Regeneration and microprojectile -mediated transformation in *Vicia faba* L.

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ABSTRACT

Ten Egyptian cultivars of faba bean (Vicia faba L.) were evaluated for their regeneration capability. Regeneration frequency varied among the cultivars from 30-92% when using embryo axes, while the shoot apex explants revealed lower regeneration frequency (12-57%). Two cultivars (Giza 716 and Giza 843) exhibited no regeneration when using the shoot apex explants. Explants were cultured on Murashige and Skoog (MS) basal salts, with 1 mg/l B5 vitamins, 4.5 mg/l BAP and 0.5 mg/l NAA for mature embryo explants, or 5 mg/l BAP and 1 mg/l NAA for shoot apex explants. The cultures were incubated for one week in the dark during the shoot induction stage to eliminate the browning caused by the accumulation of phenolic compounds. Addition of activated charcoal to the regeneration medium showed negative impact on the regeneration frequency. In vitro grafting method was successfully used for all tested faba bean cultivars. Transformation using microprojectile bombardment protocol was developed using the mature embryo as the explant. The plasmid pCGP1258, harboring the herbicide resistant gene (bar) as a selectable marker and the reporter β- glucuronidase gene (gus), was used for adapting transformation in faba bean cultivars. Mannitol at a concentration of 0.4 M, 1100-psi pressure and 6 cm shooting distance were the most efficient transformation condition. Integration and expression of the transgenes were confirmed by Southern blotting, PCR and histochemical GUS assay. The transformation frequency was as high as 2% for the cultivar Giza 40.

Key words: *Faba bean, regeneration, transformation, gus gene, bar gene, microprojectile bombardment.*

INTRODUCTION

Legumes are the third largest family of flowering plants and the most important source of plant proteins and energy. In developing countries, increased cultivation of legumes is the best hope for combating shortages in food supplies, where vegetable proteins in legume seeds are deficient in specific essential amino acids that must be made up in other ways (Jelenic *et al*., 2000). In Egypt, faba bean is the most important food legume, and area cropped to faba bean during the last five years was around 300,000 feddans annually representing about 80% of the total area devoted to cool-season food legumes (380,000 feddans).

One of the most limiting factors to faba bean cultivation is weed competition. In Egypt, broomrape (*Orobanche crenata*) is the most devastating weed attacking faba bean. Although true resistant to *Orobanche* infection in faba bean has not been identified, some

lines exhibit relativel tolerance than others (Nassib *et al.,* 1982; Hassanein *et al*., 1998).

Plant genetic transformation has become a versatile tool for cultivar improvement as well as to study gene function in plants. Successful transformation of plants demands certain criteria. Among the requirements for transformation are target tissues competent for propagation or regeneration, an efficient DNA delivery method, agents to select for transgenic tissues, the ability to recover fertile transgenic plants at a reasonable frequency, in addition to, a simple, efficient, reproducible, genotype-independent and cost-effective process (Hansen and Wright, 1999).

The major success in legume transformation was achived by methods based on transformation of the pre-existing meristems on the embryo axes, cotyledonary nodes, shoot tips or nodal plants (Hanafy *et al.*, 2005). Transformation using the direct gene transfer system was successded with soybean (McCabe *et al.,* 1988; Russell *et al.,* 1993; Sato *et al*., 1993) peanut (Brar *et al*., 1994) and *Phaseolus vulgaries* (Russell *et al*., 1993; Aragao *et al.,* 1996). Transformation using *Agrobacterium* has been successfully applied in different grain legumes such as pea (Schroeder *et al.,* 1993; Bean *et al.,* 1997) and soybean (Yan *et al.,* 2000; Olhoft *et al*., 2003). Although the most widely used method of dicotyledonous plant transformation is *Agrobacterium*, there is only two reports on *Vicia faba* using *Agrobacterium* mediated-transformation for producting transgenic faba bean plants (Bottinger *et al*., 2001; Hanafy *et al.,* 2005). The approach of the first report was based on plant regeneration from internodal stem segments, while the second report used the mature embryo discs in their regeneration system. On the other hand, transformation of *Vicia faba* using the biolestic bombardment gene delivary system was recommended by Ismail (1999) and Jelenic *et al*. (2000).

The ultimate goal of this work is to establish a reliable and stable regeneration and transformation system for Egyptian faba bean cultivars. The first step to achieve this goal was to introduced *bar* and *gus* genes into plant expression vector downstream of 35S CaMV promoter and to carry out the transformation by biolistic bombardment gene transfer methods. Genomic Southern blot hybridization and PCR strategy were performed to confirm the integration of the genes into some faba bean genomes.

MATERIALS AND METHODS

Plant material

Seeds of ten Egyptian faba bean cultivars (Giza2, Giza3, Giza40, Giza429, Giza461, Giza716, Giza834, Misr1, Sakha1 and Nubaria1) were obtained form the Field Crops Research Institute (FCRI), Agricultural Research Center (ARC) to screen their regeneration efficiency.

Regeneration of faba bean

Seed sterilization and explants preparation

Faba bean mature seeds were surface-sterilized with 20% Clorox (commercial bleach contained 5% sodium hypochlorite) plus five drops of tween-20 for 20 min, followed by rinsing several times with sterile distilled water and then soaked overnight in sterile water. The embryo axes of mature seeds were isolated, scliced to two sections and cultured on the regeneration medium.

Sterilized seeds were germinated in a pre-autoclaved wet cotton pads, placed in 10 cm glass jars and covered, then incubation at 28 °C under 16 hr photoperiod from cool white fluorescent lamp (3000 lux). *In vitro* grown seedlings of faba bean cultivars (10-13 days old) were used as a source of shoot apexes. Shoot apexes were excised using a

sharp scalpel and cultured on the regeneration medium.

Shoot formation stage

Explants were cultured on MS basal medium (Murashig and Skoog, 1962) and with 1 mg/l B5 vitamins (Gamborg *et al*, 1968), 3% sucrose and 0.3% phytagel supplemented with 4.5 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l α-naphthalene acetic acid (NAA) for mature embryos, or 5 mg/l BAP and 1 mg/l NAA, for shoot apex explants according to Tawfik (1998) and Ismail (1999). The cultures were then incubated at 28±2 °C for 3 weeks in the light (3000 lux). Subsequently, the cultures were incubated for one week in the dark for eliminating the browning problem due to the phenolic compounds. On the other hand, the effect of the activated charcoal at 3 g/l was studied. The regenerated shoots produced from the shoot apex explants were transferred into the same fresh medium as an elongation medium. Regenerated shoots were transferred to the same regeneration medium supplemented with gibberellic acid (GA_3) at a concentration of 10 mg/l when using the embryo explants. Cultures were incubated for another 4 weeks in the light.

Rooting stage

The root initiation study was carried out on four cultivars (Giza 461, Giza 429, Giza 40 and Giza 843). To select the suitable rooting condition, the shoots reached 3-5 cm height during the elongation stage were transferred to three different rooting media, i.e., M1 (MS with B5 vitamins), M2 (half strength MS) and M3 (MS medium with 0.4 mg/l NAA). All media were supplemented with 30 g/l sucrose and 7 g/l agar. In the parallel, *in vitro* grafting method was studied in which mature seeds were germinated on hormone-free MS medium. After 10 days, seedling decapitated and the proliferated shoots obtained from elongation stage were grafted on the cut seedling of germinated faba bean. The cultures were incubated for 3-4 weeks in the light.

Transformation *via* biolistic gene gun

In this investigation, the faba bean cultivar Giza 40 was used in the bombardment transformation. Mature embryo axes were used as explants and cultured in the middle of plates containing the regeneration medium directly before the bombardment with the plasmid pCG1258 (has been kindly provided by ICARDA) containing *bar* and *gus*-intron genes (Fig. 1).

Bombardment conditions

The embryo axes from mature seeds were longitudinal dissected into two sections and cultured into the middle of plates containing the regeneration medium. In order to develop the optimum bombardment protocol, the distance of explants in relation to carrier membrane (6 and 9 cm) and the helium pressure (1100 and 1350 psi) were studied. Each treatment has 5 replicates and the number of explants in each treatment was 50 embryo discs per plate. Bombardment was carried out as described by Jefferson *et al.* (1987). Osmotic treatment was studied by adding mannitol at concentrations of 0.0, 0.2 and 0.4 M. The bombarded explants were incubated for two days or for one week on mannitol. Data were statistically analyzed using the analysis of variance as outlined by Gomez and Gomez (1984) based on MSTATC program. The differences between means were compared using Duncan's multiple range test (Duncan, 1955).

Selection and regeneration of transformed tissues

To establish the selection protocol, it was necessary to study bialaphos sensitivity on the growth of faba bean mature embryo non-

transformed explants. Different concentrations of bialaphos (0, 0.5, 0.75, 1, 1.25, 1.50, 1.75, 2.0 mg/l) were added to the regeneration medium and shoots formation ability was scored after 4 weeks. The highest concentration was the best to kill non-transformed tissues and consequently was recommended selected for the selection of transformed tissues.

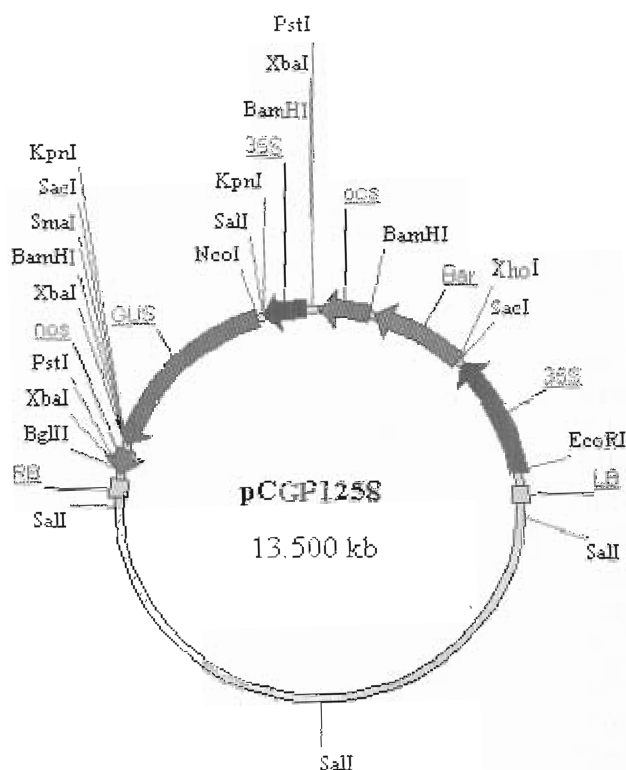


Fig. (1): Restriction map of the pCGP1258 binary vector used in faba bean transformation, with bar and gus genes.

For the production of transgenic faba bean plants, five experiments have been performed, each experiment has 20 explants with a total number of 100 explants. The explants (embryo axes) were prepared and cultured on regeneration medium supplemented with 0.4 M mannitol for two days.The explants were bombarded under helium pressure of 1100 psi at a distance of 6 cm. The bombarded explants were incubated on the bialaphos-free medium for 2-3 days in the dark. Thereafter, the cultures were transferred to the selection medium composed of regeneration medium with 2 mg/l Bialaphos. Plates were kept in the dark for another 3-5 days for eliminating the browning phenomenon, and then transferred to the light for 4 weeks. Subsequently, produced shoots were transferred to the elongation medium for another 4 weeks in the light.

Histochemical *gus* assay

Histochemical *gus* assay was carried out on transformed explants to detect the transient *gus* activity. Tissues were immersed in *gus* buffer containing 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) from Clonetech and incubated overnight at 37°C as described by Jefferson *et al.* (1987).

PCR analysis

The presence of both genes in the phosphinothricin (PPT) resistant shoots was

determined by PCR. Total DNA was isolated from *in vitro* leaves of putatively transformed and non-transformed faba bean lines according to Delaborta *et al*. (1983). The extracted DNA was amplified according to Sambrook *et al.* (1989) and PCR was done with the synthetic specific primers for both genes: *gus* (5'-CCTGTAGAAACCCCAACCCG-3'; 5'-TGGCTGTGACGCACAGTTCA-3') and *bar* (5'-GCAGGAACCGCAGTGGA-3'; 5'-AGCCCGATGACAGCGACCAC-3'). Thirty five cycles of amplification were carried out under denaturation condition of 95°C for 30 sec, annealing at 66°C for either gene, or extension at 72°C for 2 min. There was a denaturation cycle at the beginning of the PCR at 95°C for 5 min and one final extension cycle at 72 °C for 7 min.

Southern blot analysis

Genomic DNAs (10 µg) extracted from *bar*-positive regenerated and control plants were digested with *BamH1* and *Xho1* and subjected to electrophoresis (0.8% agarose gel). The nucleic acid bands were transferred onto positively-charged Nylon membrane as recommended by Sambrook *et al*. (1989). The plasmid pCGP1258 was used to amplify a fragment of 264 bp from the *bar* gene. The fragment was labeled with digoxigenine (DIG; Boehringer Mannheim, Roche) and was used as a probe for Southern hybridization.

RESULTS AND DISCUSSION

Attempts have been carried out to study the regeneration frequency of ten faba bean Egyptian cultivars for establishing an efficient regeneration system.

Establishment of a regeneration system in faba bean

Despite early interest in *Vicia faba* as an experimental organism for molecular biology, the application of biotechnology to faba bean improvement was slower than other economically important crops. However, advances are made in tissue culture and genetic transformation which could help rejuv-enate interest in this crop. In this study, regeneration frequency of ten faba bean Egyptian cultivars was studied using two kinds of explant (mature embryos and shoot apices). After 7-10 days of seed germination, mature embryo explants enlarged approximately 3 times and started to produce callus and shoots. Whereas, the shoot apex explants stared to enlarge and produce calluses and shoots after 10-15 days. It was observed that the regeneration efficiency varied among the cultivars and also between the two explants. Mature embryos exhibited a higher regeneration frequency than the shoot apex explants. The percentages of shoot formation obtained from mature embryos ranged from 30 to 92%. While, shoot formation varied from 0 to 57 % in the case of shoot apex explants (Table 1 & Fig. 2). It was also found that cultivar Giza 461 revealed the highest regeneration frequency (92%), followed by Giza 40 (89%), while Nubaria 1 gave the lowest frequency (30%). However, the addition of the activated charcoal into the regeneration medium gaves a negative impact in both explants, as it reduced the number of shoots per explant to 0-1 shoot/ explant compared to 2-5 shoots /explant in the absence of charcoal. The negative effects of charcoal could be related to the capacity of charcoal to absorb the growth regulators in the medium. The shoot apices that produced shoots were then transferred to the same medium for elongation. While, the embryo explants were transferred to the elongation medium, consisting medium with GA_3 at a concen-tration of 10 mg/l for another 3 weeks. This period allows proliferation and elongation of the regenerated shoots. The use of embryonic axes explants cultivated on a high concentration of

BAP with a low concentration of IAA was adopted by Bottinger *et al.* (2001). They reported that the high cytokinin concentration in combination with low auxin concentration not only enhances the direct shooting without intermediate callus phase, but also reduces the possibility of somaclonal variation to a minimal level.

Table (1). Regeneration frequencies of ten faba bean Egyptian cultivars using two types of explants (mature embryo & shoot apex).

Cultivars	Shoot formation from mature embryo (%)	Shoot formation from shoot apex (%)
Giza 3	54	54
Giza 461	92	50
Giza 716	52	0
Sakha 1	72	12
Giza 40	89	28
Giza 2	52	52
Giza 843	86	0
Giza 429	84	56
Misr 1	80	57
Nubaria 1	30	24

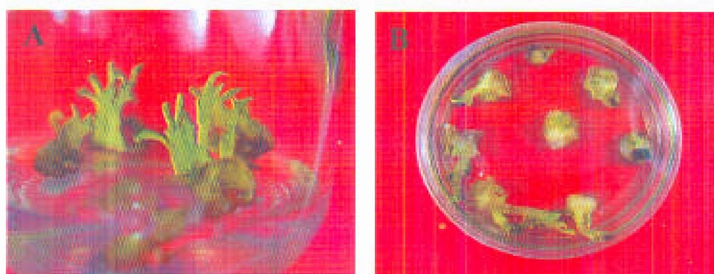


Fig. (2): (A) Shoots developed and proliferated from the mature embryos, (b) Shoots produced from the shoot apex explants, grown on regeneration medium for 2 weeks.

Fig. (3): Regenerated shoots grafted in vitro on the 10-day-old faba bean seedling (Giza 40) grown on hormone-free MS medium.

Shoots with a height of 3-5 cm were transferred to 3 different rooting media. Some other shoots were in vitro grafted on the decapitated faba bean seedlings. These shoots were incubated for 3-4 weeks. Results showed that the medium M2 containing half-strength MS medium exhibited very low frequency of root formation as it gave a percentage of 1%. However, there is no root formation observed on the other rooting media (M1 & M3). While, it was found that grafting revealed a high survival percentage (50-66%) for all cultivars tested, i.e., Giza 461, Giza 716, Giza 40 (Fig. 3) and Giza 843. Therefore, grafting was recommended during the rooting stage. Similar results were reported by Hanafy et al. (2005) as they grafted faba bean shoots on seedlings.

Establishment of a transformation system in faba bean

In this investigation, faba bean cultivar Giza 40 mature embryo discs (as explants) were utilized in the trial to produce transgenic faba bean plants by the bombardment gene delivery system.

The selectable marker *bar* gene of *Streptomyces hygroscopicus* encodes phosphinothricin acetyl-transferase (PAT), which inactivates bialaphos (PPT), the active component of BASTA by acetylation (Thompson *et al.*, 1987). Therefore, biolaphos was used to select the faba bean transformed cells during tissue culture. To study the suitable selection conditions for the faba bean cultivars, the mature axes explants were incubated on regeneration medium containing different concentrations of bialaphos for 3 weeks. Results showed that bialaphos at a concentration of 2 mg/l, totally suppressed the growth of the embryo explants. While, in the absence of the bialaphos, the explants were regenerated as in the preliminary regeneration experiments (Table 2 & Fig. 4). Hanafy *et al.*, (2005) also used 2 mg/l bialaphos when they developed a transgenic Mythos and Albatross faba bean cultivars. They suggested that this low concentration might favor the cells reaching a critical size, which is necessary for shoot development and increases the probability of obtaining transgenic plants.

Table (2): Number of suppressed mature bean embryo explants by different bialaphos concentrations.

	Bialaphos concentrations (mg/l)							
Cultivars	0	0.5	0.75	1.00	1.250	1.500	1.750	2.00
G40	48	28	27	21	18	16	8	0
G461	41	29	25	22	19	14	5	0
G843	44	30	27	24	20	13	3	0
G716	42	27	20	15	10	8	4	0

Each treatment contains 50 explants

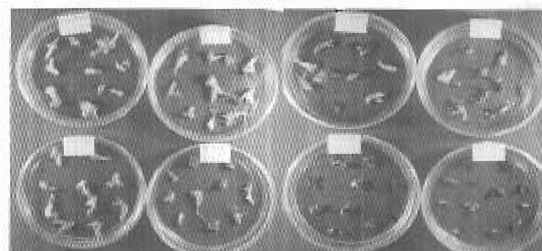


Fig. (4): Shoots proliferation of the bean mature embryo explants on different concentrations of bialaphos.

Table (3): Effects of different bombardment pressures (1100 or 1350 psi) and time after bombardment on number of blue spots of faba bean embryonic axes.

Days after bombardment	Pressure (psi)				
	1100		1350		1100
	6 cm	9 cm	6 cm	9 cm	6 cm and 9 cm
Two days	7.6 a	6.96 ab	5.42 abc	4.7 bc	7.8 a
One week	6.08 ab	6.04 abc	5.3 abc	4.2 cd	7.5 a
Mean	6.84	6.5	5.36	4.45	7.65

Table (4): Effect of different concentrations of mannitol (0, 0.2 and 0.4 M) and time after bombardment on number of blue spots of embryonic axes.

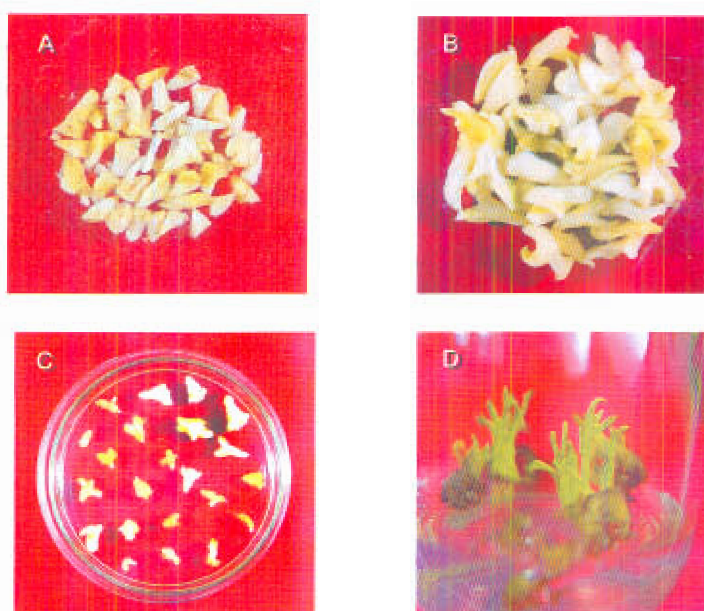
Days after bombardment	Osmotic concentration (M)					
	0		0.2		0.4	
	6cm	9 cm	6 cm	9 cm	6 cm	9 cm
Two days	16.2 bc	12.59 c	28.1 abc	26.4 bc	39.6 a	32.4 ab
One week	17.3 bc	12.5 c	22.8 abc	16 bc	37.4 a	31.9 ab
Mean	16.75	12.55	25.45	21.3	38.5	32.15

For developing a proper bombardment protocol, mature embryo explants of cultivar Giza 40 were subjected to the bombardment conditions of two distances (6 and 9 cm) between the explants and the carrier membrane, two helium pressures (1100 and 1350 psi) and osmotic treatments by adding the mannitol at two concentrations; 0.2 and 0.4 M. The explants were cultured on the mannitol medium (mannitol free medium used as a control) and incubated for two days or one week. Results in Table (3) revealed no significant difference in number of blue spots, when the explants were subjected to one shot at 6 cm or two shots at 6 or 9 cm distance under 1100 psi which were much higher than those at 1350 psi. Therefore, the optimum bombardment protocol chosen is a pressure of helium gas at 1100 psi and the distance of explants in relation to carrier membrane at 6 cm. Adding mannitol at the concentration of 0.4 M to the regeneration medium for two days, significantly increased the number of tissue spots (Table 4).

Regeneration of transformed faba bean tissues

The mature embryo explants of faba bean cultivar Giza 40 were cultured on the middle of plates containing the regeneration medium, subsequently, these explants were bombarded with the plasmid pCG1258 with *bar* and *gus*-inton genes. The cultures were kept in the dark for one week. Thereafter, the bombarded explants were transferred to the selection medium supplemented with the 2 mg/l bialaphos, subsequently incubated under light. It was observed that after 7-15 days of culturing on selection medium, mature embryo explants were enlarged 2-3 times and started to produce shoots. Out of 100 explants, only 89 shoots were produced under bialaphos selection. After 3-4 weeks, these explants were transferred to the same selection medium with GA_3 at a concentration of 10 mg/l for proliferation and elongation. A number of 47 shoots were only elongated out of the 89 produced shoots. The elongated PPT resistant shoots (4-5 cm) were then grafted to the root of decapitate faba bean seedlings and the rest of shoots that did not reach the suitable height were transferred to a fresh elongation medium. Fig. (5) Illustrates the different tissue culture stages.

Fig. (5): ***Transformation and selection of mature embryos of faba bean Giza 40 cultivar.***
A): Mature embryo on osmotic treatment before bombardment.
B): Callus induction on MS medium supplemented with 2 mg/l bialaphos.
C): Callus induction after 7 days.
D): Shoots elongation .



Histochemical *gus* assay

Histochemical *gus* assay was carried out to examine transient expression of the treated explants. In addition, herbicide resistant shoots were subjected to histochmical assay (Fig. 6). All the tested plantlets developed a blue color, showing a correlation between *gus* enzymatic activity and herbicide resistance.

Fig. (6): Histochemical gus assay in embryo explants of faba bean Giza 40 cultivar bombarded with plasmid pCG1258.

PCR analysis

To confirm the presence of both *gus* and *bar* genes in the phosphinothricin (PPT) resistant faba bean plants, PCR was performed using specific primers for each gene. Fragments of 750 and 264 bp for *gus* and *bar* genes, respectively, were amplified from the DNA of the transgenic tissues (Figs. 7 & 8). It was observed that out of 47 PPT-resistant shoots, only 18 revealed the presence of *bar* and *gus* genes.

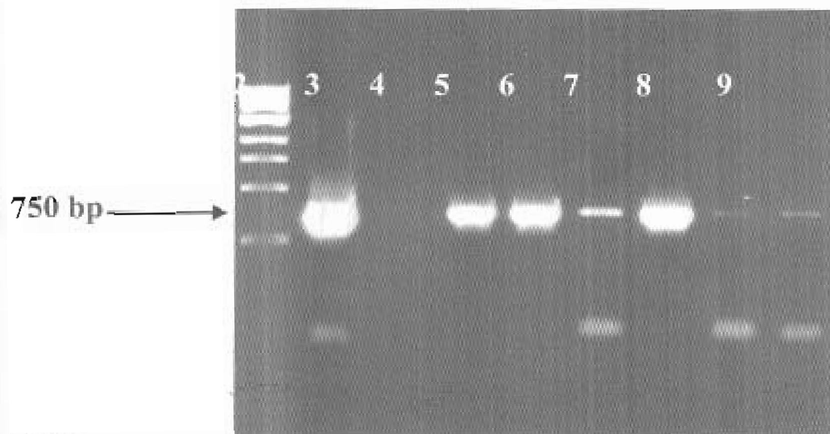


Fig. (7): PCR reaction with gus gene fragment (750 bp) was amplified from the transgenic faba bean lines.

Lane 1	*: Marker (I kb ladder)*
Lane 2	*: Positive control (plasmid pCGP1258)*
Lane 3	*: Non-transgenic faba bean plant*
Lanes 4 to 9	*: Transgenic faba bean lines*

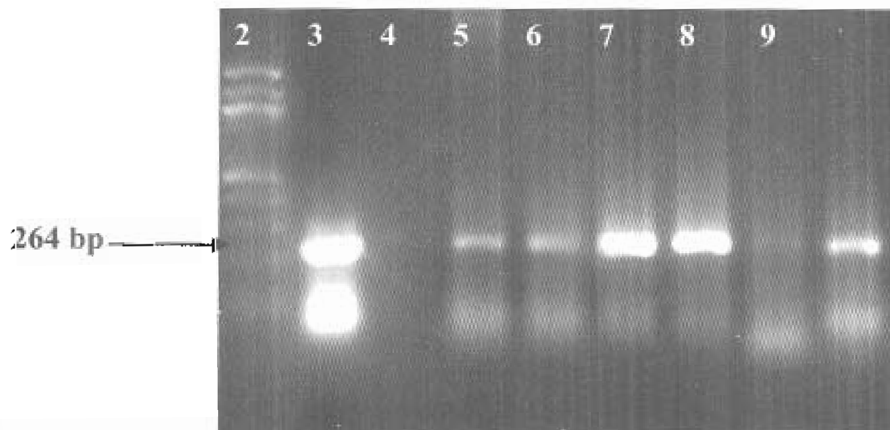


Fig. (8): PCR-amplified bar gene.

Lane 1 : Marker (100 bp ladder)
Lane 2 : Positive control (plasmid pCGP1258)
Lane 3 : Non transgenic faba bean plant
Lanes 4 to 9: Transgenic faba bean lines

Southern blot analysis

PCR-positive plants were further analyzed to determine the integration of *bar* gene in the plant genome. Southern blot analysis of *BamHI* / *XhoI* -digested genomic DNA was carried out using PCR amplified *bar* fragment (264 bp) as a probe. Results shown in Fig. (9) Confirmed the integration of the *bar* gene into the genomic DNA of two transformants whereas no hybridization signal was detected from the non-transformed control. Data illustrated in Table (5) represent the

number of treated explants, the survival explants, explants produced calli and shoots and finally the regenerated shoots on the bialaphos condition. In addition, the number of putative transformants was based on PCR screening, while the number of the transformants based on the Southern blot hybridization was two lines.

Table (5): Transformation efficiency of Vicia faba cultivar Giza 40 .

Number of explants treated	Number of survival explants	Number of shoot but regenerated	Number of elongated shoots	Number of putative transformants based on PCR analysis	Number of transformants based on Southern blot hybridization	Transformation efficiency %
100	95	89	47	18	2	2

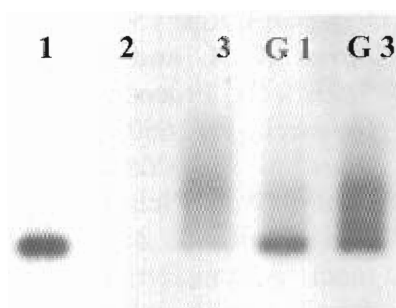


Fig. (9): Southern blot analysis of bar–intron gene in transgenic faba bean .
Lane 1: Positive control (plasmid pCGP1258).
Lane 2 : Non transgenic faba bean lines .
Lane 3 : Putative transgenic event that was negative
Lanes 4&5: G1 and G3 are transgenic faba bean lines .

These results demonstrate that the transformation percentage is 2% for the faba bean cultivar Giza 40. Hanafy *et al.* (2005) reported that the mature and immature embryonic axes of six elite faba bean cultivars, included Mythos, Albtross, Giza 2, Giza 716 and Giza 429 and Giza blanka were carried out for production of transgenic plants resistant to PPT using the *Agrobacterium* mediated transformation. They reported that the transformation frequencies ranged between 0.15% to 2.0%. It is important to mention that the transformation frequency was 0 % of the Egyptian cultivars, i.e., Giza2, Giza 429, Giza 716 and Giza Blanka.

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REFERENCES

Aragâo, F. J. L.; Barros, L. M. G.; Brasileiro, A. C. M.; Ribeiro, S. G.; Smith, F. D.; Sanford, J. C., Faria, J. C. and Rech, E. L. (1996). Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed *via* particle bombardment. Theor. Appl. Genet., 93: 142–150.

Bean, S. J.; Gooding, P. S.; Mullineaux, P. M. and Davies, D. R. (1997). A simple system for pea transformation. Plant Cell Rep., 16: 513-519.

Bottinger, P.; Steinmetz, A.; Schieder, O. and Pickardt, T. (2001). *Agrobacterium*-mediated transformation of *Vicia faba.* Molecular Breeding. 8: 243-254.

Brar, G. S.; Barry, C. A.; Carole, V. L. and Johnson, W. (1994). Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from

elite cultivars utilizing ACCL technology. The Plant Journal., 5: 745-753.

Delaborta, S. L.; Wood, J. and Hicks J. B. (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep., 4: 19-21.

Duncan, D. B. (1955). Multiple range and multiple F-tests. *Biom.* 11: 1-42. Experimental Botany, 40: 813-817.

Gamborg, O. L. ; Miller, R. A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp., Cell. Res. 151-158.

Gomez, K. A. and Gomez, A. A. (1984). Statistical Procedures for Agricultural Research. Pp. 680, (2nd ed) J. Wiley and Sons Ltd., New York.

Hanafy, M. ; Pickardt T. ; Kiesecker, H. and Jacobsen, J. (2005). *Agrobacterium*-mediated transformation of faba bean (*Vicia faba* L.) using embryo axes. Euphytica ,142: 227-236.

Hansen, G. and Wright, M. S. (1999). Recent advances in the transformation of plants. Trends in Plant Science, 4: 226-231.

Hassanein, E. E. ; Salim, A. A. ; Mohamed, A. A. and El-Khanagry, S. S. (1998). Weed survey in winter crops in Menofia governorate. 6th Annual Coordination meeting. Nile Valley Program for wild oats and other weeds control in winter cereals and some other winter crops, Cairo 6-11 Sep.

Ismail, M. R. (1999). Genetic Engineering Studies in Some Plants. Ph. D. Thesis, Ain Sham Univ., Egypt.

Jefferson, R. A. ; Kavanaugh, T. A. and Bevan, M. W. (1987). GUS fusion beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO* J., 6: 3901-3907.

Jelenic, S. ; Mit, P. T. ; Papes, D. and Jelaska, S. (2000). *Agrobacterium*-mediated transfor-mation of broad bean *Vicia faba* L. Food Tech., 38: 167-172.

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

Murashige, T. and Skoog, F. (1962). A revised medium for a rapid growth and bioassay with tobacco tissue culture. Physiologica Plantarium, 15: 473-479.

Nassib, A. M. ; Iberahim, A. A. and Saber, H. A. (1982). Breeding for resistance to orobanche.In: G. Hawtin, C. Weeb (eds). Faba bean improvement, ICARDA Workshop of Seed Legumes, Aleppo, Syria, ICARDA. 133-136.

Olhoft, P. M. ; Flgel, L. E. ; Christopher, M. and Somers D. A. (2003). Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta, 216: 723-735.

Ressell, D. R. ; Wallace, K. M. ; Bathe, J. H., Martinell, B. J. and McCabe, D. E. (1993). Stable transformation of *Phaseolus vulagris via* electric-discharge mediated-particle acceleration. Plant Cell Rep., 12: 165-169.

Sambrook, J. ; Fritsh, E. F. and Maniatis, T. (1989). Molecular Cloning: A laboratory manual 2ed. Cold Spring Harbor Laboratory Press. NY, USA.

Sato, T.; Newell, C.; Kolaez, K.; Tredo, L.; Finer, J. and Hinchee, M. (1993). Stable transformation *via* particle bombardment in two different Soybean regeneration systems. Plant Cell Rep., 12: 408-413.

Schroeder, H. E.; Schotz, A. H.; Wardley-Richardson, T.; Spencer, D. and Higgins, T. J. V. (1993). Transformation and regeneration of two cultivars of pea (*Pisum sativum* L). Plant Physiology, 101: 751-757.

Tawfik, M. S. (1998). Some physiological and biotechnological studies on *Vicia faba.* M.SC. Thesis, Cairo University.

Thompson, C. J.; Movva, N. R.; Tizard, R.; Crameri, R. ; Davies, J. E. ; Lauwereys,

M. and Botterman, J. (1987). Characterization of the herbicide-resistance *bar* gene from *Streptomyces hygroscopicus*. EMBO J., 6: 2519–2523.

Yan, B.; Roddy, M. S.; Collins, G. B. and Dinkins, R. D. (2000). *Agrobacterium tumefaciens*-mediated transformation of soyabean (*Glycine max* L.) using immature zygotic cotyledon explants. Plant Cell Rep., 19: 1090-1097.

الملخص العربي

إعادة التمايز والنقل الوراثى باستخدام جهاز الدفع المباشر لاصناف من الفول البلدى

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تم تقييم إعادة التمايز لعشرة اصناف من الفول البلدى وتراوحت النسبة المئوية لاعادة التمايز بين ٣٠-٩٢% وذلك باستخدام الاجنة المقطوعة بينما كانت النسبة المئوية اقل عند استخدام القمم النامية حيث تراوحت بين ١٢-٥٧% . وقد كانت نسبة إعادة التمايز معدومة باستخدام القمم النامية بالنسبة لصنفى الفول البلدى جيزة ٧١٦ و جيزة ٨١٣ . وتم زراعة الاجنة المقطوعة على بيئة الاملاح مورشيجى وسكوج مضاف اليها ١ ملجرام / لتر من فيتامين B5 وايضا ٤,٥ مليجرام / لتر من بنزيل ادنين ٠,٥ مليجرام / لتر من نفثالين اسيتك اسيد. وتمت زراعة القمم النامية على بيئة الاملاح مورشيجى وسكوج مضافا اليها ١ ملجرام / لتر من فيتامين B5 وايضا ٥ مليجرام / لتر من بنزيل ادنين ١ مليجرام / لتر من نفثالين اسيتك اسيد مع تحضين المزارع لمدة اسبوع فى الاظلام على ٢٦± ٢ درجة مئوية وذلك لحين تكوين النمو الخضرى كى يعمل على تقليل المواد الفينولية . وقد اتضح ان اضافة الفحم النباتى الى بيئة إعادة التمايز يعطى نتائج سلبية لنسبة إعادة التمايز الناتجة. وقد نجحت مرحلة التجذير باستخدام التطعيم. واستعمل جهاز الدفع المباشر لنقل الجينات الى الاجنة المقطوعة وذلك باستخدام البلازميد pCGP1258 الذى يحتوى على جين الbar وجين gus. وكانت افضل النتائج لاحداث التحول الوراثى عند استعمال تركيز من المانيتول ٤و مولر مليجرام/لتر تحت ضغط 1100psi وعند ارتفاع ٦ سم. وقد كانت نسبة التحول الوراثى فى صنف جيزة ٤٠ ٢%. وتم التاكد من ذلك بالكشف عن الجين المنقول باستخدام تفاعل البلمرة المتسلسل وطريقة الكشف الهستوكميائية عن جين gus وكذلك التهجين النووى لجين bar .