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Transformation and regeneration of the legume *Lotus corniculatus*: A system for molecular studies of symbiotic nitrogen fixation

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Summary. A procedure for transformation and regeneration of the legume species *Lotus corniculatus* (Bird's-foot trefoil) has been developed. The *Agrobacterium rhizogenes* 15834 and 8196 strains were used to transform plant cells in wound site infections and transformed roots were propagated in vitro. Transformation was monitored by hybridization with pRi T-DNA sequences and by detection of agropine and mannopine. Transformation frequencies of up to 90% were obtained. Shoots spontaneously formed on hairy root cultures were excised, rooted and inoculated with *Rhizobium*. Root nodules formed on transformed plants had nitrogenase activities comparable to untransformed nodules. Transcript levels from the nodule-specific leghemoglobin genes and the constitutive ubiquitin genes were similar in transformed and untransformed root nodules. Transformed plants responded to *R. loti* and *Bradyrhizobium* sp. (*Lotus*) strains with phenotypes identical to phenotypes for untransformed plants.

Key words: *Agrobacterium rhizogenes* – Plant transformation – Transgenic legumes – Nitrogen fixation

Introduction

Transformation and regeneration systems are available for a number of plant species including tobacco, petunia, tomato and potato (Krens et al. 1982; De Block et al. 1984; Horsch et al. 1985; Ooms et al. 1985). The most intensively used transformation system is the *Agrobacterium tumefaciens* T-DNA system, but transformation with naked DNA has also been reported (Paszkowski et al. 1984; Caboche and Deshayes 1984; Hain et al. 1985). Transformed plants with the *A. rhizogenes* pRi T-DNA stably integrated have been obtained with carrot, *Convolvulus saepium*, potato and tobacco (Chilton et al. 1982; David et al. 1984; Tepfer 1984; Petit et al. 1986). Single plant genes have been reinserted into plant genomes using *A. tumefaciens* T-DNA derived vectors (Herrera-Estrella et al. 1983; Fraley et al. 1983; Bevan 1984; De Block et al. 1984) and correct expression patterns were observed such that studies of the regulatory mechanisms involved in plant gene expression could be initiated (Herrera-Estrella et al. 1984; Broglie et al. 1984). DNA sequences required for promoter activity have been defined for the light-induced ribulose-1,5-biphosphate carboxylase small subunit gene *rbc*S-E9 (Morelli et al. 1985), the pea chlorophyll a/b binding protein gene (Simpson et al. 1985) and the chalcone synthase gene (Kaulen et al. 1986). Enhancer-like sequences are present in the 5′ regulatory regions of the ribulose-1,5-bisphosphate-carboxylase small subunit gene *rbc*Sss 3.6 (Timko et al. 1985) and the chalcone synthase gene (Kaulen et al. 1986). These studies take advantage of the well characterized tobacco and petunia transformation systems. A major obstacle in the study of the molecular genetics of symbiotic nitrogen fixation is the lack of suitable transformation and regeneration systems for legumes. Various species including alfalfa, white and red clover, and *Lotus* have been regenerated after tissue culture (Flick et al. 1983) but so far regeneration of genetically engineered legumes has only been described for *Medicago varia* (Deak et al. 1986).

We have used *A. rhizogenes* to establish a simple, reliable transformation and regeneration system for the legume *Lotus corniculatus*. Transformed plants responded to inoculation with *R. loti* and *Bradyrhizobium* sp. (*Lotus*) strains with phenotypes comparable to untransformed plants. We have investigated whether root nodules formed on transformed roots are functionally equivalent to nodules on untransformed plants. Both plant and bacterial encoded symbiotic functions were tested and appeared to be undisturbed in transformed plant tissue, as also recently demonstrated by Stougaard et al. (1986). The accompanying paper describes vectors developed for this plant transformation system (Stougaard et al. 1987).

Materials and methods

Bacterial strains. Two *Agrobacterium rhizogenes* strains were used. The mannopine-type 8196 wild type originating from J.A. Lippincott, Dept. of Biological Sciences, Northwestern University, Ill 60201 and the agropine-type C58C1 rif^r pRi 15834 strain from J. Schell, Max-Planck Institut für Züchtungsforschung, D-5000 Cologne.

Wound site infections. Surface sterilized seeds, soaked 15 min in 2% NaClO, rinsed in sterile water were germinated and grown on 1.2% agar plates. The medium consisted of Monier's salts (1976) plus vitamins of Morel and Wetmore (1951), both in half concentration. Ten-to twelve-day-old plantlets were wounded on the hypocotyls using a needle or scalpel dipped in *A. rhizogenes*. Bacteria were

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taken directly from fresh colonies grown on LA plates at 30° C for 2 days. Plants were left two weeks in sealed petri dishes for roots to develop. Growth conditions were 26° C, 70% humidity, continuous light. Seeds of *L. corniculatus* "Rodéo" were kindly supplied by Cooperative Agricole Mathieu, Sainte-Christie, 32390 Montestruc/Gers, France.

In vitro culture of roots. Stem fragments with hairy roots were excised and transferred to agar plates containing Mo/2 medium (salts and vitamins as above plus sucrose 15 g/l) containing the antibiotic claforan 500 µg/ml. Root cultures were established 8–10 days later on new Mo/2 claforan 500 µg/ml plates from individual fast-growing roots. Growth conditions were as above but in the dark.

Regeneration. Well developed root cultures were subcultured on Mo/2 and transferred to continuous light. Three-to four-cm-long shoots arising from spontaneous organogenesis were subcultured on Mo/2 to form roots. Rooted plants were transferred to pots containing a mixture of vermiculite and "leca", covered with plastic bags and moved to growth cabinets. Plants were watered with B & D+2 mM KNO_3 (Broughton and Dilworth 1971) for 2 weeks, then only with nitrogen-free B & D. *Rhizobium loti* NZP2037 was inoculated 2 days after transfer. Plastic bags were removed stepwise after 1–2 weeks culture. Conditions in growth cabinets were: 22° C, 70% humidity, day-night cycle of 16/8 hours.

Opine analysis. High voltage paper electrophoresis was performed as described (Petit et al. 1983), except that extracts were prepared by boiling samples in water rather than in 1% HCl and concentrated by evaporation in a vacuum centrifuge.

RNA analyses. Extraction and Northern blot analysis was as described by Marcker et al. (1984).

Test of Bradyrhizobium *sp.* (Lotus) *strains and* R. loti. Sterile rooted plants were transferred to sterile growth jars containing B & D+1 mM KNO_3. Plants were inoculated with the various strains and covered with plastic bags. Bags were removed after 1–2 weeks. Control plants were started from surface sterilized seeds. Nodules were harvested after 4–5 weeks. Acetylene reduction assays and chloramphenicol acetyl transferase assays were as described by Stougaard et al. (1986).

Results

Transformation-regeneration

Legumes are generally susceptible to *Agrobacterium*, and *A. rhizogenes* transformed tissue can be obtained from e.g. soybean, pea, and alfalfa (unpublished data). *Lotus corniculatus* develops roots from wound sites after *A. rhizogenes* infections on hypocotyls (Petit et al. 1986). Roots did not develop from uninfected wounds or wounds infected with the disarmed *A. tumefaciens* 3850 strain (Zambryski et al. 1983). Transformed roots were cultivated in vitro (Petit et al. 1986) and root cultures, some with a "hairy root" appearance were established (Fig. 1a). Roots were freed from *A. rhizogenes* by including antibiotics (claforan) in growth media. Organogenesis occurred spontaneously on *L. corniculatus* hairy root cultures as illustrated in Figs. 1 and 2. The frequency of bud formation was enhanced by light. Shoots 4- to 5-cm-long were excised and transferred to culture tubes containing the same medium for rooting. Rooted plants were moved to pots and transferred into growth cabinets. The transformation and regeneration protocol is outlined in Table 1. Regenerated plants were inoculated with *Rhizobium loti* and root nodules developed approximately 3 weeks later. The time required for a transformation-regeneration-nodulation cycle was approximately 5 months.

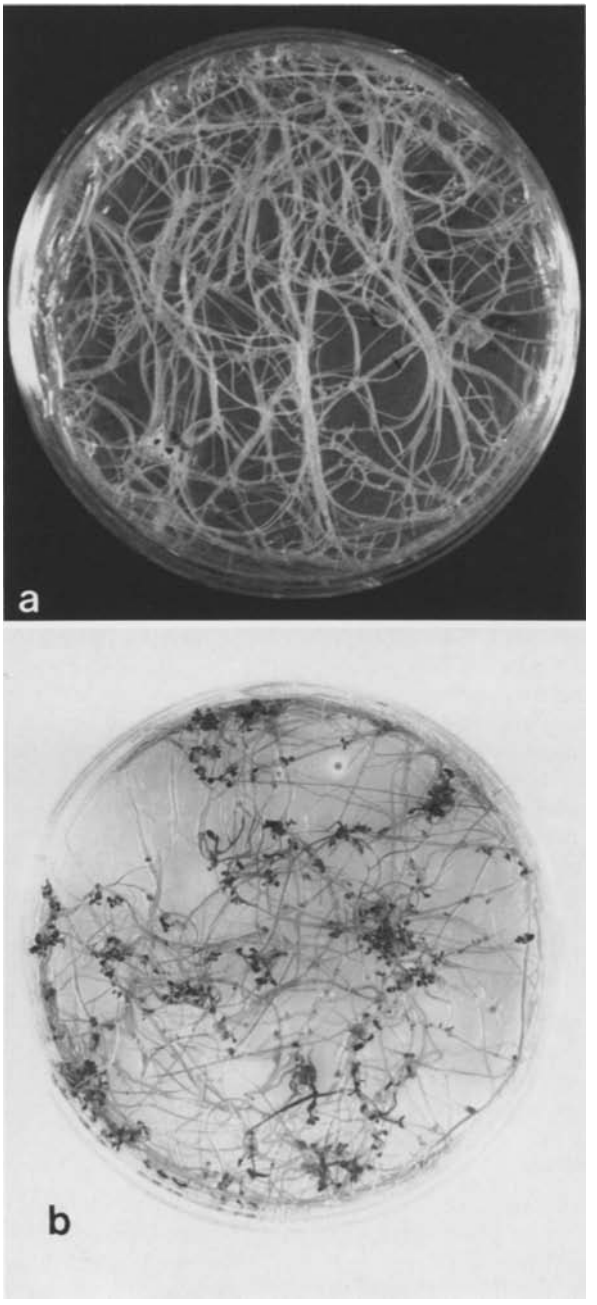

Fig. 1a, b. Stages in regeneration of *L. corniculatus* from transformed roots. **a** Root culture; **b** root culture with "green spots" and shoots emerging after transfer into light

Transformation frequencies

Regenerated plants were tested for transformation by both opine analysis and Southern blot analysis. Figure 3 shows

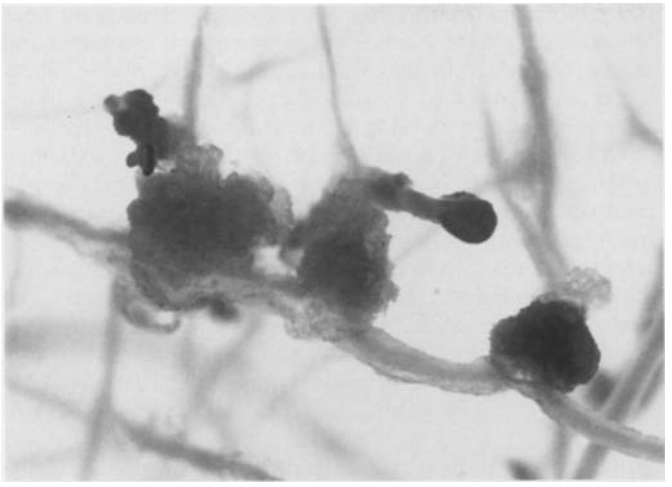

Fig. 2. Organogenic structures appearing on transformed root cultures. The picture shows shoots developing from small calli; however organogenesis more directly from roots is also seen

Table 1. Transformation, regeneration and nodulation scheme for *L. corniculatus*

Surface sterilised seeds placed on Mo/2	
	8–10 days light
Wound site infection of *A. rhizogenes* on hypocotyls	
	14 days light
Stem pieces with developed "hairy roots" transferred to Mo/2 + claforan	
	8–10 days dark
Individual roots excised from pieces of stems moved to Mo/2 + claforan	
	20–30 days dark
Root culture subdivided on new Mo/2 moved into light	
	20–30 days light
Organogenesis, shoot elongation	
	20–30 days light
Rooting of excised shoots	
	20–30 days light
Plant transferred to pots, moved to growth cabinets and inoculated with *Rhizobium*	
Transformed plants with nodules	

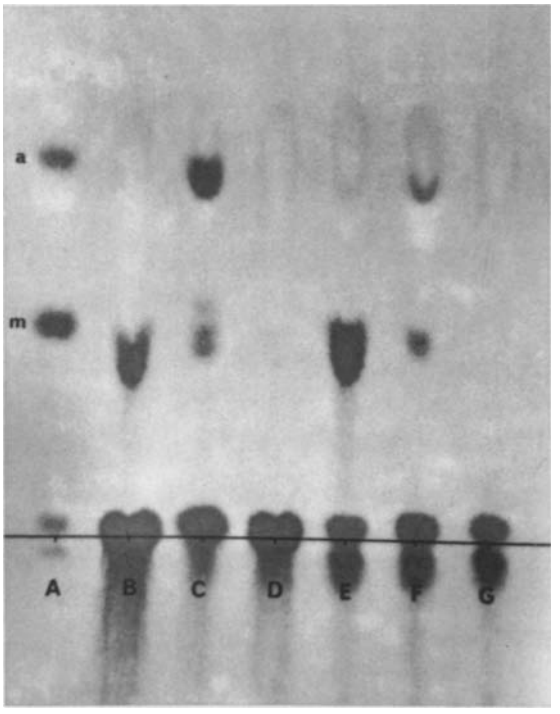

Fig. 3. High voltage paper electrophoresis of opines from *A. rhizogenes* transformed *L. corniculatus* root cultures and leaves. Lane A, standard containing agropine (a) and mannopine (m). Lanes B and C, extracts from in vitro grown root cultures; lanes E and F, extracts from leaves of transformed plants; lanes B and E, 8196 transformed; lanes C and F, 15834 transformed. Lanes D and G, extracts from roots or leaves of untransformed control plants. The electrophoresis buffer was formic acid/acetic acid/water (30/60/910, v/v/v), and staining was with silver nitrate. The amounts of extracts spotted corresponded to approximately 5 mg of fresh material

the agropine and mannopine synthesized in plants transformed with the 15834 and 8196 pRi T-DNA. Other independent lines were analysed directly for pRi T-DNA. In the analyses of more than 30 lines approximately 90% were transformed (Stougaard et al. 1987).

Analysis of root nodules

The pRi T-DNA region confers the hairy-root phenotype to transformed roots and plants. To test whether the presence of T-DNA in transformed plants would perturb processes involved in symbiotic nitrogen fixation, bacterial and plant functions were assayed. First the expression of endogenous *Lotus* leghemoglobin genes was determined in transformed and untransformed root nodules. The cDNA for the soybean Lba gene was used to probe the steady-state level of leghemoglobin mRNA in 4-week-old nodules. Total RNA was extracted and subjected to Northern analysis. Figure 4a shows the steady-state level of Lb transcripts in nodules. A cDNA probe for the human ubiquitin (Wiborg et al. 1985), a histone associated protein, was used to assay transcripts from constitutively expressed genes. Figure 4b shows the steady-state levels of transcripts in roots and root nodules. No major changes in steady-state levels of Lb or ubiquitin mRNA was detected. The presence of T-DNA in transformed plants, roots and root nodules does not affect the transcription rate or half lives of mRNA present. If these parameters are changed it appears to be in a way that does not disturb steady-states levels of transcripts. Enzymatic activity from the rhizobial encoded nitrogenase enzyme was also determined. Similar levels of nitrogenase activity, measured as acetylene reduction, were found in nodules from untransformed and transformed plants (see Table 2). Correct developmental induction of leghemoglobin and nitrogenase in root nodules of transformed plants has been demonstrated previously (Stougaard et al. 1986).

Response of transformed plants towards R. loti *and* Bradyrhizobium *sp.* (Lotus)

Two different species of *Rhizobiceae* can nodulate *L. corniculatus*. The fast growing *R. loti* nodulates *L. corniculatus*

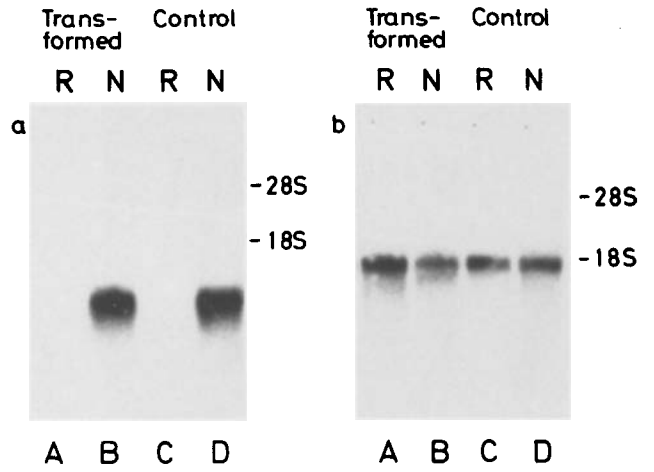

Fig. 4a, b. Steady state levels of leghemoglobin and ubiquitin mRNAs in transformed and untransformed *L. corniculatus*. Total RNA (5 μg) was analysed in Northern blots. **a** Probed with the soybean Lba cDNA; **b** probed with the cDNA for ubiquitin. Lane A, RNA from transformed roots (R); lane B, transformed nodules (N); lane C, untransformed control roots; lane D, untransformed control nodules

Table 2. Symbiotic effectiveness of *R. loti* NZP2037 and *Bradyrhizobium* sp. (*Lotus*) strains NZP2076, NZP2243, NZP2309, NZP2257, NZP2183 on transformed and untransformed *Lotus corniculatus* plants

Strain	Phenotype	Acetylene reduction nmol ethylene/μg protein per hour		CAT activity Cpm/μg protein per hour
		transformed	untransformed	
NZP2037	wild-type E fast	3.5	3.1	185070
NZP2076	I slow	1.4	1.7	89596
NZP2243	I slow	0.8	0.4	239237
NZP2309	I, PE slow	3.2	1.9	115391
NZP2257	PE slow	3.4	2.2	181175
NZP2183	PE(E) slow	4.1	3.0	344402

E, effective; I, ineffective; PE, partly effective; fast, fast growing strain; slow, slow growing strain
Nitrogenase activity measured as acetylene reduction was used to determine the effectiveness of root nodules

effectively but most strains develop ineffective nodules on *Lotus pedunculatus* plants. Slow growing *Bradyrhizobium* sp. (*Lotus*) strains have the reverse relationship to the two host species (Jarvis et al. 1982). To determine whether these two *Rhizobium* species would interact in a similar way with transformed *L. corniculatus* plants, *Bradyrhizobium* sp. strains NZP2076, NZP2243, NZP2309, NZP2257, NZP2183 (Bailey et al. 1971) and the *R. loti* strain NZP2037 were inoculated onto two different transformed plant lines and control plants. Nodule development was evaluated and acetylene reduction measured (Table 2). No major differences were observed in size, shape, numbers, or colours between nodules developed on transformed or untransformed plants. All strains tested formed red or red/white nodules. The level of nitrogenase activity for untransformed and transformed nodules was also comparable on plants nodulated with the same strain. Plants were similarly green and well developed in effective symbiotic associations while pale and underdeveloped in ineffective associations. The transgenic plant lines used were transformed with the chimeric soybean Lbc_3 *5′3′-CAT* gene consisting of the chloramphenicol acetyltransferase coding sequence flanked by the 5′ and 3′ soybean Lbc_3 regions (Stougaard et al. 1986). CAT activity from the chimeric Lbc_3 *5′3′-CAT* gene was found in all nodules tested from transformed plants. All bacterial strains tested seemed to induce comparable levels of CAT activity. As untransformed nodules do not have any CAT activity no quantitative comparison could be made.

Insertion site effects on expression levels

Chloramphenicol acetyl-transferase activity from the Lbc_3 *5′3′-CAT* gene was determined in effective root nodules of ten independent transgenic *Lotus corniculatus* lines and the variation in CAT activity was about sixfold. The minimal copy number for the CAT gene was determined in the same lines and accounts for approximately threefold differences. Integration site effects therefore seems to influence expression levels.

Discussion

We describe here an *A. rhizogenes* derived transformation system characterized by high transformation frequencies, easy identification and propagation of transformed tissue, without the use of selectable markers. A further characteristic is a high plant regeneration frequency and a relatively rapid transformation-regeneration cycle. Regeneration and subsequent nodulation of transformed *L. corniculatus* plants allowed the organ-specific expression of the Lbc_3 *5′3′-CAT* gene in root nodules (Stougaard et al. 1986). *Agrobacterium rhizogenes* mediated transformation of *Lotus* is therefore useful for the study of the molecular genetics of symbiotic nitrogen fixation. We have further tested the fidelity of the system. Both plant and bacterial functions are unperturbed in nodules of transformed plants. Transcripts from the endogenous Lb genes were present in similar amounts in transformed and untransformed nodules. Comparable steady-state levels of the mRNA from the constitutively expressed ubiquitin genes are found in nodules and roots from transformed or untransformed plants, respectively.

Several *Bradyrhizobium* sp. (*Lotus*) strains and a *R. loti* strain were tested on transformed plants. No major differences in nodule morphology, nodulation pattern or level of nitrogenase activity were found between transformed and untransformed plants inoculated with the same strain. Known strain differences were thus maintained and the interaction between the microsymbiont and transformed plants is therefore not altered in any obvious way in transformed tissue.

Expression of foreign genes was obtained with the chimeric Lbc_3 *5′3′-CAT* gene. The variation in expression levels measured as CAT activity in root nodules of ten independent lines of *L. corniculatus* was sixfold. Jones et al. (1985) reported variation in transcription rates of up to 200-fold after a pTi transformation. The difference could be explained as modulation of expression at the post-transcriptional level. Plant roots that are slow growing due to T-DNA integration site effects might also be lost under in vitro culture or might not develop from the transformed plant cells in the wound sites.

Although hairy-root plants have a characteristic phenotype, which is more or less marked depending on the particular hairy-root line from which they have been regenerated, the presence of T-DNA in engineered plants does not seem to interfere with expression of the Lbc_3 *5′3′-CAT* gene. Furthermore the hairy-root T-DNA and foreign gene(s) transferred as unlinked sequences (Petit et al. 1986; Stougaard et al. 1987) could easily be segregated in the sexual progeny of hairy-root transformants. Thus, the *Lotus corniculatus-A. rhizogenes* genetic engineering strategy provides an efficient means of studying expression of plant genes involved in symbiotic nitrogen fixation.

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