

Short Communications

Agrobacterium-mediated Transformation of Lucerne (*Medicago sativa* Linn.): Optimizing Biological and Physical Parameters

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An optimized protocol for the transformation of Indian cultivars of lucerne (*Medicago sativa* Linn.) using *Agrobacterium tumefaciens* has been developed. It involved a selectable marker gene encoding hygromycin phosphotransferase (*hpt II*) which confers resistance to the antibiotic hygromycin, together with a reporter gene (*uid A*) encoding β -D glucuronidase (GUS); cotyledonary and hypocotyl explants from *in vitro* grown 5 days -old seedlings raised from mature seeds of genotype L-2 and IL-75 as target tissues, and co-cultivation of explants with *A. tumefaciens* strain LBA-4404 harbouring plasmid pCAMBIA-1301. Cotyledonary explant was found to be more responsive for transformation. Bacterial density of 5×10^9 cells/ml and co-cultivation for three days were found to be most effective for stable GUS expression in 91% of the hygromycin-resistant calli.

Keywords: lucerne, *Agrobacterium*, genetic transformation, forage quality improvement

Lucerne or alfalfa (*Medicago sativa* Linn.; family-Fabaceae; Papilionaceae) is one of the most important leguminous forage crops of India. Widely known as the "queen of the forage", it is cultivated throughout the world in diverse environments (Bolton *et al.*, 1972; Hanson *et al.*, 1988). It contains protein, 15-20; calcium 1.5 and phosphorus, 0.2%, and also is a rich source of vitamin A, B and D for animal feeding (Bickoff *et al.*, 1972; McCoy & Walker, 1984). The deficiency in sulphur containing amino acids and saponins, which reduce its nutritional value, make it a candidate crop for forage quality improvement (McCoy & Walker, 1984; Bickoff *et al.*, 1972). Introduction of gene for ovalbumin (which has balanced amino acids profile including 6.5% cysteine and methionine) in lucerne cultivars would be a right

strategy for improvement (Schroeder *et al.*, 1991). Similarly, introduction of genes controlling lignin (reduce digestibility) and tannin production (which acts as the anti-bloat factor), would improve quality (Baucher *et al.*, 1999). Lucerne has a number of inherent problems for its improvement through conventional breeding methods. biotechnological tools provide tremendous scope for its genetic improvement. Methods for transformation of higher plants employing *Agrobacterium* are well-established; produce stable transgenic plants (Baucher *et al.*, 1999); do not require complicated protocols or sophisticated equipments; result in higher transformation efficiency as predictable pattern of foreign DNA integration (Chan *et al.*, 1993). Using this technique, optimization of biological and physical parameters for transformation of Indian cultivars of lucerne is described in this paper.

Mature seeds of lucerne genotypes (L-2 & IL-75) obtained from germplasm collection available at Indian Grassland and Fodder Research Institute, (IGFRI) Jhansi, were surface-sterilized in 0.1% mercuric chloride solution with a drop of Tween-20 for 5 min and washed 4-5 times with sterile distilled water. They were germinated and grown on half strength MS agar medium (Murashige & Skoog, 1962) under 16 hrs photo-period at $25 \pm 2^\circ\text{C}$. Cotyledon and hypocotyl segments (0.5 to 1.0 cm) from 5 days-old axenically grown seedlings were used as explants for transformation. Several wounds were made on the cotyledon surface, while thin cylindrical hypocotyl segments were having wounds on the cut ends only. *A. tumefaciens* (LBA-4404 bearing plasmid construct pCAMBIA-1301), reporter genes for GUS (*uid A* gene interrupted by catalase intron) and hygromycin resistance (*hpt II*), both of them driven by CaMV 35S promoters with *nos* terminators (Fig. 1), was grown in Yeast Extract Mannitol (YEM) medium supplemented with Kanamycin (100 mg/l) and Rifampicin (10 mg/l) at $28 \pm 2^\circ\text{C}$ for 2-3 days. The bacterial pellet was obtained by centrifugation (5000 rpm, 5 min at room temperature) and resuspended in YEM medium (density, 5×10^7 - 5×10^{10} cells/ml). The explants were immersed in it for 15 min, excess bacterial suspension was blot dried and explants transferred onto MS callusing medium

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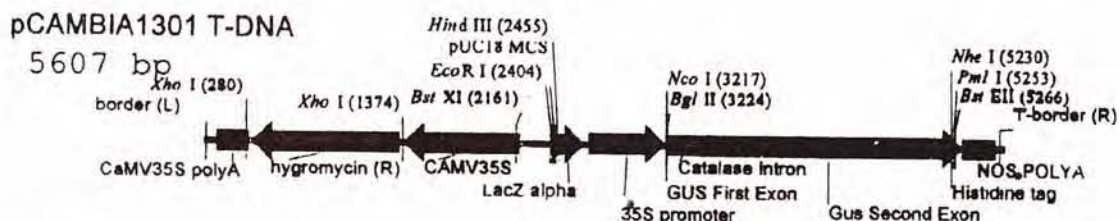


Fig. 1—T-DNA region of the plasmid pCambia-1301 used for *Agrobacterium*-mediated transformation of lucerne.

(CLM), supplemented with 9.0 μM 2,4-D, 5.0 μM NAA and 0.8 μM BAP, with or without 50 μM acetosyringone (3',5'-dimethoxy-4'-hydroxy-acetophenone; Aldrich Chemical Co, USA). Co-cultivation on this medium was continued at $28 \pm 2^\circ\text{C}$ for 2-5 days in dark. The explants were rinsed thoroughly with 250 mg/l Cefotaxime solution (Melford Lab Ltd, UK), to get rid of bacterial cells from the surface of the explant, blot dried and transferred on CLM medium containing hygromycin 50 mg/l and Cefotaxime 250 mg/l (ClmHC). Embryogenic calli induced from the explants were subcultured at two weeks interval on freshly prepared ClmHC medium. The *uid A* gene expression was determined by a histochemical assay (Jefferson, 1987) in randomly selected calli, 4-5 weeks after co-cultivation. GUS assay staining solution was prepared by dissolving 5 mg X-gluc (5-bromo, 4-chloro, 3-indolyl- β -D-glucuronide; Sigma Chem Co, USA), 359 mg sodium dihydrogen phosphate dihydrate and 0.01 ml of Tween-20 in 20 ml of double distilled water (pH 7.0), filter sterilized and stored at 4°C . GUS gene expression was observed with Nikon-SMZ-2T binocular stereozoom microscope as blue spots. Transformation frequency is the number of GUS expressing hygromycin-resistant calli divided by the number of calli assayed and expressed as percentage. The experimental data (replicated three times), were analyzed using analysis of variance and significant differences among treatment means at 5 per cent level of significance were calculated using Duncan's multiple range test (IRRISTAT module).

Cotyledonary and hypocotyl explants of the test genotypes, L-2 and IL-75, were co-cultivated with *A. tumefaciens* strain LBA-4404, and hygromycin 50 mg/l for proliferation of transformed cells. GUS assay was performed in callus after 4-5 weeks co-cultivation. While GUS gene expression was not detected in control (Fig. 2), genotype IL-75 responded better for both *in vitro* culture and transformation. Significant differences observed in transformation efficiency of the L-2 and IL-75 might be purely due to

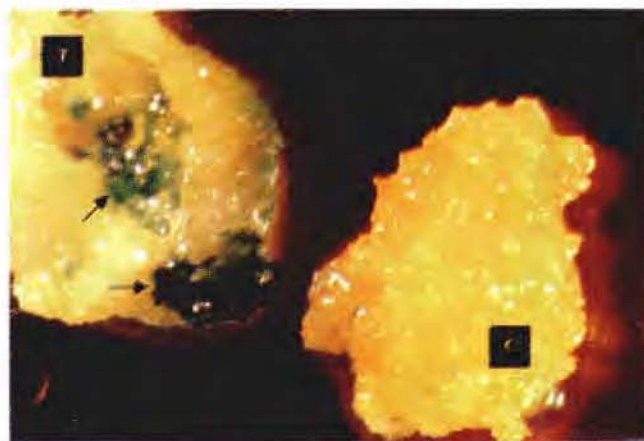


Fig. 2—Stable GUS gene expression in transformed hygromycin-resistant calli, 4 weeks after co-cultivation (T). Unstained chimeric region on the calli lost the gene expression. No expression in control (C).

their genotypic behaviour. GUS gene expression was observed in calli from 82 cotyledonary and 48 hypocotyl explants, out of 90 from each in a set of experiment with IL-75 genotype. The difference might be due to more wounded surface on cotyledon (wounding was easier) than that on hypocotyl and wounded cotyledons offered comparatively more sites for infection by *Agrobacterium*. Wounded leaves of *M. varia* plants gave four times higher transformation frequency than petioles (Chabaud *et al.*, 1988).

Although the effects of plasmid-vector/*Agrobacterium* strain on transformation efficiency were not examined by the authors, such effects of genotype, bacterial strain and vector combination have been observed by others (Byrne *et al.*, 1987; Desgagnés *et al.*, 1995; Du *et al.*, 1994). GUS gene expression was observed in 91% randomly selected calli proliferating on selection medium (ClmHC) in the form of blue spots (Fig. 2).

Significant difference in transformation efficiency was also observed with different concentration of bacterial cells used for co-cultivation. The optimal transformation efficiency of 91.3% ($\sigma = 3.05$) was obtained with 5×10^9 cells/ml (Table 1). Bacterial

Table 1—Effect of bacterial cell density on transformation efficiency

Explant/cells/ml	Transformation efficiency (%)			
	5×10^7	5×10^8	5×10^9	5×10^{10}
Cotyledons	43.3 ^d	66.6 ^c	91.3 ^a	76.3 ^b
Hypocotyl	21.3 ^c	36.3 ^b	53.6 ^a	42.3 ^b

Co-cultivation was continued for three days. Figures are the mean of three replications showing percentage of calli with GUS positive response. In a row, means followed by a different letter are significantly different at 5 per cent level of significance.

Table 2—Effect of duration of co-cultivation on transformation efficiency

Explant/Co-cultivation	Transformation efficiency (%)			
	2 days	3 days	4 days	5 days
Cotyledons	21.3 ^d	91.3 ^a	53.3 ^b	41.6 ^c
Hypocotyl	11.3 ^d	53.6 ^a	41.6 ^b	31.3 ^c

Optimal density (5×10^9 cells/ml) was used for co-cultivation. Figures are the mean of three replications showing percentage of calli with GUS positive response. In a row, means followed by a different letter are significantly different at 5 per cent level of significance.

concentration is an important parameter for efficient transformation (Chabaud *et al*, 1988). Further, co-cultivation of explants for three days recorded significantly higher (optimal) transformation efficiency (Table 2). When acetosyringone was added in the co-cultivation medium to induce bacterial *vir* genes, no significant difference in transformation efficiency was observed.

It is hoped that optimized parameters for *Agrobacterium*-mediated transformation of Indian cultivars of lucerne will be useful for the development of transgenic plants harbouring genes of interest, particularly for forage quality improvement.

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