OPTIMIZATION OF *Agrobacterium*-MEDIATED TRANSFORMATION CONDITIONS FOR EGYPTIAN BREAD WHEAT CV. G164

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Agrobacterium-mediated transformation was firstly used to transform dicot species. Nevertheless, this highly efficient and successful method was not used for cereal transformation as monocots were widely considered to be outside the natural host range of *Agrobacterium*. Fortunately, a few individuals continued to defy the conventional wisdom, and were finally rewarded with success, first with rice and then all of the major cereals. One of the major barriers to the use of *Agrobacterium* to transform cereals was the absence of wound response and the associated activation of virulence genes. These problems were overcome with the use of actively dividing, embryogenic cells, such as immature embryos and calli induced from scutella, which are co-cultivated with *Agrobacterium* in the presence of acetosyringone, which is a potent inducer of virulence genes (Vasil, 2005).

Wheat is one of the most important field crops worldwide, with the largest harvested area and production levels. As a monocotyledonous plant, wheat has lagged behind dicotyledonous plants in ease and efficiency of transformation using *Agrobacterium*-based technique. Immature embryos have long been known to be a good regenerable explant source for wheat and there are effective protocols using biolistics for transformation and regeneration of this tissue. However, despite considerable interest there are few publications describing successful *Agrobacterium*-mediated transformation of wheat (Cheng *et al.*, 1997; Weir *et al.*, 2001; Wu *et al.*, 2003). However, research to make the *Agrobacterium*-based transformation method amendable to cereal crops has continued as the system is perceived to possess several advantages over other forms of transformation including: the ability to transfer large segments of DNA with minimal rearrangement; the precise insertion of transgenes resulting in fewer copies of inserted genes; and it is a simple technology with lower cost (Amoah *et al.*, 2001). Moreover, it allows for the stable integration of a defined segment of DNA into the plant genome and generally results in an improved stability of expression over generations than the direct DNA delivery methods (Smith and Hood, 1995). In addition, *Agrobacterium* transformation may facilitate removal of plant selectable marker genes by segregation. These are important

considerations, particularly when creating genetically manipulated lines in crop species for field testing; when the presence of unnecessary DNA and transgene arrangement/copy number are scrutinized as part of the regulatory processes (Wu *et al*., 2003).

The majority of wheat transformation investigations that have been reported (Vasil *et al*. 1992, 1993; Weeks *et al*. 1993; Nehra *et al*. 1994; Becker *et al*. 1994; Zhou *et al*. 1995; Zhang *et al*. 2000, Bahieldin *et al*., 2000) utilized microparticle bombardment technology. Cheng *et al*. (1997) first reported the success of *Agrobacterium*-mediated transformation in wheat with transformation efficiency 1-4%, but these results were limited to small-scale experiments and selection for the neomycin phosphotransferase II gene (*nptII*). Since then, some advances have been achieved (Jones *et al*., 2005). *Agrobacterium*-mediated wheat transformation, however, has not yet become an established and robust method of genetic transformation because the ability to routinely transform wheat using *Agrobacterium tumefaciens* is currently restricted to a few well-resourced laboratories worldwide (Jones *et al.*, 2005).

A critical step in the development of *Agrobacterium tumefaciens*-mediated transformation is the establishment of optimal conditions for T-DNA delivery into tissues from which whole plants can be regenerated (Amoah *et al*., 2001). Success in wheat transformation using *Agrobacterium* requires the identification of a model tissue culture system with a high capacity for producing regenerable cells, the optimization of parameters for gene transfer into those cells and tailoring selection and regeneration procedures to recover transgenic plants (Jones *et al*., 2005). In the present investigation, we optimized the conditions for genetic transformation of wheat cv. Giza 164 by examining three variables influencing T-DNA delivery and the regeneration of fertile plants in one of the commercially important Egyptian wheat variety.

MATERIALS AND METHODS

Plant material

A spring wheat, *Triticum aestivum* cv Giza164, was grown in the field to be used as source of explants. Immature caryopses were collected approximately two weeks post anthesis, surface-sterilized with 20% bleach (5.25% sodium hypochlorite) and 0.1% Tween 20 for 30 min, and then washed five times with sterile double-distilled H_2O. Immature embryos were isolated and placed with the scutellum side up on a callus induction medium as described by Weeks *et al*. (1993) with 1.5 mg/l of 2,4-D as the auxin source. Immature embryos either used directly after dissection or incubated on callus induction medium for five days before being used for transformation.

Agrobacterium strains and binary vector

The *Agrobacterium tumefaciens* strains EHA101 (Hood *et al*., 1986), C58 and LBA4404 have been used. All strains

contained the standard binary vector system pTF102 (12.1 kb), containing a *P*35S *bar* selectable marker gene cassette (phosphinothricin acetyltransferase gene driven by the cauliflower mosaic virus [CaMV] 35S promoter) and a *P*35S-*gus*-int reporter gene cassette (β-glucuronidase [GUS] gene with an intron driven by the CaMV 35S promoter). In all experiments, bacterial cell densities were adjusted to an optical density (OD_{600}) to 0.40 using a spectrophotometer immediately before embryo infection.

Tissue culture media and conditions

Infection medium composed of MS basal salts (Murashige and Skoog, 1962) with 150 mg/l L-asparagine, 1.5 mg/l 2,4-D, 68.4 g/l sucrose, 36.0 g/l glucose (pH 5.2) and supplemented with 100 μM acetosyringone (AS) before use. Co-cultivation medium composed of MS basal salts with 150 mg/l L-asparagine, 1.5 mg/l 2,4-D, 20 g/l sucrose (pH 5.8) and solidified with 3.0 g/l phytagel. Resting medium composed of MS basal salts supplemented with 150 mg/l L-asparagine, 1.5 mg/l 2,4-D, 30 g/l sucrose, 0.85 mg/l $AgNO_3$, 250 mg/l carbincillin (pH 5.8) and solidified with 8 g/l solidified agar. Selection medium was identical to resting medium with the addition of 1.5 or 3 mg/l bialaphos. Infection medium was filter sterilized, whereas all other media were autoclaved.

Inoculation and co-cultivation

Wheat immature embryos or five-days-old embryogenic calli were washed twice in bacteria-free infection medium. Wheat explants were inoculated with *A. tumefaciens* strains EHA101, C58 or LBA4404 harboring the plasmid pTF102 and suspended in infection medium. The tubes were incubated at room temperature for 5 min or 30 min. After infection the explants were transferred to the surface of co-cultivation medium. Embryos were oriented with the embryo-axis side in contact with the medium. Plates were wrapped and incubated in the dark at 22°C for four days after which explants were transferred to resting medium. The number of embryogenic calli was determined as the number of co-cultivated immature embryos or calli that had initiated embryogenic calli after two weeks.

Selection and regeneration

Selection of resistant clones was carried out by transferring wheat calli to selection medium containing 1.5 mg/l bialaphos. Calli were incubated on this medium for two weeks before they were transferred to selection medium containing 3 mg/l bialaphos. Putatively transformed events were regenerated on regeneration medium containing MS basal salts and vitamins, 2 mg/l TDZ, 250 mg/l carbincillin and solidified by 2 g/l phytagel. After two weeks, young wheat shoots were transferred to a rooting medium which contains half-strength MS basal salts and vitamins with 250 mg/l carbincillin for 2-3 weeks. Well-rooted plants were transferred to the greenhouse potting mix soil: beatmos: sand (1:1:1). The greenhouse day/night temperatures

were 25 ± 2°C/19°C under a 16-h photoperiod. Leaf painting assay was performed according to Weeks *et al*. (1993), using a 0.1% aqueous solution of Basta herbicide.

GUS activity assays

For *in vitro* experiments, both histochemical staining and fluorometric measurement of GUS activity in transformed immature embryos and calli were carried out as described by Jefferson (1987). Histochemical GUS assay was used to asses the transient expression of the *uidA* (GUS) gene in wheat explants two days after co-cultivation. Five explants were collected from each plate and incubated at 37°C overnight in 500 µl GUS buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. Blue foci were counted and evaluated with the aid of a microscope. Embryos or calli displaying blue foci or blue stain on the scutellum side were considered as positive. The explants were then categorized as follows: (+) weak expresser, (++) moderate expresser, (+++) strong expresser and (-) non-expresser or blue stain on the embryo axis.

Fluorometric assay of GUS activity was performed essentially as described by Jefferson (1987), by mixing pure β-glucuronidase enzyme with tissue extracts of putative transgenic as well as non transgenic plants. In both cases, 1 g tissue samples were ground in 0.5 ml phosphate buffer (50 mM KH_2PO_4, pH 7.0) at 0°C using a mortar and pestle. The samples were centrifuged at 12,000 xg for 15 min at 4°C, and protein concentrations of the supernatants were adjusted to 1 mg/ml for each sample. MUG assay conditions included 10 µg of purified enzyme (0.2 U), 250 µl x 2 GUS buffer, various volumes of tissue supernatants, and phosphate buffer to a final volume of 500 µl. After incubation at 37°C for 4 h, the reactions were stopped by addition of 2 ml of 0.2 M Na_2CO_3. Samples were illuminated at 360 nm and fluorescence at 455 nm was measured. Fluorometer records were statistically analyzed using the analysis of variance as outlined by Gomez and Gomez (1984) using MSTATC program. The differences between means were compared using Duncan multiple test (Duncan, 1955).

Herbicide leaf painting assay

For each plant tested, one approximately healthy looking and fully extended leaf was selected for leaf painting. The herbicide Basta was applied at 0.1% concentration with Tween-20 (0.1%) by painting the upper and the lower surface of about 5 cm part of the selected leaves. Control plants were also painted with the herbicide at the same time. After three days, Basta resistance was determined according to the percentage of necrosis suffered over the area painted with the herbicide solution in comparison to the control plants.

Molecular analysis

Genomic DNA was isolated by homogenizing 100-200 mg of leaf tissue and extracting essentially according to Stacey and Issac (1994). PCR was used to confirm the presence or the absence of transgenes in the primary transformants and their progeny (Pastori *et al*., 2001). The primer sequences were: *bar* - 5'-GTCTGCACCA TCGTCAACC- 3' and 5'-GAAGTCCAGCTGCCAGAAAC-3'; *uidA* - 5'-AGTGT ACGTATCACCGTTTGTGTGAAC-3' and 5'- TCGCCGCTTTGGA CATACCAT CCGTA-3'. Annealing temperatures and the approximate product length were 57°C, 484 bp respectively for *bar*, and 62°C, 750 bp respectively for *uidA*. At least two replicates were carried out for each PCR analysis.

RESULTS AND DISCUSSION

Factors affecting T-DNA delivery

In a preliminary experiment, a number of Egyptian wheat varieties have been tested for their embryogenic callus induction and regeneration frequencies. It was found that scutellum size strongly influenced culture response in all varieties tested. The highest embryogenesis and shoot regeneration frequencies were obtained from immature embryos with scutella of 0.75-1.0 mm size. Wheat variety Giza 164 revealed the highest percentage of embryogenic callus induction and regeneration frequency. This line has been chosen to test different transformation parameters in establishing an *Agrobacterium*-mediated transformation system for Egyptian wheat.

Three factors were tested for their influence on the transient and stable transformation of the elite wheat genotype G164, i.e. *Agrobacterium* strains (C58, EHA101 and LBA4404), type of explant (freshly isolated immature embryo and four-days-old callus) and infection time (5 and 30 minutes). Experiments were carried out in completely randomized design. Three replicates were carried out for each factor under investigation, each replica composed of five plates with fourty explants in each plate. The T-DNA delivery was assessed by determining the extent of GUS expression in explants. Two assays were used to detect GUS expression, i.e., visual histochemical GUS screening and the quantitative fluorescent MUG assay (Jefferson, 1987). Five explants, sampled at random after three days on resting medium (seven days from the inoculation) from each inoculated plate, were used for each assay.

Effect of Agrobacterium strain

Comparing three *Agrobacterium* strains, transgenic plants were only recovered from embryos infected with C58 and EHA101, while no transient or stable *gus* gene expression or transgenic plants were recovered from embryos infected with LBA4404 strain. This result is in contrast to that obtained by Debasis *et al.* (2006), where the *Agrobacterium* strain LBA4404 has been used for the transformation of hexaploid bread wheat (*Triticum aestivum*) and tetraploid pasta

wheat (*Triticum durum*). They achieved successful generation of transgenic plants at a transformation frequency ranging from 1.28 to 1.77% following 2-3 days co-cultivation using mature embryos and also mature embryo-derived calli with binary *Agrobacterium* strain LBA4404 (pBI101 :: *Act1*) and LBA4404 (p35SGUSINT), respectively.

Transient GUS expression assays

Visual screening of transient GUS expression by histochemical assay revealed that the highest expression was obtained when the *Agrobacterium* strain C58 was used to inoculate immature embryos for 5 min while inoculation of calli with the same conditions revealed lower value (Table 1). *Agrobacterium* strain EHA101 revealed lower number of immature embryos or calli with high GUS expression. Although the number of embryos with medium GUS expression was relatively higher with C58 for 5 min infection, the inoculation of calli for 5 or 30 min did not show any differences with different *Agrobacterium* strains (EHA101 or C58), (Fig. 1).

Quantification of GUS activity by fluorescent MUG assay, reflecting the proportion of cells transiently expressing GUS gene, from immature embryo inoculated with different *Agrobacterium* strains is illustrated in Table (2). The statistical analysis of the mean number for GUS activity of the two strains indicated that GUS activity obtained by *Agrobacterium* strain C58 (925.8) was significantly higher than that obtained by EHA101 (261.3). While, there was no significant difference between the two tested inoculation time (5 and 30 min). Statistical analysis indicated significant difference in the interaction between *Agrobacterium* strain and inoculation time. *Agrobacterium* strain C58 inoculated for 5 minutes revealed the best value (1070.0) followed by the same strain inoculated for 30 minutes (781.5).

GUS Fluorometric assay data confirmed the results of the histochemical assay which indicates that the use of *Agrobacterium* strain C58 gave higher expression of *gus* gene in the transformed embryos. While the inoculation time (5 and 30 min) was not a significant factor.

Effect of the type of explant

The choice of starting material (explant) has proved to be crucial in successful *Agrobacterium*-mediated wheat transformation. The scutellum of cultured immature seed embryos has long been known to be a good regenerable explant source for wheat (Ozias-Akins and Vasil, 1982). In the present study, when four-days old-wheat calli were infected with *Agrobacterium* strains C58 or EHA101, moderate to low transient GUS expression has been obtained but no transgenic plants have been recovered. Embryogenic calli and stably transformed wheat plants have been obtained only from transformed immature embryos. In this context, Wu *et al*. (2003) stated that, the duration of pre-culture and the explant-*Agrobacterium* incubation period was found to affect explant survival,

transient GUS expression and response in tissue culture. The shorter pre-culture times resulted in higher transient expression although explants pre-cultured less than one hour had slightly lower survival rates. Transient GUS expression increased with inoculation time, but survival rate and regeneration capacity were both dramatically reduced at the longer immersion periods. Our results for the effect of explants type on transformation and regeneration frequency are in consistence with other investigations. It has been reported by Jones *et al*. (2005) that immature embryos of Bobwhite, pre-cultured between 1 and 6 days on CM4C medium, are the most commonly used explant, although the use of 9 day pre-cultured immature embryos of cv. Fielder and callus derived from immature embryos of Bobwhite and cv. Veery 5 has also been reported.

Regeneration of putative transgenic wheat plants

The steps of tissue culture, transformation, selection and regeneration of wheat plantlets are illustrated in Fig. (2). Percentage of embryogenic calli on selection medium varied with different treatments (Table 3). The highest percentage of embryogenic calli was 37.6 formed by the immature embryos transformed with the *Agrobacterium* strain C58 with infection time of five min while increasing the infection time to 30 min lowered the amount of embryogenic calli formed by the transformed wheat immature embryos to 25.0%. Using callus as transformation explants had a negative effect on the type of calli recovered on selection medium where the percentage of embryogenesis was 14.0% and 12.8 % for calli transformed using the C58 and EHA101 for 5 min infection time, respectively. While the percentage of embryogenic calli was only 4.5% and 4.4% for calli transformed with C58 and EHA101 for 30 min, respectively. These results revealed that infection time is very critical for maintaining and recovering embryogenic calli capable for regeneration. Increasing the inoculation time reduced the efficiency of embryogenesis and consequently, reduced the regeneration capacity of transgenic plants. In this context, other infection times have been used by different investigators. Wu *et al*. (2003) achieved successful transformation after incubation of freshly isolated immature embryos with *Agrobacterium* suspension for three hours. While, Debasis *et al*. (2006) recovered transgenic plants by immersing the excised mature embryos or three-week-old mature embryo-derived calli in bacterial suspension and incubating them for 1 hour.

Transformation efficiency

The highest transformation efficiency of 5.1% was obtained when C58 *Agrobacterium* strain was used for the infection of immature embryos for 5 min, while only 2.7% was obtained when the *Agrobacterium* strain EHA101 was used for immature embryo infection for 5 min. Callus infection with *Agrobacterium* strain C58 for 30 min revealed the lowest

transformation efficiency (0.2%). While no wheat transformed events were recovered by infection of wheat calli with EHA101 with infection time 5 or 30 min.

All T_0 plants surviving bialaphos and basta selection (Fig. 2F & G) were screened for the presence of marker gene sequences by PCR analysis. Lines which did not show PCR products for both marker genes were considered 'escapes' and discarded. PCR analysis for *bar* gene in putatively transgenic plants confirmed the presence of *bar* and *uidA* genes in wheat plants (Fig. 3a & b). The number of positive lines produced by using *Agrobacterium* strain C58 was more than the number of lines produced as result of using EHA101 (Table 3).

In the present study, we investigated some factors that influence *Agrobacterium*-mediated transient expression of *uidA* in wheat tissue. The most successful *Agrobacterium* transformation of wheat has been achieved using the *A. tumefacins* strain C58 with freshly isolated immature embryos. We also compared the transformation efficiency with other *Agrobacterium* strains, i.e. EHA101 and LBA4404. The ability of particular *Agrobacterium* strains to transform plant cells is defined by their chromosomal and plasmid genomes which between them must encode all the machinery necessary for attachment and DNA-transfer. The *Agrobacterium* strains that have been successfully used for wheat transformation are based on only two chromosomal backgrounds, LBA4404 (Ach5) and C58 but these have been used with a wide range of Ti and binary plasmids (Jones *et al*., 2005). In consistence with our results, Cheng *et al*. (1997) reported the successful transformation of wheat immature embryos, precultured immature embryos and embryogenic calli using the *A. tumefaciens* strain C58 harboring a binary vector pMON18365 containing the *gus*-intron gene and the neomycin phosphotransferase II gene. Although the weakly virulent *Agrobacterium* strain LBA4404 was successful with the transformation of elite maize genotypes (Assem *et al*., 2006), it has been reported that it could be successful in transforming wheat only when augmented by the superbinary plasmid pHK21 which possessed extra copies of *vir* B, C and G genes from pTiBo542 (Jones *et al*., 2005). Moreover, Guo *et al*. (1998) investigated several factors influencing the T-DNA transfer from *Agrobacterium* into wheat and barley cells. Using an intron-GUS gene as a marker, they found a supervirulent strain, EHA101 (pIG121Hm), which as the most efficient strain among three strains studied. They also confirmed that acetosyringone (AS) was found to be vital for this process and that the frequency of T-DNA transfer, as measured by GUS expression, was greatly increased by longer and repeated co-cultivation. Other investigators studied the role of various factors responsible for virulence of *Agrobacterium* to establish better transformation systems. Ali *et al*. (2007) screened different factors affecting the transformation of rice. They studied the

effect of callus induction medium, the amount of acetosyringone, pH value of infection medium, the carbon source, bacterial cell density and the incubation conditions during co-cultivation period.

The establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated is an important step in the development of *Agrobacterium*-mediated transformation system. In the present investigation, some factors that influence T-DNA delivery into wheat tissue have been investigated using cv. Giza 164. The combination of using the *Agrobacterium* strain C58 with freshly isolated immature embryos at inoculation time of five min. is recommended for production of stably transgenic wheat lines. This is an important first step to utilize *Agrobacterium* in the transformation of immature wheat inflorescence tissue.

SUMMARY

Gene transfer by *Agrobacterium* is the method of choice for the genetic transformation of most plant species. The development of *Agrobacterium*-mediated transformation protocol for recalcitrant species like bread wheat requires the optimization of T-DNA delivery conditions. We have used explants from one of the recalcitrant bread wheat varieties (i.e. cv. Giza 164) and the *Agrobacterium* strains harboring a plasmid with a T-DNA incorporating the *bar* gene and *uidA* (β-glucuronidase) gene to investigate and optimize major T-DNA delivery and tissue culture variables. Factors that produced significant differences in T-DNA delivery and regeneration including the *Agrobacterium* strain, type of explants and inoculation time were studied. Activity of *uidA* gene has been evaluated by histochemical and fluorometric MUG assay. Putative transgenic wheat plantlets have been tested by PCR and herbicide leaf painting. The transformation frequency ranged between 0.2 to 5.1%.

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Table (1): Effect of *Agrobacterium* strain (C58, EHA101) and type of explants (immature embryo, 4-days-old calli) and inoculation time (5, 30 minutes) on the transient expression of *uidA* (GUS*)* gene.

Strain	Explants	Inoculation time (min)	GUS expression		
			+	++	+++
C58	Immature embryos	5	26	45	49
		30	32	44	24
	4-days-old calli	5	24	37	16
		30	22	15	12
EHA101	Immature embryos	5	15	23	23
		30	6	30	24
	4-days-old calli	5	13	12	16
		30	10	7	11

+: week, ++: moderate, +++: strong expression of *uidA* (GUS) gene in the transformed wheat tissues.

Table (2): Fluorometric assay for *uidA* (GUS) gene expression in transformed embryos infected for 5 or 30 minutes with two *Agrobacterium* strains.

Strain	5 min	30 min	Mean
C58	1070.0a	781.5a	925.8A
EHA101	289.2b	233.3b	261.3B
Mean	679.6^{A}	507.4^{A}	

Means within rows and/or column, followed by the same letter(s) are not significantly different by Duncan's New Multiple Range Test ($P<0.05$).

Table (3): Effect of *Agrobacterium* strain (C58, EHA101), type of explants (immature embryo, 4-days-old calli) and inoculation time (5, 30 minutes) on the embryogenesis and transformation efficiency.

Strain	Explants	Inoculation time (min)	Embryogenesis %	No. putative transgenic plants/no. explants infected	Transformation efficiency (%)
C58	Immature embryos	5	37.6	26/511	5.1
		30	25.0	3/460	0.6
	4-days-old calli	5	14.0	2/498	0.3
		30	4.5	1/440	0.2
EHA101	Immature embryos	5	23.8	4/147	2.7
		30	11.5	1/173	0.6
	4-days-old calli	5	12.8	0/140	0.0
		30	4.4	0/157	0.0

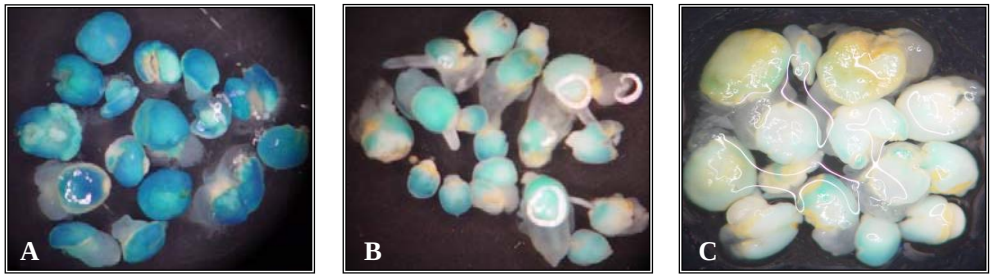

Fig. (1): The expression of *uidA* (GUS) in transformed wheat calli. (A) strong expression, (B) moderate expression, and (C) week expression.

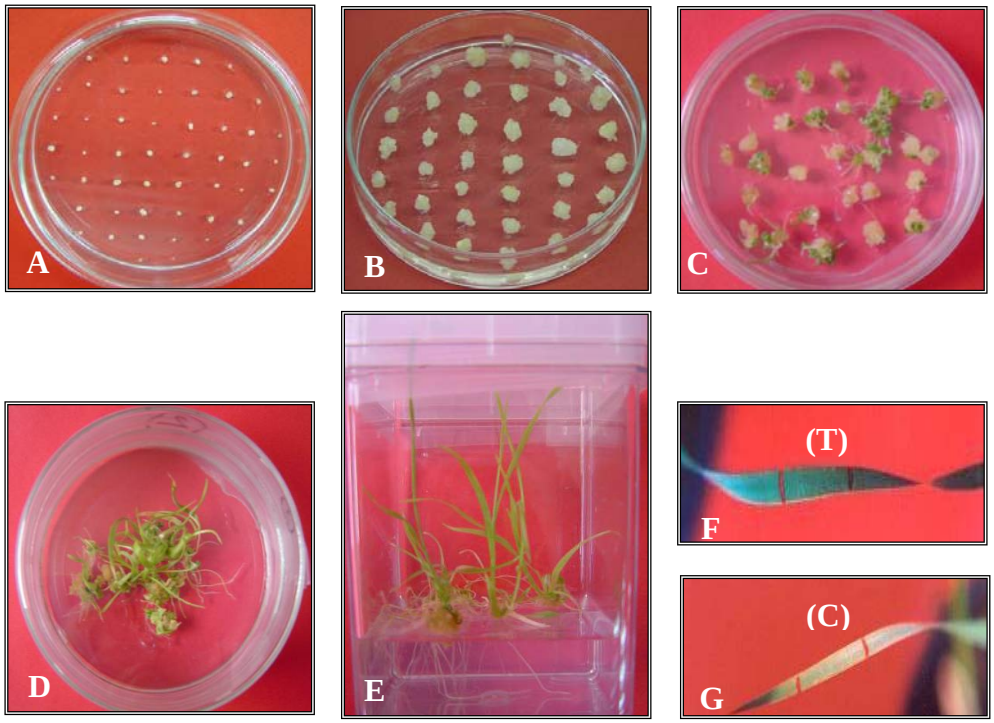

Fig. (2): Different tissues culture steps of wheat cultivar Giza 164. (A) immature embryos on callus induction medium, (B) induced calli (C & D), regenerated calli on regeneration medium, (E) regenerated wheat plantlets, (F & G) wheat leaves painted with Basta (transgenic-T and control-C plantlets).

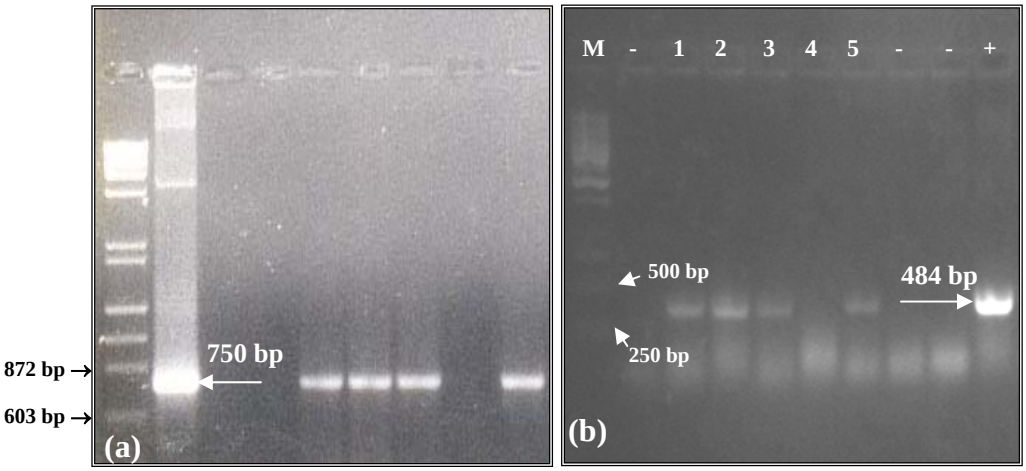

Fig. (3): PCR analysis of *gus* (a) and *bar* (b) genes in transgenic wheat plants. M: DNA molecular weight size marker, +: Plasmid positive control, - : Non-transformed wheat DNA, 1-5: DNA samples of putatively transgenic wheat plants.