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ORIGINAL PAPER

Agrobacterium tumefaciens-mediated transformation of *Lotus tenuis* and regeneration of transgenic lines

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Abstract A protocol for the production of transgenic plants was developed for *Lotus tenuis* via *Agrobacterium*-mediated transformation of leaf segments. The explants were co-cultivated (for 3 days) with an *A. tumefaciens* strain harbouring either the binary vector pBi RD29A:oat arginine decarboxylase (ADC) or pBi RD29A:glucuronidase (GUS), which carries the neomycin phosphotransferase II (*nptII*) gene in the T-DNA region. Following co-cultivation, the explants were cultured in Murashige and Skoog medium supplemented with naphthalenacetic acid (NAA) and benzyladenine (BA) and containing kanamycin (30 μg ml^{-1}) and cefotaxime (400 μg ml^{-1}) for 45 days. The explants were subcultured several times (at 2-week intervals) to maintain the selection pressure during the entire period. About 40% of the explants inoculated with the pBiRD29:ADC strain produced eight to ten adventitious shoots per responsive explant through a direct system of regeneration, whereas 69% of the explants inoculated with the pBi RD29A:GUS strain produced 13–15 adventitious shoots per responsive explant. The selected transgenic lines were identified by PCR and Southern blot analysis. Three ADC transgenic lines were obtained from 30 infected explants, whereas 29 GUS transgenic lines were obtained from 160 explants, corresponding to a transformation efficiency of 10 and 18.1%, respectively. More than 90% of the in vitro plantlets were successfully transferred to the soil. The increase in the activity of arginine decarboxylase from stressed ADC- *Lt*19 lines was accompanied by a significant rise in the putrescine level. The GUS transgenic line driven by the RD29A promoter showed strong signals of osmotic stress in the leaves and stem tissues. All of the transgenic plants obtained exhibited the same phenotype as the untransformed controls under non-stress conditions, and the stability of the gene introduced into the cloned materials was established.

Keywords *Agrobacterium tumefaciens* · Arginine decarboxylase · Direct shoot regeneration · *Lotus tenuis* · Polyamines · Transformation

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Introduction

Lotus tenuis Waldst and Kit ex Willd. (ex *Lotus glaber* Mill.) (Fagaceae) is an exotic forage legume that can be found scattered throughout the Flooding Pampa of Argentina (Quinos et al. 1998). Due to its high nutritive value and adaptive plasticity to the edaphic and environmental stresses characterised by the presence of heavy and saline soils, this species is considered an important alternative source of cattle feed. Genetic improvement of abiotic stress tolerance in *L. tenuis* through conventional breeding methods has been limited due to the heterozygous nature of the existing wild populations (Kade et al. 2003). However, in recent years, emphasis has been placed on the use of in vitro culture approaches for the multiplication and improvement of this genus through genetic transformation techniques. *Agrobacterium*-mediated transformation of different *Lotus* species, including *L. angustissimus* (Nenz et al. 1996), *L. corniculatus* (Armstead and Webb 1987;

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Belluci et al. 2000; Damiani et al. 1993, 2008), *L. japonicus* (Aoki et al. 2002; Lohar et al. 2001; Stiller et al. 1997) and *L. tenuis* (Damiani et al. 1993), has been reported. All of the transformation protocols developed to date are based on the sensitivity of most *Lotus* species to *Agrobacterium*-mediated gene transfer to the plant, using either *A. tumefaciens* (Aoki et al. 2002; Armstead and Webb 1987; Lohar et al. 2001) or *A. rhizogenes* (Damiani et al. 1993; Nenz et al. 1996; Stiller et al. 1997). In the case of *L. tenuis*, hairy root transformation has only been performed from hypocotyl inoculation using a hypodermic needle due to the lack of an efficient protocol for direct plantlet regeneration.

To study how the steady-state content of free polyamines can be influenced by the stress-inducible overexpression of arginine decarboxylase (ADC), we constructed transgenic *L. tenuis* plants. Here we report an efficient regeneration and transformation system via organogenesis from leaf cultures, followed by the scaling-up production of the transgenic lines. To the best of our knowledge, this is the first report of transgenic *Lotus tenuis* plants being regenerated from leaf explants via *Agrobacterium tumefaciens*-mediated transformation.

Materials and methods

Plant material

Thirty-day-old plants obtained by in vitro germination of *L. tenuis* cv. INTA Pampa seeds (diploid, $2n = 2x = 12$) were used as a source of explants. The seeds were pretreated with an aqueous solution of NaOH (2.5 mM) for 15 min, surface-sterilised with 1.2% NaOCl and 0.1% Triton X-100 for 15 min and thoroughly washed with sterile distilled water. The seeds were then cultured in 11-ml glass tubes (3 seeds/tube) containing 3 ml of MS (Murashige and Skoog 1962) medium supplemented with 30 g l^{-1} sucrose. The medium was adjusted to a pH of 5.8 prior to autoclaving (20 min at 121°C, 1.4×10^4 kg m^{-2}) and solidified using 6.5 g l^{-1} agar (A-1296; Sigma, St. Louis, MO). The cultures were incubated in a growth room at 27 ± 2°C under a 14/10-h light/dark photoperiod with light provided by white fluorescent lighting at an intensity of 116 μmol m^{-2} s^{-1} PPFD.

Organogenesis and plant regeneration

Adventitious bud formation was induced from roots, cotyledons and leaves of 30-day-old aseptically grown seedlings. Each primary root (without the root meristem) or cotyledon was divided by two or three cuts transversely to the mid-rib, and the upper three leaves of the shoot tip were harvested and sliced into each foliole. Each explant was cultured in 11-ml glass tubes containing MS medium (3 ml) supplemented with naphthaleneacetic acid (NAA; 0.5 μM), 6-benzyladenine (BA; 5, 10 or 20 μM) and thidiazuron (TDZ; 2.5, 5, 10 or 20 μM) for 45 days. To stimulate bud sprouting and subsequent stem elongation, we subcultured the regenerative leaves from the best induction medium in MS medium without plant growth regulators (PGRs) for 30 days. The elongated shoots were rooted by pre-treatment in an aqueous solution of indolebutyric acid (IBA; 500 μM) for 2 h and then transferred to MS medium lacking PGRs. All cultures were incubated under the same physical conditions as described above.

Histological analysis

Samples of cultured explants were fixed in a formalin:ethanol:acetic acid (FAA) solution and dehydrated through a Biopur series as described by González and Cristóbal (1997). Transverse and longitudinal serial sections 8–10 μm thick were stained with safranin (C.I.50240)–Astra blue and mounted in Canada balsam. The photomicrographs were taken with an Olympus CH30 photomicroscope (Olympus, Tokyo, Japan) and a Sony ExwaveHAD (Sony, Tokyo, Japan) camera. Samples were taken 30 days after the beginning of the induction phase.

DNA constructs and *Agrobacterium*-mediated transformation

We used the constructs developed by Chiesa et al. (2004) for the transformation of *L. tenuis* plants. Leaf discs were inoculated with *A. tumefaciens* strain GV3101 (pMP90). The binary vector used for transformation contained the RD29A osmotic stress-inducible promoter from *Arabidopsis thaliana* and either the ADC gene from oat (pBiRD29:ADC) or the *β*-glucuronidase reporter gene (GUS) (pBiRD29:GUS); it also carried the selectable marker neomycin phosphotransferase II gene (*nptII*), which confers resistance to kanamycin.

The *Agrobacterium* strains harbouring the binary vectors were grown overnight in the dark in 50 ml of Luria–Bertani medium (LB; Sambrook et al. 1989) containing 30 μg ml^{-1} kanamycin, 30 μg ml^{-1} streptomycin and 100 μg ml^{-1} rifampicin at 28°C on a gyratory shaker set at 200 rpm. One millilitre of bacterial suspension was subcultured in fresh medium and incubated under similar conditions until absorbance at 600 nm reached 0.8. Bacteria from this culture were centrifuged at 5,000 rpm for 5 min at 4°C, and the pellet was resuspended in 50 ml regeneration medium and cultured in the dark for 60 min (until the OD_{600} reached 0.8) at 180 rpm at 28°C. This

Agrobacterium culture was competent for leaf disc infection.

Young leaves were harvested from aseptically grown plants, submerged in the bacterial culture for 20 min and blotted dry on sterile filter paper. Leaf pieces were then placed on regeneration semi-solid medium composed of MS medium plus 0.5 μM NAA and 20 μM BA for co-cultivation for 3 days in the dark and then transferred to a fresh regeneration medium containing kanamycin (30 μg ml^{-1}) and cefotaxime (400 μg ml^{-1}). The infected explants were regularly subcultured in fresh medium at 14-day intervals to maintain the selection pressure. After 45 days of culture under the light conditions mentioned above, the regenerative explants were transferred to the elongation and rooting medium.

Selection and analysis of transgenic plants

The pBi RD29A:GUS- and pBi RD29A:oatADC-transformed plants were analysed for the presence of transgenes by PCR. Template DNA was extracted from young tissues using a modified CTAB method (Yamamoto et al. 2000). An internal fragment (0.67 kb) of the *gus*A gene was amplified using primers GUSA F (5′-GCAACGTCTGG TATCAGCGC-3′) and GUSA R (5′-ACGGTTTGTGGT TAATCAGG-3′). The cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension of 10 min at 72°C. For the oat*adc* gene, a 1.8-kb fragment was amplified using primers oatADC F (5′-CACCTATCATGGCCAAGAACTAC-3′) and oatADC R (5′ CAGCCAGCTAGGCCATTG-3′) (Chiesa et al. 2004). These cycling conditions comprised an initial denaturation at 95°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min and a final extension of 5 min at 72°C.

To detect a potential latent *Agrobacterium* contamination in transformed tissues, we performed a PCR-based amplification of the *A. tumefaciens* 23S rRNA gene using the specific primers UF and B1R developed by Pulawska et al. (2006).

DNA gel blot analysis

A selected number of primary transformed plants were analysed by Southern hybridisation to confirm the stable integration of the foreign gene. Genomic DNA (15 μg) isolated from leaves was digested with *Eco*RI, separated by gel electrophoresis and blotted onto Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by the capillary method (Sambrook et al. 1989).

Membranes were probed with [^{32}P]dATP-labelled *adc* and *gusA* fragments obtained by PCR with primers sets oatADCF/oatADCR and GUSA F/GUSA R, respectively. After 16 h of hybridisation at 65°C, the membranes were washed three times at 65°C with 2× SSC plus 0.1% sodium dodecyl sulphate (SDS), 1× SSC plus 0.1% SDS for 20 min and finally transferred to 0.1× SSC plus 0.1% SDS for 10 min. The washed membranes were wrapped in Saran wrap and subjected to autoradiography at −70°C for 10 days (Sambrook et al. 1989).

Determination of putrescine

To determine free putrescine (Put) levels in leaf extracts, plant material (300 mg) was ground in liquid nitrogen, extracted in 600 ml 5% (v/v) perchloric acid and incubated overnight at 4°C. After centrifugation at 10,000*g* for 15 min, 10 ml of 100 mM 1,7-heptanediamine (ICN Biomedicals, Costa Mesa, CA) was added as an internal standard to 200-ml aliquots of leaf extracts, followed by the addition of 200 ml saturated Na_2CO_3 and 400 ml dansyl chloride (10 mg/ml in acetone); the mixture was then incubated overnight in the dark at room temperature. The reaction was stopped by adding 100 ml of Pro (100 mg ml^{-1}), and dansylated Put was extracted in 500 ml of toluene. The organic phase was vacuum-evaporated, and dansylated Put was dissolved in 200 ml of acetonitrile and analysed by reversed phase high-performance liquid chromatography as described previously by Garriz et al. (2004).

Enzyme activity assays

The ADC was extracted by homogenisation of shoot (stem and leaves) tissues (500 mg fresh weight) in 2 volumes of 100 mM, pH 7.5, phosphate buffer containing 0.5 mM EDTA, 10 mM dithiothreitol, 1 mM pyridoxal phosphate and 20 mM sodium ascorbate. The crude extracts thus obtained were clarified by centrifugation at 10,000*g* for 10 min. All of the above-described procedures were carried out at 4°C. The protein concentration in the supernatants was determined using the standard method of Bradford (1976). Enzyme activities were determined by mixing 190 ml of the extract with 10 ml of the substrate solution in a glass tube fitted with a rubber stopper and a filter paper disc soaked in 2 N KOH. Substrate solutions for the determination of ADC activities contained 1 mM nonradioactive substrate amended with 5 nCi ml^{-1} L-[$^{14}C_1$]arginine. After a 1-h incubation at 37°C, the reaction was stopped, and $^{14}CO_2$ was released by adding 200 ml of 10% (v/v) perchloric acid. Following a 1-h distillation of $^{14}CO_2$ at 37°C, the paper was immersed in 200 ml scintillation cocktail (4 g Omnifluor in toluene), and radioactivity was determined in a Beckman LS 5000 scintillation counter (Beckman Coulter, Brea, CA) .

β-Glucuronidase activity

For GUS histochemistry, shoots and roots were immersed in a solution of 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D glucuronide (X-Gluc) in 100 mM sodium phosphate buffer (pH 7.2), 0.1% (v/v) Triton X-100, 10 mM EDTA and 1 mM potassium ferricyanide (Stomp 1992). A visible colorimetric reaction was obtained in a time range of a few minutes to an overnight incubation at 37°C in the dark.

Chromosome counting

Since regenerants of many species, including *Lotus corniculatus* (Webb and Watson 1991), undergo both morphological and cytological changes as a result of tissue culture, roots of plants regenerated from leaves were examined for evidence of cytological variation. Root tips were collected from regenerated plants at 1:30 p.m. and pretreated in saturated aqueous 8-hydroxyquinoline for 3 h at room temperature, then fixed in 3:1 ethanol/acetic acid for 24 h and finally stained with Schiff's reagent. Isolated meristems were macerated in a drop of 2% acetic orcein solution and squashed in 45% acetic acid. Chromosomes were counted in the metaphase stage of mitosis.

Scaling-up production of transgenic plants

Stem segments containing one axillary bud obtained from transgenic plants were cultured in MS medium supplemented with indoleacetic acid (IAA, 0.2 μM) and BA (2.5, 5 or 10 μM) for 30 days. The protocol described above was used for the induction of rooting.

Acclimatisation

Plantlets obtained in vitro were carefully washed under running water, placed in 200-ml pots filled with a mixture of sterile soil and sand (1:1 v/v) plus 0.5 g of controlled-release micro-fertiliser (Osmocote; N:P:K, 9:45:15; 180-day release) and covered with transparent polyethylene, which was subsequently lifted to reduce humidity. The plantlets were then grown for 6–8 weeks under a day/night air temperature of 25–27/20–22°C and a substrate temperature of 22–25°C. A 14/10-h light/dark photoperiod was maintained, and light was provided by 16 cool-white fluorescent lamps (40 W; distance above plants 1.6 m) at an intensity of 345 μmol m^{-2} s^{-1} PPFD in the wavelength range of 400–700 nm.

Experimental design and statistical analysis

All of the experiments were arranged in a completely randomised design. Each treatment consisted of ten explants, except for GUS, for which the trial was performed with 100 explants. The experiment was repeated three times, and the treatments were arranged randomly on the shelves in the growth room. The results presented are the means of the replications with the standard error (±SE). The number of shoots is presented as the mean number of shoots regenerated per morphogenic explant. The regeneration rate is expressed as the average percentage of roots, cotyledons or leaves that differentiated shoots from the total number of cultivated explants. Selected RD29A:oat ADC transgenic lines were analysed for ADC activity induced by osmotic treatment (200 mM NaCl) for 30 days. The analysis of *adc* gene expression was completed by the determination of free Put. Data were subjected to analysis of variance (ANOVA) (GraphPad Software, San Diego, CA) using Tukey's multiple comparison test. To assess statistical significance, we chose a probability level of 0.05.

Results and discussion

Protocol for in vitro organogenesis and plant regeneration

Our first aim was to develop an efficient protocol for the regeneration of *L. tenuis*. After 45 days in culture, all explants tested were examined for adventitious bud regeneration (Table 1). The number of shoots formed per responsive explant increased from 1 to 33 (Table 2) as the percentage of explants producing shoots increased from 7 to more than 97%. In general, the regeneration rate was similar in all cases, but leaves were chosen as the explant source because of the high quality and growth rate of the regenerated shoots and the ease in obtaining a profuse explant source from them. In this context, either BA or TDZ alone at low concentrations (5 and 2.5 μM, respectively) produced the highest regeneration rate; however, the inclusion of a low concentration of NAA plus BA in the culture medium promoted the normal growth of shoots. Although the regeneration rate was not significantly different between any of the NAA/BA concentrations evaluated, the addition of either 5 or 10 μM of BA was discarded due to the proliferation of calli. The best result was obtained when the basal medium was supplemented with 0.5 μM NAA and 20 μM BA, in which 67 ± 8.8% of the leaves formed 10 ± 4.4 shoots per responsive explant through a direct system of regeneration without callus proliferation. In general, the addition of TDZ to the culture medium caused strong vitrification accompanied by abnormal development during the regeneration phase, which negatively affected the sprouting of adventitious buds and subsequent shoot elongation. This aberrant

Table 1 Effects of NAA, BA and TDZ on the regeneration of adventitious shoots of *Lotus tenuis* explants cultured on MS medium

Plant growth regulators (μM)			Shoot regeneration (responsive explants in %)		
NAA	BA	TDZ	Roots	Cotyledons	Leaves
–	5	–	47 ± 24	86 ± 3.2	93 ± 3.3
–	10	–	73 ± 6.7	86 ± 7	60 ± 5.8
–	20	–	73 ± 17.6	74 ± 12.8	80 ± 10
–	–	2.5	62 ± 21.7	63 ± 6.7	97 ± 3.3
–	–	5	60 ± 11.5	87 ± 8.8	81 ± 5.9
–	–	10	60 ± 11.5	75 ± 12.5	53 ± 3.3
–	–	20	47 ± 13.3	60 ± 5.8	35 ± 7.4
0.5	5	–	55 ± 10.4	90 ± 5.8	87 ± 8.8
0.5	10	–	63 ± 8.8	93 ± 6.7	83 ± 6.7
0.5	20	–	73 ± 17.6	80 ± 5.8	67 ± 8.8
0.5	–	2.5	57 ± 3.3	83 ± 6.7	70 ± 10
0.5	–	5	62 ± 21.7	83 ± 8.8	70 ± 5.8
0.5	–	10	65 ± 5	60 ± 5.8	77 ± 3.3
0.5	–	20	58 ± 10.1	63 ± 6.7	70 ± 15.2
0.5	5	2.5	73 ± 17.6	80 ± 11.5	67 ± 6.7
0.5	5	5	51 ± 14.6	70 ± 11.5	57 ± 8.8
0.5	5	10	44 ± 8.1	58 ± 7.6	43 ± 12
0.5	5	20	27 ± 17.6	52 ± 1.7	43 ± 3.3
0.5	10	2.5	55 ± 8.1	73 ± 8.2	43 ± 3.3
0.5	10	5	60 ± 23	55 ± 7.9	47 ± 14.5
0.5	10	10	74 ± 3.8	62 ± 7.9	40
0.5	10	20	27 ± 17.6	45 ± 17.2	27 ± 2.7
0.5	20	2.5	7 ± 6.7	75 ± 12.6	70 ± 10
0.5	20	5	42 ± 21.3	63 ± 18.6	33 ± 3.3
0.5	20	10	36 ± 19.5	62 ± 7.3	24 ± 3.3
0.5	20	20	58 ± 19.5	60 ± 2.9	24 ± 4.3
Analysis of variance					
F value			1.34	0.97	7.78
P value			0.18	0.51	< 0.0001

NAA, Naphthaleneacetic acid, BA, 6-benzyladenine, TDZ thidiazuron; MS, Murashige and Skoog medium (1962)

Values are given as the mean ± standard error of the mean (SEM) of three replicates with ten explants in each replication

Table 2 Effects of NAA, BA and TDZ on the proliferation of adventitious shoots of *L. tenuis* explants cultured on MS medium

Plant growth regulators (μM)			Mean number of buds per responsive explant		
NAA	BA	TDZ	Roots	Cotyledons	Leaves
–	5	–	33 ± 4	27 ± 4.6	28 ± 2.9
–	10	–	20 ± 4.9	17 ± 1.5	10 ± 1
–	20	–	9 ± 0.8	20 ± 1.3	19 ± 3.4
–	–	2.5	12 ± 4.2	13 ± 4.6	12 ± 1.3
–	–	5	9 ± 3.2	13 ± 3.3	9 ± 2.3
–	–	10	16 ± 11	7 ± 0.2	12 ± 3.2
–	–	20	11 ± 7.4	12 ± 3.4	7 ± 1
0.5	5	–	15 ± 3.1	31 ± 7.1	25 ± 6.8
0.5	10	–	14 ± 2.1	22 ± 4	10 ± 1.5
0.5	20	–	16 ± 1.4	12 ± 3.7	10 ± 4.4
0.5	–	2.5	9 ± 2.9	8.8 ± 0.5	13 ± 1.4
0.5	–	5	11 ± 3.5	11 ± 1.4	10 ± 3.6
0.5	–	10	9 ± 1.8	7 ± 3.7	8 ± 0.9
0.5	–	20	10 ± 2.6	8 ± 1.8	13 ± 2.6
0.5	5	2.5	12 ± 0.1	9 ± 1.8	10 ± 1.3
0.5	5	5	13 ± 2.8	8 ± 0.9	7 ± 2.2
0.5	5	10	9 ± 3.5	10 ± 0.8	11 ± 1.8
0.5	5	20	12 ± 2.1	10 ± 2.3	12 ± 3.5
0.5	10	2.5	11 ± 2.2	8 ± 1.8	7 ± 3
0.5	10	5	12 ± 2.3	8 ± 1.8	12 ± 2.2
0.5	10	10	13 ± 1.8	9 ± 3	11 ± 2.6
0.5	10	20	6 ± 3	6 ± 1.6	13 ± 0.3
0.5	20	2.5	1	9 ± 0.8	10 ± 2.7
0.5	20	5	12 ± 0.5	11 ± 2.7	12 ± 2.2
0.5	20	10	6 ± 3.4	7 ± 0.8	8 ± 3.2
0.5	20	20	5 ± 0.3	7 ± 1.3	17 ± 9.1
Analysis of variance					
F value			1.66	5.02	2.88
P value			0.069	0.0001	0.0007

Values are mean ± SEM of three replicates with ten explants in each replication

growth form causes significant losses to the micropropagation system because of the poor survival rate of plantlets when they are transferred from tissue culture to the ex vitro environment during the rustification phase (Gribble et al. 2003). Additionally, the explants cultured in medium with TDZ in combination with BA produced fewer shoots due to the high proliferation of calli than explants cultured in medium containing a cytokinin derived from adenine alone.

Finally, taking into account that *Lotus tenuis* has a compound leaf with three folioles, we performed an experiment to study the effect of topophysis on the growth and differentiation of adventitious buds from the upper three leaves of the shoot tip sliced into each foliole. No statistical differences were observed after 45 days of culture in MS medium supplemented with 0.5 μM NAA and 20 μM BA (data not shown).

Transgenic plant regeneration

Using the protocol mentioned above, leaf discs of *L. tenuis* were inoculated with *A. tumefaciens* carrying one of the two binary vectors. The best regeneration medium was chosen with the aim of developing the transformation protocol. After 45 days of culture in MS supplemented with 0.5 μM NAA and 20 μM BA, 40 ± 15% of the leaf

explants inoculated with the pBiRD29:ADC strain (ADC) had produced eight to ten adventitious shoots per responsive explant (Fig. 1a) through a direct system of regeneration. The meristemoid was originated by periclinal divisions of epidermal cells, resulting in a meristematic zone emerging from the leaf surface that could differentiate to produce shoots (Fig. 1b). Likewise, 69.3 ± 6.1% of the explants inoculated with the pBi RD29A:GUS strain (GUS) produced 14 ± 0.8 adventitious shoots per responsive explant. The developing shoots were transferred to fresh MS medium without PGRs for elongation (Fig. 1c). We confirmed the gene integration of the transgenic lines from the leaf sections of *L. tenuis.* PCR analysis using the primer sets for *adc* yielded amplification products of the expected size. All of the transformed lines tested showed the 1.8-kb predicted band for the *adc* gene (Fig. 2). Three ADC transgenic lines were obtained from the 30 explants infected with pBiRD29:ADC, and 29 transgenic lines were obtained from the 160 explants infected with pBi RD29A:GUS based on our analysis using the specific primers for the *gus*A gene. These results correspond to a transformation efficiency of 10 and 18.1%, respectively.

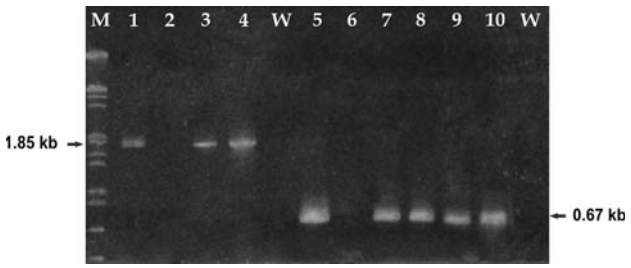

Fig. 2 PCR analysis of putative transgenic lines using the specific primers for either the arginine decarboxylase (ADC; *lanes 1–4*) or glucuronidase (GUS; *lanes 5–10*) genes. *Lanes*: *M* Molecular weight marker (λDNA *Eco*RI/*Hin*dIII), *1*, *5* plasmids pBiADC and pBiGUS (positive controls), *2*, *6* non-transformed plants, *3*, *4* ADC putative transgenic lines, *7–10* GUS putative transgenic lines, *W* water

The absence of latent *Agrobacterium* contamination in transformed tissues was confirmed by PCR-based amplification of the *A. tumefaciens* 23S rRNA gene (Fig. 3).

Southern blot analyses were performed to verify the integration of the transgenes and to determine the respective copy number. When DNA from *adc* transgenic plants was digested with *Eco*RI, Southern blot analysis indicated

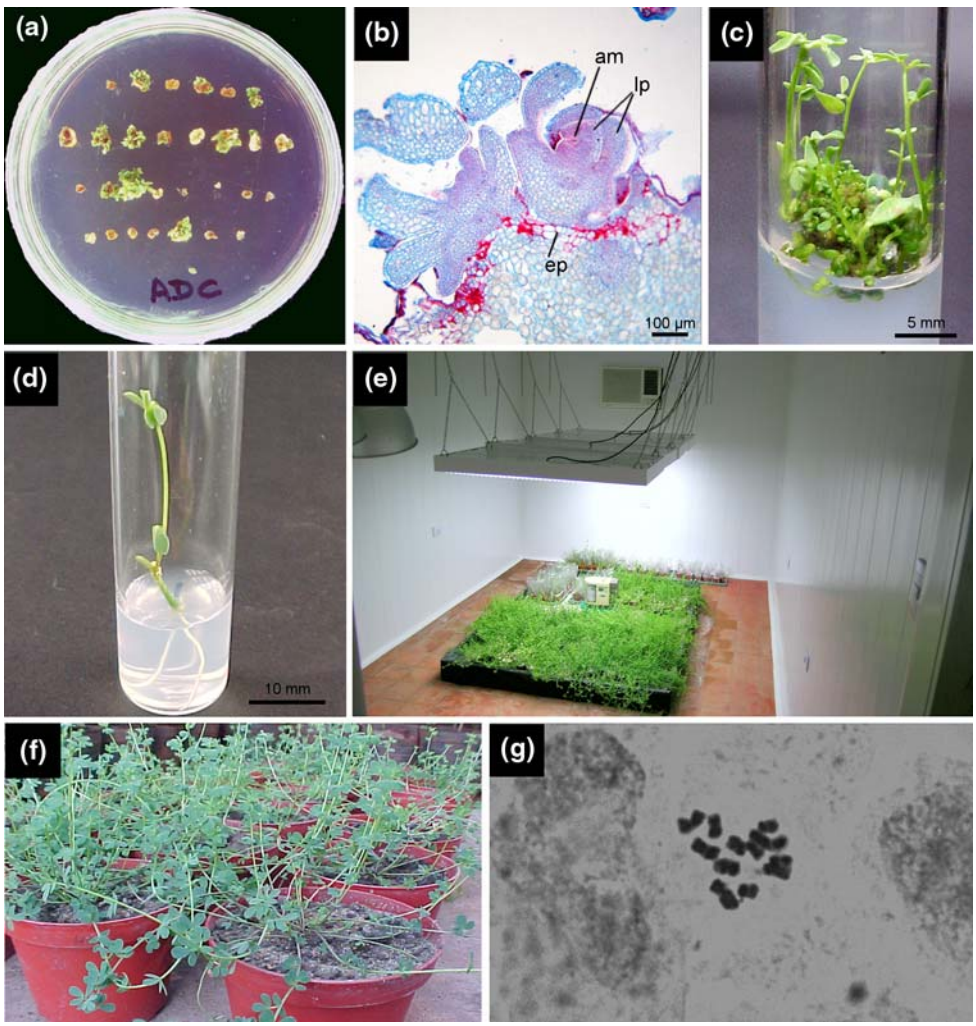

Fig. 1 *Agrobacterium*-mediated transformation and plantlet regeneration through leaf disc culture of *Lotus tenuis* Mill. **a** Shoot regeneration from co-cultivated leaf discs grown in kanamycin-containing selective medium. The photograph was taken 45 days after the co-cultivation with *A. tumefaciens* GV3101 PMP90 harbouring the binary vector pBiRD29:ADC. **b** Direct bud formation from leaf tissues. *am* Apical meristem, *ep* epidermis, *lp* leaf primordia. **c** Shoot elongation by culture in MS medium without plant growth regulators (PGRs). **d** Direct root formation and full-grown in vitro plantlet. **e** Plantlet grown under controlled environmental conditions as described in the Material and methods. **f** Plantlet grown in pots under greenhouse conditions. **g** Mitotic chromosomes at metaphase stage, $2n = 2x = 12$

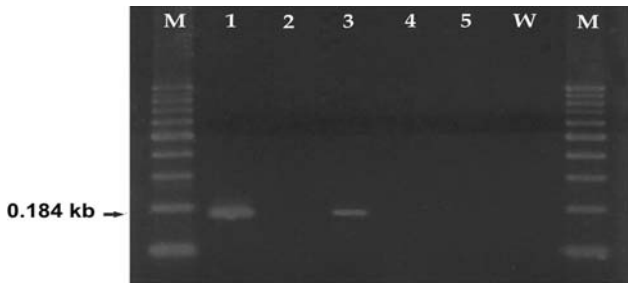

Fig. 3 Analysis of ADC putative transgenic lines to detect contamination with *Agrobacterium* cells. DNA from transgenic lines was used as PCR templates for the amplification of the 23S rRNA gene from Agrobacteria. *Lanes*: *M* 100-bp DNA ladder (Promega, Madison, WI) as marker, *1 A. tumefaciens* total DNA, *2* non-transgenic plant DNA, *3* non-transgenic plant contaminated with *Agrobacterium* cells, *4*, *5* transgenic plant DNA (lines 19 and 21, respectively). The lack of amplification of the 23 s rRNA gene in *lanes 2*, *4* and *5* indicates the complete elimination of *A. tumefaciens* by the antibiotic treatment

a single insertion of pBiRD29:ADC (lines *Lt*19 and *Lt*21), as judged from the single hybridising band detected (Fig. 4). In contrast, two bands were identified in all pBiRD29:GUS transgenic lines tested, thus suggesting that two copies of the T-DNA were integrated into the plant genome. No hybridisation bands were detected in non-transgenic control plants.

All transformed shoots were subsequently identified and propagated. After 30 days of culture, the most effective treatment appeared to be a culture medium containing only 2.5 μM BA, in which a micropropagation rate of 14 ± 2.6 shoots per regenerative explant was obtained. Ultimately, more than 60% of the IBA-treated shoots formed adventitious roots by a direct pattern of root organogenesis without callus proliferation (Fig. 1d). Plantlets with fully expanded leaves and well-developed roots were acclimatised in pots with transparent covers that were subsequently lifted to reduce humidity (Fig. 1e). The acclimatised

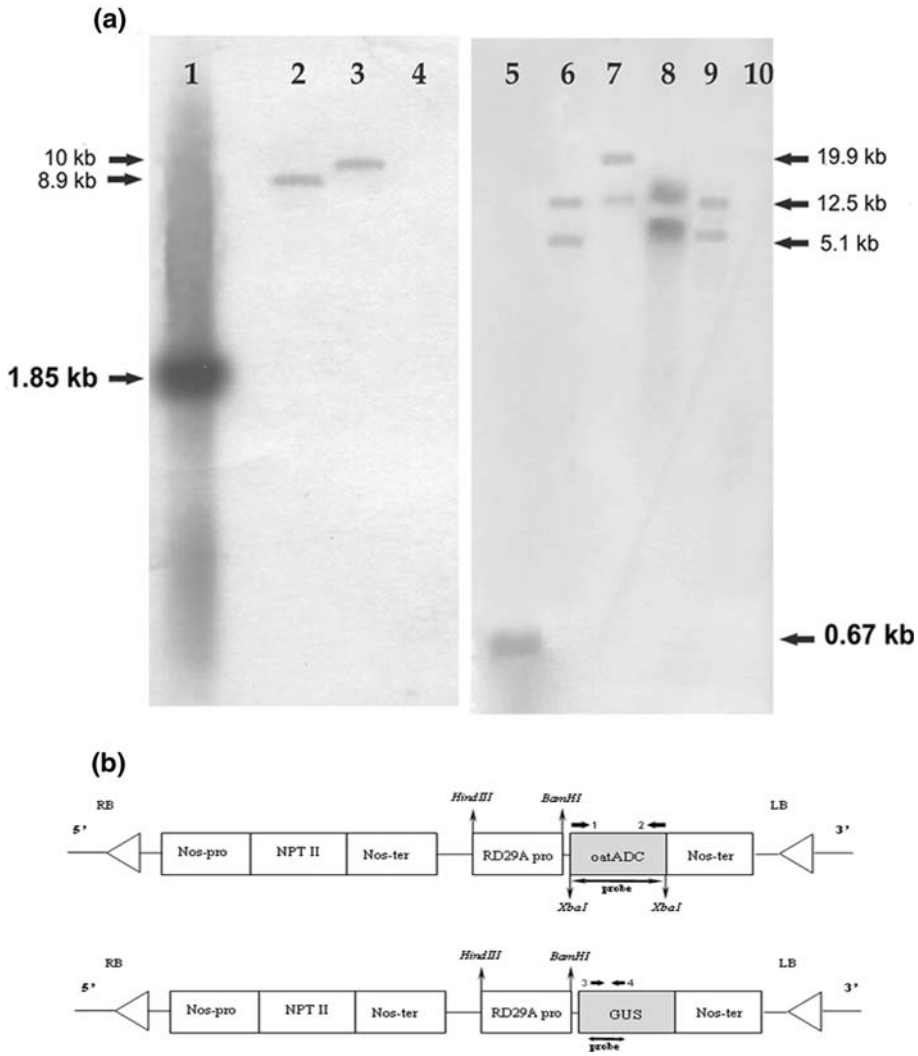

Fig. 4 **a** Southern hybridisation analysis of ADC and GUS transgenic lines probed for *adc* (*lanes 1–4*) and *gus* (*lanes 5–10*) sequences, respectively. Genomic DNA (10 μg) was cut with the restriction enzyme *Eco*RI to show integration into the plant genome and the number of integrations. *Lanes*: *1*, *5 adc* (1.85 kb) and *gus* (0.67 kb) fragments used as positive controls, *2*, *3*, ADC-transformed plants, *4*, *10* non-transformed control plants, *6–9* GUS-transformed plants. **b** Map of the T-DNA with the restriction sites *Hin*dIII, *Bam*HI and *Xba*I used to digest plant DNA, and the position of the gene probe. The binary vector contained the RD29A osmotic stress-inducible promoter and either the ADC gene (pBiRD29:ADC) or the *β*-GUS reporter gene (pBiRD29:GUS) and carried the selectable marker neomycin phosphotransferase II (*nptII*). *LB* Left border, *NOS-pro* nopaline synthase promoter, *NOS-ter* nopaline synthase terminator, *RB* right border

plantlets were successfully established in the soil (Fig. 1f). The developed protocol for regeneration did not produce any alterations in the karyotype, and all lines tested revealed the normal chromosome number $2n = 2x = 12$ (Fig. 1g). The stability of the gene introduced in the cloned materials was established by PCR analysis. All transgenic plants obtained in this study exhibited the same phenotype as that of the non-transformed controls under non-stress conditions.

The RD29A:oat ADC *Lt*19 line and the wild-type (wt) plants were analysed for ADC activity induced by a 30-day osmotic treatment (200 mM NaCl) (Fig. 5). The *Lt*19 line showed a 73-fold increase in ADC activity compared with the untreated control of the same genotype, while the stressed wt plants showed a 23-fold increase relative to the control. We could not observe significant differences in ADC activity between unstressed and stressed wt plants. The stressed *Lt*19 line, based on its induction of ADC activity, accumulated almost twofold more Put than the unstressed samples.

In further confirmation of the expression patterns, the GUS transgenic plants driven by the RD29A promoter showed strong signals of osmotic stress in leaves and stems (Fig. 6).

Most of the procedures for transforming *Lotus* species via *A. tumefaciens*-mediated transformation are based on indirect plant regeneration from root-, hypocotyl-, and cotyledon-derived calli (Aoki et al. 2002; Lohar et al. 2001; Lombardi et al. 2003). In our study, we developed a simple procedure for transforming *L. tenuis* that yields transformed plants through a direct system of bud regeneration

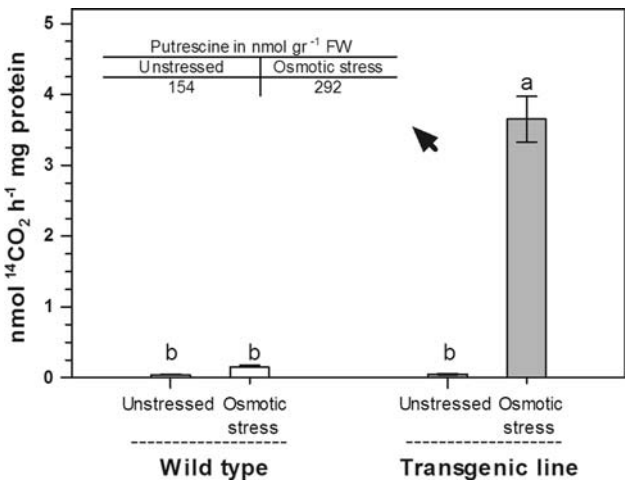

Fig. 5 Mean ADC activity and shoot putrescine content of the osmotic-elicited pBiRD29A:oat ADC *Lt*19 line grown in pots. For the osmotic stress treatment, plants were irrigated for 30 days with 200 mM NaCl, starting with 50 mM. *Error bars* ± standard error of the mean of triplicate trials. *Means* followed by the *same letter* do not differ significantly at $p \leq 0.05$ (Tukey's multiple comparison test)

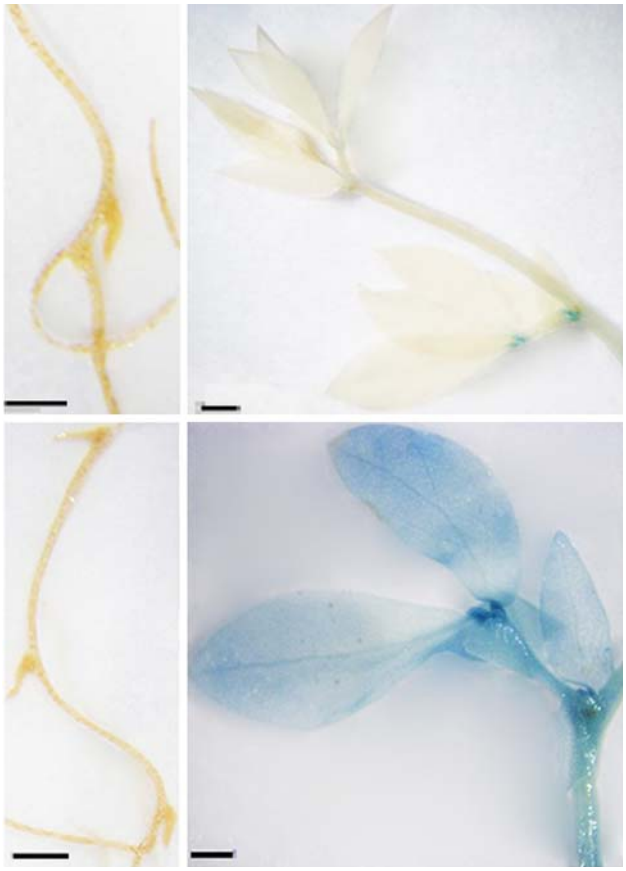

Fig. 6 Histochemical localisation of GUS activity in transgenic *L. tenuis* tissues. Expression patterns of the GUS gene in the roots, stems and leaves of unstressed (*upper*) and osmotically stressed (*bottom*) transgenic lines. For the osmotic stress treatment, plants were irrigated for 30 days with 200 mM NaCl, starting with 50 mM. In all cases, *bars* indicate 1 mm

without callus proliferation. This fact, in addition to the use of leaf segments as a source of explants, may reduce the risk of producing somaclonal variants (Webb and Watson 1991).

Conclusions

The findings of our study provide the first evidence for the genetic transformation and transgenic plant regeneration of *L. tenuis* Mill. via *A. tumefaciens*-mediated transformation. The system consists of the inoculation of leaf segments with the *A. tumefaciens* strain GV3101 (pMP90) harbouring either the binary vector pBiRD29:ADC or pBiRD29:-GUS in the presence of 30 μg ml^{-1} kanamycin, 30 μg ml^{-1} streptomycin and 100 μg ml^{-1} rifampicin, co-cultivation for 3 days and regeneration in MS medium containing NAA (0.1 μM), BA (20 μM), kanamycin (30 μg ml^{-1}) and cefotaxime (400 μg ml^{-1}). The elongated shoots from the transformed material should be then cloned by growth in

MS containing BA (2.5 μM). The transgenic lines obtained do not display any phenotypic alterations and are currently under molecular and physiological evaluation.

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