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IMPROVED TRANSFORMATION OF ALFALFA SOMATIC EMBRYOS USING A SUPERBINARY VECTOR

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Increased transformation efficiency of alfalfa (*Medicago sativa* L. cv. Zaječarska 83), was achieved using *Agrobacterium tumefaciens* LBA4404 carrying the superbinary vector pTOK233 and the *hpt* gene for hygromycin resistance. Evidence for transformation was based on GUS activity and the presence of a fragment of the *uidA* gene in transformed embryos and regenerated plants, as demonstrated by the polymerase chain reaction. Efficiency was 1% (LBA4404/pBI121) to 14% (LBA4404/pTOK233) higher than that of some other vectors used in previous work with the same cultivar. The higher efficiency may be due to the presence of an extra set of *vir* genes in pTOK233. Moreover, the presence of the *hpt* gene, enabling the use of hygromycin instead of kanamycin for selection, was better for the development of secondary somatic embryos, thus contributing to a higher final number of transgenic plants.

Key words: *Agrobacterium tumefaciens*, *Medicago sativa* L., hygromycin, somatic embryogenesis, superbinary vector.

INTRODUCTION

Since the first successful *Agrobacterium tumefaciens*-mediated transformation of alfalfa (Shahin et al., 1986; Deak et al., 1986) there have been many published reports on different aspects of the procedure. Most of them showed that the efficiency of transformation depended on the genotype (Mariotti et al., 1984; Du et al., 1994) and bacterial strain (Chabaud et al., 1988; Desgagnes et al., 1995, Chabaud et al., 2003). The efficiency of transformation also depends on the ability to select transformants and the frequency of shoot regeneration (Aoki et al., 2002).

The alfalfa cultivar Zaječarska 83 used in this experiment is a newly registered cultivar selected from the autochthonous populations from eastern Serbia. It has already been shown that somatic embryos of this cultivar are amenable to *A. tumefaciens*-mediated transformation (Ninković et al., 1995, 1998) but the regeneration ability of transformed embryos was quite low. The negative effect of kanamycin on the regenerative potential of alfalfa has been suggested (Pezzotti et al., 1991; Desgagnes et al., 1995; Trinh et al., 1998), and *npt*II screenable marker is not recommended for alfalfa transformation. We studied other vectors for genetic transformation.

Here we report results obtained with the use of *A. tumefaciens* LBA4404 carrying a so-called superbinary vector with *vir* genes from pTiBo542 plasmid and hygromycin-resistance gene (*hpt* gene).

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MATERIALS AND METHODS

Single somatic embryos of alfalfa (*Medicago sativa* L. cv. Zaječarska 83) obtained after many generations of recurrent embryogenesis on MS medium with B_5 vitamins (Parrott and Bailey, 1993) were used for transformation. Embryogenic cultures initially were obtained from immature embryos collected from field plants (Ninković et al., 1998). The cultures were maintained at 25 ± 2°C under a 16 h photoperiod (irradiance 47 µmol m^{-2} s^{-1}).

The bacterial strain used in this work was *Agrobacterium tumefaciens* LBA4404 carrying the superbinary vector pTOK233 containing the following genes in the T-DNA region: *npt*II gene under the control of nopaline synthase promoter, *hpt* gene under the control of 35S promoter, and intron -*gus* gene with the same promoter (Hiei et al., 1994). Plasmid pTOK233 also contains *vir*B, *vir*C and *vir*G genes from pTiBo542. The *Agrobacterium* strain was grown at 28°C on AB medium (Chilton et al., 1974) supplemented with 50 mg/L hygromycin.

Secondary somatic embryos in the early cotyledonary stage were longitudinally split into halves and immersed in a late log suspension of the *Agrobacterium* strain (10^9 cells/mL). After 5–10 min inoculation, the embryos were gently blotted on filter paper and then placed on top of culture media (MS with B_5 vitamins) for 3 days at 25°C in the dark. Following this period they were transferred to the same medium, supplemented with 250 mg/L cefotaxime and 50 mg/L hygromycin. Secondary embryos formed within 3 weeks on this medium were moved to medium containing 100 mg/L hygromycin. Embryo clones surviving 15 days of this selection were cultured on medium with 50 mg/L hygromycin for another 3 months. For plant regeneration, clamps of embryos were cultured for a week in Petri dishes with 1 cm^3 medium without antibiotic selection and then transferred to medium supplemented with 50 mg/L hygromycin or 50 mg/L kanamycin. The regenerated plantlets were then placed on non-selective medium for further propagation, rooted and then transferred to greenhouse conditions.

For PCR analysis, DNA was isolated from somatic embryos and regenerated plants, using DNAzol™ (Molecular Research Center, Inc.). The analysis was performed with primers 5' CCCGGCAATAACATACGGCGTG 3' and 5' CCTGTAGAAACCCCAACCCGTG 3', which delimit a 366 bp fragment from the *uid*A coding region. PCR reactions were carried out with 100 ng plant DNA. The GUS assay was performed as described by Jefferson et al. (1987).

TABLE 1. Embryo regeneration on media with different antibiotics

Clone	Antibiotic (50 mg/L)	Cultured embryos	Regenerated plantlets	Regeneration efficiency [%]*
1	Hygromycin	30	17	56.6 ± 3.3
	Kanamycin	30	7	23.3 ± 4.1
2	Hygromycin	20	3	15.0 ± 5.0
	Kanamycin	20	0	0

* Regeneration efficiency was calculated as the percentage of total number of regenerated plantlets per total number of cultured embryos ± SE.

For histological studies, the initial explants (control and transformed embryos) were collected every day up to 21 days after the beginning of culture on MS + B_5 medium. For light microscopy the material was fixed in FAA and embedded in paraffin, and sections 10–15 µm thick were stained with Delafield hematoxylin. Single transformed and control somatic embryos were also observed with a scanning electron microscope (JEOL CX) without pretreatment.

RESULTS AND DISCUSSION

A total 150 somatic embryos were isolated from the embryogenic alfalfa tissue and cocultivated with *A. tumefaciens* LBA4404/pTOK233 for 3 days as described. The first signs of secondary somatic embryogenesis on some embryos appeared in the hypocotyl region by the 5th day, on medium with 50 mg/L hygromycin (Fig. 1b), at the same time as in control embryos (Fig. 1a). By day 12, secondary embryos in different stages of development were seen by SEM on the surface of the hypocotyledonary region. The embryos were attached to the parental tissue by their radical pole. They frequently had more than two cotyledons, and very often two or more embryos were fused (Fig. 1c–d). After 3 weeks on selective medium, secondary embryogenesis occurred in 21 (14%) inoculated embryos, while no embryos appeared in the controls. Inhibition of chlorophyll synthesis along with necrosis of embryos was observed in the rest of the clones. All 21 clones survived 2 weeks of stronger selection pressure (100 mg/L hygromycin). Surviving clones were tested for GUS activity. All embryogenic clones displayed a positive reaction, but among them the intensity of color varied (data not presented). The two most intensely colored clones were chosen for further work, and were multiplied at 50 mg/L hygromycin.

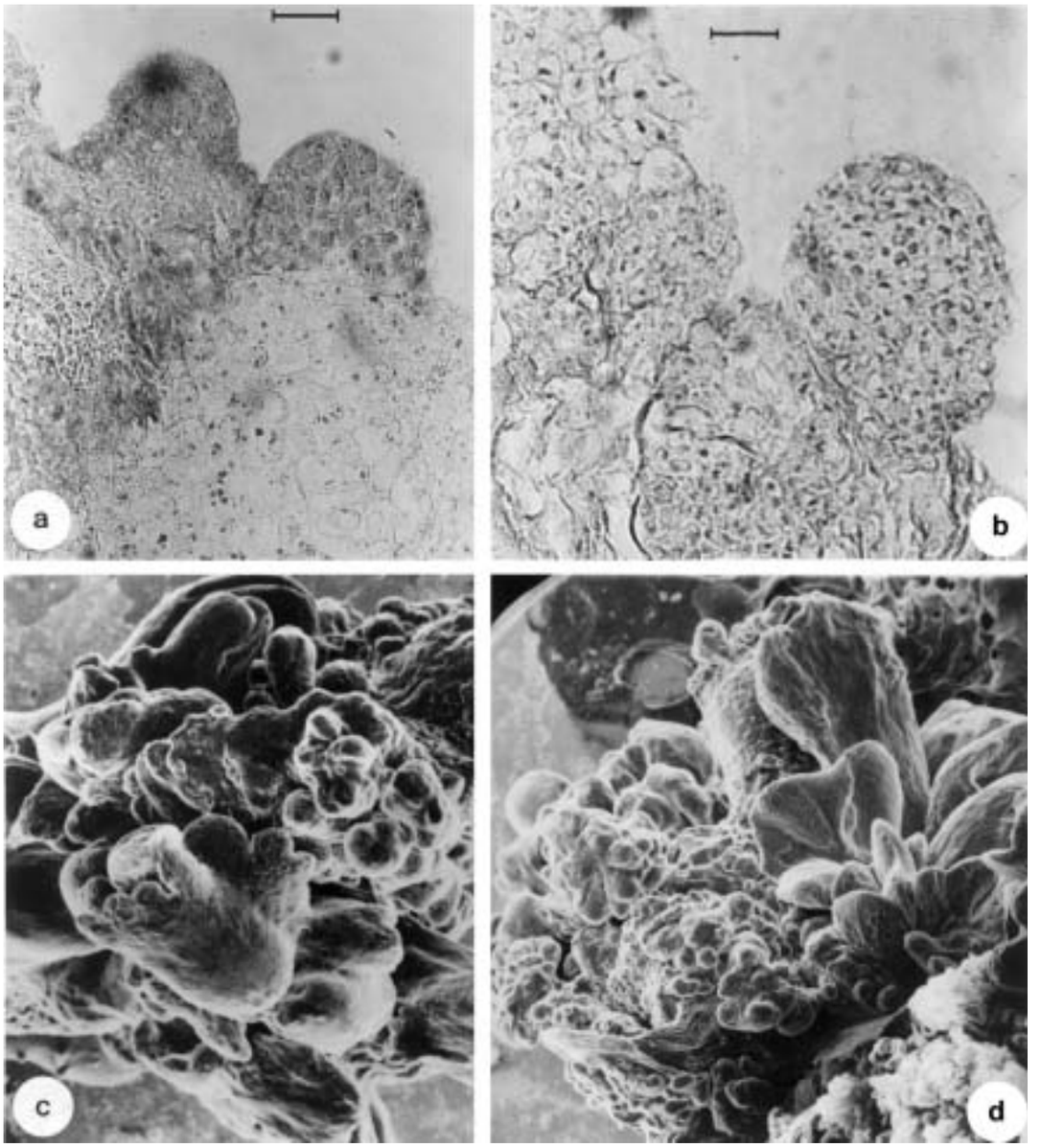

Fig. 1. Histology showing synchronized appearance of secondary somatic embryogenesis on control (**a**) and transformed (**b**) embryos of *M. sativa* cv. Zaječarska, after 5 days in culture. Bar = 120 µm, (**c–d**) SEM showing no difference between secondary somatic embryos seen on surface of control (**c**) and transformed (**d**) primary embryos, after 12 days in culture. × 300

Single embryos from these clones were repeatedly tested for GUS reaction and the results were positive. In untransformed clones the GUS reaction was always negative.

For plant regeneration, somatic embryos were exposed to drought conditions (see: Materials and Methods). After drying embryos were transferred to medium containing 50 mg/L hygromycin, 56% of transformed embryos in clone No. 1 and 15% in clone No. 2 started to grow into rooted plantlets. The number of regenerated plantlets on medium with the same concentration of kanamycin was significantly less, 23% and 0% respectively (Tab. 1). The difference in the number of regenerated plants between the two clones is probably due to the position of the inserted genes. The regenerated plantlets

Fig. 2 (**a**) Transformed (left) and control (right) plants of *M. sativa* cv. Zaječarska after 2 months of acclimation, (**b**) PCR analyses of LBA4404/pTOK233 transformed alfalfa embryos and regenerated plants, *lane 1* – 1 Kb DNA ladder; *lane 2* – blank; *lane 3* – LBA4404/pTOK233 as positive control; *lane 4* – transformed embryos (clone 1); *lane 5* – transformed embryos (clone 2); *lane 6* – transformed plants (clone 1); *lane 7* – transformed plants (clone 2); *lane 8* – nontransformed embryos as negative control.

were then placed on non-selective medium for further propagation, or else transferred to the greenhouse. Within a few months, 60 plantlets were raised in soil (Fig. 2a).

The presence of the *uidA* gene in the cells of the two transformed clones was confirmed by PCR analysis. The appearance of the 366 bp amplification product indicated the presence of GUS gene sequences in the genome of the transformed embryos and plants. The reaction was negative with the nontransformed alfalfa embryogenic clone (Fig. 2b, *lane 8*) and in the case where DNA was omitted, to confirm that the PCR products were not artefacts (Fig. 2b, *lane 2*).

A comparison of these results with already published results on transformation of *Medicago sativa* cv. Zaječarska 83 (Ninković et al., 1995, 1998) suggests that transformation efficiency is improved using *Agrobacterim tumefaciens* LBA4404/pTOK233 (14%) instead of *A. tumefaciens* LBA4404/pBI121 (1%), C58C1/pGKB5 (2%) or A281/pGA472 (5%). The presence of an extra set of *vir* genes in pTOK233 may have contributed to the higher efficiency. Similar results have been obtained for *Medicago truncatula* cv. Jemalong with strain AGL1, the hypervirulence of which derives from disarmed pTiBo542 (Chabaud et al., 2003). Improvement of transformation frequencies with a superbinary vector has been reported in wheat (Khanna and Daggard, 2003) and loblolly pine (Tang, 2003). Superbinary vector seems to be a generally useful vector for plant transformation.

Kanamycin resistance is a screening tool commonly used in alfalfa transformation. In some cases, kanamycin affected callus development (Desgagnes et al., 1995) and regeneration of alfalfa plants (Pezzoti et al., 1991). Trinh et al. (1998) reported that kanamycin selection is not efficient in *M. sativa* ssp. falcata: 80% of regenerated plants were transformed using a construct containing hygromycin, and only 2% using the same construct containing kanamycin resistance. Hygromycin apparently is a more reliable selective agent than kanamycin. In our work, hygromycin selection had no influence on secondary somatic embryogenesis. Regeneration of transformed embryos was more successful when selected on hygromycin than when selected on kanamycin.

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