants. Most of the induced buds developed into shoots 3 cm tall when transferred to MSBIA with PPT. Eight GUS-positive, putative transgenic plants were obtained from three different explants. Transformation efficiency (TE), defined as the percentage of initial explants that regenerated at least one transgenic plant, was 2.1 (Table 3)

The reproducibility of this transformation protocol was corroborated in an independent experiment (TF=1.9, Table 3). Note that the selection efficiency (SE—the number of confirmed transgenic events divided by the

Table 3 Effect of several factors on transformation frequency of cotyledon explants of *R. pseudoacacia*. Cotyledons were sonicated or not (treatment control) with a suspension of *A. tumefaciens* AGL1vir carrying the binary vector pTAB16. *n* Explant number, *SE* selection efficiency (% of selected explants that developed transgenic shoots), *TF* transformation frequency (% of initial explants that developed at least one transgenic plant)

Experiment	Preculture (days)	Coculture (days)	Selection applied at (days)	*n*	Explants developing shoots (%)	Individual transformation events	SE	TF[c]
1	3	3	0	140	8 (5.7)	3	37.5	2.1a
2	3	3	0	160	13 (8.1)	3	23.0	1.9a
3	0	3	0	316	6 (1.9)	0	0.0	0.0b
4	4	3	3	354	19 (5.4)	7	36.8	2.0a
Control[d]	3	3	0	130	0 (0.0)	0	0.0	0.0b

[c] Data followed by the same lower case letter are not significantly different according to Tukey's test ($P \leq 0.05$)
[d] Control without sonication

Fig. 4 GUS expression on leaf explants from a transgenic (*left*) and control (*right*) plant after histochemical GUS assay. *Bar* 1 cm

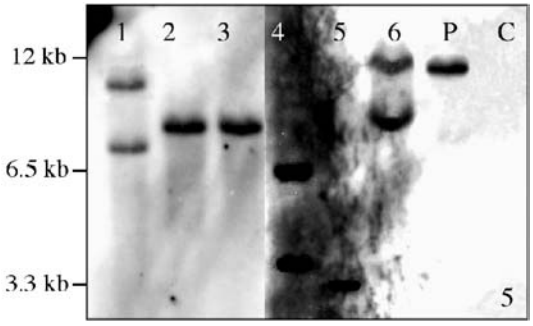

Fig. 5 Southern analysis of putative transformed plants. Genomic DNA was digested with *Eco*RI and probed using a digoxigenin-11-dUTP-labelled *bar* probe, prepared from the plasmid pTAB16. Lanes: *1–6* Different transgenic lines, *P* plasmid, *C* negative control plant

number of selected explants that developed PPT-resistant shoots expressed as a percentage) was 23–37%, which implies a high occurrence of escapes. This phenomenon could be explained by either contamination with endogenous *Agrobacterium* or by a high efficiency of shoot formation opposed to a low transformation frequency, which in turn results in a protective effect of non-transformed cells by the surrounding transformed cells (Peña et al. 1995). In some species, avoiding preculture decreases escapes (Sales et al. 2003). In our experiments, non-precultured cotyledons did not expand before infection, were physically disrupted after sonication, and transgenic plants were not obtained (Table 3). Increasing preculture up to 4 days and delaying application of PPT for 3 days after coculture produced transgenic plants and did not affect SE (Table 3). Representative transgenic plants from six independent PPT^+ lines are being maintained in vitro by sequential shoot-tip and axillary shoot subcultures as described in Arrillaga and Merkle (1993). Twenty transgenic plants were grown ex vitro and transferred to the greenhouse. These plants exhibited normal development as compared to control plants. The survival rate was 90%. The time required to obtain a transgenic plant growing in the greenhouse was 4 months. Plants cannot be expected to flower for approximately 2 years.

Biochemical and molecular characterisation of transgenic plants

Histochemical GUS assays

Leaves from regenerated plants were assayed for GUS activity. Histochemical analysis showed two different patterns of gene expression. Most of the leaves were deeply blue-stained while others presented a weak blue precipitate only in the vascular tissue. GUS activity was not detected in control plants (Fig. 4).

Molecular analysis of transgenic plants

To detect the presence of the *bar* and *gus*A genes in putative transformants, PCR analyses were performed on at least one plant from each of the transgenic lines. Only those plants with strong GUS activity throughout the leaf amplified the predicted 550 bp DNA fragment for the *bar* gene and the 600 bp DNA fragment for the *gus*A gene (data not shown). No amplification was obtained from DNA of control plants. Southern analysis of six plants from six different explants confirmed independent events for integration of the *bar* gene. *Eco*RI cleaves one site outside of the *bar* gene yielding predicted hybridisation

Fig. 6 Chlorophenol red (CR) assay. Control plant (*left*) and *bar* positive transgenic black locust plant (*right*)

bands larger than 1.7 kb (corresponding to the fragment right T-DNA border–*Eco*RI). Three out of the six plants analysed showed a single insert of the *bar* gene (Fig. 5, lanes 2, 3 and 5), while lanes 1, 4 and 6 showed two hybridisation bands for this gene. No bands were observed in the control plant (Fig. 5). The presence of the transgenes in the micropropagated transgenic plants was confirmed periodically through PCR analyses.

Chlorophenol red assays

All PCR-positive plants induced change of CR from red to orange-yellow (as pH decreases from 6.0 to 5.0) (Fig. 6). This indicated that phosphinothricin acetyltransferase (PAT), the enzyme encoded by the *bar* gene, was able to detoxify PPT, and that ammonium was not accumulated in the culture medium.

Herbicide resistance

Regenerated plants were examined for their resistance to PPT. The rate of herbicide application was similar to that recommended for foliar spray in the field (100–500 mg/l, You and Barker 2002). The control plants died 4 days after herbicide application while transgenic plants showed complete tolerance to the herbicide (Fig. 7). After 14 days of herbicide application these plants showed no symptoms of damage. These results demonstrated that the *bar* gene was expressed in the transgenic plants and the level of PAT activity was sufficient to confer resistance to the herbicide.

An *Agrobacterium*-mediated transformation procedure has been developed for black locust cotyledons. Evidence

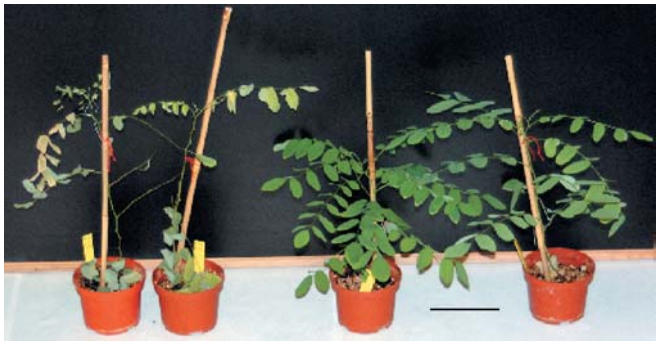

Fig. 7 Herbicide assay. Two control (*left*) and two *bar*-positive (*right*) black locust plants 4 days after herbicide application. *Bar* 0.9 cm

for stable transgene integration was obtained by Southern hybridisation, expression of the *gus*A gene, CR assays and herbicide application. In our experiments no transgenic plants were recovered when conventional co-culture procedures were employed, probably due to inefficient *Agrobacterium* delivery (Table 3). In contrast, SAAT permitted the efficient delivery of bacteria to a large number of black locust cotyledon cells. The efficacy of the SAAT method to increase transformation has been reported for many species including other legumes (Trick and Finer 1997). In comparison with other work on black locust, Igasaki et al. (2000) applied vacuum infiltration to leaf- and stem-explants to obtain transgenic plants for the species at an efficiency of 14.6 and 24.1%, respectively.

The above results indicate that a suitable transformation protocol using *Robinia pseudoacacia* cotyledons would include preculture of explants for 3–4 days, 1 min sonication with AGL1 strain, a co-culture period of 3 days, and regeneration on selection medium with 4 mg/l PPT. Preculture, sonication and co-culture were performed in the presence of 100 μ*M* AS. The efficiency of transformation using our method did not improve on that previously described by Igasaki et al. (2000), probably due to the genetic variability of explant source, explant type, *Agrobacterium* strain and/or selectable marker. This work demonstrated, however, that the *bar* gene can be used for selection in this species and is the first report of transgenic black locust plants from cotyledon explants. The availability of protocols and selectable markers for gene transfer along with the small genome size of the black locust (1C=0.65 pg) provides the species as a model to study the biochemistry and molecular biology of trees.

Acknowledgements The financial support of the former IVEI (Generalitat Valenciana, Spain, Project 002/075) is gratefully acknowledged. We thank Drs. S. González-Nebauer (UPV) and C. Roig for their technical support.

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