TRANSFORMATION SYSTEM OF MATURE EMBRYO OF SOME EGYPTIAN BARLEY GENOTYPES

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Barley transformation is a genotype-dependent process. In addition, the type of *Agrobacterium* strain, co-cultivation conditions and timing, selection system and plant regeneration system are important for successful transformation. *Agrobacterium* strains; AGL1 or AGL0 are commonly used but LBA 4404 has also been successfully used. In many reports of barley transformation, the *AFP* gene conferring resistance of fungal was used for selection together with hygromycin phospho transference (*hpt*) and β-glucuronidase (*GUS*) genes, as selectable and marker genes, respectively. *Agrobacterium*-mediated barley transformation procedure from growth of donor plants to provide immature embryos was applied to confirm the transgenic status of the regenerated plants (Harwood *et al.,* 2009). Moreover, Dahleen (1999) and Sharma *et al*., (2005) reported that commercial barley cultivars are difficult to transform because of the lack of an efficient method. Regeneration ability in barley depends on the donor plant material, genotype, media and environment.

A number of investigations were carried out to circumvent limitations in transformation frequency by improving tissue culture techniques to increase regeneration rates (Temel *et al.*, 2008). Various tissue culture protocols have been developed by using immature embryos and regeneration system of mature embryos (Gozukirmizi and Temel, 2011). Optimizing culture protocols for specific cultivars of commercial interest may facilitate practical application of *in vitro* genetic manipulation in barley. The frequency of embryogenic calli, the relative growth rate of embryogenic calli, and regenerability are influenced by genotype, culture media and genotype with culture medium interaction (Bregitzer, 1992; Manoharan and Dahleen, 2002; Abumhadi *et al*., 2005).

The objectives of this study were to assess of the efficiency of *Agrobacterium tumefaciens* transformation with three types of mature embryos of three barley genotypes (El-Kasr, Giza126 and Giza130) and to confirmthe most appropriate

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parameters for *Agrobacterium* transformation by PCR analysis.

MATERIALSAND METHODS

Plant materials

Transformation experiments were performedusing barley mature embryos of the three genotypes (El-Kasr, G126 and G130).

Bacterial strain and plasmid

Agrobacterium tumefaciens LBA4404 strain was used. This strain harbors the pITB-*AFP* plasmid vector which contains defensin (*AFP*) gene under the transcriptional control of cauliflower mosaic virus 35S-promoter (CaMV-35S) and nopaline synthase, NOS as terminator, hygromycin phosphor-transferase (*hpt*) gene and *GUS* (*uidA*-β-glucouronidase) as a reporter gene. Bacteria were grown overnight on LB medium supplemented with antibiotics streptomycin at 30 mg/l.Transformation experiments were performed according to Harwood *et al*., (2009).

Plasmid DNA preparation (large scale preparation)

Preparation of plasmid DNA was performed according to Sambrook *et al*., (1989) procedure with some modifications.

Agrobacterium mediated transformation

Bacterial culture medium

Two types of bacterial culture medium were used, YEB medium (Table 1) for *Agrobacterium*, and LB (Table 2) medium for either *E. coli* or *Agrobacterium*.

Preparation of glycerol stocks of bacteria

Glycerol stocks of bacteria were prepared in a ratio of 1:3, where one colony was picked from the master plate, dissolved in 10 ml LB medium and inoculated for 2-3 hours on shaker at 250 rpm, then transferred to 25 ml LB medium containing the necessary amounts of antibiotics and incubated on a shaker at 250 rpm, 28°C or 37°C in the dark for 15 h. The stock solution was prepared using 500 µl glycerol (86%) and 1000 µl of growing bacterial-suspension in 1.5 micro tube, stored at -80°C for further use.

Agrobacterium-mediated transformation protocol of barley

Day 1: Collected barley mature embryos were sterilized by rinsing 70% ethanol and kept in 4.5% sodium hypochlorite for 5 minutes. Then seeds were rinsed five or six times with sterile distilled water. Isolated mature embryos (1.5-2 mm diameter) from the seeds, with removed axis and was transferred to callus induction medium (Medium M1 (Table 3) was an improved medium described by Abumhadi *et al*., (2005); 20 embryos per plate), scutellum was side down and incubated in the dark at 24°C.

For preparation of the bacterial suspension culture, single colony of the bacteria was grown overnight (16 h.) on a shaker at 28°C and 180 rpm in LB liquid

medium containing appropriate antibiotics 20 µl/20ml Kanamycin and 20 µl/20 mlstreptomycin. LB liquid medium containing 1:1 (LB liquid medium: distilled water).

Day 2: using a pipette drips full strength *Agrobacterium* suspension onto each embryo. The embryo was dragged gently across the surface of the medium to remove any excess of *Agrobacterium* and transferred to fresh callus induction medium, scutellum was side down. Incubated in the dark at 24°C and co-cultivated for 3 days. Any damaged embryos were discarded.

Day 5: After co-cultivation the explants were subsequently washed thoroughly with sterile distilled water and placed on solidified MS (Murashige and Skoog, 1962). MS media were supplemented with 30 g/l maltose and 3.7 g/lgelrite, pH was adjusted to 5.7, 2 mg/l 2,4D, 1g/l casein, 690 g/l proline, 5 mg/l thiamine and 500 mg/l inositol, 500 µg/lcefotaxime and 60 µL hygromycin for 2 weeks without selection pressure. The developing calli were subcultured every 14 days, for a period of 12-18 weeks, following suitable shoot regeneration programmed (Harwood *et al*., 2000). Only resistant embryogenic lines were transferred to regeneration medium and any material stained with oxidized polyphones were discarded. Any explants which become overgrown with *Agrobacterium* were immediately discarded.

Genetic transformation

Optimization of Agrobacterium* conditions *via GUS transient expression

The transient expression of PITB-*AFP* gene was investigated as a preliminary test of the transformation efficiency. *GUS* gene expression was determined few days after transformation of the explants; 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) was the best substrate for histochemicheal localization of the β-glucuronidase activity in the tissues and cells (Stangeland and Zhian, 2002). This substrate produces a blue precipitate at the site of enzyme activity. Transformation efficiency was expressed as the number of blue spots per *Agrobacterium* transformation parameters. The transformed explants were incubated overnight at 37°C in the substrate buffer which contains 0.5 mg X-Gluc/mL. After staining, the explants were soaked in 70% ethanol to allow the detection of the blue stain (Harwood *et al*., 2009).

The mature embryos were then transferred to plates containing a selection medium (M1) supplemented with 60 µl-1 hygromycin. The plates were sealed with parafilm and explants were left to regenerate at 22°C, with a 16/8 h (light/dark) photoperiod in the culture room. Hygromycin-resistant calli obtained after a second round of selection were transferred to a fresh selection medium.

The survived calli were transferred to maintain and regeneration medium (described previously in Tables1 and 2). Once every two weeks, the fresh and healthy looking hygromycin resistant calli were sub-cultured in a fresh selection medium (Mb). The direct regeneration appeared in two weeks on M1 (Table 3). Approximately 6-8 weeks' indirect regeneration appeared. A set of explants which were not co-cultivated with *Agrobacterium* was also prepared, as described above, as a negative control (Abumhadi *et al*., 2005).

The experiment was applied in three replications in a completely randomized design. The number of surviving calli in each case was determined and the data was subjected to analysis of variance. Transformation frequency was expressed as a percentage of the number of shoots recovered from hygromycin-resistant calli relative to the total number of incubated hygromycin-resistant calli. Any rooting and shooting on the selection medium containing 60 µl-1 hygromycin was considered as transformants after two subcultures.

Recovery phase

After co-cultivation, the explants were subsequently washed thoroughly with sterile distilled water and placed on solidified MS media (Murashige and Skoog, 1962). MS medium was supplemented with 30 g/l maltose and 3.7 g/lgelrite, pH was adjusted to 5.7, 2 mg/l 2,4-D, 1 g/l casein, 690 g/l proline 5 mg/l thiamine and 500 mg/l inositol, 500 mg/l cefotaxime and 60 µl hygromycin for 2 weeks.

Evaluation of putative transgenic plants

Histochemical Gus assay

GUS activity was analyzed in putative transformed primary explants, shoots and leaves from regenerated plants for 6-8 weeks after inoculation by Agrobacterium.The transformed explants were incubated overnight at 37°C in the substrate buffer which contains 0.5 mg X-Gluc/ml. After staining, the plant explants were soaked in 70% ethanol to allow the detection of the blue stain.

Histochemical localization of the GUS activity in the transgenic tissue was performed according to the method described by Zhang *et al*., (1996).Transgenic mature embryos or calli or any explants were subjected to the *GUS* assay. They were incubated overnight in the GUS substrate mixture at 37°C in the dark. Blue spots were visualized on tissues.

Molecular analysis for AFP gene transformation

Genomic DNA extraction

DNeasy TM Plant Mini Kit (Qiagen Inc.) was used for DNA isolation from putative transgenic and their progeny as well as non-transgenic control plants.

Polymerase chain reaction (PCR) analysis

The presence of *AFP* gene was investigated by PCR amplification. The sequences of the specific oligonucleotide primers for *AFP* gene were as follows:

Forward: 5'-CGCGGATCCATGGCGAGGTGTGAGAATTT-GGCT-3'
Reverse: 5'-TGCTCTAGAATGGCGAGGTGTGAGAATTT-GGCT-3'

The *GUS* primers were as follows:

Forward: 5'-CCTGTAGAAACCCCAACCCG-3'
Reverse:5'-TGGCTGTGACGCACAGTTCA-3'

Each PCR reaction was performed in 25 µl (total volume) of the reaction mixture that consisted of IX reaction buffer, 10 ng plant DNA from a putative transgenic plant as a template.DNTPs, 1.5 mM $MgCl_2$, 2 mM of each primer and 0.5 unit of TaqDNA polymerase enzyme (Promega) (Table 3).Amplification was carried out in a Thermal Cycler Biometra (Table 4).

Amplified DNA fragments were electrophoresed on 1.0% agarose gel, detected by ethidium bromide staining and photographed under ultraviolet light. The presence of the target band in the transformants and its absence in the untransformed plants is considered as a proof of successful transformation.

RESULTS AND DISCUSSION

The *Agrobacterium tumefaciens* transformation was used with three types of explants of mature embryos of the three barley genotypes (El-Kasr, Giza126 and Giza130).In addition, confirmation of the most appropriate parameters for *Agrobacterium* transformation was investigated by PCR analysis (Fig. 1).

Transient *GUS* gene expression was determined three days after transformation of the explants. 5-bromo-4-chloro-3-indolyl glucuronidase (X-Gluc) is the best substrate for histochemical localization of the β-glucuronidase activity in the tissues and cells (Stangeland and Zhian, 2002). Mature embryo axis explants infected with *Agrobacterium* showed blue spot (positive Gus expression). Conversely, negative Gus expression was observed with untransformed embryo axis of barley mature embryos (Fig. 2).

Indirect shoots and roots organogenesis

Callus formation and initiation of transformed mature embryos in Agrobaterium started 3-5 days after culturing of mature embryos (Fig. 3 stage 1). The optimum mature embryo size (1-2 mm) for maximumcallus induction was obtained from 14-16 day-old embryos (Fig. 3 stage 1). The control of mature embryo no contained any callus induction and dead after one to two month (Fig. 3 stage 2). Direct regenerationinitiation for one month appeared in (Fig. 3 stage 3). Indirect regeneration initiated for two to three months (Fig. 3 stage 4).

The indirect regeneration of shoots and roots formation (Fig. 3 stage 4) obtained by culturing mature embryos (intact) of three genotypes on three tested media indicated that mature embryos (in-

tact) of the three genotypes; El-Kasr, Giza130 and Giza126 gave the highest percentages of embryo-genic calli when cultured on medium M1 in comparison with the other two media, while the lowest percentage of embryo-genic calli revealed by the explants of these two genotypes were in all media. Similar results were obtained on medium 2 by cultivating the three genotypes. This result agreed with Ren *et al*., (2010) who showed that the efficiency of mature embryo culture was significantly influenced by the genotypes, sugar types and 2,4-D concentrations. 4 mg/L 2,4-D proved to be the best effective for inducing embryogenic callus and also gave the highest proportion of plants regeneration across the two cultivars.

The result of Table (5) showed high mean percentage of shoot formation formed by intact mature embryos of genotype (El-Kasr) on the three media (22%). This was followed by the mean percentage of shoots of the two genotypes (Giza126 and Giza130), respectively, (11% and 5%), on the three media. The high mean percentage of root formation formed was recorded by intact mature embryos of genotype (El-Kasr) on the three media (16%). This was followed by the percentage of roots of the two genotypes (Giza126 and Giza130), respectively, (9% and 0.1%) on the three media. This indicated that the genotype El-Kasr is the best genotype for callus, shoots and roots formation followed by Giza126, then Giza130 as the best three genotypes in this study.

Molecular analysis

PCR analysis

Total genomic DNA from leaf samples of putatively transgenic plants resulting from transformation experiments of mature embryos or calli explants were analyzed by PCR using AFP-specific primers. The expected PCR fragment (300 bp), appeared in transformation which was not observed in untransformed (negative control) plants (Fig. 4). This result clearly demonstrated the presence of the *AFP* gene in barley plantlets co-cultivated with *Agrobacterium* containing the AFP-disarmed plasmid construct.

Using the polymerase chain reaction technique (PCR) as indicator for the presence of transgenes in the genomic DNA of the putatively transgenic plants has been reported by many investigators; Cho *et al*. (1998) confirmed the presence of the introduced gene in putatively transformed plants by PCR analysis of genomic DNA. The PCR showed bands corresponding to the expected size 300 bp for partial length of the *AFP* gene, and the expected size 300 bp for partial length of *GUS* gene (Fig. 4 and Fig. 5, respectively)

As conclusion, this study proved that barley *Agrobacterium*-mediated transformation using mature embryos is genotype-dependent, providing the choice of the right media and following the appropriate protocols at the phenotypic and molecular genetics levels could enhance the

degree of success of obtaining transformed barley with desirable agronomic traits. Hussein *et al.,* (2003) obtained embryogenic callus formation and plant regeneration from immature embryos of some barley genotypes, while Gozukirmizi and Temel (2011) reported that barley transformation studies using mature embryos have been successfully used to acquire biotic and abiotic resistance.

SUMMARY

Transformation of barley based on the infection of mature embryos with *Agrobacterium tumefactions* system used for transformation of explants El-Kasrgenotype using the strain harbors the pITB-AFP plasmid vector which contains defensin (*AFP*) gene, hygromycin phosphor transferase (*hpt*) and β-glucuronidase (*GUS*) genes as selectable and marker genes. The Transient expression of *AFP* gene was investigated as a preliminary test of the transformation efficiency. Transformation experiments were carried out with different parameters and the one barley genotype El-Kasr. The tested transformation parameters included: the type of explants mature embryos based on the results of *GUS* transient expression. PCR analysis using primers specific for the *AFP* and *GUS* genes with specific probe was applied.

The results revealed that mature embryo axis explants infected with *Agrobacterium* showed blue spot (positive *Gus* expression). Conversely, negative *GUS* expression was observed with untransformed embryo axis of barley mature embryos (intact) of the three genotypes gave the highest percentages of embryo-genic calli when cultured on medium M1 in comparison with the other two media. This was followed by the percentage of roots of the two genotypes (Giza126 and Giza130), respectively, (9% and 0.1%). PCR showed bands corresponding to the expected size of 300 bp for partial length of the *AFP* gene, and the expected size of 300 bp for partial length of *GUS* gene by intact mature embryos of genotype (El-Kasr) on the three media (16%). This was followed by the percentage of roots of the two genotypes (Giza126 and Giza130), respectively. Evidently, El-Kasr is the best genotype for callus, shoots and roots that were amenable totransformation.

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Table (1): YEB-medium component and concentration.

Component	Concentration
Beef extract	5 g/L
Yeast extract	1 g/L
Bacto – Tryptone	05 g/L
Sucrose	sucrose 5 g/L
Mg_4SO_4 X 7 H_2O	0.495 g/L

Table (2): LB medium components

Component	Concentration
Bacto–Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

Table (3): Components of maintenance indirect regeneration media.

Media code	Maintenance	Indirect regeneration
M1	Control	MS media, 1mg/L 2,4-D, supplemented with 0.5 mg/L BAP
M2	4mg/L 2,4-D, 50µg/L BA	MS medium free hormones
M3	6 mg/lL 2,4-D, 100 µg/L BA	half-MS medium supplemented with 0.5 mg/L 2,4-D and 1.0 mg/L zeatin

Table (4): PCR reaction mixture.

Compound	Amount per reaction
Double distilled water	7.5 µL
Forward primer	1.0 µL
Reverse primer	1.0 µL
20-50 ng genomic DNA	3.0 µL
Maser mix	12.5L
Total volume	25 µL

Table (5): PCR program temperature, time and No. of cycles.

PCR step	Temperature (°C)	Time (min)	No. of cycles
Initial Denaturation	94°C	3	1
Denaturation	94°C	2	35
Annealing	54°C	1	
Extension	72°C	2	
Final extension	72°C	10	1
Cooling down	4°C	Until use	

Table (6): Means transformation (%) of shoots and roots formation obtained by culturing mature embryos (intact) of three genotypes on three tested media.

Geno-types	Transformation (%)							
	Shoot formation (%)			Means	Root formation (%)			Means
	M1	M2	M3		A	B	C	
El Kasr	20	33	13	22a	17	17	13	16a
Giza 126	13	20	0.1	11b	13	13	0.1	9b
Giza 130	0.1	13	0.1	5c	0.1	0.1	0.1	0.1c

Means with the same letters are not significantly different.

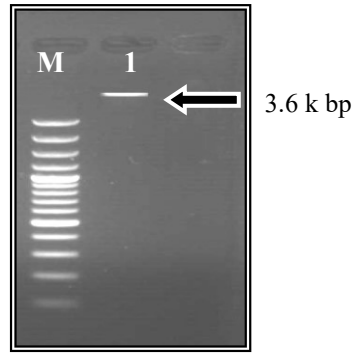

Fig. (1): PCR confirmations of pITB-AFP plasmid vector which contains defensin (*AFP*) gene under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S).

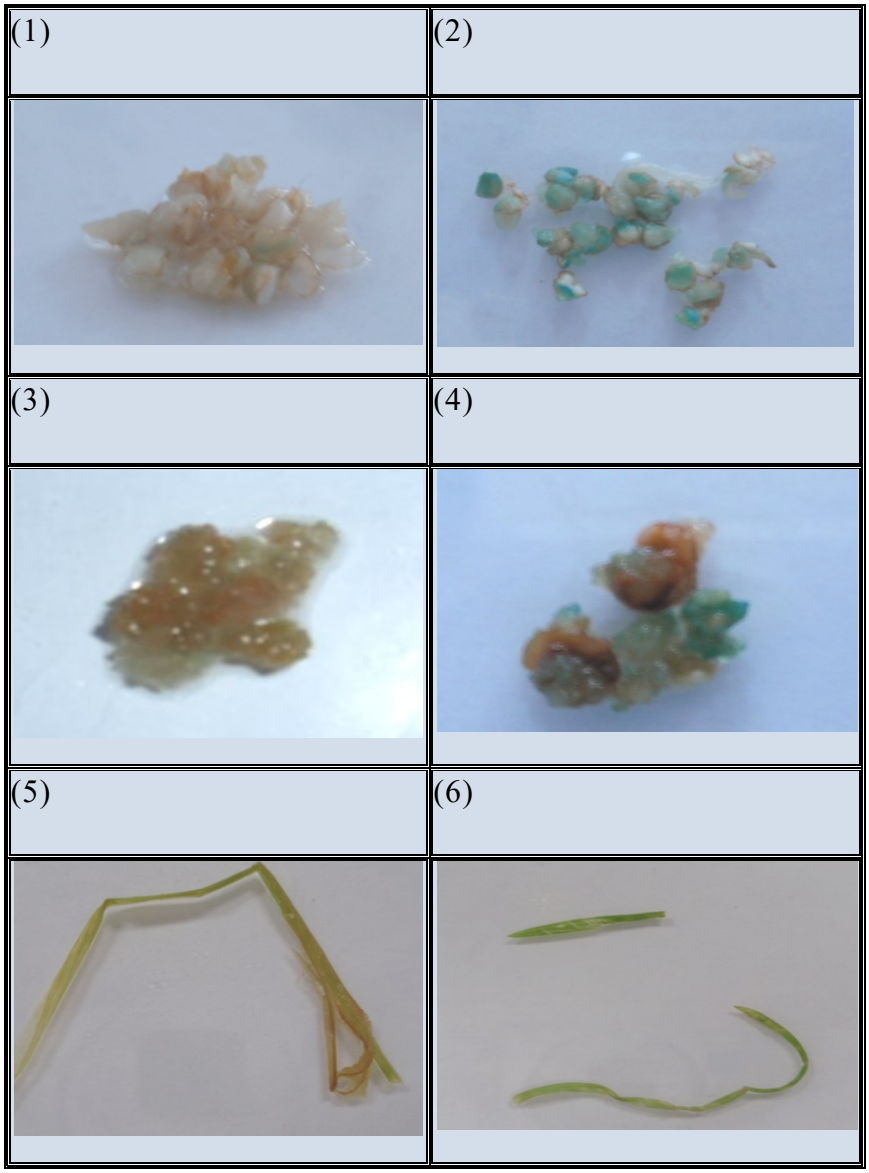

Fig (.2): *GUS* gene expression in transformed mature embryos and callus: (1) Control mature embryos, (2) positively transformed mature embryos, (3) control callus, (4) permanent positively transformed callus, (5) control shoot regeneration, (6) permanent positively transformed shoot regeneration.

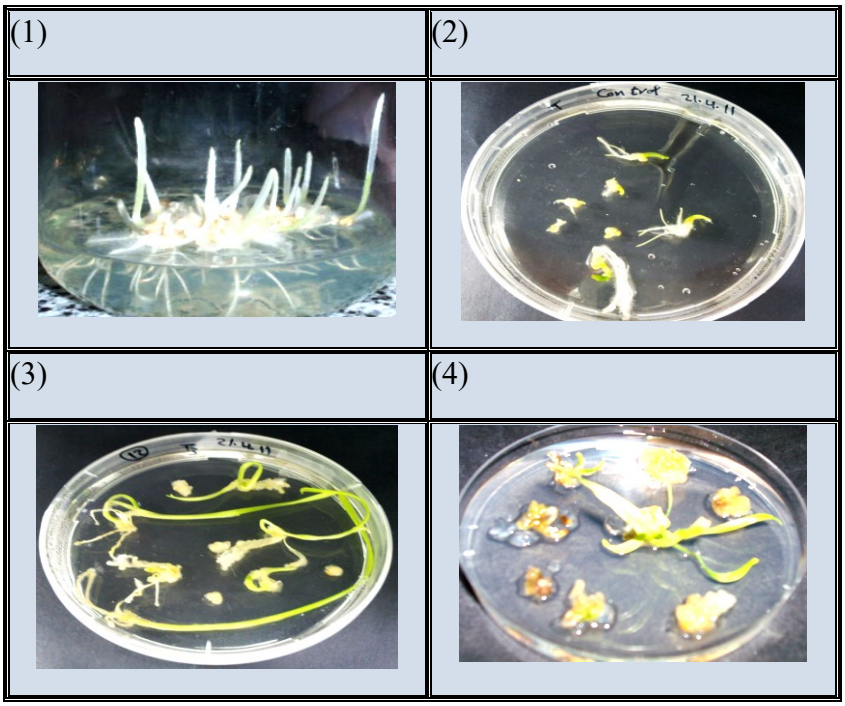

Fig. (3): (1) Initiation of transformed mature embryos (2) Control (3) Direct regeneration (4) Indirect regeneration.

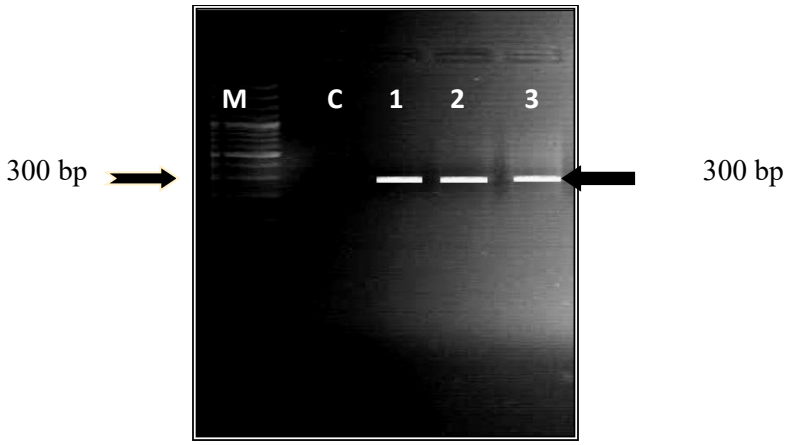

Fig. (4): PCR confirmations of regenerated transformed plants using specific primers of *AFP* gene. (Lanes1): M 1 kb marker; (Lane2): Negative control; (Lane3 to Lane 5) was Transformed plants genotype (G126, G130 and Elkasr).

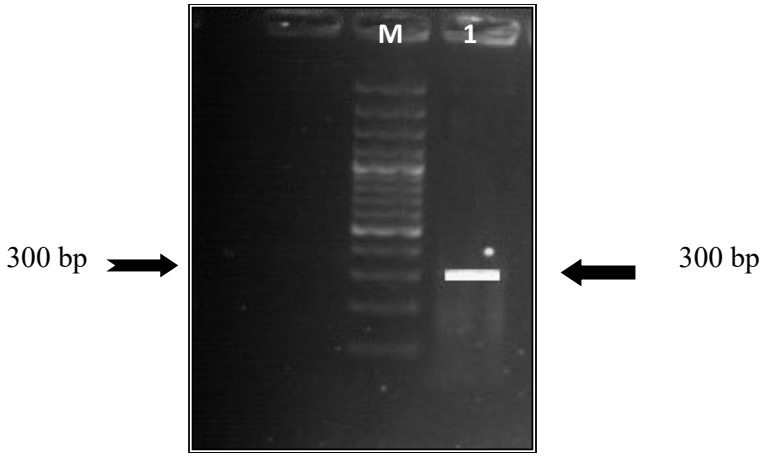

Fig. (5): PCR confirmations of regenerated transformed plants using *GUS* gene specific primers. (Lanes1): M 1 kb marker; (Lane2):