Arch. Biol. Sci., Belgrade, 59 (4), 311-317, 2007 DOI:10.2298/ABS0704311N

EFFICIENT GENETIC TRANSFORMATION OF *LOTUS CORNICULATUS* L. USING A DIRECT SHOOT REGENERATION PROTOCOL, STEPWISE HYGROMYCIN B SELECTION, AND A SUPER-BINARY *AGROBACTERIUM TUMEFACIENS* VECTOR

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Abstract — Cotyledons from 6-day-old *Lotus corniculatus* cv. Bokor seedlings, transversally cut into two halves, were capable of regenerating buds without intervening callus formation. The explants were co-cultivated with the *Agrobacterium tumefaciens* LBA4404/pTOK233 superbinary vector carrying the uidA-intron gene and the genes hpt and nptII. They were cultured for 14 days on a regeneration medium, then subjected to a stepwise hygromycin B selection procedure consisting of gradually increasing antibiotic concentrations (5-15 mg L-1) over 21 weeks. Transformed shoots were obtained within 5 months after co-cultivation. Out of 124 initially co-cultivated explants, 52 (42%) plants survived hygromycin B selection. The presence of transgenes in regenerated plants was verified by β-glucuronidase histochemical assays and PCR analysis for the presence of uidA gene sequences. Hygromycin B-resistant and PCR-positive T0 plants were cultured in the greenhouse to produce flowers and seeds. The obtained data demonstrate that the reported transformation protocol could be useful for introducing agriculturally important genes into the new L. corniculatus cultivar Bokor.

Key words: *Agrobacterium tumefaciens,* genetic transformation, β-glucuronidase, hygromycin B, *Lotus corniculatus*

UDC 581.6:633.2
577.2:582.998:579.64

INTRODUCTION

Bird's foot trefoil (*Lotus corniculatus* L.) is a perennial forage legume which in many areas is preferred to alfalfa because of its tolerance to adverse environmental conditions and high nutritive value. The productivity of bird's foot trefoil could be increased by introducing stably inherited traits such as pest, disease, and herbicide resistance, or improved protein quality. Since the indicated traits are not available in natural *L. corniculatus* populations, biotechnological approaches could be used to achieve these goals. The local cultivar Bokor, selected by the polycross method at the Center for Agricultural and Technological Research in Zaječar, Serbia (Mijatović et al., 1986), was used in the study. This cultivar displayed a high morphogenic potential *in vitro* (Nikolić et al., 1997, 2006) and was successfully transformed with *A. rhizogenes* (Nikolić et al., 2003/4). Here we report on some modifications of the protocols used before which could possibly improve the efficiency of transformation and the quality of transgenic plants. The modifications are based on the experience of other authors working with *L. corniculatus* or related species (e.g., Webb et al., 1996., Aoki et al., 2002., Olhoft et al., 2003). They concern: (a) the elaboration of a direct regeneration system which circumvents development of a callus; (b) the use of a superbinary *A. tumefaciens* vector such as LBA4404/pTOK233, which has been reported to improve the transformation of soybean (Olhoft et al., 2003) and alfalfa (Ninković et al., 2004) somatic embryos, and (c) a selection procedure based on hygromycin B resistance, which may be more suitable for transformant regeneration than the previously used kanamycin.

MATERIAL AND METHODS

Agrobacterium tumefaciens strain and vector

A. tumefaciens strain LBA4404 carrying the super-binary vector pTOK233 (Hiei et al., 1994) was used for transformation. Within the T-DNA borders, pTOK233 contains: the kanamycin-resis-

tance gene (*nptII*), under control of the *nos* promoter; the hygromycin-resistance gene (*hpt*) and the β-glucuronidase (GUS-intron, *uidA*) gene, both fused to the CaMV 35S promoter (Fig. 1); and extra copies of the *virB*, *virC,* and *virG* genes on the vector backbone. The latter gene copies were sequences isolated from the virulent pTiBo542 plasmid (An *et al.,* 1985). The LBA4404/pTOK233 culture was maintained on agar-solidified AB medium (Chilton et al., 1974), supplemented with 50 mg L^{-1} hygromycin B (Sigma Co.). Prior to inoculation, *A. tumefaciens* culture was grown for 3 days at 25°C on AB medium with 50 mg L^{-1} hygromycin B. One full loop (3 mm) of bacterial culture was scraped from a 3-day-old plate and suspended in 5 ml of a liquid infection medium in a 50-ml flacon tube. The tube was fixed horizontally to a Vortex platform and shaken at a low speed for 3-5 h at room temperature.

Plant material, transformation, and selection

Seeds of *L. corniculatus* L. cv. Bokor were rinsed in 70% ethanol for 1 min, surface sterilized in 20% commercial bleach (4-6% NaOCl) for 1 hour, rinsed five times in sterile distilled water, and germinated on 0.7% plain agar (Torlak, Belgrade) for 6 days. For infection, the cotyledons were excised from 6-day-old seedlings and cut transversally to obtain two explants with competence for regeneration from each cotyledon. The protocol for *A. tumefaciens*-mediated transformation and selection is presented in Fig. 2. The explants were immersed in a bacteria-containing infection medium for 1 min, then transferred to a co-cultivation medium for 3 days, and finally subcultured on a regeneration medium containing BA, NAA, and 300 mg L^{-1} cefotaxime for 2 weeks. Cotyledonary explants with small emerging shoots were subcultured several times on hormone-free selection media containing 300 mg L^{-1} cefotaxime and hygromycin B. The concentrations of hygromycin B were increasing (5-15 mg L^{-1}) in the first four subcultures and decreasing (10-5 mg L^{-1}) in the last three subcultures. The transfer intervals between subcultures were two to four weeks.

Media and culture conditions

The basal culture medium (BM) contained MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, and 0.7% agar, except that agar was omitted in the liquid infection medium. The regeneration medium (also used for infection, co-cultivation, and induction) contained 0.5 mg L^{-1} each of benzylaminopurine (BA) and 1-naphtaleneacetic acid (NAA). Upon co-cultivation, 300 mg L^{-1} cefotaxime was added to supress bacterial growth. The selection media were hormone-free BM with added hygromycin B, while the rooting medium consisted of BM supplemented with 0.2 mg L^{-1} indole-3-butyric acid (IBA). Cultures were maintained in a growth room under conditions of 16/8-h day/night cycles, 25 ± 2°C, and light intensity of 47 μmol m^{-2} s^{-1}.

ß-*Glucuronidase assays and PCR analysis*

ß-Glucuronidase enzyme activity (GUS-assay) was determined histochemically in leaves and roots excised from plants that survived the selection procedure (Jefferson, 1987). Histochemical assays were performed immediately after the selection procedure was finished and repeated two years after the beginning of experiments.

PCR analyses were also performed two years after co-cultivation. Genomic DNA was extracted from leaves of the putative transformed and untransformed control plants using a CTAB extraction method (Xiaomei et al., 1994). The presence of the *uidA* gene was demonstrated by PCR-amplification of a 366-bp fragment using the primer 5'-CCCGGCAATAACATACGGCGTG-3' and the reverse primer 5'-CCTGTAGAAACCCCAACCCGTG-3'. Primers specific for amplification of the *virG* gene (Melchers et al., 1986) amplified a fragment of 390 bp; the primer forward sequence was 5'-GCCGACAGCACCCAGTTCAC-3', the reverse sequence 5'-CCTGCCGTAAGTTTCACCTCACC-3'. Thermocycling conditions were as follows: denaturation at 94°C for 5 min; and 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The program was terminated by a final extension step at 72°C for 10 min. PCR products were separated on 0.9% agarose gels and visualized by ethidium bromide staining (0.5 μg ml^{-1}).

RESULTS

In order to optimize the regeneration procedure, half-cotyledon explants from 6-day-old *L. corniculatus* seedlings were cultured on the regeneration medium in a preliminary experiment. Bud initials (Fig. 3) appeared all around the explant rim, especially at the cut edge. Ninety six percent of explants produced shoots (Fig. 4); after 45 days in culture, the number of regenerated shoots *per* explant ranged from 21 to 51. This procedure was therefore judged as to be highly efficient for regeneration and was adopted in further experiments.

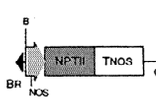
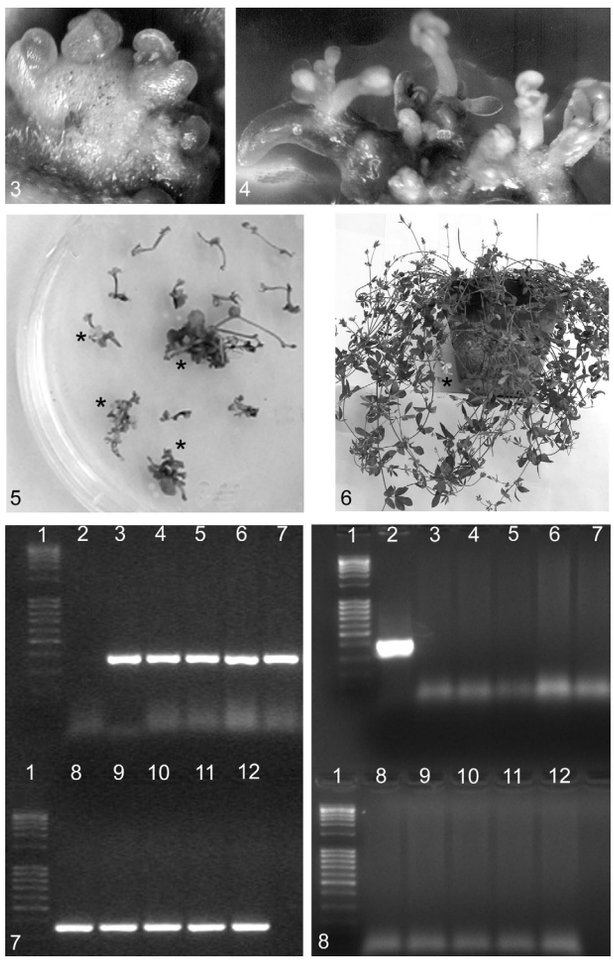


Fig. 1. Schematic map of T-DNA region of the super-binary vector pTOK233 (H i e i et al., 1994)

Cotyledons (6-day-old seedlings)
↓
Co-cultivation with LBA4404/pTOK233 (3 days)
Regeneration medium BA + NAA, 0.5 mg L^{-1}, each
↓
Regeneration medium (2 weeks)
300 mg L^{-1} Cef + BA + NAA
↓
I Selection medium (2 weeks)
300 mg L^{-1} Cef + 5 mg L^{-1} Hyg
↓
II Selection medium (2 weeks)
300 mg L^{-1} Cef + 10 mg L^{-1} Hyg
↓
III Selection medium (2 x 3 weeks)
300 mg L^{-1} Cef + 15 mg L^{-1} Hyg
↓
IV Selection medium (3 weeks)
300 mg L^{-1} Cef + 10 mg L^{-1} Hyg
↓
V Selection medium (2 x 4 weeks)
300 mg L^{-1} Cef + 5 mg L^{-1} Hyg
↓
Rooting
IBA 0.2 mg L^{-1}
↓
Transfer of plantlets to soil

Fig. 2. Flow chart of the transformation procedure. Note that all selection media were hormone-free.

Agrobacterium-mediated transformation was performed by co-cultivation of 124 half-cotyledon explants, derived from 31 seedlings (= genotypes). Instead of starting selection immediately, the explants were cultured for the first 14 days on



Figs. 3-8. Initiation of direct shoot regeneration on untransformed cotyledonary explant after two weeks of culture on medium containing BA + NAA (0.5 mg L-1, each). Fig. 4. As in Fig. 3, after three weeks in culture. Fig. 5. Proliferating hygromycin B-resistant shoots (asterisks) on selection medium III, containing 15 mg L-1 hygromycin B. Fig. 6. Transformed *L. corniculatus* plant (clone 3) in early flowering phase (asterisk), acclimated in the greenhouse. Fig. 7. PCR analysis of genomic DNA to detect the presence of the uidA gene in transformed plants. Lane 1 - DNA ladder; Lane 2 - DNA from untransformed control plant; Lane 3 - pTOK233 as positive control; Lanes 4 - 12 - DNA samples from transformed plant clones 1 (plant no. 1 and plant no. 2), 2, 3, 7, 9, 10, 8, and 4. Fig. 8. PCR analysis of *A. tumefaciens* virG gene detection showing the absence of contamination with *Agrobacterium* in all plant samples tested. Lane 1 - DNA ladder; Lane 2 - positive control for virG gene (DNA of pTOK233); Lane 3 - DNA from untransformed control plant; Lanes 4-12 - DNA samples from transformed plant clones 1 (plant no. 1 and plant no. 2), 2, 3, 7, 9, 10, 8, and 4.

a non-selective regeneration medium until shoot initials emerged. Delaying the onset of selection for two weeks permitted regeneration in 65% of explants. Explants with bud initials were transferred to selection medium I (Fig. 2), containing 5 mg L^{-1} hygromycin B for two weeks. Most explants were apparently chimeric with respect to hygromycin B sensitivity. During the cultivation on selection medium I, non-transformed shoots stopped growing and the cotyledon blades turned brown. Green bud initials were grouped in small clusters that were subsequently subcultured on medium II with 10 mg L^{-1}, followed by 2 x 3 weeks on medium III with 15 mg L^{-1} hygromycin B. On media II and III, apparently all hygromycin B-sensitive shoots became completely necrotic (Fig. 5). After two subcultures on 15 mg L^{-1} hygromycin B (6 weeks), no more necrotic shoots appeared, so the hygromycin B content was gradually lowered to 10 and 5 mg L^{-1}. After five subcultures on selection media, lasting for 13 weeks, 4.9% of inoculated regenerating explants survived (Table 1) and further multiplied. Finally, 52 hygromycin B-resistant shoots were obtained, meaning that the efficiency of transformation was 42%.

The regenerated plants belonged to four genotypes. The obtained shoots differed in their development in such a way that 10 shoot clones were singled out. Clones 1, 2, 3, 4, and 10 developed well on a non-selective, hormone-free medium. Shoots 2-4 cm long were transferred to the rooting medium, and 73% shoots took root. The plantlets were subsequently acclimated and grown in the greenhouse (Fig. 6). They exhibited the normal phenotypic characteristics of wild-type *L. corniculatus* plants. However, clones No. 5, 6, 7, and 9 were characterized by poor growth and a scarce rooting response. Experiments aimed at improving their development are in progress and will be reported later (Nikolić et al., in preparation).

Analysis of expression of the GUS-intron gene was performed in shoots that survived selection procedure, and 100% plants showed blue staining in their shoot tissue. Two years after co-cultivation, the leaves and roots of clones cultivated *in vitro* on a hormone-free medium were repeatedly tested for the GUS reaction. In all ten plant clones, the GUS reaction was positive and the tissue was colored intensely blue. Shoots with a positive GUS reaction were not found in untransformed control plants.

PCR analysis was performed 2 years after the beginning of experiments using leaves of eight clones maintained in culture. DNA was extracted from hygromycin B-resistant and control plants. The results indicated the presence of the 366-bp sequence of the *uidA* gene from the super-binary vector LBA4404/pTOK233 in all transformed clones (Fig. 7, lanes 4-12). The reaction was negative in DNA from a tested untransformed plant (Fig. 7, lane 2). The absence of contamination with *Agrobacterium* was verified in all samples tested, as no *virG*-specific signal was detected (Fig. 8, lanes 3-12). As a control for this reaction, DNA from *A. tumefaciens* LBA4404 was amplified (Fig. 8, lane 2).

Table 1. Transformation efficiency and shoot regeneration in half-cotyledon explants of *L. corniculatus* cv. Bokor.

Treatment	No. of isolated explants	No. (%) of surviving regenerating explants*	No. of surviving shoots**	Transformation efficiency (%)***	No. (%) of rooted plants
LBA4404/pTOK233	124	6 (4.9)	52	42	38 (73)
Control	28	0	0	0	0

* Surviving half-cotyledon explants after five subcultures on hygromycin B-containing media
** Surviving shoots after seven subcultures on hygromycin B-containing media
*** Transformation efficiency = (No. of survived shoots/No. of isolated explants) x 100

DISCUSSION

While genetic transformation of many crop species with well-known marker genes has become a routine procedure, the introduction of transgenic lines into agricultural practice has still been met with unsolved difficulties. One of them is the occasional instability of morphological traits, other than those encoded by the inserted transgenes in transgenic plants. *L. corniculatus* is known as a species readily amenable to *in vitro* regeneration, though at the same time subject to chromosomal aberrations, which may result in grossly changed phenotypes (Webb and Watson, 1991). The reasons for these aberrations are not clear, though somaclonal variations in regenerating callus cells caused by the excessive use of growth regulators are likely to be responsible. Rybczyński and Badzian (1987) first reported direct shoot regeneration in root segments on a hormone-free medium. Likewise, hairy root segments produced plants that were superior to controls in some respects and even lacked the typical traits encoded by *rol* genes (Nikolić et al., 2003/2004). In the transformed regenerants described here, we did not notice morphological traits different from the control plants. Three factors may have contributed to the stability of our cultures: (a) The shoots were regenerated directly from cotyledon tissue, without any intervening callus; (b) multiplication of putative hygromycin-resistant shoots occurred by branching of axillary meristems and not by *de novo* regeneration; and (c) BA and NAA were applied only for the first two weeks as an inductive treatment and were omitted from all subsequently used media.

How many host cells will be transformed by *A. tumefaciens* during co-cultivation is hard to predict, but it can be supposed that virulent strains are more efficient. *A. tumefaciens* LBA4404/pTOK233 was designed to transform recalcitrant cereal species (Hiei et al., 1994) and was hitherto used successfully in transformation of many other species, including legumes (Olhoft et al., 2003; Ninković et al., 2004).

Ensuring the ability of transformed cells to divide and organize apical meristems or embryonic structures is probably the most critical step in genetic transformation. If selection pressure comes too early, the transformed cells, surrounded by dead or damaged cells, may fail to form the meristematic centers. Delaying antibiotic selection for two weeks after co-cultivation facilitates organogenesis. However, the disadvantage of that procedure consists in the greatly augmented chances of generating chimeric transformants. Stepwise increasing the concentrations of hygromycin B may perhaps alleviate that inconvenience, as it did in soybean (Olhoft et al., 2003) and cotton (Meng et al., 2007). In our experiments, the putative transformants were maintained on selection media for 21 weeks, including 8 weeks on media with the highest hygromycin B concentration of 15 mg L^{-1}. Hygromycin has been reported to interfere with polypeptide elongation by cytoplasmic ribosomes (González et al., 1978). It is therefore assumed that the transformed cells are not likely to protect the untransformed ones by synthesizing a metabolite that would counteract the activity of hygromycin (Olhoft et al., 2003). Hence, the death of untransformed cells occurs rather quickly. Meng et al. (2007) evaluated the sensitivity to hygromycin B of various cotton tissues. They found that cotyledons were the most sensitive, since hygromycin B concentrations of 2.5 to 5 mg L^{-1} completely stopped callus growth and caused cotyledon necrosis within 3 weeks. Callus induction on the least sensitive parts, hypocotyl segments, was blocked for 1-2 months on media containing 15-20 mg L^{-1} hygromycin. We therefore feel that our prolonged selection with hygromycin B was efficient in removing all untransformed cells. The undoubtedly strong positive GUS reactions and results of PCR analyses two years after co-cultivation support this conclusion.

Acknowledgment – The present work was supported by the Ministry of Science of the Republic of Serbia (Grant No. 143026).

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ЕФИКАСНА ГЕНЕТИЧКА ТРАНСФОРМАЦИЈА *LOTUS CORNICULATUS* L. ПОМОЋУ ДИРЕКТНЕ РЕГЕНЕРАЦИЈЕ БИЉАКА, ПОСТЕПЕНЕ СЕЛЕКЦИЈЕ СА ХИГРОМИЦИНОМ Б И *AGROBACTERIUM TUMEFACIENS* СУПЕР-БИНАРНОГ ВЕКТОРА

РАДОМИРКА НИКОЛИЋ, НЕВЕНА МИТИЋ, СЛАВИЦА НИНКОВИЋ и МИРЈАНА НЕШКОВИЋ

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Котиледони, изоловани са 6 дана старих клијанаца *Lotus corniculatus,* сорта Бокор, су се показали као погодни експлантати за регенерацију биљака без интермедијарне калусне фазе. Котиледони су пресецани трансверзално на две половине и ко-култивисани са *Agrobacterium tumefaciens* супербинарним вектором LBA4404/pTOK233, који носи *uidA*-интрон, *hpt* и *nptII* гене. После ко-култивације експлантати су првих 14 дана гајени на регенеративном медијуму без селективног агенса, а затим су 21 недељу били подвргнути селективној процедури током које је концентрација антибиотика постепено повећавана (5-15 mg L^{-1}). Трансформисани изданци добијени су 5 месеци након ко-култивације. Од 124 иницијално ко-култивисана експлантата, добијене су 52 (42%) биљке које су преживеле селекцију на хигромицину В. Присуство *uidA* трансгена у регенерисаним биљкама је потврђено помоћу β-глукуронидазне хистохемијске реакције и PCR анализом. Т0 биљке, резистентне на хигромицин В и PCR позитивне, гајене су у стакленику у циљу продукције и растења цветова и семена. Добијеним резултатима је јасно указано да би саопштени протокол за трансформацију могао несумњиво бити употребљен за интродукцију агрономски значајних гена у *Lotus corniculatus*, сорта Бокор.