



Transformed roots of *Lupinus mutabilis*: induction, culture and isoflavone biosynthesis

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Abstract

Transformed roots of *Lupinus mutabilis* cv. Potosi induced by *Agrobacterium rhizogenes* strain R1601 were cultured on Murashige and Skoog-based medium lacking kanamycin sulphate, or with this antibiotic at 40 mg l⁻¹. The neomycin phosphotransferase gene in the genome of transformed roots was confirmed by non-radioactive Southern hybridisation. Neomycin phosphotransferase protein was detected by ELISA. Transformed roots synthesised isoflavones, but not quinolizidine alkaloids; the latter are typical secondary metabolites of lupin normally produced in aerial parts of the plant. Genistein and 2'-hydroxygenistein, were the main secondary metabolites in cultured, transformed roots, whereas the glycoside genistin was more abundant in roots of non-transformed plants. Wighteone concentrations in transgenic roots were higher than those of non-transformed roots. Transformed roots produced twice the concentration of isoflavones compared with roots from non-transformed plants, indicating that Ri plasmid T-DNA genes modified isoflavone concentration and pattern of biosynthesis.

Abbreviations: DIG – digoxigenin; f. wt. – fresh weight; GC-MS – gas chromatography-mass spectrometry; GFP – green fluorescent protein; GUS – β -glucuronidase; *gus* – β -glucuronidase gene; HPLC – high performance liquid chromatography; MS – Murashige and Skoog (1962); m. wt. – molecular weight; NPTII – neomycin phosphotransferase; *nptII* – neomycin phosphotransferase gene

Introduction

There are few reports of the induction of transformed roots by *Agrobacterium rhizogenes* on grain legumes. Those grain legumes which do respond to *A. rhizogenes* include members of the genera *Cicer* (Siefkes-Boer et al., 1995), *Glycine*, *Phaseolus*, *Pisum*, *Vicia* and *Vigna* (Porter, 1991) and *Lupinus* (Mugnier, 1988). In assessments of a range of bacterial strains, Porter (1991) concluded that genera from certain plant families were more susceptible to *A. rhizogenes* than others, while naturally occurring bacterial strains were superior to engineered strains in effecting transformation. Plants are diffi-

cult to regenerate from transformed roots of grain legumes, although there are examples in the wild soybeans, *G. canescens* (Rech et al., 1989) and *G. argyrea* (Kumar et al., 1991) following transformation with *A. rhizogenes* R1601, the same strain as used in the present investigation. In a review of genetic transformation of plants by *A. rhizogenes*, Tepfer (1990) cited the regeneration of plants from transformed roots of *Vigna aconitifolia*, but did not report the bacterial strain used for transformation. Saalbach et al. (1994) transformed roots of *Pisum sativum* and *Vicia faba* with *A. rhizogenes* strain 15834 harbouring the binary vector pGSGGLUC1-2S carrying the Brazil nut 2S albumin gene with the CaMV 35S promoter. Although

transformed roots expressed the 2S albumin gene and several shoots were regenerated, the latter did not root and fertile plants were not obtained. Inoculation of cotyledon explants of soybean (*G. max*) cultivars with *A. rhizogenes* strain K599 resulted in transformed roots expressing genes for GUS, NPTII and GFP (Cho et al., 2000), but plant regeneration from these roots was unsuccessful.

In lupin, transformed roots were established in culture from *L. albus* and *L. polyphyllus* following inoculation of stem explants with *A. rhizogenes* strain A4 (Mugnier, 1988). Later, Berlin et al. (1991a) cultured transformed roots of *L. polyphyllus* and *L. hartwegii* induced by *A. rhizogenes* strain 15834. The nature of the explant (e.g., hypocotyl or stem sections) and bacterial virulence influenced the transformation frequency. Attempts to regenerate shoots from transformed roots of lupin have failed, although limited success in shoot regeneration was reported following transformation of *L. angustifolius* (Molvig et al., 1997; Pigeaire et al., 1997), *L. luteus* (Li et al., 2000) and *Lupinus mutabilis* (Babaoglu et al., 2000a, b) with *A. tumefaciens*. Another problem with grain legumes is the rooting of regenerated transgenic shoots for which grafting may be the only option to obtain fertile plants for seed production (Krishnamurthy et al., 2000; Li et al., 2000). Interestingly, *A. rhizogenes* has been used to induce roots on regenerated shoots (Damiano and Monticelli, 1998), including shoots of *L. mutabilis* transformed by *A. tumefaciens* (Babaoglu et al., 2000b).

An attraction of transformed roots is their potential for biomass production with synthesis of secondary metabolites in the absence of exogenous growth regulators (Baiza et al., 1999). Indeed, transformed roots have a role as model systems in plant metabolite engineering (Shanks and Morgan, 1999), as in the manipulation of shikonin biosynthesis in *Lithospermum erythrorhizon* (Boehm et al., 2000). Berlin et al. (1991b) reported that cultured transformed roots of lupin may provide a useful supply of secondary products, such as isoflavones. This paper reports on the induction of transformed roots in *L. mutabilis* and analysis of isoflavones.

Materials and methods

Plant material

Seeds of *L. mutabilis* cv. Potosi, kindly provided by Dr P. Römer, Südwestdeutsche Saatzucht, Im

Rheinfeld 1-13, 7550 Rastatt, Germany, were immersed in 20% (v/v) 'Domestos' bleach (Diversey Fabergé, Sutton-on-Thames, UK) for 20 min, before rinsing five times with sterile reverse osmosis water. Seeds were sown on the surface of 50 ml aliquots of growth regulator-free, semi-solid [0.8% (w/v) agar] MS-based medium containing 30 g l⁻¹ sucrose, pH 5.8 (designated MS0 medium), in 175 ml screw-capped glass jars (Beatson Clark and Co., Ltd., Rotherham, UK; 4 seeds/jar). Axenic seedlings were cultured with a 16-h photoperiod (37 μmol m⁻² s⁻¹, Cool White fluorescent tubes; Thorn EMI, Hayes, UK) at 22 ± 2 °C. Cultures were established by excising shoot tips, each approx. 1 cm in height, and their transfer to MS0 agar medium. Shoots were maintained by transfer of apices to MS0 medium every 28 days.

Twenty-one-day-old seedlings were sub-cultured to MS0 agar medium containing kanamycin sulphate at 0, 10, 15, 20, 25, 30 and 50 mg l⁻¹ to assess their natural resistance to this antibiotic.

Axenic shoots of *L. mutabilis* cv. Potosi, already transformed by *A. tumefaciens* strain 1065 carrying the *nptII* and *gus* genes (Babaoglu et al., 2000b), were also inoculated with *A. rhizogenes* strain R1601.

Bacterial cultures

Agrobacterium rhizogenes strain R1601 carried pRiA4b with a chimaeric *nptII* gene co-integrated into *HindIII* fragment 21 of the TL-DNA, together with pTVK291 *in trans*, conferring a supervirulent phenotype (Pythoud et al., 1987). Cultures were established in 5–10 ml of APM medium [5.0 g l⁻¹ yeast extract, 0.5 g l⁻¹ casein hydrolysate, 2.0 g l⁻¹ (NH₄)₂SO₄, 3.0 g l⁻¹ NaCl and 8.0 g l⁻¹ mannitol, pH 6.6] with 100 mg l⁻¹ kanamycin sulphate and 100 mg l⁻¹ ampicillin in 25 ml flasks on a rotary shaker (150 rpm, dark, 28 ± 2 °C, 16–24 h). Bacteria were grown to an OD₆₀₀ of 1.0–1.2 before harvesting (10 min, 10,000 rpm, 7000× g). The medium was discarded and the bacterial pellet resuspended in the same volume of MS0 liquid medium.

Inoculation of explants with *A. rhizogenes*

Root, hypocotyl, stem and petiole explants, and hypocotyls and epicotyls with attached shoot apices, were excised from 7- and 21-day-old seedlings. Explants were inverted (basal ends up), inserted into MS0 agar medium (3 explants/50 ml medium/175 ml jar) and inoculated by placing one loopful of bacterial suspension onto the cut surface of each explant.

Leaf explants (each 1 cm² in size) were immersed in the bacterial suspension for 30 min, followed by blotting with sterile filter paper. Explants were laid on MS0 agar medium with their lower surfaces in contact with the medium (5 explants/20 ml medium/9 cm Petri dish). The bacterial suspension was used undiluted or after dilution 1:1, 1:5 or 1:10 (v:v) with MS0 liquid medium. Ninety explants were inoculated at each bacterial dilution.

Shoots of *L. mutabilis* cv. Potosi transformed by *A. tumefaciens* strain 1065, carrying the *nptII* and *gus* genes, were also inoculated with *A. rhizogenes* R1601 to generate 'double' transformed roots. The procedure involved removal of the stem bases, inversion of the intact shoots (each 2–3 cm in height) and insertion of their apices into MS0 agar medium, followed by inoculation of the cut stems. Inoculated explants were incubated under the same conditions as for the growth of seedlings.

Culture of transformed roots

Roots initiated after 14–28 days at inoculation sites were excised from the explants and transferred to semi-solid MS0 medium containing 300 mg l⁻¹ cefotaxime and 40 mg l⁻¹ kanamycin sulphate. Approximately 100 mg f. wt. samples were sub-cultured to MS0 agar medium (50 ml aliquots of medium in 175 ml jars) every 21 days and incubated in the light (55 μmol m⁻² s⁻¹; Daylight fluorescent tubes) or the dark at 22 ± 2 °C. The cefotaxime concentration was reduced (250, 200, 150, 100, 50, 0 mg l⁻¹) during subsequent sub-culture, with kanamycin maintained at 40 mg l⁻¹. Following completion of cefotaxime treatment, 100 mg samples of roots selected randomly from the cultures were macerated and incubated in 20 ml aliquots of Luria Bertani medium (Sambrook et al., 1989) at 28 °C for 48 h to confirm removal of the agrobacteria. When free of agrobacteria, roots transformed by strain R1601 ('single' transformed roots) and those transformed by strain 1065 followed by R1601 ('double' transformed roots) were cultured on MS0 agar medium with kanamycin at 40 mg l⁻¹ or in the absence of the antibiotic, in the light or the dark.

Growth of 'single' transformed roots was recorded at days 15 and 21 after sub-culture for 5 and 26 passages in the dark, in the absence or presence of kanamycin at 40 mg l⁻¹. The growth of 'double' transformed roots was compared with that of 'single' transformed roots after 6 passages in the dark in the

absence or presence of the same concentration of antibiotic. Twelve replicates were assessed in all cases.

NPTII ELISA, GUS analysis and DNA–DNA hybridisation

A double sandwich ELISA immunoassay was used to quantify NPTII protein in cultured, transformed roots, using the manufacturer's instructions (5 Prime → 3 Prime, Inc.[®], Boulder, Co 80303, USA) and as by Babaoglu et al. (2000b). The kanamycin resistance of such roots was also evaluated. GUS activity in 'double' transformed roots was assayed histochemically (Jefferson et al., 1987). Roots were harvested at day 5 during passage 8 on MS0 medium containing 40 mg l⁻¹ kanamycin and DNA extracted (Dellaporta et al., 1983). DNA was also isolated from non-transformed seedlings. Ten 10 μg of DNA was restricted with *HindIII* or *EcoRI*, and samples loaded onto 0.8% (w/v) agarose (Sea-Kem GTG, BioWhittaker Molecular Applications, Rockland, USA) gels, which were run for 16 h at 25 V. DNA was blotted onto positively charged nylon membrane (Boehringer-Mannheim Ltd., Lewes, UK) and the *nptII* gene was detected non-radioactively (McCabe et al., 1996; Babaoglu et al., 2000b).

Analyses of isoflavones by HPLC

Roots cultured for 10 passages ('single' transformed roots) or 8 passages ('double' transformed roots) on MS0 medium in the dark with 40 mg l⁻¹ kanamycin, were freeze dried and ground to a fine powder. Samples of 500 mg were extracted with dimethyl formamide/methanol followed by addition of MeOH to the combined extracts to a volume of 100 ml. This solution was used for HPLC (gradient system with two model 64 pumps, 20 μl injection volume and Eurochrom 2000 software; Knauer, Berlin, Germany) with an endcapped RP-18 column (5 m, 250-4, Merck, Sharp and Dohme G.m.b.h., Haar, Germany). Separation was achieved using gradient elution with acetonitrile/water/phosphoric acid. Detection was with a 1040A photodiode array detector (Hewlett Packard, Waldbronn, Germany) at 260/200 nm. Mean values were based on two independent determinations. Isoflavone standards were genistin (Roth, Karlsruhe, Germany), genistein (Fluka, Munich, Germany), wighteone and 2'-hydroxygenistein (Dr M. Roberts, School of Pharmacy, University of London).

Approximately 1 g of dry powdered plant material was incubated with 15 ml 0.5 N HCl overnight,

followed by homogenisation for 30 min. Homogenates were adjusted to pH 10 with ammonia (25% aqueous solution). Alkaloids were isolated by solid phase extraction using Extrelut columns (Merck) and dichloromethane as an eluent. Extracts were subjected to capillary GC and GC-MS, as described below.

Quinolizidine alkaloid extracts were separated with a Carlo Erba ICU 600 GC (CE Instruments, Wigan, UK) equipped with FID and spectra physics integrator and a DB1-30W column (15 m, 0.317 internal dia.). Helium was the carrier at 2 ml min⁻¹. Other conditions were a detector temperature of 300 °C, an injector temperature of 25 °C and an oven temperature initially of 150 °C for 2 min (isothermal), followed by 150–250 °C at 15 °C min⁻¹, 250–300 °C at 25 °C min⁻¹ and 300 °C for 5 min (isothermal). Retention indices: Kovats (1958) indices were calculated with respect to a set of co-injected even numbered hydrocarbons (C₁₀–C₂₈).

GC-MS employed a Carlo Erba HRGC 4160 GC equipped with a fused silica column (OV1; 30 m, 0.3 mm ID). The capillary column was coupled directly to a Finnigan MAT 4500 quadrupole mass spectrometer (Thermo-Finnigan, San Jose, USA). Electron Impact-Mass Spectra were recorded at 40 eV. The conditions were injection at 250 °C, with a temperature programme of 70–300 °C at 6 °C min⁻¹ or 150–300 °C at 6 °C min⁻¹ in a split ratio of 1:20 (v:v). Helium was the carrier at 0.5 bar.

Results

Induction and culture of transformed roots

Hypocotyl, epicotyl and stem explants which responded to the hypervirulent *A. rhizogenes* strain R1601 produced callus within 15–21 days of inoculation, from which roots emerged by 28 days of culture. Bacterial concentration influenced hairy root induction, with the highest frequency of induction at a dilution of 1:5 (v:v) of the bacterial suspension with MS0 liquid medium. Hypocotyl sections from 7-day-old seedlings were more responsive than those of 21-day-old seedlings. Thus, at bacterial dilutions of 1:1, 1:5 and 1:10 (v:v), the percentages of hypocotyls from 7-day-old seedlings which produced roots were 13, 30 and 7%, respectively, compared to 7, 23 and 3% for explants from 21-day-old seedlings. The response of hypocotyl explants was increased approx. twofold when these explants remained attached to

shoots. Petiole, leaf and root explants failed to respond to *Agrobacterium* inoculation, undergoing necrosis after 6–7 days of co-cultivation.

Roots excised from inoculated explants were vigorously growing, branched and negatively geotropic when cultured on MS0 agar medium with cefotaxime initially at 250 mg l⁻¹ followed by reduction during subsequent passages, either with kanamycin at 40 mg l⁻¹ or without this antibiotic, in the light or dark. Twenty-eight-day-old non-transformed seedlings failed to grow on MS0 agar medium containing kanamycin at or above 15 mg l⁻¹, with cefotaxime at 250 mg l⁻¹ or without the latter antibiotic. Roots excised from non-transformed seedlings failed to grow even on MS0 agar medium lacking antibiotics.

'Single' transformed roots maintained a high growth rate over 14 months (26 passages) when sub-cultured every 21 days on MS0 agar medium, with or without kanamycin and in the light or dark. Roots exhibited a sigmoid growth pattern with lag (0–5 days), exponential (5–15 days) and stationary phases (Figure 1). The mean growth in terms of biomass at day 15 for roots cultured during passage 26 was not significantly different to that for cultures in passage 5 (Table 1). The growth of roots was not significantly different in the light or the dark. However, it was essential to sub-culture dark-grown roots to new medium at day 21, since growth ceased after this time.

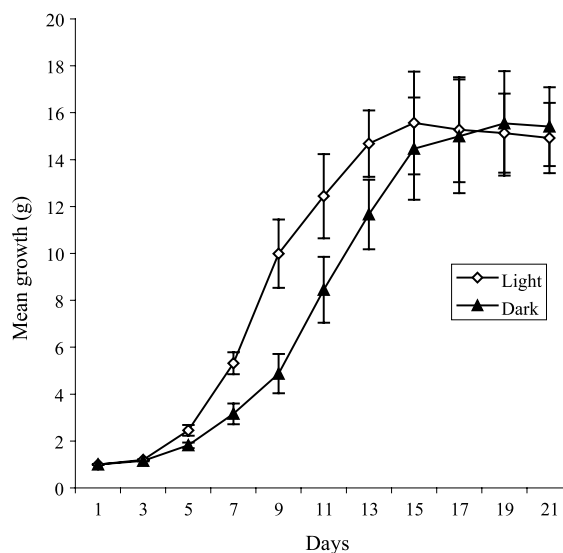


Figure 1. Growth in the light or dark on MS0 agar medium with 40 mg l⁻¹ kanamycin of roots of *L. mutabilis* transformed by *A. rhizogenes* strain R1601. Bars = SEM; *n* = 4 throughout.

Table 1. Growth of transformed roots of *L. mutabilis* during short term (passages 5, 6) or long term (passage 26) culture on MS0 agar medium with or without kanamycin

Root type	Passage No.	f. wt. (g) – Kanamycin	f. wt. (g) + Kanamycin (40 mg l ⁻¹)	NPTII protein (ng mg ⁻¹ total protein)	Kanamycin resistance (mg l ⁻¹)
'Single' transformed	5 dark	16.7 ± 1.0	14.6 ± 1.0		
	26 dark	16.0 ± 0.6	14.2 ± 0.7		
	6 light	15.6 ± 1.6	14.9 ± 1.3		
	6 dark	14.6 ± 1.0	15.4 ± 0.8	11.0 ± 3.5	400
'Double' transformed	6 dark	12.3 ± 0.8	10.3 ± 0.7	5.1 ± 3.3	200

Inoculum = 1.0 g f. wt.; *n* = 12 throughout.

'Double' transformed roots produced less biomass than 'single' transformed roots from the same weight of inoculum (1.0 g f. wt.). The main axes of 'double' transformed roots were thicker with shorter lateral roots compared to 'single' transformed roots under all conditions of culture. 'Single' and 'double' transformed roots failed to regenerate shoots when transferred to medium with a range of growth regulators.

NPTII protein content, kanamycin resistance, *GUS* activity of DNA–DNA analysis of *A. rhizogenes*-induced roots

Significant differences were observed between 'single' transformed and 'double' transformed roots with respect to their NPTII protein content and kanamycin resistance (Table 1). NPTII protein was not detected in roots excised from non-transformed seedlings. 'Double' transformed roots exhibited intense indigo staining following the GUS histochemical assay, indicative of the expression of the *gus* gene carried by *A. tumefaciens* strain 1065 used in the initial transformation of lupin seedlings.

Genomic DNA from 'single' transformed roots cultured on MS0 medium with 40 mg l⁻¹ kanamycin and restricted with *Hind*III hybridised to the DIG-labelled *npt*II probe giving a fragment of approx. 10 kb in size (Figure 2). DNA from shoots transformed by *A. tumefaciens* strain 1065 used as targets to generate 'double' transformed roots, gave fragments of 4.8, 6.0 and 7.8 kb. 'Double' transformed roots also contained the *npt*II gene as a 1.6 kb *Eco*RI fragment. DNA from roots of non-transformed plants did not hybridise to the *npt*II gene probe.

Secondary product synthesis by lupin roots

Quinolizidine alkaloids were not detected by GC or GC-MS in cultured kanamycin resistant lupin

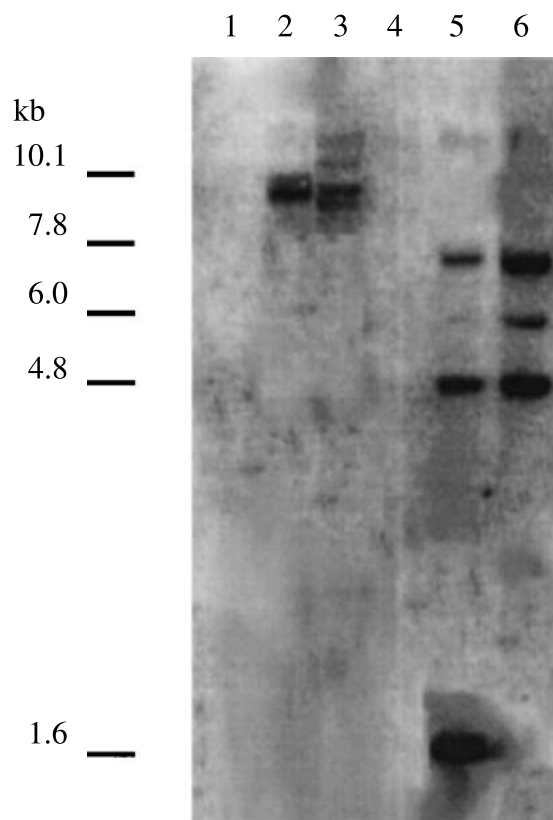


Figure 2. Hybridisation of DNA from transformed roots of *L. mutabilis* with a DIG-labelled *npt*II probe. Lanes 1, 4 – DNA from roots of non-transformed plants. Lanes 2, 3, respectively – DNA from roots transformed by *A. rhizogenes* strain R1601 and cultured on MS0 agar medium with 40 mg l⁻¹ kanamycin or without this antibiotic. Lanes 5, 6, respectively – DNA from a 'double' transformed root and from a shoot transformed by *A. tumefaciens* strain 1065 prior to inoculation with *A. rhizogenes* strain R1601.

roots or in roots excised from non-transformed seedlings.

HPLC analyses of transformed roots revealed that genistein was the dominant isoflavone in both 'single'

transformed and 'double' transformed roots, while genistin concentration was higher in roots from non-transformed seedlings (Figure 3; Table 2). Similarly, wighteone and 2'-hydroxygenistein were also 5–10-fold higher respectively, in transformed roots than non-transformed roots. Other isoflavones were not detected. In general, the total isoflavone concentration in transgenic roots was double that produced by roots of non-transformed plants.

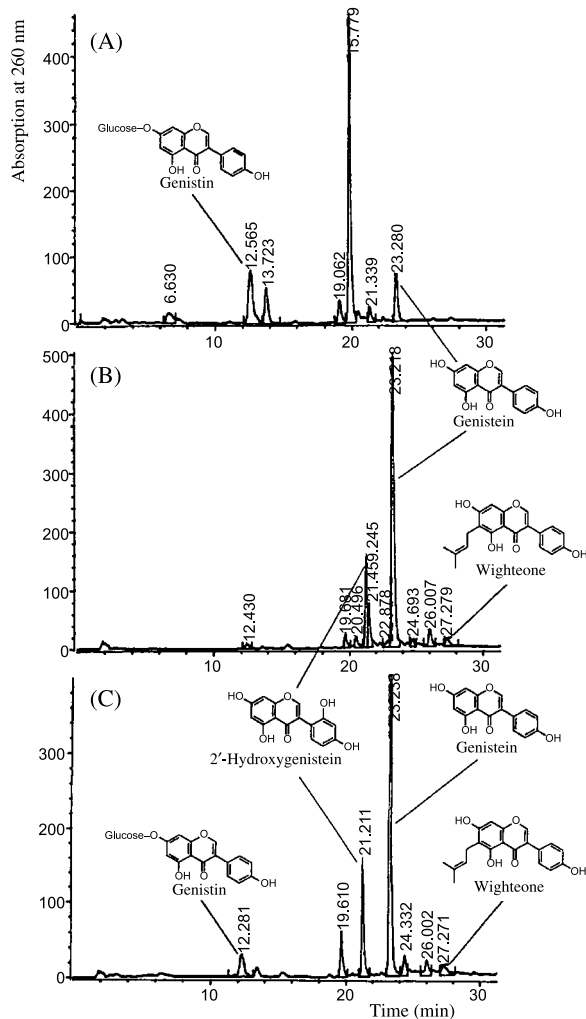


Figure 3. HPLC profiles of isoflavones produced by roots of non-transformed seedlings (A), 'double' transformed roots (B) and 'single' transformed roots (C). Detection was at 260 nm. Isoflavones which were unequivocally detected were genistin, genistein, 2'-hydroxygenistein and wighteone. The major peak in roots from non-transformed seedlings with a retention time of 19.7 min was not an isoflavone.

Table 2. Isoflavone concentrations in transgenic and non-transformed roots of *L. mutabilis* as determined by HPLC

Flavonoids	Non-transformed roots	'Double' transformed roots	'Single' transformed roots
Genistin	5.5	0.5	2.2
Genistein	2.2	14.0	10.7
2'-hydroxygenistein	0.4	3.1	3.8
Wighteone	0.1	0.6	0.8
Total of four isoflavones	8.2	18.3	17.5

Concentrations are mean values in mg g^{-1} dry weight of roots. All determinations were made at least twice. 'Single' transformed roots were assessed after 10 passages and 'double' transformed roots after 8 passages on MSO agar medium with 40 mg l^{-1} of kanamycin.

Discussion

The superiority of *A. rhizogenes* R1601 compared to other strains to induce transformed roots on host plants has been discussed previously (Rech et al., 1989; Joao and Brown, 1994), the supervirulence of strain R1601 being attributed to the presence *in trans* of pTVK291 containing part of the *vir* region of pTiBo4542. Indeed, attempts to induce transformed roots on *L. mutabilis* cv. Potosi by wild-type strains of *A. rhizogenes*, such as A4T (Moore et al., 1979), were unsuccessful (Babaoglu et al., unpublished data). Berlin et al. (1991a) indicated the responsiveness of hypocotyl and stem explants of *L. polyphyllus* and *L. hartwegii* to oncogenic strains of *A. rhizogenes*. Similar explants were also more responsive, in the present investigation with *L. mutabilis*, than explants of leaves, petioles and roots. Hypocotyl and stem explants showed an even better response when the shoot apex was attached. This may have been due to the contribution of these tissues to the inoculation sites through the provision of assimilates or polarly transported endogenous growth regulators, such as indole acetic acid.

Hybridisation of genomic DNA to the *nptII* probe confirmed the transgenic nature of roots induced by *A. rhizogenes* strain R1601. A 1.6 kb *EcoRI* fragment of the *nptII* gene confirmed the second, independent integration by strain R1601 of the *nptII* gene into transgenic roots induced on stem explants of *L. mutabilis* already carrying the *nptII* and *gus* genes through earlier transformation by *A. tumefaciens* strain 1065. This fragment, related to the second transformation, was of the expected size, since in *A. rhizo-*

genes R1601, the *nptII* gene is on a 1.6 kb *EcoRI* fragment in the TL-DNA (Taylor et al., 1985; Pythoud et al., 1987). 'Double' transformed roots had a lower concentration of NPTII protein and were less tolerant of kanamycin in the medium, possibly through a reduction in gene expression following insertion of the extra copy of the *nptII* gene.

Previous biochemical analyses have demonstrated that quinolizidine alkaloids are typical secondary metabolites of lupins which, although accumulated in all parts of the plant (Wink, 1993), are synthesised in aerial chlorophyllous tissues and transported via the phloem to other organs. Lupin roots do not produce alkaloids (Wink, 1987), as confirmed in the present investigation by GC and GC-MS analyses. However, Baumel et al. (1995) came to a different conclusion after studying alkaloids in phloem and xylem sap of lupin. They detected small concentrations of alkaloids in the xylem, which led them to believe that the roots must be the site of alkaloid formation. The present data, together with previous results (Wink, 1987, 1993), contradict their conclusions and confirm that roots are not the site of quinolizidine alkaloid formation in lupin.

Joao and Brown (1994) emphasised that long-term stability of transformed roots is of commercial importance, as in the synthesis of recombinant proteins which may have pharmaceutical and agrochemical potential. Among such compounds, genistein (C₁₅H₁₀O₅; 5,7,4'-trihydroxyisoflavone, m. wt. 270.2), an isoflavone secreted by roots of many large seeded legumes, including lupins, has antimicrobial and fungistatic activities, besides serving as a signalling compound that stimulates *nod*-genes in the initiation of the legume–*Rhizobium* symbiosis. Genistin is the glycoside form of genistein, with the former compound predominating in non-transformed plants. Genistein has anti-cancer and phyto-oestrogenic activities, based on the inhibition of tyrosine kinases and/or inhibition of DNA topoisomerase II (Alhasan et al., 2001; Win et al., 2002). Analyses showed that genistein and 2'-hydroxygenistein were the predominant compounds in transformed roots of *L. mutabilis*, indicating that expression of bacterial genes on the T-DNA of the Ri plasmid, particularly those involved in the synthesis of growth regulators in transformed plant cells, may also modify secondary product biosynthesis. Other examples of the modification of secondary product biosynthesis, following transformation by *A. rhizogenes*, have been reported in *Trigonella foenum-graecum* (Merkli et al., 1997),

Nicotiana tabacum and *Zea mays* (Yu et al., 2000) and *Arabidopsis thaliana* (Jung et al., 2000).

Kneer et al. (1999) proposed that hydroponically grown roots of *L. luteus* (yellow lupin) can be an excellent source for the secretion of isoflavones for commercial production and demonstrated that the rhizosecretion of genistein was stimulated by treatment of the roots with soluble chitosan, salicylic acid or potassium cyanide. Most of the elicitor-induced secretion occurred during the first day of treatment, followed by a gradual decline. However, more information is still needed on product secretion in relation to elicitors and growth conditions. The production of isoflavones in plants, such as soybean, was reported to depend on the genotype and environmental factors (Hoeck et al., 2000). The concentrations of genistein and genistin, as determined by HPLC, in soybean and soybean products, were in the range of 4.6–18.2 and 200.6–968.1 µg g⁻¹ (Fukutake et al., 1996), respectively, much lower than recorded here for *L. mutabilis*. Hence, cultured transformed roots of lupin, as established in the present investigation, can be an excellent source for the continuous production of genistein and other isoflavones over long periods without loss of growth rates, with more than 10–15 mg being synthesised per gram of dry roots.

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