Establishment of a transformation system in some Egyptian cultivars of *Vicia faba* L.

(Accepted: 06.06.2001)

Roba M. Ismail*, F.M. El-Domyati, A.S. Sadik*,***, T.M. Nasr El-Din* and A.Z.E. Abdelsalam****
* Agricultural Genetic Engineering Research Institute (AGERI), ARC, P.O.Box 12619, Giza, Egypt
** Department of Genetics, Faculty of Agriculture, Ain Shams University, Shubra El-Kheima, Egypt
*** Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shubra El-Kheima, Egypt

ABSTRACT

The present investigation was designed to establish a transformation systems for two cultivars of faba bean (cv. G 461 and G 674). In this study, the cotyledons and shoot apex explants of the two cultivars were transformed via Agrobacterium-mediated gene transfer. The Agrobacterium strain (EHA101) was transformed by introducing the pBI121 plasmid carrying NPTII and GUS genes, which was produced in a large-scale from a recombinant E. coli. The explants were then transformed by co-cultivation with the recombinant Agrobacterium in VSI liquid medium for 24 and 48 hr. Using microprojectile bombardment, the cotyledon explants and calli derived from shoot apex explants were transformed by shooting with different concentrations of the recombinant DNA plasmid at a distance of 6 cm and 1100 or 1350 psi. The transformants were selected on VSI medium with 100 mg/l kanamycin. The results showed that the DNA concentration of 500 ng/ul and 1100 psi were the most suitable conditions for explant transformation. The transformants were evaluated for the presence of NPTII by PCR and Southern blot hybridization with a DNA probe labelled with ^{32}P, while the expression of the introduced GUS gene was determined by GUS assay.

Key words: *Faba bean, Agrobacterium-mediated gene transformation, microprojectile bombardment, GUS assay, NPTII gene, PCR, Southern blot.*

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important legumes worldwide. It is grown as a winter annual crop in warm temperate and subtropical areas and it is now introduced and cultivated in North and South America, China and Uganda. Egypt is reported to be the third country in the production of faba bean as we produce 444,800 metric tons (Anonymos, 1997). Faba bean is not only important for human nutrition but also for animal feed due to its high protein content, as the dry seeds contain 25-30% protein with almost 50-60% soluble carbohydrates, 10-15% fibers and 5% minerals especially phosphorus and potassium (Saleh, 1998).

A dramatic reduction in seed yield/ha and total production of faba bean is mainly due to the infection by different pathogens that decrease the crop. Traditional plant breeding so far has not overcome this problem. The recent development in recombinant DNA technology (Antonio *et al.*, 1988) and plant transformation (Depicker *et al.*, 1983; Selva *et al.*, 1989) may allow alternative approaches to enhance plant resistance to such pathogens.

Recent advances in genetic engineering have clearly demonstrated the possibility of incorporating foreign genes for desired agronomic traits, while preserving the existing characteristics of improved genotypes (Powell-Abel *et al.*, 1989).

The interest in tissue culture research on legumes has increased considerably, as an important initial step in introducing new genes through recombinant DNA technology (Schroeder *et al.*, 1984; Thomashow, 1984., Dhir *et al.*, 1991). Through this technique, improving nutritional quality, resistance to environmental stress, resistance to viral diseases and increasing the yield of legume crops can be achieved (Hussey *et al.*, 1989).

This work was designed to establish a transformation system for *V. faba* cvs. Giza 461 and Giza 674 *via Agrobacterium*-mediated gene transfer system and/or microprojectile bombardment using neomycin Phospho-transferase (*NPTII*) and -glucuronidase (*GUS*) genes and to evaluate the transformed tissues and/or plants by polymerase chain reaction (PCR), Southern blot hybridization and histochemical assay.

MATERIALS AND METHODS

The present work was carried out at the gene transfer laboratory (GTL), Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Giza, Egypt.

Source and preparation of explants

Two cultivars of *Vicia faba* L. (G 461 and G 674) were obtained from the Field Crops Research Institute, ARC, Giza, Egypt and used as a source of explants. Mature seeds were surface sterilized by soaking them in 70% ethanol for 30 seconds, then rinsed in sterile distilled water (d.H_2O) before transferring to 20% commercial household bleach for 10-15 min with 1-2 drops of Tween 20 followed by washing 5-6 times with d.H_2O. Seeds were germinated in a pre-autoclaved wet cotton pads and mature cotyledon and shoot apices were excised and scratched using a sharp scalpel from the *in vitro* grown seedlings of the two cultivars (10-13 days old).

Bacterial strains and plasmids

Agrobacterium tumefaciens strain EHA101 was kindly obtained from Dr. Stan Gelvin (Department of Biological Science, Purdue University, West Lafayette, USA). *Escherichia coli* strain DH5α and the plasmid pBI121 containing *NPTII* and *GUS* genes were obtained from CLONTECH Inc. (CA, USA). A single colony of *E. coli* strain DH5α carrying the plasmid pBI121 was inoculated in a 5 ml liquid LB medium containing kanamycin (100 µg/ml) and grown to late-log phase.

Kanamycin sensitivity

In this experiment, different concentrations of kanamycin : 25, 50, 75, 100, 125 and 150 mg/l were used. For each concentration, 40-50 explants were tested. The percentage of surviving explants was recorded after 4 weeks from culture.

Isolation, purification and digestion of the plasmid DNA

The DNA pBI121 plasmid was isolated and purified according to the method of Sambrook *et al.* (1989) from a volume of about 750 ml of LB medium containing kanamycin sulfate and inoculated with 5 ml of the late-log phase culture. The DNA pellet(s) was dried and resuspended in 500 l of 1X TE (pH 8.0). The plasmid DNA (1g) was subjected to digestion with 1 unit of each of *Bam*HI+*Eco*RI (for *GUS* gene+NOS-ter) or *Pst*I (for *NPTII*) enzymes as given by Khalil (1999). The digested plasmids were

electrophoresed on an agarose mini gel (1%) and stained with ethidium bromide. The bands were checked for the completion of digestion using UV transilluminator.

Transformation of *Agrobacterium*

The 3-keto-lactose test was used for detection of *A. tumefaciens* as described by Bernaertd and Deley (1960; 1963) and Khalil (1999). The *Agrobacterium* competent cells were prepared as described by Grumet (1990) and Khalil (1999). The pBI121 plasmid was introduced into the cells of the *Agrobacterium* strain EHA101 by adding 0.5 µg DNA of recombinant pBI121 plasmid to 100 µl of competent *Agrobacterium* cells according to the method of Khalil (1999). The transconjugated *Agrobacterium* cells containing the plasmid were selected on a solidified LB medium (LB+15 g/l Bactoagar) supplemented with 100 mg/l kanamycin + 25 mg/l rifampicin and incubated for 2 days at 28°C. The PCR technique was also used for detection of *NPT II* gene as reported by Khalil (1999).

Agrobacterium* transformation of *V. faba

A single colony from *A. tumefaciens* EHA101 strain, transformed with the pBI121 construct was cultured overnight in 5 ml of LB liquid medium supplemented with 50 mg/l kanamycin at 28°C in a shaking incubator. Sixty cotyledon and shoot apex explants from *V. faba* cvs. G 461 and G 674 were used for transformation. The explants were placed into 200 ml glass jars containing 30 ml of broth MS medium free of bacteria on a rotary shaker (120 rpm) at 25°C for 24 hr. The jars were then inoculated with 0.3 ml of the culture of *A. tumefaciens* strain EHA101 10-fold diluted in LB broth medium (OD 600nm = 0.1) and incubated for 24 and/or 48 hr. The treated materials were then washed three times with d.H_2O and placed after 24 or 48 hr on a suitable regeneration medium supplemented with 100 mg/l kanamycin and 300 mg/l carbincillin. After three weeks, the number of kanamycin resistant explants that produced calli as well as the survived calli were determined. As control, a similar number of explants was treated as described above with no *Agrobacterium* inoculation.

Transformation of *V. faba via* micro-projectile bombardment

Particle sterilization and coating

Tungsten particles (M10) provided by Sylvania Chemicals /Metols Towanda, PA and 50 mg of particles in 500 µl absolute ethanol in a 1.5 microfuge tube were used. Particles were rinsed 3 times in sterile d.H_2O by repeated vortexing, centrifugation and resuspension in 500 µl d.H_2O. The particles were then coated with DNA by mixing 25 µl of tungsten particle suspension (2.5 mg) with 5-7 µg of DNA, 25 µl of 2.5 M $CaCl_2$ and 10 µl of 100 mM spermidine (free base). After allowing the particles to settle for 5 min at 4°C, 50 µl of the supernatant were removed. For precipitation, the microfuge tube was vortexed after the addition of each component and the total procedure was performed as fast as possible. The concentrated pellet mixture was gently resuspended and 2 µl were used for bombardment (Finer and McMullen 1990; Oard 1993; Vain *et al.*, 1993, Chlan *et al.*, 1995).

Preparation of explants for bombardment

Prior to bombardment, the shoot apex and cotyledon explants were stored on a suitable solidified vicia shoot induction (VSI) medium for 24 hr prior to bombardment. About 30 tissue sections with apical meristems were placed in the center of a 10-15 mm disposable Petri dish containing the solidified shooting culture medium. The tissues were stored at 25°C in a moderate light until

bombardment within 3 to 24 hr (Chlan *et al.*, 1995).

Bombardment of explants

Embryogenic calli derived from shoot apex and cotyledon explants were bombarded using the Biolistics Particle Delivery System (Bio-Rad PDS/1000/He gene gun). In this experiment, the following conditions were used: microcarrier (tungsten), 1.1 and 1.6 μM (60 mg/ml d.H_2O); rupture disk macrocarrier (6 mm), microcarrier travel distance (8 mm); chamber vacuum (25 in Hg); Helium pressure (1350 kg/cm^2, and 1100 Kg/cm^2). Fifteen l of the tungsten/DNA complex were placed on a macrocarrier and placed into the acceleration tubes, and then acceleration was loaded and became ready for firing. When the sample chamber was evacuated till the chamber pressure was at vacuum (28 inches Hg), the PDS-1000 was fired. Samples were bombarded 3-5 times according to Sautter (1993) and Becker *et al.* (1994).

Selection of transgenic tissues

The explants transformed with *Agrobacterium* or with microprojectile bombardment technique were placed on antibiotic-free VSI medium for 3 days. Then, proliferating cultures were subcultured onto the same medium containing 100 mg/l kanamycin for two weeks at 28°C2 in the dark, then transferred to the light for 12 hr photoperiod with the second subculture. The kanamycin resistant tissues were subcultured on a selection medium (VSI + kanamycin) every two weeks. Dead tissues (non-transgenic tissues) were cut off in each subculture and yellow-green tissues were transferred to a fresh selection medium. The explants were then subjected to histochemical and molecular tests.

Histochemical assay of transgenic tissues

For GUS assay, three days after transformation, a number of transformed explants or calli were soaked in 1 ml GUS buffer (Daniell *et al.*, 1991) containing 1 mg X-Gluc (5-bromo-4-chloro-3-indolyl) glycuronide and rapped with aluminum foil to prevent light effect (Jefferson *et al.*, 1987). The mixtures were then incubated for about 24 hr on a rotary shaker (160 rpm) at 37°C for color development. Samples were treated with 10% commercial bleach solution to remove pigments such as chlorophyll from shoot apices.

PCR detection of transformed plant materials

Samples collected from transformed explants or calli of *V. faba* and the corresponding control (non-transformed explants) were subjected to isolation of DNA according to the method described by Xu *et al.* (1993). Two specific primers were designed based on the nucleotide sequence of *NPTII* gene (Khalil, 1999) and synthesized at AGERI in DNA/RNA Synthesizer. The nucleotide sequences of the primers were, **KI**, 5' AGA CAA TCG GCT GCT CTG ATT 3' and **K2**, 5' GAC TAC GAG AAG CAG GTC TAA 3'. The PCR reaction was conducted in a volume of 50 l as described by Khalil (1999) using 1 l DNA template (100-150 ng). This mixture was covered with 50 l mineral oil and then pre-heated for 4 min at 94°C and subjected to 35 cycles: 94°C /1 min, 55°C /2 min and 72°C /1 min. The reaction was then exposed to 72°C /5 min for one cycle. The PCR products were detected in the presence of standard DNA marker by electrophoresis in 1% agarose gel for 1 hr at 60 volt and the bands were visualized by staining the gel with ethidium bromide and exposing to UV-transilluminator.

Southern hybridization detection

DNA blotting

The previous gel representing the PCR detection was rinsed with d.H_2O and the PCR DNA product was transferred onto a nitrocellulose membrane as described by Sambrook *et al.* (1989). The DNA was fixed by exposure of the membrane in a Saran Wrap to UV for 5 min (Harding *et al.*, 1991).

Labeling the DNA probe

In this experiment, Gene Clean purification system (BIO 101) (Bio-Rad) was used to purify PstI- digest DNA fragment (obtained from pBI 121) from the agarose gel. Radioactive labeling system has been used to analyze Southern blot by Ready-to-go DNA labeling system from dCTP Pharmacia P-L Biochemicals.

Hybridization

Pre-hybridization and hybridization were carried out either in heat sealed polyethylene bags contained in plastic boxes or in hybridization tubes based on the method given by Sambrook *et al.* (1989) and Khalil (1999). For detection of ^{32}P signals, membranes were wrapped in Saran Wrap and exposed to an X-ray film (Kodak X-Omat) in a light-proof cassette with intensifying screen and incubated for 3 or 6 hr at -80°C. The film was then developed and replaced with another one for long exposure.

RESULTS AND DISCUSSION

The production of transgenic plants in legumes has been reported in a few number of species using delivery systems based on *A. tumefaciens* and *A. rhezogenes* (Francesco and Arcioni, 1991) and also by electroporation (Arago *et al.*, 1993; Collen and Jarl, 1999).

Kanamycin sensitivity

Kanamycin sensitivity should be determined in the initial stages of developing a plant transformation system (Hauptmann *et al.*, 1988). In this experiment, kanamycin sensitivity of growth and differentiation of *V. faba* tissues, which were used in the establishment of transformation was determined. Sensitivity affects the recovery of the transformed plant, which varies widely among tissues and species. It was also important to apply kanamycin selection to well grown rootless shoots, as the lack of kanamycin is essential in root production from transgenic shoots as recommended by Fontana *et al.* (1993). James *et al.* (1989) reported that the use of kanamycin for selection has been shown to be effective especially in the root development phase.

In this investigation, shoot apex and cotyledon explants were cultured under the same conditions used for regeneration. The results showed that increasing the kanamycin concentration was accompanied by a parallel decrease in the percentage of the surviving explants. In the case of cv. G 461, a concentration of 25 mg/l kanamycin decreased survival to 70% with a percentage 50% of callus formation. However, a percentage of 35% surviving was recorded at 50 mg/l kanamycin and the formation of callus was decreased to about 16%. No surviving calli were observed when 100 mg/l kanamycin was used. The concentration of 100 mg / l kanamycin was then chosen as a selection criterion to test the transformed tissues in the two cultivars (G 461 and G 674) using the two explants (shoot apex and cotyledon). In the case of cv. G 674, a concentration of 25 mg/l decreased survival to 60% with a percentage of 30% of callus formation. However, a percentage of about 40% survival was recorded at 50 mg/l kanamycin and the formation of calli was decreased to about 10%.

A higher concentration of kanamycin (75 mg/l) decreased the survival percentage to 20% with no callus formation. Furthermore, increasing kanamycin concentration (up to 200 mg/l) resulted in no surviving explants and no callus formation. No surviving explants or callus formation were observed when 100 mg/l kanamycin was used (Fig. 1). The obtained results were confirmed by statistical analysis (data not shown).

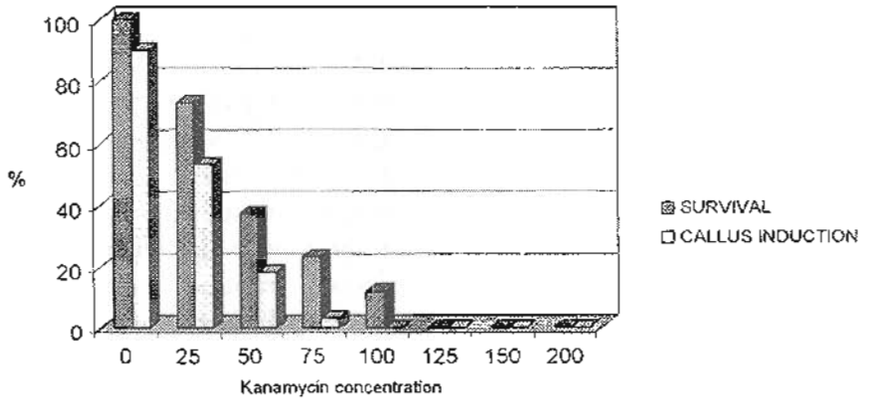

Fig. (1): Effect of kanamycin concentration on non-transformed tissues of V. faba.

Transformation of *Agrobacterium*

Testing the *Agrobacterium* strain before conducting the transformation is one of the most important steps in transforming *V. faba* or any other plant. As studied by Bernaertd and DeLey (1958), 3-ketolactose derivative is an intermediate in bacterial carbohydrate metabolism. Bernaertd and DeLey (1963) found that *Agrobacterium* is characterized by excreting a disaccharide that can be oxidized in the glycosyl radical at carbon 3. In this respect, the production of 3-ketolactose was reported to be a successful test for this step. In the present study, *A. tumefaciens* strain EHA101 was re-tested for 3-ketolactose production and showed a positive result (yellowish color in and around the bacterial growth was seen with the 3-ketolactopositive reaction due to the production of copper ioxide from Benedict solution).

The pBI121 plasmid containing the *GUS* and *NPTII* genes was confirmed by digestion using *Bam*HI /*Eco*RI restriction endonucleases. Results in Figure (2) showed that two fragments with a size of about 2205 and 10795 bp were fractionated. These fragments belong to the *GUS* gene with the NOS terminator and the rest of the plasmid, respectively. The plasmid pBI121 was then introduced into the disarmed *A. tumefaciens* strain EHA101.

The transformed *Agrobacterium* colonies were firstly cultured on LB medium containing 50 mg/l kanamycin and 25 mg/l streptomycin to confirm their capacity to resist the antibiotics. While the non-transformed *Agrobacterium* colonies failed to grow on the same medium. The second test was PCR, which was used to detect the presence of the construct in the *Agrobacterium* colonies resisting kanamycin. Using two primers specific to *NPTII* gene, a fragment with a size of about 870 bp was amplified from transformed *Agrobacterium* as shown in Figure (3), but no PCR product was obtained from the non-transformed *Agrobacterium* (control).

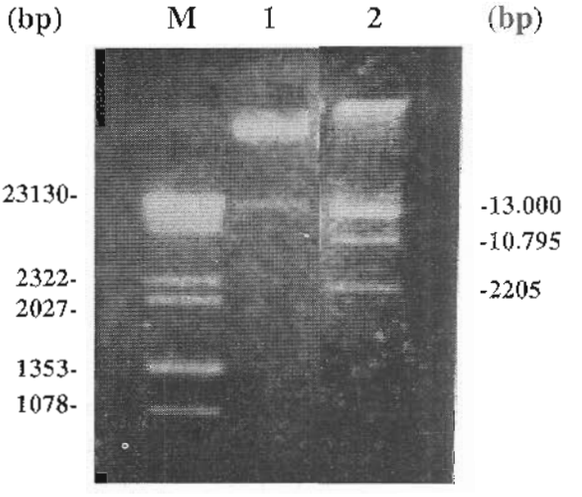

Fig. (2) : Electrophoresis separation of the digestion of plasmid pBI121 with different restriction enzymes. Separation was carried out on 1% agarose gel and stained with 0.5 μg/ml ethidium bromide and visualized by ultraviolet irradiation. M, λ / HindIII-ΦX174/HaeIII (DNA Marker). Lane 1, undigested plasmid. Lane 2, digested plasmid (pBI121) using EcoRI and BamHI.

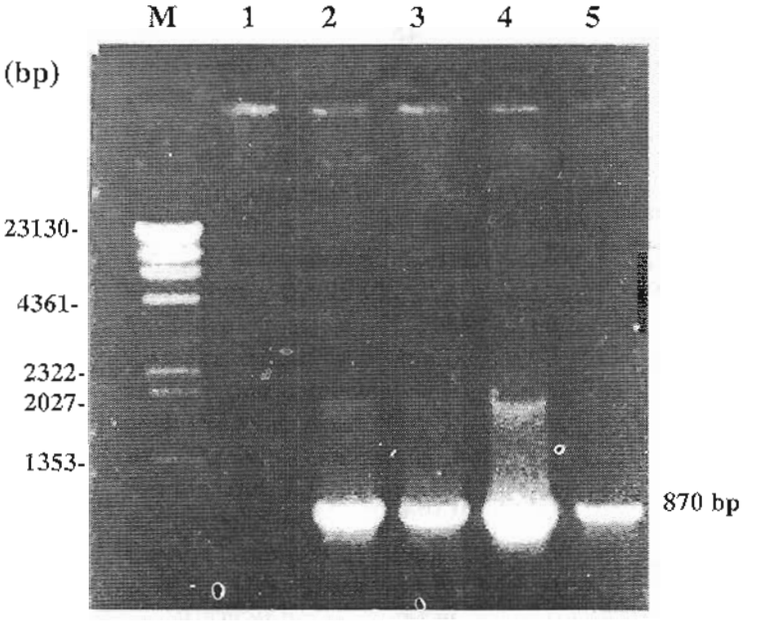

Fig. (3): PCR detection of NPTII gene in the A. tumefaciens strain EHA101 carrying pBI121 plasmid. The PCR product was fractionated on 1% agarose gel and stained with 0.5 ug/ml ethidium bromide. M, λ / HindIII-ΦX174/HaeIII (DNA Marker). Lane 1, negative control (non-transformed Agro.). Lane 2, positive control (pBI121 plasmid). Lanes 3-4, transformed Agro. by pBI121 plasmid.

Agrobacterium* transformation of *V. faba

It is well known that one of the requirements for a successful plant transformation using *Agrobacterium* is the ability to regenerate plants from the explants, which are difficult to obtain in legumes (Fontana *et al.*, 1993). Data in Table (1) show that 48 hr co-cultivation time was found to be more effective than 24 hr for transformation of cotyledon explants from cv. G 461 as 56.6% of the explants showed kanamycin resistance. On the other direction, 24 hr period was found to be effective than 48 hr for cv. G 674, as 53.1% of the treated explants were resistant to kanamycin (100 mg/l). In the case of shoot apex explants, only 2 (3.3%) and 1 (1.6%) out of the 60 transformed explants were found to be resistant to kanamycin after 24 hr from co-cultivation with *Agrobacterium* for cvs. G 461 and G 674, respectively, as shown in Table (1). Data also show that no resistant calli were found after 48 hr post co-cultivation with *Agrobacterium* for both cultivars.

Table (1): Selection of kanamycin-resistant explants co-cultivated with A. tumefaciens carrying pBI121 plasmid containing NPTII and GUS genes.

Cultivar	Explant	Co-cultivation time (hr)	Kanamycin-resistant explants	
			No.	%
G 461	Cotyledons	24	30	50.0
		48	43	56.6
	Shoot apex	24	02	3.3
		48	00	0.0
G 674	Cotyledons	24	32	53.1
		48	29	45.1
	Shoot apex	24	01	1.6
		48	00	0.0

Sixty explants were used per treatment.

Ramsay and Kumer (1990) studied the transformation of cotyledon and stem slits of *V. faba*. They found that the transformed cotyledon explants were more regenerable than stem slits. Mohamed (1999) reported that the highest transformation efficiency, using *A. tumefaciens* strain EHA101, was obtained with 0.8 optical density when cotyledonary node explants were incubated for one hour with the bacterial suspension. In the present study, the calli obtained from shoot apices were found to be regular. Comparable results were reported by Pickardt *et al.* (1991), who obtained approximately 18% regular calli using shoot tips as explants of *V. narbonensis* inoculated with *A. tumefaciens*.

Biolistic gun transformation of *V. faba*

Klein *et al.* (1989) and McCabe *et al.* (1988) initially demonstrated the biolistic approach. In this technique, DNA is introduced into plant tissues for studies of tissue-specific gene expression. However, studies are still required to evaluate the applicability of this technique in bean cultivars. In the present study, two concentrations of DNA, two sizes of rupture disks, and two types of explants (shoot apex and cotyledon) for the two cultivars (cvs. G 461 and G 674) were used as shown in Table (2).

The obtained data show that the transformation of *V. faba* cvs. G 461 and G.

674 was more successful using 500 ng/µl DNA and 1100 psi than using 250 ng/µl DNA and 1350 psi using cotyledon explants, as of transformed explants about 90.9 and 95% were obtained for G 461 and G 674, respectively, with the previous optimum conditions. Data also show that the use of shoot apex explants for transformation of the two cultivars *via* microprojectile bombardment was not effective, as explants as well as calli showed no kanamycin resistance. A similar relationship between DNA concentration and transformation frequency was obtained by Chowrira *et al.* (1995), who revealed that the increase of DNA concentration from 100 to 200 mg/ml had increased the transient expression from 13 to 25% of plants electroporated. This result may be supported by the fact that high pressure could cause severe injuries to the cells, depending on the type of tissue as reported by Dekeyser *et al.* (1990).

Table (2): Effect of different DNA concentrations and pressures on transformation of V. faba cotyledon explants and calli from shoot apices using biolistic gun.

Cultivars	Treated tissues	DNA concentration (ng/µl)	Pressure vacuum (psi)	% TE
G 461	Cotyledon	250	1100	50.0
			1350	37.6
		500	1100	90.9
			1350	78.6
	Callus	250	1100	0.00
			1350	0.00
		500	1100	0.00
			1350	0.00
G 674	Cotyledon	250	1100	93.8
			1350	66.6
		500	1100	95.0
			1350	65.5
	callus	250	1100	0.00
			1350	0.00
		500	1100	0.00
			1100	0.00

Fifty explants were used per treatment %TE: percentage of transformed explants

Histochemical detection of transgenic tissues

Explants (from cotyledons) and calli (from shoot apices) co-cultivated with *Agrobacterium* were tested for *GUS* expression after 3 days as the first evidence of transformation. The highest expression was mainly localized in the cotyledon explants (26 out of 50 explants), while untreated calli did not show any activity (Fig. 4). On the other hand, calli derived from shoot apices showed a very little expression, as 2 out of 50 calli for each of cv. G 461 and cv. G 674 showed blue color in the GUS assay (Fig. 5). This result agrees with that found by Hussey *et al.* (1989) who reported that the younger the tissue the more rapidly pea shoots can be transformed by the wild type of *Agrobacterium*.

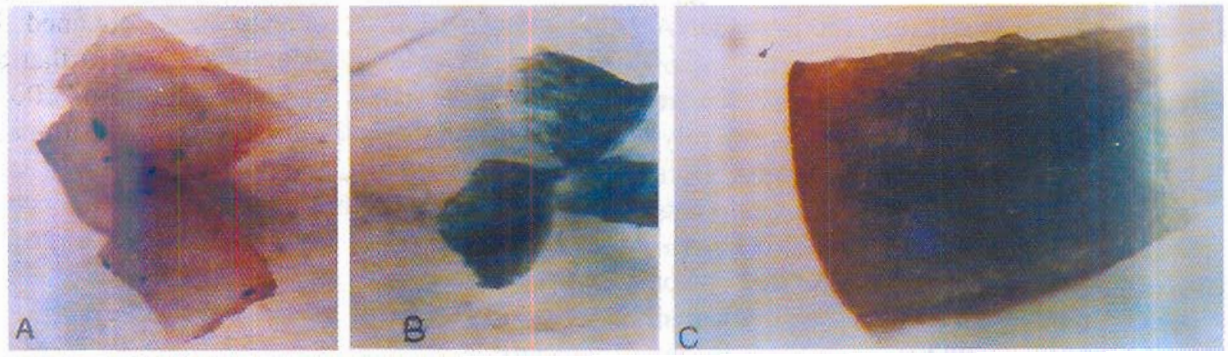

Fig. (4): Histochemical assay of GUS gene in transformed cotyledon explants in V. faba via Agrobacterium transformation. (A) non-transformed explants.(B) transformed explants of cv. G 461 showing blue color. (C) transformed explants of cv. G 674 showing blue color.

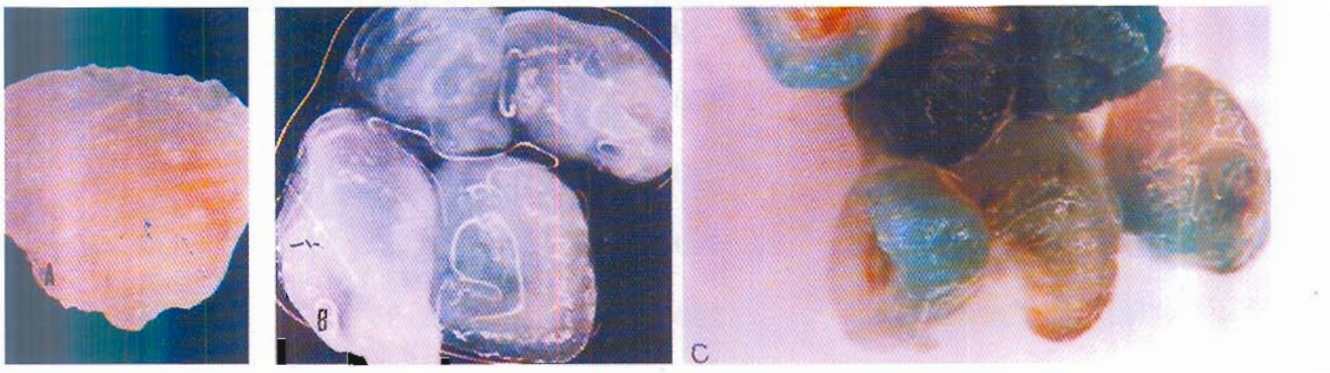

Fig.(5): Histochemical assay of GUS gene in transformed calli derived from shoot apex explants in V. faba via Agrobacterium transformation. (A) non-transformed callus. (B) transformed callus tissues of V. faba cv. G 461 showing blue color. (C) transformed callus tissues of V. faba cv. G 674 showing blue color.

It was noticed that for cotyledon explants, the *GUS* assay reaction was obvious 24 hr post incubation with GUS buffer at 37 C, while the transformed shoot apex tissues were blue stained after 48 hr, for the two cultivars G 461 and G 674. *GUS* expression of bombarded explants obtained from the two *V. faba* cultivars was determined by histochemical assay, after 3 days from bombarding. All tissue sections of stained and unstained regions exhibited *GUS* activity, as microscopic observations revealed localized blue color inside the tissue cells. How, staining was never seen in the whole tissues being assayed (Figure 6). In bean (*Phaseolus vulgaris* L.), Arago *et al.* (1993) reported the parameters influencing the expression of the *GUS* gene in bean embryonic axes, cotyledons, apical meristems and leaves followed by evaluation after gene delivery with an electrical particle acceleration device. They illustrated that bean gave low level of *GUS* expression in cotyledons.

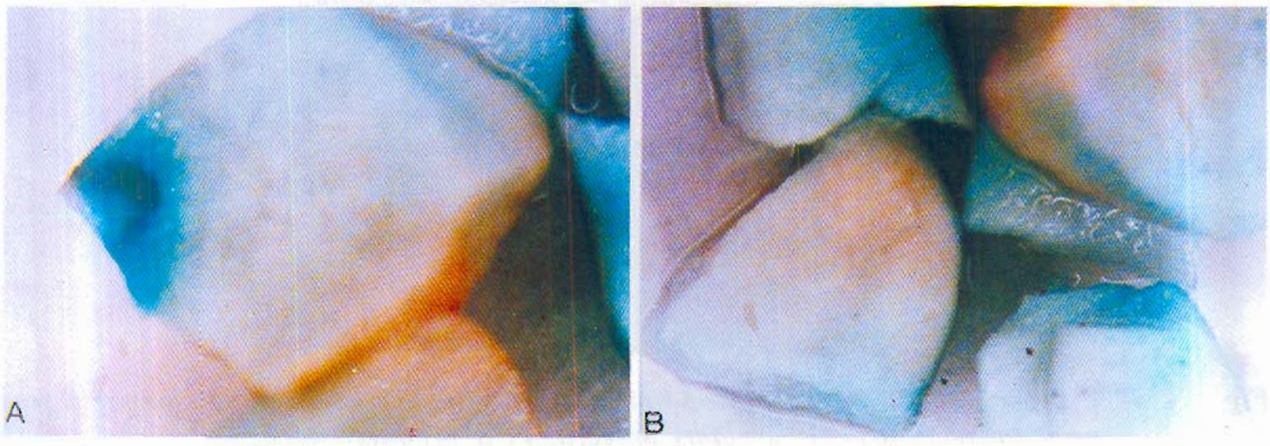

Fig. (6): Histochemical assay of the GUS gene in transformed cotyledon explants of V. faba after 48 hr from transformation by bombardment. (A) explants of the cv. G 461 with 500 ng/ µl DNA plasmid. (B) explant(s) of the cv. G 461 with 250 ng/µl DNA plasmid.

PCR detection

The PCR is a sensitive technique allowing single-copy genes to be amplified and extracted out of a complex mixture of genomic sequences. Amplified DNA is visualized as distinct bands on agarose gels (Innis and Gelfand, 1990). PCR was utilized in this investigation for the rapid screening of *NPTII* gene in the kanamycin-resistant explants. For screening total genomic DNA was isolated from explants of several transformants. Using two primers specific to *NPTII* gene (K1 & K2) a PCR product with a size of about 870 bp was amplified when the total nucleic acid extracts prepared from the transformants were used as templates. The obtained result confirmed the presence of *NPTII* in the transformants. DNA of non-transformed explants was used as a negative control and DNA of pBI121 was used as a positive control, as shown in Figure (7).

Southern blot hybridization

To confirm that the PCR amplified fragment is considered as a typical *NPTII* coding sequence, the fragment labelled with ^{32}P obtained from the pBI121 plasmid digested with Pst1 enzyme was used as a DNA probe. The PCR product was resolved by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with the ^{32}P -labeled DNA probe. The probe did not show any hybridization signals with the negative control nucleic acids, but hybridized with the PCR products derived from the transformants as illustrated by Figure (8). This result confirmed the integration of the insert into the plant DNA genome.

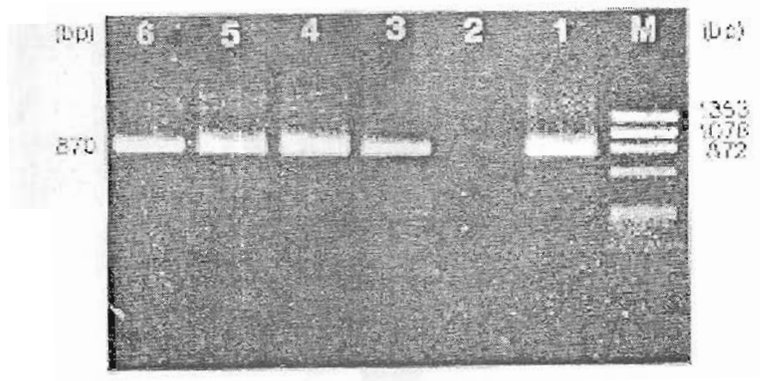

Fig. (7): PCR products (870 bp) amplified from the total nucleic acid extracts prepared from some putative transgenic explants or calli. PCR products were fractionated on 1% agarose gel stained with ethidium bromide. M, ΦX174 DNA standard marker digested with HaeIII. Lanes 1&2, positive and negative controls, respectively. Lanes 3-4, PCR products amplified from putative transgenic explants. Lanes 5-6, PCR products amplified from putative transgenic calli.

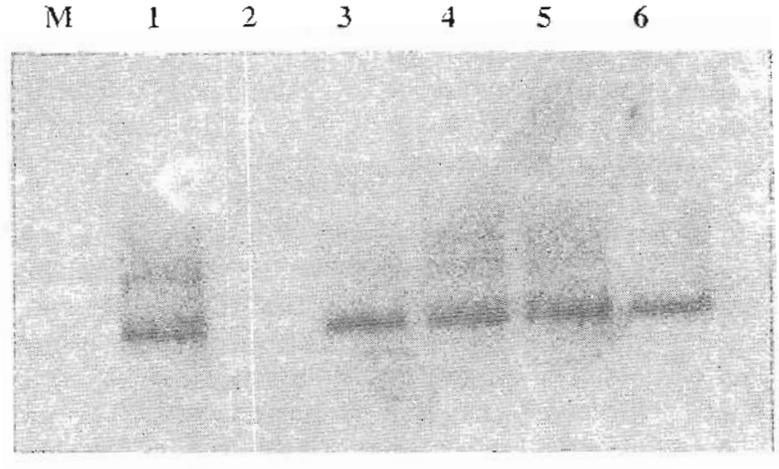

Fig. (8): Southern blot detection of PCR products amplified from the total nucleic acid extracts prepared from some putative transgenic explants or calli. The hybridization was done using a DNA probe labelled with ^{32}P. M, ΦX174 DNA standard marker digested with HaeIII. Lane 1, positive control, Lane 2, negative control, Lanes 3-4, putative transgenic explants, Lanes 5-6, putative transgenic calli.

In conclusion, using *A. tumefaciens* binary vector pBI121 carrying the *NPTII* and *GUS* genes, transgenic calli of *V. faba* expressing B-glucuronidase activity was obtained. The presence of the *NPTII* gene was confirmed by PCR analysis and kanamycin resistance appeared as a reliable marker in faba bean transformation. The present results also indicated that the cotyledon explants of *V. faba* cvs. G 461 and G 674 were more susceptible to be transformed than the calli derived from shoot apex explants.

ACKNOWLEDGMENTS

The authors would like to express their cordial thanks and sincere indebtedness to Prof. Dr. Magdy A. Madkour, Director of AGERI and Prof. Dr. Gharib A. Gad El-Karim, Head of Research and Head of Biocomputing and Network Unit, AGERI for their sincere help throughout this study.

REFERENCES

Anonymous (1997). Ministry of Agriculture and Land Reclamation (MALR), Agriculture Research Center (ARC), Field Crops Research Institute Annual Progress Rep. 1997.

Antonio, C., E. Selva, M. Briquet and M. Boutry (1988). Transformation of *Vicia faba* explants with *Agrobacterium tumefaciens*. Archives Internationals de Physiologie et Biochimie 96: 8153.

Arago, F.J.L., M.F. Grossidesa, M.R. Davey, A.C.M. Brasleiro, J.C. Faria and E.L. Rech. (1993). Factors influencing transient gene expression in bean *(Phaseolus vulgaris* L.) using an electrical particle acceleration device. Plant Cell Rep.12: 483-490.

Becker, D., R. Brettschneider and H. Lorz (1994). Fertile transgenic wheat from microprojectile bombardment of scuttelar tissue. Planta 5:299-307.

Bernaertd, M.J. and J. DeLey. (1958). Biochem. Biopys. Acta 30:661-662.

Bernaertd, M.J. and J. DeLey (1960). Microbial formation and preparation of 3-ketoglycosides from disaccharide s. Gen. Microbiol. 42:572-606.

Bernaertd, M.J. and J. DeLey (1963). A biochemical test for crown gall bacteria. Nature 197: 406-407.

Chlan, C.A., J. Lin, J.W. Cery and T.E. Cleveland (1995). A procedure for biolistic transformation and regeneration of transgenic cotton from meristematic tissue. Plant Mol. Biol. 13: 31-37.

Chowrira, G.M., V. Akella and P.F. Lurquin (1995). Electroporation-mediated transfer into intact nodal meristems in plants. Molecular Biotechnology, 3: 549-557.

Collen, A.M.C. and Jarl, C.I. (1999). Comparison of different methods for plant regeneration and transformation of the legume *Galega orientalis* Lam. (goat s rue). Plant cell Rep., 19: 13-19.

Daniell, H., M. Krishnan and B.F. Mcfadden (1991). Transient expression of β-glucuronidas in different cellular compartments following biolistic delivery of foreign DNA into wheat leaves and calli. Plant Cell Rep., 9: 615- 619.

Dekeyser, R.A., B. Claes, R.M.U. De Rycke, V. Habets, M.E. Montagu, M.C. and A.B. Caplan (1990). Transient expression in intact and organized rice tissues. Plant Cell, 2: 591-602.

Depicker, A., M.V. Montaga and J. Schell (1983). Plant cell transformation by *Agrobacterium* plasmid. Basic Sci. 26:143-176.

Dhir, S.K., S. Dhir and J.M. Widholm (1991). Plantlet regeneration from immature cotyledon protoplast of soybean (*Glycine max* L.). Plant Cell Rep. 10:39-43.

Finer, J.J. and M.D. McMullen (1990). Transformation of cotton *(Gossypium hirsutum* L.*) via* particle bombardment. Plant Cell Rep. 8: 586-589.

Fontana, G., L. Santini, S. Caretto, G. Furgis and D. Mariotti (1993). Genetic transformation in the grain legume chickpea (*Cicer arietinum* L.*).* Plant Cell Rep. 12:194-198.

Francesco, D. and S. Arcioni (1991). Transformation of *Medicago arborea* L. with an *Agrobacterium rhizogenes* binary vector carrying the hygromycin resistance gene. Plant Cell Rep. 10:300-303.

Grumet, R. (1990). Genetically engineered plant virus resistance. HortiScience 25: 508-513.

Harding, R. M.; T.M Burns. and J.L. Dale (1991). Virus-like particles associated with banana bunchy top disease contain small single-stranded DNA . J. Gen. Virol. 72: 225-230.

Hauptmann, R.M., V. Vasil, P.O. Zias-A-Kins, Z. Tabaeizaadah, S.G. Rogers, R.T. Fraley, R.B. Horsch and I.K. Vasil (1988). Evaluation of selectable markers for obtaining stable transformation in the Gramineae. Plant Physiol. 86:602-606.

Hussey G., R.D. Johnson and S. Warren (1989). Transformation of meristematic cells in the shoot apex of cultured pea shoots by *Agrobacterium tumefaciens* and *A. rhizogenes.* Protoplasma 148: 101-105.

Innis, M.A. and D.H. Gelfand (1990). In PCR protocols: A guide to methods and applications. Baston. T.J. (eds.), Academic Press Inc., San Diego, New York, pp. 3-12.

James, D. J., A.J. Passey, D.J. Barbara and M. Vevan (1989). Genetic transformation in legumes. Plant Cell Rep.7: 658-661.

Jefferson, R.A., T.A. Kavanagh and M. Bevan (1987). GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3201-3207.

Khalil, S.M. (1999). Production of transgenic cucumber plants resistant to zucchini yellow mosaic p*otyvirus via* genetic engineering approaches. Ph. D. Thesis, Cairo Univ., Egypt, pp.125.

Klein, T.M., L. Kornstein, J.C. Sanford and M.E. Framm (1989). Genetic transformation of maize cells by particle bombardment. Plant Physiol. 91:440-444.

McCabe, D.E., W.F. Swain, B.J. Martinell and P. Christow (1988). Stable transformation of soybean *(Glycine max L.)* by particle acceleration. Bio/Technology 6:923-926.

Mohamed, Sahar A. (1999). Transformation and expression of methionin rich 2S- albumin gene into *Vicia faba.* M. Sc. Thesis, Helwan University, Egypt, pp. 109.

Oard, J. (1993). Development of an air gun device for particle bombardment. Plant Cell, Tissue and Organ Culture 33: 247-250.

Pickardt, T., M. Martin, S. Verena and S. Otto (1991). Transformation of *Vicia narbonensis via Agrobacterium*-mediated gene transfer. Plant Cell Rep. 9: 535-538.

Powell-Abel, P., R. S. Nelson, B. De, N. Hoffmann, S. G. Rogers, R. T. Farley and R. N. Beachy (1989). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat-protein. Gene Sci. 232:738-743.

Ramsay, G. and A. Kumar (1990). Transformation of *Vicia faba* cotyledon and stem tissues by *Agrobacterium rhizogenes* infectivity and cytological studies. Plant Cell Rep. 12:483-490.

Saleh, T.M. (1998). Some physiological and biotechnological studies on *Vicia faba* L. M. Sc. Thesis, Cairo Univ., Egypt, pp. 112.

Sambrook, J., E.F. Fritsch and T. Maniatis (1989). Molecular cloning: A laboratory Manual. Cold Spring Harbor Lab. Press, New York, USA, 2nd Ed. pp. 1626.

Sautter, C. (1993). Development of microtargeting for particle bombardment of plant meristems. Plant Cell, Tissue and Organ Culture 33:251-257.

Schroeder, G., S. Waffenschmidt, E. Weiter and J. Schroder (1984). The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. 138:387-391.

Selva, E., B. Stouffs and M. Briquet (1989). *In vitro* propagation of *Vicia faba* L. by micro-culturing and multiple shoot induction. Plant Cell, Tissue and Organ Culture 18:167-179.

Thomashow, L. (1984). Crown gall oncogenesis: Evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid P TiA6 encodes an enzyme that catalyzes synthesis of indole acetic acid. Pro. Nat. Acad. Sci. USA. 81:5071-5075.

Vain, P., N. Keen, J. Murillo, C. Rathus, C. Nemes and J.J. Finer (1993). Development of the particle inflow gun. Plant Cell, Tissue and Organ Culture 33:237-246.

Xu, Y.S., M.S. Clark and E. Pehu (1993). Use of RAPD markers to screen somatic hybrids between *Solanum tuberosum* and *S. brevidens.* Plant Cell Rep. 12:107-109.

الملخص العربي

تشييد نظام للتحول الوراثي لبعض أصناف الفول البلدي المصرية

ربي مدحت اسماعيل*، فتوح محمد الدمياطي ،عاطف شكري صادق*،*****
تيمور محمد نصرالدين*، علي زين العابدين عبدالسلام**
*: معهد بحوث الهندسة الوراثية الزراعية، مركز البحوث الزراعية، ص.ب.١٢٦١٩، جيزة، مصر
**: قسم الوراثة، كلية الزراعة، جامعة عين شمس، شبرالخيمة، مصر
***: قسم الميكروبيولوجيا الزراعية، كلية الزراعة، جامعة عين شمس، شبرالخيمة، مصر

يعتبر نبات الفول البلدي من أهم النباتات ذات القيمة الاقتصادية في جمهورية مصر العربية . وقد صممت هذه الدراسة لتأسيس نظام التحول الوراثي (Transformation) في بعض أصناف الفول البلدي وهما جيزة ٤٦١، و جيزة ٦٧٤. تم استخدام نظام (*Agrobacterium*-mediated gene transfer) و ذلك باستخدام سلالة الأجروباكتريم المعروفة باسم *A. tumefaciens* strain EHA101 ، و المورثات *NPTII* and *GUS* genes ، كما استخدمت الفلقات و القمة النامية كمصدر للأجزاء النباتية (Explants). و لهذا الغرض فقد ثبت أن التركيز المميت كان ١٠٠ مليجرام/لتركاناميسين حيث أدى الى موت جميع الأجزاء النباتية غير معدلة التركيب الوراثي. تم عمل تحول وراثي للاجزاء النباتية المختارة باستخدام بكتريا الأجروباكتريوم المحولة وراثيا و ذلك بتحضين الأجزاء النباتية مع الأجروباكتريم في بيئة VSI السائلة لمدة ٢٤ و ٤٨ ساعة. وباستخدام نظام بندقية الجينات فقد تم عمل تحول وراثي للأجزاء النباتية المستخدمة والكالس (Calli) المنتجة من الأجزاء النباتية المتحصل عليها من القمة النامية البلازميد pBI121 المحتوي على المورثات *NPTII* and *GUS* genes علي مسافة ٦سم وضغط ١١٠٠ أو ١٣٠٠. تركيز ٥٠٠ نانوجرام/ميكرولتر من الدنا(DNA) مع ضغط ١١٠٠ في التحول الوراثي في كلا الجزأين النباتيين. وقد تم الكشف عن تواجد مورث *NPTII* باستخدام تكنيك تفاعل البلمرة المتسلسل و المعروف باسم (PCR) وأيضا باستخدام تكنيك Southern blot بينما تم الكشف عن التعبير الجيني لمورث *GUS* كجين كاشف (Reporter gene) وذلك باستخدام اختبار الـ *GUS* .