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BRIEF COMMUNICATION

Efficient genetic transformation of *Lotus corniculatus* L. and growth of transformed plants in field

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Abstract

An efficient protocol for shoot regeneration and genetic transformation was applied to root segments of a new *Lotus corniculatus* L. cultivar Bokor. The shoots, that regenerated on root segments, were inoculated with *Agrobacterium rhizogenes* A4M70GUS, and produced hairy roots, which on media with 0.2 mg dm^{-3} benzylaminopurine, regenerated shoots. After rooting and acclimation, the transformed plants were planted in the experimental field. Their morphological traits were compared to controls. No signs of the *rol* genes phenotype were present. The transformants were significantly taller than controls, while there were no significant differences in the leaf area. The glucuronidase activity and the presence of *uidA* gene was demonstrated in transformed plants of T_0 and in seedlings of T_1 generations. It is concluded that *A. rhizogenes* could be a vector of choice for the transfer of desirable genes into the bird's foot trefoil genome.

Additional key words: *Agrobacterium rhizogenes*, hairy roots, regenerated shoots, GUS activity, *uidA* gene.

Bird's foot trefoil (*Lotus corniculatus* L.) is an important forage legume, which is in many areas replacing white clover and alfalfa, for its tolerance to adverse environmental conditions and high nutritive value. *L. corniculatus* is easily amenable to tissue culture techniques (early review by Arcioni *et al.* 1988), and to *Agrobacterium*-mediated transformation (*e.g.* Damiani *et al.* 1993, Webb *et al.* 1999). These techniques were used for the improvement of economically important traits. These include studies on the competence for nodulation and nitrogen fixation in response to *Rhizobium* infection (Petit *et al.* 1987, Stiller *et al.* 1997), and on the capacity to synthesize flavonoid and isoflavonoids, as dependent on the activity of chalcone synthase gene (Colliver *et al.* 1997). As a model plant to study condensed tannin metabolism (Robbins *et al.* 1992), *L. corniculatus* may be the source of genes that in sense or antisense orientation modify the tannin content in other forage crops (Carron *et al.* 1994, Bavage *et al.* 1997). Experiments aimed at increasing the nutritive value of *L. corniculatus* (Belluci *et al.* 2002), by introducing genes coding for sulphur amino acids, seem very promising. We were interested in introducing the herbicide resistance into a recently released *L. corniculatus* cultivar Bokor (Mijatović *et al.* 1986). The tissue culture-derived progeny displayed satisfactory performance in field conditions (Nikolić *et al.* 1997). We have further studied the use of a few vectors for genetic transformation, and the results obtained with an *A. rhizogenes* strain, that was most efficient, have been presented in this report.

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Abbreviations: BAP - benzylaminopurine; BM - basal medium; GUS - glucuronidase; MS medium - Murashige and Skoog medium; PCR - polymerase chain reaction.
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Regenerated shoots, arising on primary root segments of aseptically germinated seeds (Rybczinsky and Badzian 1987), were used for transformation. The shoots were cultured on a basal medium (BM), containing MS (Murashige and Skoog 1962) mineral salts, MS vitamins, 0.7 % agar, 3 % sucrose, and supplemented with 0.2 mg dm^{-3} benzylaminopurine (BAP). To induce hairy roots, 3 - 4 cm tall shoots were punctured by a needle, dipped into the overnight bacterial suspension, about 1 cm above the medium. The bacterial strain was *A. rhizogenes* A4M70GUS, comprising the *uidA* gene integrated in the TL region of pRiA4 plasmid (Tepfer and Casse-Delbart 1987). Five mm tips of hairy roots were cultured in hormone-free liquid BM medium, supplemented with 50 mg dm^{-3} cefotaxime; when root segments were transferred to solid medium supplemented with 0.2 mg dm^{-3} BAP, the putative transformed shoots regenerated and were cultured afterwards on hormone-free BM. *A. rhizogenes* cells were eliminated with 50 mg dm^{-3} cefotaxime (*Jugoremedia*, Zrenjanin, Yugoslavia). The cultures were maintained at temperature of 25 ± 2 °C and 16-h photoperiod (irradiance of 47 µmol m^{-2} s^{-1}). The rooted shoots were acclimated in a greenhouse and transferred subsequently to the experimental field. In primary transformants and in seedlings of T1 generation the expression of glucuronidase (GUS) was assayed with 5-bromo-4-chloro-3-indolyl-glucuronid (X-gluc), according to Jefferson *et al.* (1987). Genomic DNA was isolated from leaves and roots of the same samples (Xiaomei *et al.* 1994). Polymerase chain reaction (PCR) was performed using the primers GUS 392 and GUS 22, which amplified a 366 bp fragment of the *uidA* coding region. PCR was carried out with 100 ng of plant DNA in 0.1 cm^3 of 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 200 pM of each primer and 2.5 U native Taq DNA polymerase.

The entire procedure starting from shoot inoculation to completed acclimation took 70 - 85 d. Initially, shoots derived from 51 seeds (= genotypes) were inoculated in three experiments, and 25 (51 %) genotypes produced adventitious roots at the inoculation site after 3 weeks. In liquid medium the mass of hairy roots increased to about 100 % in 27 d of culture. After several cycles of root culture, some roots spontaneously regenerated buds, while in the rest regeneration was induced by transferring segments (0.5 - 1.0 cm) to solid medium with 0.2 mg dm^{-3} BAP. Usually 6 - 9 buds per segment were regenerated. From the initial 25 genotypes that produced adventitious roots, a total of 177 shoots were obtained. On the hormone-free medium these shoots elongated, branched by tillering and spontaneously rooted, thus producing whole plants. When potted, 136 (77 %) plants survived the treatment. The genotypes 2 and 5 were chosen for further work and thirty five plants were planted in the experimental field, where they flowered (Fig. 1) and set T_1 seeds. Their morphological traits were compared with controls (Table 1). Untransformed control plants from the same genotypes, derived from *in vitro* cultures, were planted in the same field, but in the required distance to prevent cross pollination. None of the transformants displayed the phenotype typical for the presence of *rol* genes, such as plagiogravitropic roots, short internodes and wrinkled leaves. The transformed plants differed significantly from controls by having longer stems, less shoots and a lower number of flowers per plant. They did not differ significantly in the leaf length and width, growth habit, and date of flowering (data not shown). The average number of seeds per transformed plant was 84, 65 and 56 in the genotypes 2, 3, and 5, respectively.

Fig. 1. A transformed T_0 flowering plant of the genotype 2, after 83 d of acclimatization.

Table 1. Some morphological traits of the transformed *L. corniculatus* plants, grown in the experimental field (differences significant at * - $P \leq 0.05$; ** - $P \leq 0.01$; ns - non-significant).

Trait	Genotype	Transformants	Controls
Average height	2	35.2**	28.8
[cm]	5	35.0*	31.4
Number of stems	2	32.0**	40.0
[$plant^{-1}$]	5	29.6**	34.8
Longest stem	2	61.6**	31.8
[cm]	5	55.8**	41.6
Number of internodes	2	12.6*	11.2
[longest $stem^{-1}$]	5	12.6ns	12.2
Number of flowers	2	272.0**	340.2
[$plant^{-1}$]	5	264.0**	308.4

GUS activity was histochemically demonstrated in the leaves of all plants regenerated from hairy roots. In the seedlings of T_1 generation of genotypes 2, 3, and 5, the positive reaction was observed in 50 % of leaves. Due to the low number of seedlings tested, the proportion of positive tests could not be precisely determined. Control plants did not stain blue with the reagent. The presence of the *uidA* gene segment was proved by PCR analysis in T_0 plants of the genotypes 2, 5, and 8 (Fig. 2*A*), and in the T_1 generation of genotypes 2 and 5 (Fig. 2*B*). The reaction was negative with control plants.

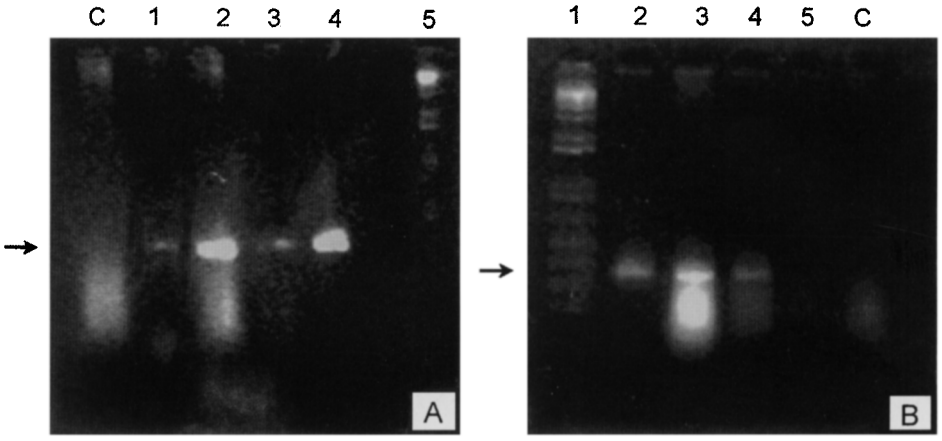

Fig. 2. PCR analyses of transformed T_0 plants (*A*) and of theirs T_1 progeny (*B*). *A*: *lane C* - DNA sample from nontransformed control plant; *lanes 1, 2 and 3* - DNA samples from transformed T_0 plants (genotypes 2, 5 and 8) contained *uidA* gene; *lane 4* - A4M70GUS as a positive control; *lane 5* - DNA standard. *B*: *lane 1* - DNA standard; *lane 2* - A4M70GUS as a positive control; *lanes 3 and 4* - DNA samples from T_1 progeny (genotypes 2 and 5) contained *uidA* gene; *lane 5* - blank; *lane C* - DNA sample from nontransformed control plant.

From the results presented here it can be concluded that the *L. corniculatus* cv. Bokor is very susceptible to *A. rhizogenes*, and that the transformation protocol results in plants with good agronomic qualities. Most important is the fact that no morphological alterations, due to the presence of *rol* genes were observed. Since cv. Bokor was selected for its high green mass production, high crude protein yield and good tolerance to local climatic conditions of eastern Serbia, the prospects of its genetic modification using *A. rhizogenes* as a vector for desirable genes seem recommendable.

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