

# Genetic transformation of mature embryos of bread (*T. aestivum*) and pasta (*T. durum*) wheat genotypes

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The objective of the present study is to develop an efficient protocol for regeneration of transgenic wheat plants using *Agrobacterium*-mediated transformation of mature embryos of hexaploid bread wheat (*Triticum aestivum*) and tetraploid pasta wheat (*Triticum durum*). The data indicated that embryogenic calli were formed within 7 days in the presence of 2 mg l<sup>-1</sup> 2,4-D. Adventitious shoots emerged from the embryonic calli in the presence of 2 mg l<sup>-1</sup> BA. Shoot regeneration frequency varied between wheat cultivars according to their genetic background differences. Regeneration frequency was higher in the cultivar Gemmiza 10 (95%) compared with the other cultivars tested. Mature embryos derived callus of the cultivars Gemmiza 10 and Gemmiza 9 were co-cultivated with *A. tumefaciens* strain LBA4404 harboring a binary vector pBI-121 containing the neomycin phosphotransferase-II gene (*npt-II*). The resulted putative transgenic plantlets were able to grow on kanamycin containing medium. A successful integration of the transgene was confirmed by analyzing the plantlets using Southern hybridization and PCR amplification. The *gus* gene expression can be detected only in the transgenic plants. The reported protocol is reproducible and can be used to regenerate transgenic wheat plants expressing the genes present in *A. tumefaciens* binary vectors.

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## Introduction

Wheat is one of the most abundant sources of energy and nourishment for mankind. Ninety-five percent of the cultivated wheat cultivars are hexaploid which are used for the preparation of bread and other baked products and the remaining 5% are durum (tetraploid) wheat, which is used essentially for making pasta and macaroni.<sup>1</sup>

Wheat production potential in Egypt is assessed only under irrigated conditions, both for areas already irrigated and for those which may come under irrigation in future. Small areas of land that could grow wheat with irrigation from local groundwater sources such as springs are left out of consideration because of their limited extent and preferred alternative uses. (www.fao.org).

Environmental stresses such as drought and salinity some of the most limiting factors for agricultural productivity worldwide.<sup>2,3</sup> Not only do they greatly decrease the potential yield of current crop species, but they also restrict the area where production can take place and hinder introduction of crop plants into new areas.<sup>4</sup> Salinity in particular is a major problem, affecting crop production on nearly one-third of the world's irrigated agricultural land.<sup>5,6</sup> There is a shortage of arable land area and this area is steadily decreasing, as farming practices cause cultivated land to become more saline.<sup>7</sup> Wheat (*Triticum aestivum*) is

a moderately salt-tolerant crop.<sup>8</sup> In the field, where the salinity rises to 100 mM NaCl (about 10 dS m<sup>-1</sup>), wheat will produce a reduced yield. Durum wheat (*Triticum turgidum* ssp. durum) is less salt tolerant than bread wheat.<sup>8</sup>

The regeneration of whole plants from selected transgenic tissues is required for the successful application of biotechnology in wheat improvement. Tissue culture responses which includes callus induction and regeneration capacity of wheat are influenced by the genotypes explants source, geographical origin and physiological status of the donor plants, the culture medium and the interactions between them.<sup>9</sup>

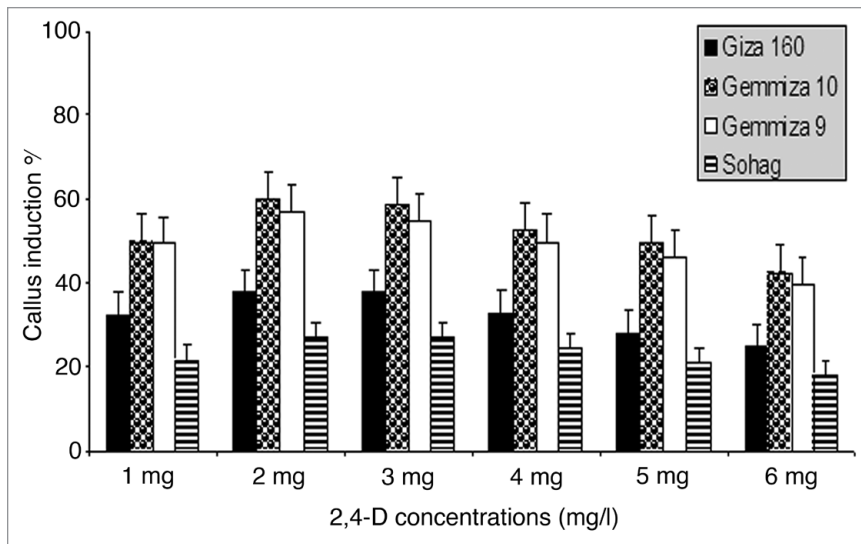
Establishing an efficient tissue culture technique is difficult in monocotyledonous species particularly in Gramineae family.<sup>10</sup> As a member of the family, wheat is also a recalcitrant crop that limits the utilization of tissue culture technique for crop improvement.<sup>11</sup> If a suitable protocol for plant regeneration from mature embryogenic callus is available, research can be carried out on wheat transformation throughout the year.

In vitro regeneration of wheat have been successfully achieved using different explant types such as mature and immature embryos, seeds, endosperm, leaves, shoot bases and root tips.<sup>12</sup> From the above mentioned explants, the immature embryo was proved to be the best for callus induction and shoot regeneration.<sup>12-14</sup> But the availability of immature embryo is limited by

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**Figure 1.** The calli induction frequency of four wheat cultivars under different 2,4-D concentrations.

wheat growing season or requires expensive and sophisticated growth chambers. On the other hand mature seeds of wheat are readily available throughout the year, hence can be used for plant regeneration in any convenient time. High frequency of callus induction can be also obtained by using mature embryo culture in wheat.<sup>15</sup>

Genetic transformation is an indispensable tool for functional studies of genes and the use of exotic genes that cannot be transferred by sexual means in crop improvement.

Wheat was considered as a recalcitrant to *Agrobacterium*-mediated transformation. However, since the first successful report of fertile transgenic wheat plants,<sup>16</sup> there has been considerable progress. Various factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat have been explored by several workers in a quest to achieve higher transformation efficiency.<sup>17-20</sup> The *Agrobacterium*-mediated gene delivery system has distinct advantages over the other methods, involves the ability to transfer large segments of DNA with minimal rearrangement, with fewer copies at a higher efficiency incurred with minimal cost. Thereby, such an approach results in simple segregation pattern, better transformation frequency and also the absence of vector backbone sequences. Furthermore, in *Agrobacterium*-mediated transformation DNA generally inserts into transcriptionally active regions via illegitimate recombination, thereby ensuring the active transcriptional expression of the transgenes.<sup>21</sup>

The main objective of this study was to develop a procedure for transformation and regeneration of viable shoots from mature seeds of commercial Egyptian wheat varieties. Three commercial cultivars of bread wheat (*T. aestivum*) and one cultivar of the pasta wheat (*T. durum*) were successfully regenerated. Differences in the regeneration capacity among the cultivars were observed. The system described here can be used as a basis for the future development of commercial scale production of regenerated wheat

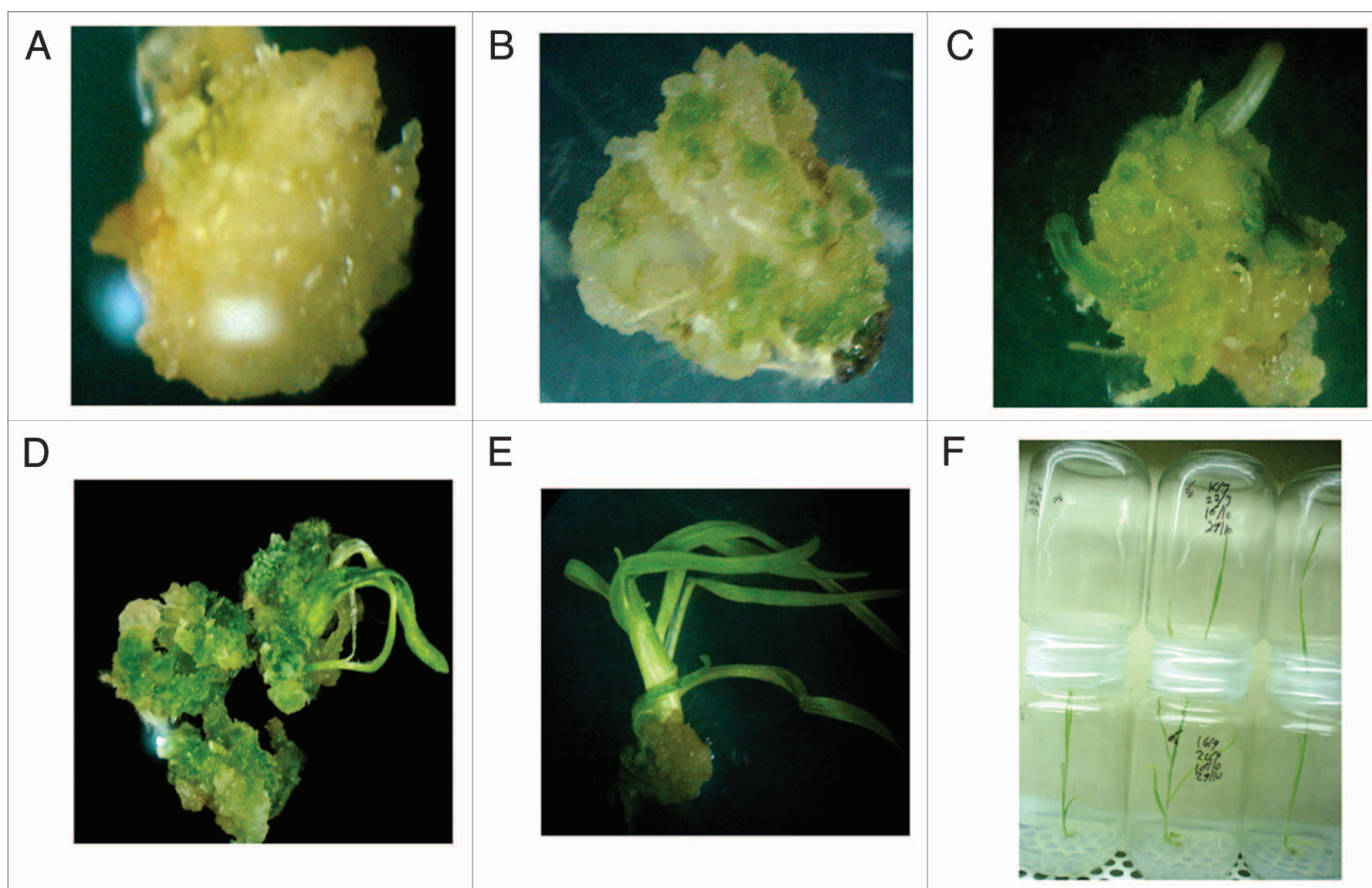
plants. In addition, the co-transformation of a foreign gene with T-DNA in the Ti plasmid of *Agrobacterium tumefaciens* delivery system leads to an improvement of plant properties.

## Results

In the present study an efficient system for shoot regeneration in some wheat cultivars was established as a prerequisite for wheat transformation. For callus induction from wheat mature seeds, six different concentrations of 2,4-dichlorophenoxyacetic acid were used. Explants exhibited an initial swelling followed by callus formation within one week of incubation. The number of calli increased with increasing 2,4-D concentration from 1 mg l<sup>-1</sup> to 2 mg l<sup>-1</sup> in all cultivars. However, raising the concentration to 3, 4, 5 or 6 mg l<sup>-1</sup> produced an opposite effect. The cultivars also differed for callus induction. The data presented in **Figure 1** indicate that at 2 mg l<sup>-1</sup> 2,4-D the cultivar Gemmiza 9 and showed the highest number of calli/explant followed by Gemmiza 10 and then Giza 160 while the lowest number was obtained from the cultivar Sohag.

Explants-derived calli were placed on regeneration medium containing 2 mg/l benzyladenine. The nodular structures developed into adventitious shoots when the embryogenic calli were sub-cultured in the medium supplemented with BA within two weeks. The somatic embryos directly emerged from the calli explants. **Figure 2** shows the different developmental stages of the plantlets. The regeneration frequencies of the mature embryo explants derived from four wheat cultivars are listed in **Table 1**. The data indicated that the cultivars differed in the number of regenerated plants. The cultivar Gemmiza 10 showed the highest regeneration percentage (95%) followed by Gemmiza 9 (87.5%) while the cultivar Sohag induced the lowest regeneration frequency (72%). The initiated shoots were matured and germinated on half strength MS medium. The seedlings were acclimatized and transferred to the greenhouse.

For adapting transformation system in wheat the cultivars Gemmiza 10 and Gemmiza-9 that show higher regeneration frequency were used for transformation experiments. Embryonic calli derived from mature seeds from the cultivars Gemmiza 10 and Gemmiza 9 was co-cultivated with *Agrobacterium tumefaciens* LBA-4404 harboring the binary vector pBI-121.<sup>23</sup> After co-cultivation period (3 days), the infected calli were placed on MS medium with low selection pressure. During the process of selection, the successfully transformed calli continued to grow vigorously to produce shoot initiations, whereas the untransformed ones failed to form shoots and eventually bleached and became necrotic within 3 weeks. Putative transgenic shoots were regenerated within 4 to 6 weeks on the MS medium containing 2 mg/l BA. During the selection culture, the explants were sub-cultured into fresh medium containing 50 mg/l kanamycin which greatly reduced the number of escapes. The transformation percentages



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**Figure 2.** Induction of somatic embryogenesis in wheat. (A–E) are the different developmental stages of somatic embryos in wheat [(A) embryonic calli, (B) globular stage, (C) torpedo stage, (D and E) shoot regeneration and (F) Elongation process of regenerated plant].

**Table 1.** Shoot regeneration frequencies on mature explants of four wheat cultivars

Cultivar	Total number of explants	Explants forming					
		Callus inducing explants	Callus induction (%)	No. of shoot initiation	Shoot induction (%)	No. of regenerated plants	Regeneration %
Gemmiza 10	300	228	76	184	82	176	95
Gemmiza 9	300	240	80	216	90	189	87.5
Giza 160	300	152	50.6	100	65.7	75	75
Sohag	300	108	36	68	62	53	72

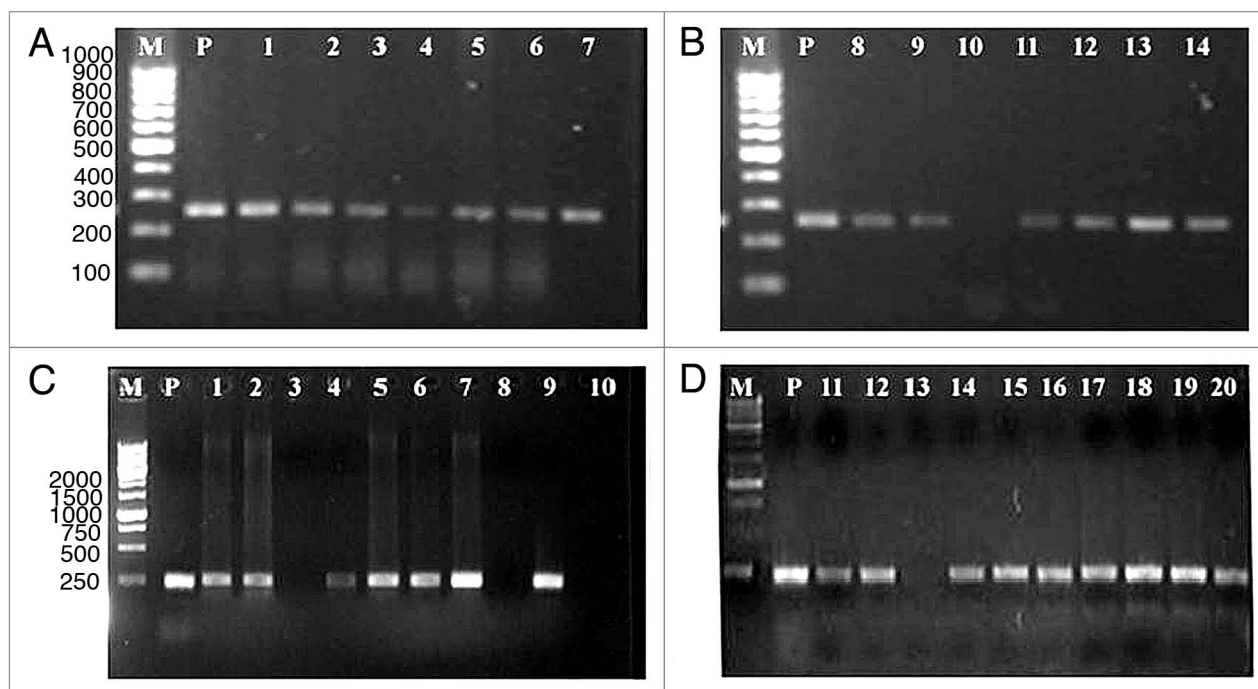
**Table 2.** The transformation percentage of wheat cultivars infection with agrobacterium

Cultivar	Number of explants used	Agrobacterium transformation					
		Callus inducing explants	Shoot induction	GUS positive	PCR positive for <i>NPT-II</i>	PCR positive for 35 S promoter	Transformation %
Gemmiza 10	300	240	912	80	80	80	8.7
Gemmiza 9	300	228	720	50	50	50	6.9

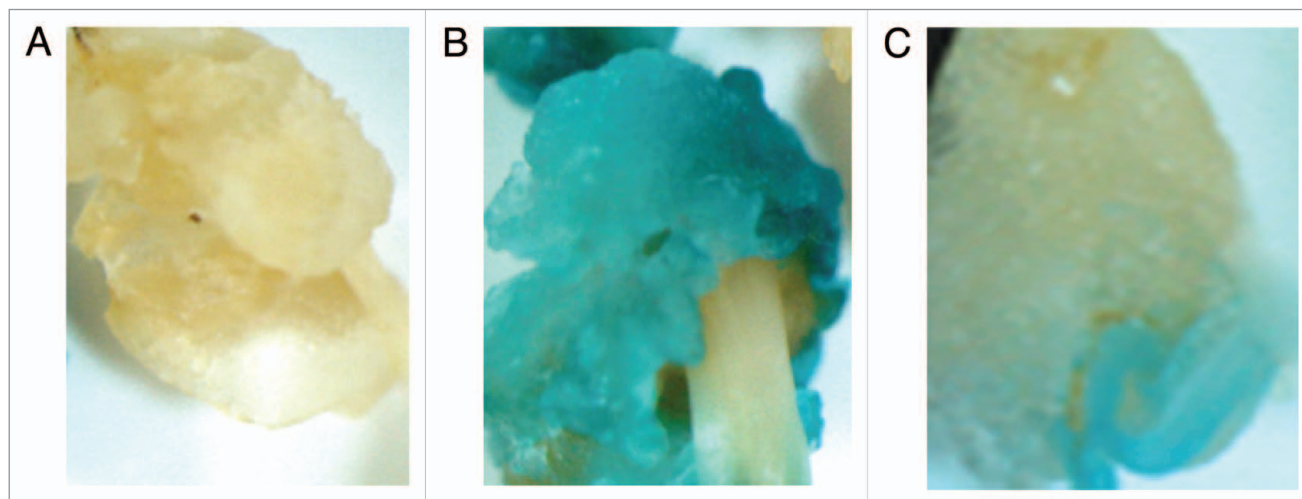
for the two tested wheat cultivars after *Agrobacterium* infection and selection were 6.9% and 8.7% for the cultivars Gemmiza 9 and Gemmiza 10, respectively (Table 2).

To confirm the presence of the *T-DNA* in the regenerated plants, all  $T_0$  plants were subjected to PCR analysis with the primers specific for the *npt-II* gene and also for the 35 S-promoter.

The PCR analysis indicated that all the regenerated plants examined showed a clear band corresponding to the relevant sequence of both of the *npt-II* gene and the 35-S-promoter (Fig. 3). In the present study the kanamycin resistant plants showed GUS expression while the non-transgenic did not show any GUS activity (Fig. 4).



**Figure 3.** PCR analysis confirming the transformation of *T-DNA* into wheat genome. (A and B) The detection of the 35-S-promoter in the transformed plants. (C and D) The stable integration of the *npt-II* gene into the genome of the regenerated plants. M: MW (XVI-250 bp ladder, Roche).



**Figure 4.** Histochemical *GUS* assay showing gene expression in transgenic plant callus (B and C) while no expression can be detected in non-transgenic plant (A).

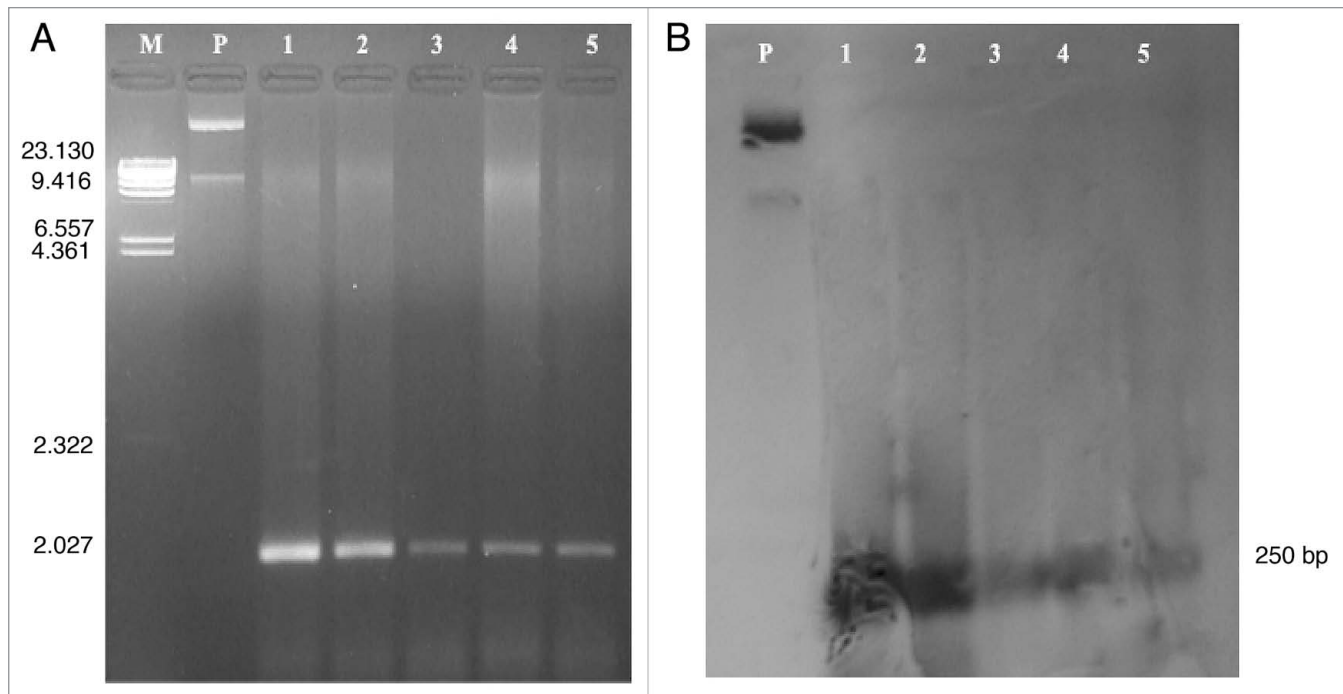
The presence of transgenes in the regenerated plants was confirmed using Southern blotting analysis. Southern blotting analysis indicated that the transgenic plant yielded 1–3 bands that were hybridized specifically with the labeled primers prepared from the *npt-II* gene (Figs. 5 and 6).

### Discussion

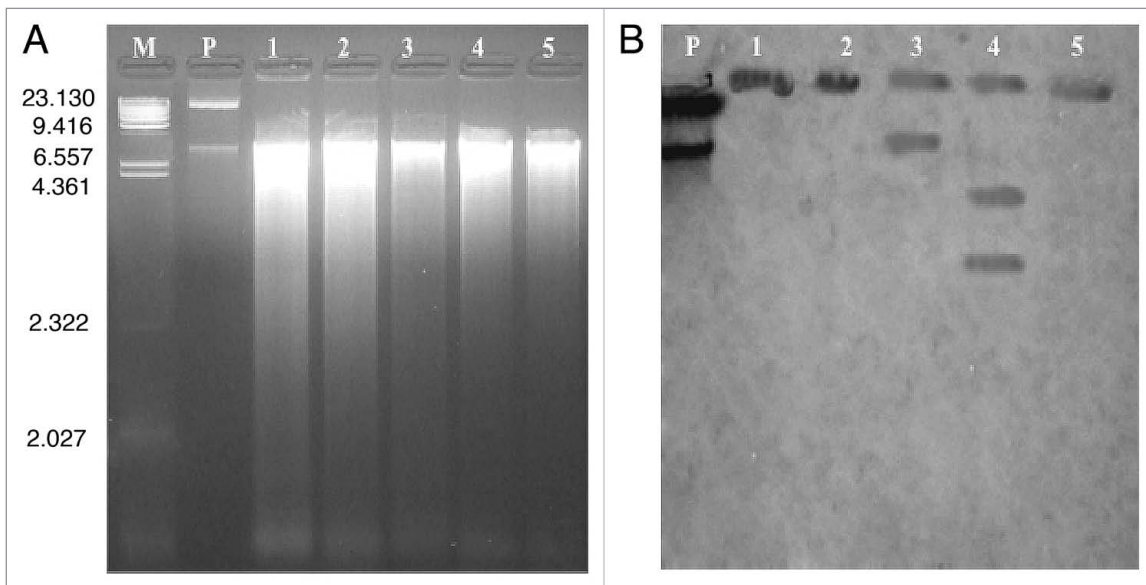
Regeneration studies in wheat have been mostly achieved using leaf bases, immature embryos, scutellum and to immature inflorescence tissue as an explants.<sup>22,23</sup> Although mature embryos have

been the explants of choice for regeneration and transformation experiments,<sup>24,25</sup> their use is not prevalent. The present investigation, described a procedure involves regeneration and transformation of mature embryos from *T. aestivum* and *T. durum* wheat genotypes; it is simple and fulfils the basic wounding requirement and does not require special media.

The degree of cellular organization and shoot meristems in most wheat genotypes can be controlled by manipulation of 2,4-D concentrations.<sup>12</sup> The response of mature seeds to culture was observed within one week on callus inducing medium. An excellent callus induction was observed at 2 mg/l 2, 4-D after a



**Figure 5.** Southern blot hybridization of DNA prepared from transgenic wheat plants, transformed by the *npt-II* gene. DNA (10  $\mu$ g) was cut by restriction enzymes *Eco RI*, separated on 2% agarose, transferred to membranes and hybridized to a labeled probe (*npt-II* gene). P; the pBI121, lane 1–5 are genomic DNA isolated from five transgenic wheat line.



**Figure 6.** Southern blot hybridization of PCR products prepared from transgenic wheat plants, transformed by the *npt-II* gene. P; the pBI121, lane 1–5 are the *npt-II* PCR product from five transgenic wheat line.

culture period of 4–5 days which is consistent with the finding reported by Mohmand, and Nabors<sup>26</sup> who obtained good callus from mature seed embryos of spring and winter genotypes of wheat. Ozias and Vasil<sup>27</sup> evaluated the effect of 2,4-D concentrations ranging from 1 to 8 mg/l on scutellar callus growth and found that a similar concentration of 2,4-D (2 mg/l) was optimal which is in agreement with our results. Malik et al.<sup>28</sup>

obtained high frequency of plant regeneration of wheat mature embryos on MS medium supplemented with 0.5 + 0.1 mg of BAP + IAA/L. Shah et al.<sup>29</sup> studied in vitro regeneration of wheat and they observed that regeneration was higher on MS medium supplemented with 4.0 mg/L of BAP alone or 2.0 mg/L of BAP in combination with 1.0 mg/L of IAA. Our data indicate that the cultivars differed in the number of regenerated plants. The bred

**Table 3.** The pedigree of the four wheat genotypes used

Cultivars	Pedigree
Gemmeiza 9	ALD"S"/HUAC"S"/CMH74A.630/5X
Gemmeiza 10	MAYA74"S"/ON//1160-147/3/Bb/4/CHAT"S"/5/CTOW
Giza 160	CHENAB-70/GIZA155
Sohag 1	GERARDO-VZ-469/3/JORI(SIB)//ND-61-130/LEEDS

wheat cultivars Gemmiza 10 percentage and Gemmiza 9 showed higher regeneration frequency (95 and 87.5%, respectively) while the pasta cultivar Sohag show the lowest regeneration frequency (72%). This finding supports the assumption that regeneration in wheat is genotype-specific.

Cereals, especially wheat were once considered as recalcitrant to *Agrobacterium*-mediated transformation. However, since the first successful report of fertile transgenic wheat plants,<sup>16</sup> there has been considerable progress. Various factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat have been explored by several workers in a quest to achieve high transformation efficiency. In the present investigation, callusing and regeneration were optimized to attain reproducible, standardized transformations.

In the present investigation, the *Agrobacterium* LBA4404 was proved to be highly virulence to the wheat genotype used, similar results was obtained by Patnaik et al.<sup>30</sup> A successful production of transgenic plants was achieved by co-cultivation of mature embryos and mature embryo-derived calluses. The vectors successfully used in this study are conventional binary vectors. The binary vector pBI121 containing the *gus* intron gene under the genetic control of 35S promoter which efficiently blocks its expression in bacteria, thus excluding false positives.

The regeneration protocol of the present study was efficiently used to produce transgenic wheat plants expressing the *npt-II* gene. The average shoot regeneration percentages for the two tested wheat cultivars after *Agrobacterium* infection and selection were 23 and 24%, with a total of 189 and 176 shoots produced for the cultivars Gemmiza 10 and Gemmiza 9, respectively (Table 2). It was greatly reduced when compared to the 95 and 87% shoot regeneration from culture without the infection of *Agrobacterium* and selection with kanamycin (Table 1).

The antibiotic resistant nature of transgenic tissues proves that the selectable marker gene used (*npt-II*) is active in wheat. The results of antibiotic resistance assays were observed to be consistent with those of molecular analysis.

In order to confirm the existence of the *T-DNA* in the regenerated plants genome, all of the T<sub>0</sub> plants obtained were subjected to PCR analysis with the primers specific for the *npt-II* gene and also for the 35 S-promoter. The PCR analysis indicated that all the regenerated plants examined showed a clear band corresponding to the relevant sequence of both of the *npt-II* gene and the 35-S-promoter. The stable integration of the T-DNA into plant genome was confirmed by Southern blot analysis.

Based on the data of the present study we can conclude that the reported regeneration system is reproducible and can be easily used to regenerate transgenic wheat plants expressing the genes present in the *Agrobacterium* binary vector T-DNA. Using this

regeneration and transformation protocol we can achieve our main goal which is the production of salt and drought tolerant wheat plants.

## Materials and Methods

In order to regenerate wheat plants from mature embryos isolated from dry seeds, three Egyptian commercial hexaploid wheat cultivars namely, Giza 160, Gemmiza-9, Gemmiza-10 and the tetraploid wheat cultivar Sohag were used. The pedigree of the cultivar (genotypes) examined is presented in Table 3. Mature seeds were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite, and rinsed in sterile distilled water. Seeds were imbibed in sterile distilled water for overnight at 25°C. For callus induction, mature embryos were cultured in 100 x 15 mm petri-dishes with modified MS medium containing MS salt, 3% (w/v) sucrose, B<sub>5</sub> vitamins supplemented with 2,4-D at graded levels (1, 2, 3, 4, 5 and 6 mg/l). The plates were sealed with parafilm and incubated at 25°C under complete dark conditions.

Somatic embryos were induced from callus by transferring the explants to MS medium supplemented with 2 mg/l BA, solidified by agar at 0.9% (w/v) at pH 5.8. The plates were sealed with Parafilm and incubated at 25°C with a 16/8-h light/dark photoperiod (70 μmol m<sup>-2</sup> s<sup>-1</sup>). Two weeks later the shoots were transferred to hormone free MS medium for roots formation. Shoots that develop used root system were grown in test tubes containing the same medium. After two or three weeks, they were transplanted to plastic pots.

**Agrobacterium mediated gene transfer in mature wheats.** The *A. tumefaciens* strain LBA4404 harboring the binary vector pBI121,<sup>31</sup> was grown overnight in 30 ml of LB medium containing 50 mg/l kanamycin sulfate (Sigma-Aldrich, Japan) at 28°C. The calli prepared from mature embryo explants of two wheat genotypes (Gemmiza 9 and Gemmiza 10) were immersed in the bacterial suspension for 5 min. Thereafter, the explants were blotted on a sterilized filter paper, placed onto a co-cultivation medium, which consisted of growth regulators free MS medium<sup>32</sup> and then were incubated under dark conditions. After co-cultivation for three days, the callus was transferred to shoot induction medium containing 2 mg/l BA, 500 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The plates were sealed with a parafilm and incubated at 25°C under a 16/8-h light/dark photoperiodic regime (1000-Lux). The explants were sub-cultured weekly on corresponding freshly prepared medium.

**PCR analysis.** In order to confirm the stable integration of the T-DNA into the plant genome, the putative transgenic plantlets were analyzed by PCR using *npt-II* gene and 35 S-promoter specific primers. DNA samples were isolated from both of the transformed and non transformed (control) plantlets according to the method described by Rogers and Bendich.<sup>33</sup> The reaction mixture (20 ml) contained 10 ng DNA, 200 mM dNTPs, 1 mM of each primer, 0.5 units of Red Hot Taq polymerase (ABgene Housse, UK) and 10X Taq polymerase buffer (ABgene Housse, UK). Samples were heated to 94°C for 5 min and then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1

min at 72°C. PCR products were separated by (2%) agarose gel electrophoresis and visualized with ethidium bromide. The forward and reverse primers for the *npt-II* gene were 5'-CGC AGG TTC TCC GGCCGC TTG GTG G-3' (position: 24–49) and 5'-TCG TCG GTC AGG GAAGGC GAA GTC-3' (position: 254–277). The sequences of the primers for the 35S promoter detection were; 5'-AAA GGA AGG TGG CTC CTA CAA AT-3' and 5'-CCTAGTAAAGTAAACCTCTCC3', respectively.

**Histochemical GUS assay.** The histochemical assay to screen for the expression of  $\beta$ -glucuronidase (GUS) activity in transgenic wheat plants was carried out according to the method of.<sup>31</sup> For analysis, callus was incubated in a reaction buffer containing 12.5 mM K<sub>3</sub>Fe (CN) 6, 12.5 mM K<sub>4</sub>Fe (CN) 6, 20% methanol,

1% Triton X-100 and 38.3 mM 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The tissue was incubated in a staining solution at 37°C for 24 h and the developed blue spots were recorded.

**Southern blotting analysis.** Total genomic DNA was isolated from both transformed and non-transformed plants according to a method described previously by Rogers and Bendich.<sup>33</sup> Restriction enzyme treatment of 10  $\mu$ g of DNA, separation in 2% agarose gels, and blotting to a nylon membrane followed standard protocols.<sup>34</sup> Labeling of the probes, hybridization and detection was carried out using the Biotin Chromogenic Detection kit #K0661, #K0662 according to instructions provided by the manufacturer (Ferments Life Sciences, USA).

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