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BRIEF COMMUNICATION

In vitro crown galls induced by *Agrobacterium tumefaciens* strain A281 (pTiBo542) in *Trigonella foenum-graecum*

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Abstract

Transformation of fenugreek (*Trigonella foenumgraecum*) was carried out with A281 oncogenic strain of *Agrobacterium tumefaciens* using root, cotyledon and hypocotyl explants excised from 1-week-old seedlings, which showed that the plant was highly susceptible to transformation. Tumors (calli) were selected on 50 mg dm^{-3} kanamycin. They were analyzed for β-glucuronidase (GUS) expression. Presence of *uidA* (*gus*) gene, was confirmed by polymerase chain reaction (PCR) amplification.

Additional key words: transformation, kanamycin resistance, GUS analysis, PCR.

The development of techniques for stably introducing foreign genes into plants has opened the way for new approaches to understanding basic plant processes and addressing agronomic problems. Manipulation of metabolic pathways (Muller-Rober *et al*. 1992), introduction of potentially useful agronomic traits such as insect, viral and herbicides resistance (Lamb *et al*. 1992, Koziel *et al*. 1993) and hybrid seed production by engineering male sterile plants (Mariani *et al*. 1990) are some examples of the potential usage of plant transformation technology. To date most of the achievements have been made in dicotyledonous species for which production of fertile transgenic plants have become routine. The range of dicotyledonous plants is wide; however, susceptibility varies between species, cultivars, explants and the bacterial strains used. The development of routine transformation protocols in fenugreek, an annual herb of legume family; is still major goal to achieve.

In order to develop a genetic transformation strategy for *T. foenum-graecum*, it is important to determine the best host pathogen combination (Lacorte and Mansur 1993). Tumor formation using wild strains of *A. tumefaciens* are an excellent model system for correlating phytohormonal regulation with structural and functional development. The purpose of this work was to determine the susceptibility of various explants of *T. foenum-graecum* to infection with wild strain of *A. tumefaciens* under *in vitro* conditions and advance the work for later transformation through non-oncogenic strains.

Seeds of *T. foenum-graecum* line No. 3 and No. 18 of were obtained from Department of Field Crops, Faculty of Agriculture, University of Ankara, Turkey. Both lines are selection from locally grown populations of fenugreek. They were surface sterilized using 100 % commercial bleach (*Axion*, Istambul, Turkey) for 15 min and subsequently washed three times with sterile distilled water. The seeds were germinated in *Magenta* vessels containing Murashige and Skoog (1962, MS) medium supplemented with 3 % (m/v) sucrose and 0.8 % (m/v) agar type A (*Sigma*, St. Louis, MI, USA). The pH of medium was adjusted to 5.6 before autoclaving at 121 °C, 1.2 kg cm^{-2} for 20 min.

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Abbreviations: GUS - β-glucuronidase; MS medium - Murashige and Skoog medium; PCR - polymerase chain reaction.
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Six to ten days old germinated seedlings were used for taking hypocotyl, cotyledon and root explants and immersed for 30 min in a 1:50 dilution (1×10^8 cm^{-3}) of overnight grown *A. tumefaciens* strain A281 (pTiBo 542)::pBI121.1 (Hood *et al*. 1986). After inoculation, explants were transferred to MS medium in glass Petri dishes and co-cultivated for 2 d. Following, co-cultivation, explants were transferred to fresh MS medium containing 500 mg dm^{-3} of bacteriostaticum augmentin (*SmithKline Beecham*, Istanbul, Turkey) and 50 mg dm^{-3} kanamycin (*Sigma*) for selection. Tumor formation on explants was monitored closely for 3 weeks.

All explants were cultured at 24 ± 2 °C with 16-h photoperiod provided by mixed daylight and radiation provided by *Sylvania* ^{R}*Grolux* fluorescent tubes (40 µmol m^{-2} s^{-1}) both for seed germination and tumor formation.

Tumorogenic *A. tumefaciens* strain A281 (pTiBo 542) was used in the study. The T-DNA of the vector for A281 (pTiBo 542) carries (*uidA*) gene coding β-glucuronidase under the control cauliflower mosaic virus CaMV 35S. Bacteria were cultured to log phase in agitated (200 rpm) NB medium (Armitage *et al*. 1988) at 28 °C for 12 - 18 h before use.

Histochemical GUS assays were based on methods described by Jefferson (1987). For histochemical staining, sliced tumors were incubated at 37 °C for 4 h to overnight in 100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1 % Triton X - 100 and 1 mM 5-bromo-4-chloro-3-indole glucuronide (X-GLUC). The tissue was then rinsed in 70 % ethanol and the presence of GUS enzyme activity was indicated by blue staining in the tissue.

DNA was isolated from transformed hypocotyls, cotyledon and root segments of 6 weeks grown tumors grown under *in vitro* conditions according to Dellaporta *et al*. (1983) and Edwards *et al*. (1991) with some modifications. They were frozen with dry ice and mashed with a disposable mortar. Following the addition of 0.4 cm^3 of extraction buffer containing 200 mM Tris-HCl (pH 7.5) 250 mM NaCl, 25 mM EDTA and 0.5 % SDS, the tissue was vortexed for 5 s. Following centrifugation for 1 min, 0.3 cm^3 of supernatant was transferred to a new tube containing 0.3 cm^3 isopropanol and incubated at room temperature for 2 min. The DNA was then collected by centrifugation, dried and resuspended in 0.1 cm^3 of double distilled water. Standard PCR techniques were used to detect *uidA* sequence in tumor samples from the selective explants following Özcan (1993). The *uidA* primers were Primer *uidA* (F): 5'-CCT TCG GTC TGT TGC CCG-3' and (R) 5'-CTG GCA GGC CTG TGG GAC TTC-3'. Polymerase chain reaction were conducted in 0.05 cm^3 reaction volumes using the following recipe: 0.005 cm^3 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl and 0.1 % Triton X-100), 0.04 cm^3 of 10 mM dNTP, 0.003 cm^3 of 25 mM $MgCl_2$, 0.002 cm^3 Taq DNA polymerase (*Promega*, Madison, WI, USA) and 0.004 cm^3 DNA. Amplification was performed using T gradient thermocycler (*Whatman Biometra,* Göttingen, Germany) that was set to run for 30 cycles at denaturation at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplified DNA was then visualized on agarose gel (Fig. 2).

A negative control was also planted for each of the genotype without treating the explants with *A. tumefaciens.* Tumor formation studies, histochemical GUS and statistical analysis along with PCR were done after 21 d to confirm the gene transfer.

Each treatment had 3 replicates containing 10 explants and was repeated twice. Significance was determined by analysis of variance (*ANOVA*) and the differences between the means were compared by Duncan's test using *SPSS 9 for Windows* computer program. Moreover standard error for the means was also calculated separately. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran 1967) before statistical analysis.

Visible tumors were detectable after 5 - 6 d of inoculation from all explants on selection media containing 50 mg dm^{-3} kanamycin and 500 mg dm^{-3} augmentin. The tumor diameter was 0.96 to 2 cm after 21 d (Table 1). The results were quite heterogeneous and very much influenced by the origin of the explant and genotype. No tumor or callus formation was observed on untreated (control) explants. Similarly no tumor was observed on root explants from line No. 18. Tumorous outgrowths were observed on all explants in line No. 3. All explants of line No. 3 were highly susceptible to *A. tumefaciens* and were tumors larger in size than those of line No. 18. Mean tumor diameter ranged between 0.76 to 0.96 cm in line No. 3 and 0 to 1.43 cm in line No. 18.

Table 1. Tumor formation induced by inoculation of *T. foenumgraecum* with *A. tumefaciens* strain A281. Means ± SE from hypocotyl, cotyledon and root explants which formed tumors. Values within a column followed by the different letters are significantly different at the 0.05 level.

Explant	Line No. 3		Line No. 18	
	formation [%]	diameter [cm]	formation [%]	diameter [cm]
Hypocotyl	100a	0.96 ± 0.09b	100a	1.11 ± 0.26ab
Cotyledon	100a	1.43 ± 0.91a	100a	1.00 ± 0.90b
Root	100a	2.00 ± 0.67a	0b	0.00 ± 0.00c

Tumors from hypocotyls and cotyledons were concentrated towards wounded edges in general; with callus formation in localized regions. However, tumors from roots resembled swellings of variable size (Fig. 1*a*,*b*,*c*). Anatomic slices (results not given) suggested that cells responding to wounding by

dedifferentiation and cell division were located with in or in the proximity of vascular tissues of hypocotyl explants. This might be explained by the fact that the T-DNA genes encode proteins which cause major alterations in the differentiation and development of the transformed plant cell and hence are responsible for neoplastic phenotype (Walden 1999) at the points of infection or in susceptible cells. *Agrobacterium* mediated tumors proliferate autonomously in the absence of the phytohormones (auxins and cytokinins) that are needed for growth of normal plant cells (Braun 1958), because of this property *in vitro* culture crown gall cells grow and form a callus even when the growth stimulating phytohormones are absent from the culture medium (Hooykaas and Schilperoort 1992) resulting in abnormal and unorganized proliferation of plant cells by causing overproduction of auxins and cytokinins (Ream 1989).

Sliced tumors from all explants stained blue which

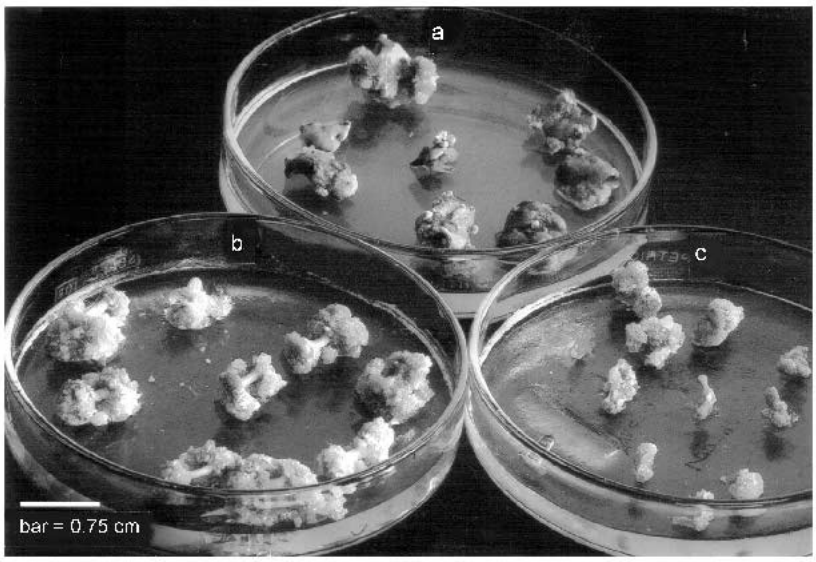

Fig. 1. Tumor formation on (*a*) cotyledon (*b*) hypocotyls and (*c*) root explants of line No. 3 of fenugreek (*T. foenum-graecum*) after 21 d of inoculation. *Bar* = 0.75 cm.

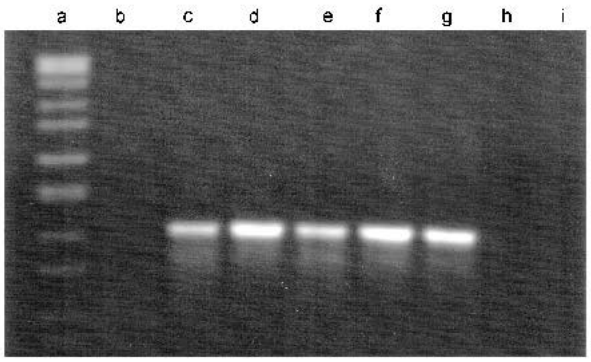

Fig. 2. PCR amplification from genomic DNA extracted from 21-d-old transformed tumors of fenugreek. *Lane a* - DNA ladder, *lane b* - non observance of band from untransformed root of line 18, *lanes c* and *d* - transformed cotyledon and hypocotyl tumors of Line 18, *lanes e* to *g* - transformed root, cotyledon and hypocotyl tumors of Line No. 3., *lanes h* and *i* - negative controls from Line No. 18 and 3. Length of amplified bands is 1 kb.

confirmed the transfer and expression of the *gus* gene in plant cells. GUS active and hence transformed cells showed irregular patchy distribution with regular growth in both genotypes. Variable staining and inconsistency between staining and tumor growth suggested an inhibition of GUS expression. GUS activity was not evident in all tumor cells especially those away from wounds had reduced or no GUS activity. This suggests that *gus* gene activity may be inhibited in some cells due to position effect at transcriptional or post transcriptional level or as result of truncated transgene sequences as suggested by (Kohli *et al*. 1999) resulting in chimeras or due to methylation of foreign genes (Rezmer *et al*. 1999). Moreover, it was felt that 35S CaMV promoter could not fully be relied for *in situ* localization of GUS expression. It also suggests that histochemical GUS assay does not enable the localization of transformed cells because the GUS expression is disturbed in some areas. To find the developmental pattern of tumors, it was considered necessary to know the exact localization of transformed cells. To prove that all tumor cells were transformed or most of them only habituated by increased phytohormone contents produced by few transformed cells, it was considered necessary to perform PCR. PCR analysis showed that all cells of the tumor were transformed and contained *uidA* gene or *gus* gene (Fig. 2), irrespective of their GUS expression. Aloni *et al*. (1995) has shown that characteristic of *Agrobacterium* induced crown gall is the setting up of a complex network of vascular tissue (globular bundle structure). The action of cytokinins in

the control of vascular differentiation particularly in the earlier steps is proposed but not demonstrated (Azmi *et al*. 2001). Most T-DNAs harbor growth inducing or growth modifying genes (Gaudin *et al*. 1994, Meyer *et al.* 2000). Among those *iaa* and *ipt* genes and their products have been studied in detail. They code enzymes that synthesize indole-3-acetic acid (IAA) and isopentyl adenine, respectively, and are the major factors responsible for tumor induction by *Agrobacterium*. Besides *iaa* and *ipt* genes, other T-DNA genes have been found to influence tumor development. Many of which belong to a highly diverged family defined on the basis of weak and partial protein homologies (Helfer *et al*. 2002).

The present study combined tumor formation with histochemical GUS analysis to investigate the process of transformation in *T. foenumgraecum* and we arrived at the following conclusions: *1*) Transformation does not automatically imply GUS expression in all cells; *2*) for fruitful transformation with non oncogenic strains of *A. tumefaciens*, cotyledon and hypocotyl explants could be relied upon fully and roots explants partially; *3*) bacteriostatic augmentin did not impair the growth and regeneration of *A. tumefaciens* treated tissues; *4*) tumors (calli) were kanamycin resistant.

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