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Transformation of *Medicago arborea* L. with an *Agrobacterium rhizogenes* binary vector carrying the hygromycin resistance gene

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Abstract: Plants of Medicago arborea have been infected with Agrobacterium rhizogenes strain LBA9402 harbouring the plasmids Ri 1855 and AGS125 carrying a gene conferring resistance to the antibiotic hygromycin. About 70% of the hairy roots showed callus formation on hygromycin-supplemented medium. Regeneration took place on antibiotic free medium only. Plantlets suitable for transfer to soil were obtained after the manual removal of most of the leaves. Plant morphology showed the usual alterations induced by the Ri plasmid; moreover, two years after soil-transfer, transformants have not flowered. Molecular analysis indicates the presence of T-DNA from both pAGS125 and p1855. The expression of the hygromycin phosphotransferase gene allowed callus and protoplasts of transformed plants to grow on media supplemented with the antibiotic. This trait will be utilized as a marker in protoplast fusion between Medicago arborea and Medicago sativa (alfalfa).

Key words: Transformation, Agrobacterium rhizogenes, forage legumes.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyaceticacid; kin, kinetin; GA3, Gibberellic acid; IAA, Indole-3-acetic acid; HPT, hygromycin phosphotransferase; NOS, nopaline synthase; MS, Murashige and Skoog (1962); B5, Gamborg et al. (1968); B5hy, B5 supplemented with 20 mg l-1 of hygromycin; YMB, yeast mannitol broth.

Introduction

Plant transformation has been successfully attempted in many species (Fraley 1989). The most efficient transformation technique utilizes the natural ability of Agrobacterium to introduce alien DNA into plants (White 1989). The two species of Agrobacterium normally used for this purpose have different characteristics: A. tumefaciens, the aetiological agent of crown gall disease, must be deleted of the tumor genes which prevent plant regeneration and produce chimaeric tissues of both transformed and untransformed cells (Yamada et al. 1985); A.rhizogenes, on the other hand produces hairy roots composed only of transformed cells (Chilton et al. 1982) but confers a modified phenotype to transformed plants (Tepfer 1984).

Experimentation on the production of transgenic plants is still limited to those species where genotypes able to regenerate have been identified, and even in these cases the efficiency of transformation can vary from species to species. For these reasons, preliminary experiments with the aim of establishing optimal conditions for transformation in a given species are recommended.

Medicago arborea, the oldest species of the genus Medicago, is an evergreen shrub used as pasture in dry lands (Corleto et al. 1980). The conditions for protoplast isolation and culture and plant regeneration have already been reported (Mariotti et al. 1984), and experiments devoted to obtaining somatic hybrids by protoplast fusion of M.sativa + M.arborea are in progress (Damiani et al. 1988; Pupilli et al. 1991).

The aim of this work was the introgression of an antibiotic resistance gene through A.rhizogenes-mediated transformation in order to identify the optimal method for genetic transformation, as well as to introduce a marker gene for in vitro selection of hybrid cells produced by protoplast fusion.

Materials and Methods

Plant material and Agrobacterium spp.

Seeds were harvested from a mesophyll protoplast-derived plant of M.arborea (Mariotti et al. 1984) and were surface sterilized with a mixture of 0.1 % (w/v) mercuric chloride plus 0.1 % (w/v) sodium lauryl sulphate (20 min), followed by 20 % (v/v) "Linda clor" (commercial bleach containing 6% sodium hypochlorite) for 20 min, rinsed 5 times with sterile distilled water and placed on hormone free MS medium (Murashige and Skoog 1962) with 0.8% agar. Seeds and plantlets were incubated at 23-24°C under fluorescent light (27 μmol m^{-2} s^{-1}, 12h photoperiod) and subcultured every 4 weeks.

The Agrobacterium rhizogenes strain LBA9402 (rinfampicin^R) was a gift of J. Hamill (Hamill et al. 1987), it harbours the wild type plasmid Ri1855 and the plasmid AGS125 (kanamycin^R) carrying the HPT (hygromycin phosphotransferase) gene under a NOS promoter (van den Elzen et al. 1985). Bacterial cultures were grown (28°C) in YMB medium (Hooykaas et al. 1977) supplemented with 50 mg l^{-1} kanamycin sulphate.

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Infection with Agrobacterium and plant regeneration

Two-day-old cultures of A.rhizogenes were centrifuged at 7000 x g for 30 min and the pellet was resuspended in the same medium to about 10^8cells ml^{-1}. The resultant suspension was directly injected, using a hypodermic syringe, into stems of 2-month-old plants (Spanò et al. 1987). After one month, adventitious hairy roots (5 mm long) developing from the infection point were excised and callus formation was induced on agar-solidified B5 medium (Gamborg et al. 1968) supplemented with 2mg l^{-1} 2,4-D, 0.1 mg l^{-1} kin, 20 mg l^{-1} hygromycin and 1 g l^{-1} carbenicillin.

Single root tip derived calluses were subcultured every week and 3-month-old callus regenerated shoots one month after transfer to agar solidified MS medium without hormones and antibiotics. Shoots (2-3 cm long) were subcultured in the same medium and formed roots after 2-3 weeks. Stem elongation was stimulated by removing most of the lateral leaves, and 8 weeks after shoot appearance plantlets were transferred into pots and maintained for two weeks in a growth cabinet (20±1°C, 216 μmol $m^{-2}sec^{-1}$, 12h photoperiod, 80% relative humidity) prior to placement in a greenhouse.

Expression assay

Six putative transformed and two seed derived (untransformed control) plants were compared for hygromycin resistance of leaf derived callus. Leaves from 3-month-old plants were surface sterilized with 10% (v/v) "Linda clor" for 10 min, rinsed with 5 changes of sterile distilled water and used for callus induction and protoplast isolation. Leaves were transversely dissected into three pieces and the middle portion (about 40 mm^2) was incubated either on B5 solid medium supplemented with 2mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin and 20 mg l^{-1} hygromycin (B5hy) or on the same medium without antibiotic. For each plant and callus induction medium, 30 explants (6 per petri dish) were used. Subculture was performed every two weeks and callus growth was evaluated by weighing after one month of culture; data were submitted to analysis of variance.

Two putative transformed plants and a 7-month-old untransformed plant were used for protoplast isolation as reported by Pupilli et al. (1991). Protoplasts were cultured (28 °C, dark) in KM8P (Kao and Michayluk 1975) semisolid medium (0.5% "sea plaque" F.M.C. Marine Colloids Divisions, Rockland, ME 04841, USA) at a density of 1.10^5 protoplasts ml^{-1} (4 dishes per plant, 4 ml each). Two weeks later, the agarose bed of each petri dish was cut into slices 5 mm wide and half of them were transferred to another dish. The slices were cultured in a mixture of KM8P and KM8 (Kao and Michayluk 1975) cell culture medium with or without 20 mg l^{-1} of hygromycin at a final density of 5.10^4 plated protoplasts ml^{-1}. The liquid medium was replaced weekly by increasing the proportion of KM8. After 6 weeks of culture the percentage of viable minicolonies (growing minicolonies/total minicolonies plus aggregates of 10-30 cells*100) was recorded. For each petri dish, 10 optical fields (original magnification 100x) were scored and the experiment was repeated twice.

Extraction of DNA and Southern analysis

Plant DNA was extracted from leaves as described by Della Porta et al. (1983), purified by CsCl gradient centrifugation (60,000xg, 13h), digested with restriction endonucleases according to the manufacturer's recommendations, electrophoresed on a 1% (w/v) agarose gel, transferred to nitrocellulose filters and hybridized (Maniatis et al. 1982) with nick translated probes (^{32}P labelled to a specific activity of 1.10^8 cpm μg^{-1}DNA). The plasmids used as probes were isolated by alkaline lysis (Maniatis et al. 1982) and purified by CsCl gradient centrifugation (60,000 x g). The probe for the HPT gene was isolated from pAGS125 as the EcoRI-BamHI fragment encompassing part of the gene (van den Elzen et al. 1985) using the DEAE technique (Maniatis et al. 1982). To probe for T_L-DNA and T_R-DNA of pRi1855, the entire plasmids MP66 and MP17 (Pomponi et al. 1983) were used.

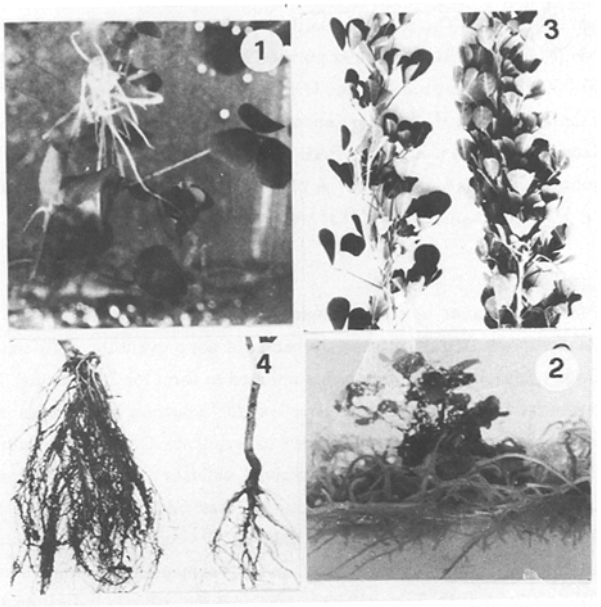
Results

A few weeks after inoculation hairy roots start to proliferate from the infection point (Fig.1). Rootlet excision did not prevent the proliferation of other adventitious roots which continued to form for 2-3 months. The concentration of hygromycin effective for selection of calluses was empirically established in preliminary experiments. On media containing 20 mg l^{-1} of hygromycin, leaf derived calluses from untransformed plants remained pale yellow, did not proliferate and died.

In 5 independent experiments, a total of 72 rootlets were individually cultured for callus induction in media containing 20 mg l^{-1} of hygromycin and 48 formed friable fast growing calluses indicating that about 70% of the hairy roots were cotransformed with the hygromycin resistance gene (HPT) from pAGS125. Two-month-old resistant calluses were transferred onto regeneration medium containing the antibiotic and since no signs of regeneration were observed after 4 weeks, they were subcultured in the same medium devoid of hygromycin. In this case shoots appeared after two weeks.

Shoots isolated from callus formed an abundant and highly branched root apparatus (Fig. 2) developping near the surface of the agar. This feature has also been observed in M.sativa plants transformed with A.rhizogenes (Spanò et al. 1987; Sukhapinda et al. 1987) and is typical of weak apical dominance both in shoots and roots. In fact, some difficulties arose for stem elongation because regenerants were characterized by very short internodes and by leaves proliferating on the top of a short axis. The addition to culture media of IAA and GA3, both separately and in combination, was unsuccessful and positive results were achieved only after manual removal of all but 2-3 leaves. After this treatment, in fact, shoots started to elongate and after 3 weeks could be transferred to soil. A total of 16 plants were grown in the greenhouse. Transformed plants showed a phenotype of moderately wrinkled leaves during the early plant stages (10-20 cm tall). Internode length was reduced and consequently the number of leaves per stem significantly increased (Fig. 3). No height difference was observed between transformants and control plants. As previously observed in M.sativa (Spanò et al. 1987), transformation completely changed the structure of the root system, which was tap-root shaped in the control and fasciculated in the transformants (Fig.4); a characteristic found in annual Medicago species growing in semiarid regions.

All putative transformed plants were grown to maturity but none flowered in the first and second years after regeneration. On the contrary, plants regenerated from leaf induced calli and maintained under the same environmental conditions flowered and set seed. This feature is not unusual and in forage legumes the expression of pRi derived T-DNA not only produces alterations at the morphological level but also interferes with the plant cycle. Some perennial species become annual and die after setting seed (M.sativa and L.corniculatus), while others such as M.arborea and L.tenuis retain their perennial nature but lose or delay the capacity to flower (unpublished data).



Figures 1-4

(1) Hairy roots induced by the infection of aseptically grown plantlets of M.arborea with A.rhizogenes, four weeks after infection.

(2) A transgenic shoot showing an abundant highly branched plagiotropic root apparatus.

(3) Stems of transformed (right) and untransformed (left) plants.

(4) Root apparatuses of transformed (left) and untransformed (right) plants.

Hygromycin resistance assay

Expression of the HPT gene in putative transformed plants was evaluated by callus and protoplast growth in the presence of hygromycin. In order to rule out the possibility of hidden

Table 1. Growth (mg fresh weight) of leaf induced callus and percentage of mesophyll protoplast derived minicolonies from putative transgenic (n° 3,5,8,9,10) and untransformed (C1,C2) plants on media supplemented with 20 mg l^{-1} of hygromycin (B5hy, KM8Phy) and on antibiotic free media (B5, KM8P).

	culture media			
Plant	callus growth		protoplasts	
tested	B5	B5hy	KM8P	KM8Phy
3	180bcd	137bc	-	-
5	168cd	140bc	-	-
8	169cd	128bc	38f	27f
9	130bc	121b	31f	24f
10	125b	117b	-	-
C1	140bc	38a	34f	3e
C2	132b	32a	-	-

values followed by the same letter do not differ per $P \leq 0.05$

contamination, leaves of transformed plants were surface sterilized, cut in small pieces (1-3 mm) and incubated in liquid and solid (0.8% agar) YMB medium at 28°C. No bacterial growth was observed after several days of culture. Table 1 reports the growth of leaf derived callus from putative transformed plants and untransformed controls evaluated on media with or without hygromycin. All plants regenerated from resistant calluses showed the same callus growth rate both in B5 and B5hy culture media while the control calluses grew only on the antibiotic free medium.

Similar results were observed in protoplast culture. Protoplasts isolated from putative transformants showed nearly the same plating efficiency in media with and without hygromycin, while protoplasts derived from untransformed control plants exhibited a very low plating efficiency in the medium containing antibiotic (Table 1).

Southern analysis

Southern blot analysis (Fig.5) was performed to determine if regenerated plants were transgenic. Total DNA of two transformed and one untransformed plant was digested with EcoRI and BamHI in order to release the internal fragment of 2.5 kbp from the transferred DNA. The HPT probe did not hybridize with DNA of the control plant (lane 2), but did with DNA of the transformed plants (lanes 3 and 4). DNA of the two transgenic plants was also digested with EcoRI, which cuts only once inside the HPT gene, at one side of the probe. The other EcoRI site observed must therefore come from the M.arborea genome. The presence of different hybridization patterns (lanes 5,6), indicates a random integration of the HPT gene. The appearance of a single band per plant indicates the insertion of the foreign DNA at only one site for each plant.

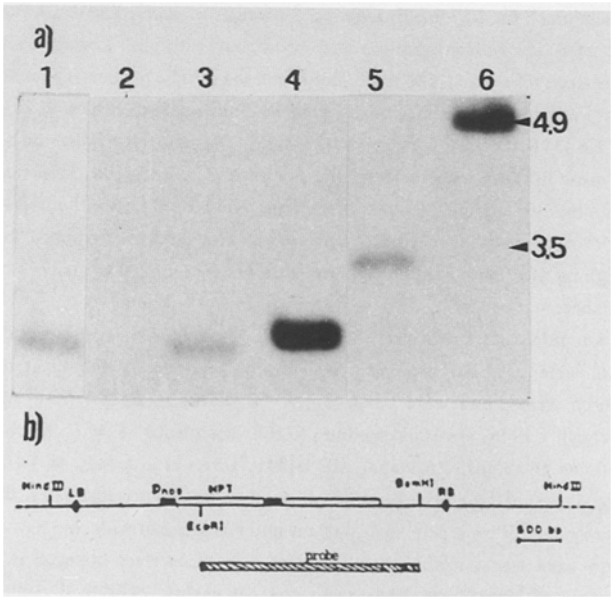


Figure 5 - Southern blot analysis of M.arborea plants transformed with A.rhizogenes containing pAGS125. a) Restricted DNA was hybridized to the EcoRI-BamHI fragment internal to pAGS125 T-DNA borders. DNA from pAGS125, one control and two transformed plants were double digested with EcoRI-BamHI (lanes 1,2,3,4, respectively). DNA from the two transformed plants restricted with EcoRI (lanes 5,6). b) Map of pAGS125 showing the EcoRI-BamHI fragment used as probe.

Lane 1 contains EcoRI restricted DNA of pAGS125 at the amount needed for a one copy reconstruction experiment, assuming that the genome of M.arborea has the same size of M.sativa. The intensity of the signal of the plant in lane 3 indicates that only one copy of the gene has been introgressed, while the plant in lane 4 shows the presence of at least 4 copies.

To verify the presence of pRi derived T-DNA, EcoRI digests of DNA from putative transformed and untransformed plants were hybridized to probes diagnostic for the T_L-DNA and T_R-DNA of Ri plasmid. Both of the tested plants were transgenic for the T-DNA of the Ri plasmid (Fig. 6).

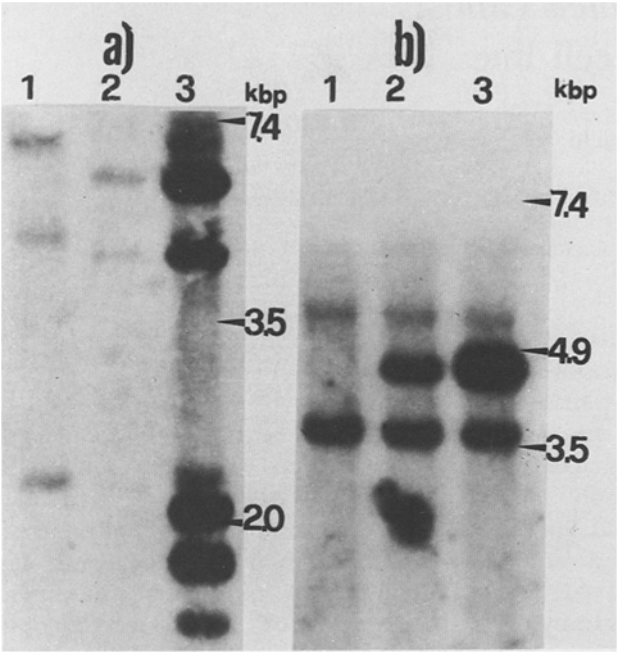


Figure 6 - Southern blot analysis of Tl and Tr T-DNA of M.arborea plants transformed with A.rhizogenes containing pAGS125. DNA from 2 transformed plants (lanes 1,2), from the plasmid MP66 containing part of T_l T-DNA (lanes 3, panel a) and from plasmid MP17 containing part of T_r T-DNA (lane 3, panel b) were restricted with EcoRI and hybridized with pMP66 (panel a) and pMP17 (panel b).

Conclusion

In this study we report the successful Agrobacterium mediated transformation of M.arborea, the only shrub species of the genus which in recent years has received particular attention as a pasture crop for dry lands of the Mediterranean Basin (Corleto et al. 1980). The introgression in M. arborea of a gene conferring resistance to hygromycin is useful for two main reasons: 1) antibiotic resistance of mesophyll protoplasts isolated from transgenic plants ensures a selection tool for hybrid cells in somatic hybridization experiments with M.sativa; 2) the optimization of conditions for the production of transgenic plants is an essential preliminary step to a subsequent transfer of genes controlling traits of economical interest.

The choice of A.rhizogenes instead of A.tumefaciens was due to a possible interference of the antibiotic in the morphogenesis process of Medicago species (Pezzotti et al. 1991; Shahin et al. 1986) and to the possibility of using the morphological alterations produced by the T-DNA of pRi 1855 for plant breeding purposes. In the transformation mediated by A.tumefaciens, transformed and untransformed cells proliferate together, and in the presence of the antibiotic, transformed cells detoxify the medium enabling neighbouring untransformed cells to survive and regenerate plants when transferred to an antibiotic free medium, making the selection process more arduous. In the transformation mediated by A.rhizogenes, the hairy root trait encoded by the pRi T-DNA was utilized for primary selection and this was very frequently associated (70%) with cotransfer of the T-DNA from pAGS 125. Thus, A.rhizogenes is a more reliable system of transformation and offers the possibility of transforming a given species without the use of a selectable gene.

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