Genetic Transformation of Egyptian Wheat Cultivars (*Triticum aestivum* L.)

*Via* Biolistic Bombardment Using Different Constructs

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**Abstract:** A transformation system for Egyptian wheat was developed by using the scutellar tissues of cultured immature embryos via particle bombardment. Two wheat cultivars (Giza 163 and Giza 164) were used to study the effect of three different constructs driven by three different promoters i.e., rice actin promoter (pAB6), maize Ubi promoter (pAH25) and CaMV 35S promoter (pB1121) on genetic transformation. In comparing the results of transformation efficiency for the three constructs across both cultivars, it can be concluded that GUS gene driven by actin promoter gave the highest transient expression followed by that driven by Ubi promoter while the lowest one was that driven by 35S promoter. Gene integration was assessed by subjecting plants to PCR analysis using specific primers for GUS and bar genes. Further confirmation by Southern blot hybridization revealed the presence of both genes. In this paper, we examined the expression of the marker gene uidA, coding for β-glucuronidase (GUS), under the control of different promoters in transgenic wheat (*Triticum aestivum* L.) from different wheat varieties. Also, we reported a transformation protocol which considered a new addition and indicates that gene(s) driven by actin promoter can result in high levels of expression in Egyptian wheat plants.

**Key words:** Wheat • cultivar • genetic • transformation • bar • gus • construct • histochemical • PCR • southern • transgenic

**INTRODUCTION**

Wheat is indisputably the most critical agricultural crop worldwide. The relatively low transformation efficiency and transgenes expression in wheat using the biolistic device calls for further improvement of the transformation conditions and selection of promoters conferring higher rates of gene expression. Use of enhanced and effective promoters to drive the genes of interest has drawn attention of the researchers, who started using a model transformation system such as of tobacco or arabidopsis. Currently, successful method used for wheat transformation is the direct delivery through particle acceleration bombardment. Becker *et al.* [1] obtained transgenic wheat Plant by bombarding with plasmid containing GUS gene under the control of the actin-1 promoter of rice and selectable marker gene bar under the control of the CaMV 35S promoter. Takumi *et al.* [2] reported the effect of six promoter-intron combinations on transient reporter gene expression in einkorn, emmer and common wheat cells by particle bombardment. Different levels of transient GUS gene activity were detected by an *in situ* enzyme assay in the three species. In these species, the level of transient GUS expression was higher in cells containing the rice Act 1 promoter, while lower in those containing the CaMV 35S promoter. Zimny *et al.* [3] obtained transgenic (*Triticale* x *Triticosecale Wittmack*) plants expressing the β-glucuronidase (uidA) and phosphinothricin acetyltransferase (bar) genes after microprojectile bombardment of scutellar tissue when the uidA gene was driven by actin-1 promoter (Act1) from rice, while and the selectable marker bar gene was driven by the CaMV 35S promoter. Chen *et al.* [4] transformed wheat with a rice chitinase gene under the control of the CaMV 35S promoter and the bar gene under control of the ubiquitin promoter as a selectable marker.

**MATERIALS AND METHODS**

Immature caryopsis of the two cultivars; Giza 163 and Giza 164 were collected approximately two weeks post-anthesis. Seeds were surface- sterilized with 20% commercial Clorox (5.25% Sodium hypochlorite)
Fig. 1: Schematic representation of plasmid pAB6

Fig. 2: Schematic representation of plasmid pAHC25

Fig. 3: Schematic representation of plasmid pBI121
supplemented with few drops of Tween 20, then washed five times with sterile d.d.H2O. Fifty immature embryos were cultured per plate with the scutelum side up onto Ms callus induction medium [S] modified for wheat cell culture with 2 mg 1^{-1} 2,4-D as a source of auxin, 0.15 g L-asparagine, 0.1 g myo-inositol, 20 g sucrose and 2.5 g phytagel. Calli were maintained in the dark at 25°C. Plant transformation was carried out by particle bombardment using the Biolistic® PDS-1000H device (Bio-Rad, USA). Three plasmids with GUS gene driven by different promoters, i.e., pAB6 with actin promoter, (Fig. 1), pAHC25 with ubi promoter, (Fig. 2) and pBI121 with 35S promoter (Fig. 3). Calli were osmotically treated with 0.4 M mannitol four hours before bombardment. Then, calli were bombarded with 1.0 μ gold particles coated with plasmid DNA. Target calli were bombarded at two distances 6 and 9 cm from stopping screen with rupture disc strength of 1100 psi. Calli were remained for additional 16 h on the same osmotic treatment, then transferred to recovery medium for two different periods, i.e., two and seven days. Calli were then assayed by histochemical GUS activity assay. The remaining calli were transferred to selective medium supplemented with 3 mg l^{-1} bialaphos. Transformed calli containing bar gene (herbicide-resistance gene) were capable to survive by detoxifying bialaphos, then maintained on selective medium. Calli showing vigorous growth were sub-cultured twice onto selection medium, then transferred onto regeneration medium supplemented with 3 mg l^{-1} bialaphos until emerging of shoots. Vigorous shoots were transferred to rooting medium with 1 mg l^{-1} bialaphos.

Regenerated putatively transgenic plants were transferred onto Magenta boxes then acclimatized by transferring into pots in bio-containment greenhouse. A number of regenerated plants resulted from transformation experiments were subjected to histochemical.

**Histochemical analysis:** Bombarded calli as well as regenerated plantlets including leaves, shoots and roots were incubated overnight at 37°C in GUS buffer with 1, 3- glucoseuronic acid.

**Molecular analysis:** Regenerated plants were subjected to molecular analysis to confirm the integration of the foreign genes (GUS and bar gene) into plant genome by PCR analysis. PCR analysis was further confirmed by southern blot hybridization.

**Statistical analysis:** Data obtained were statistically analysed based on the complete randomized design described by Snedecor and Cochran [6] in three replicates. Differences between means were tested by using Duncan’s new multiple range test as described by Duncan [7].

**RESULTS AND DISCUSSION**

The aim of using different plasmid types is to detect the influence of GUS gene under the control of different promoters, i.e., rice actin (pAB6), maize ubiquitin (pAHC25) and CaMV 35S (pBI121) promoters. The first two promoters had been isolated from monocot crops, while the third is a viral promoter. It was speculated that eukaryotic monocot promoters function better than the prokaryotic viral promoter against wheat (monocot) genomic background. Transformation efficiencies of two Egyptian wheat cultivars i.e., Giza 163 and Giza 164 with different constructs were compared and scored as number of blue spots/callus and number of blue clusters/callus after two and seven days after bombardment as represented in Tables 1 and 2 and Fig. 4 and 5. In comparison of numbers of blue spots/callus using different plasmids after two and seven days from bombardment for the two cultivars, similar results were achieved within or across cultures and within or across number of days after bombardment. In other words, it was expected that pAB6 (The smallest plasmid) had a higher chance of being integrated without the possibility of being sheared mechanically and losing function of one or more genes inserted in the plasmid. Accordingly, we expected in the present study to get higher influence of GUS gene when introduced to pAB6 when compared with the other two plasmid. In the comparison among different plasmid, it was concluded that the number of blue spots/callus was higher for pAB6 followed by pAHC25 within or across cultivars and within or across number of days after bombardment. A drastic reduction in number of blue spots/callus was shown when using pBI121 plasmid within or across cultivars and within or across the number of days after bombardment. The comparison of results of the three plasmids for the number of blue cluster/callus among the two cultivars showed that Giza 163 was significantly lower than Giza 164 for their number of blue cluster/callus across number of days after bombardment and across different plasmid. As expected, number of blue cluster seven days after bombardment was higher than that after two days only across or within cultivars and across or within the different plasmids. In other words, the longer the period after bombardment with a GUS containing plasmid, the higher the chance of cell division.
Table 1: Effect of different constructs (pAB6, pAH25 and pBH121) and time after bombardment on transient GUS expression as measured by number of blue spots in two wheat cultivars cv. Giza 163 and Giza 164

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Days after bombardment</th>
<th>Constructs</th>
<th>Constructs</th>
<th>Cultivar mean</th>
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<tr>
<td></td>
<td></td>
<td>pAB6</td>
<td>pAH25</td>
<td>pBH121</td>
</tr>
<tr>
<td>Giza 163</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13.60 aA</td>
<td>4.36 aB</td>
<td>2.52 aC</td>
<td>6.73 a</td>
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<tr>
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<td>12.56 aA</td>
<td>4.12 aB</td>
<td>2.36 aB</td>
<td>6.35 a</td>
</tr>
<tr>
<td>Day mean</td>
<td>13.80 A</td>
<td>4.24 B</td>
<td>2.44 C</td>
<td>6.55 b</td>
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<td>Giza 164</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>8.16 aB</td>
<td>3.04 aC</td>
<td>11.73 a</td>
</tr>
<tr>
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<td>6.16 bB</td>
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<td>10.14 b</td>
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<tr>
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<td>2.90 C</td>
<td>10.94 a</td>
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<tr>
<td>Treat. mean</td>
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<td>6.26 aB</td>
<td>2.78 aC</td>
<td>9.28 a</td>
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<td>2.67 C</td>
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Table 2: Effect of different constructs (pAB6, pAH25 and pBH121) and time after bombardment on transient GUS expression as measured by number of blue clusters in two wheat cultivars cv. Giza 163 and Giza 164

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Days after bombardment</th>
<th>Constructs</th>
<th>Constructs</th>
<th>Cultivar mean</th>
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<td></td>
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<td>pBH121</td>
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<td>Giza 164</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>8.30 bA</td>
<td>5.80 bB</td>
<td>2.28 aC</td>
<td>5.46 b</td>
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<tr>
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<td>5.18 B</td>
<td>2.32 C</td>
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<tr>
<td>General mean</td>
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<td>5.36</td>
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</table>

* Means followed by different capital letters in columns and those followed by different small letters in rows are significantly different at p=0.05 according to Duncan’s multiple range test.

and consequently the higher the probability of getting a cluster cells originated from one transformed GUS-containing cell. In comparing the influences of different plasmid, on the number of blue cluster/callus, it was shown that pAB6 gave the highest mean followed by pAH25 within or across cultivars and within or across number of days after bombardment. These results agreed with the findings of Fromm et al. [8] and Hauptmann et al. [9] who stated that CaMV 35S is not very effective in inducing the expression of the introduced genes in cereals. The results also agree with Callis et al. [10], Vasal et al. [11]; Christensen et al. [12] and Taylor et al. [13] who stated that substantially increases in the levels of gene expression could attain by using maize ubiquitin promoter. Dekeyser et al. [14] declared that although the CaMV 35S is used in transformation of monocot, but it is not regarded as the optimum promoter to be used in transforming monocot and also was found from transient assays of CaMV 35S promoter is only one-fourth as efficient as other promoter in rice protoplast. Rice actin promoter was shown to provide a significant increase in gene expression than other promoters when introduced into rice protoplasts. In the present study, similar results were found when pAB6 driven by actin promoter was used and gave the best results, followed by pAH25 driven by maize ubiquitin promoter and pBH121 driven by CaMV 35S promoters. These data was synchronized with Ye et al. [15], who demonstrated that wheat transformation efficiency is closely related to the constructed plasmid. There high significance between the two tested cultivars and also between the three different contracts and it could be advised to use the pAB6 construct driven by actin promoter with Giza 164 which gave the highest transformation efficiencies.

Selection and regeneration of transformed calli: Transformed calli with bar gene (herbicide resistance
Fig. 4: Effect of different constructs in transformation efficiency on Giza 163 callus a) pAB6 at 2 days b) pAB6 at 7 days c) pAHC25 at 2 days d) pAHC25 at 7 days e) pBI121 at 2 days f) pBI121 at 2 days

Fig. 5: Effect of different constructs in transformation efficiency on Giza 164 callus a) pAB6 at 2 days b) pAB6 at 7 days c) pAHC25 at 2 days d) pAHC25 at 7 days e) pBI121 at 2 days f) pBI121 at 2 days
Fig. 6: a) Calli showing vigorous growth transferred onto a fresh selection medium   b) Calli showing vigorous growth transferred to regeneration medium supplemented with 3 mg l⁻¹ bialaphos until emerging of shoots

Fig. 7: Vigorous shoots transferred to rooting medium with 1 mg l⁻¹ bialaphos   a) Side views    b) Bottom views   c) Regenerated putatively transgenic plants onto selection medium   d) Acclimatized plants grown onto pots in bio-contaminant greenhouse
Fig. 8: Histochemical GUS assay: a) root of transformed regenerated plant compared with root of non-transformed regenerated plant  
b) shoot root tissue of transformed regenerated plant compared with shoot of non-transformed regenerated plant  
c) leaf tissue of transformed regenerated plant compared with leaf of non-transformed regenerated plant  
d) shoot primordial  
e) plantlet  
f) leaf vein
Fig. 9: PCR products of GUS gene amplifying partial length (750 bp) in five putative wheat plants. Lane 1 negative control, lane 2 positive control and lanes 3-7 putative transgenic wheat plants.

Fig. 10: PCR products of bar gene amplifying partial length (450 bp) in five putative wheat plants. Lane 7 negative control, lane 6 positive control and lanes 1-5 putative transgenic wheat plants.
Fig. 11: Southern blot analysis of PCR amplified DNA for putatively transformed wheat plants. Lanes 1-5: amplified DNA from 5 putatively transformed wheat plants and lane 6 positive control for GUS gene.

Fig. 12: Southern blot analysis of PCR amplified DNA for putatively transformed wheat plants. Lanes 1-5: amplified DNA from 5 putatively transformed wheat plants and lane 6 positive control for bar gene.
gene) were capable to survive by detoxifying bialaphos and able to grow and maintained on selective medium. Calli showing vigorous growth were transferred onto a fresh selection medium 3 mg 1⁻¹ bialaphos for two subcultures and then transferred to regeneration medium supplemented with 3 mg 1⁻¹ bialaphos until emerging of shoots. Fig. (6a-b). Vigorous shoots were transferred to rooting medium with 1 mg 1⁻¹ bialaphos Fig. (7a-b). Putatively transgenic regenerated plants were transferred onto Magenta® boxes Fig. (7c), then acclimatized by transferring onto pots in bio-containment greenhouse (Fig. 7d).

**Histochemical GUS assay:** GUS enzyme activity was detected in different explants of transgenic wheat cultivars; Giza 163 and Giza 164 by histochemical GUS assays. Results indicated that GUS activity in transgenic Egyptian wheat cultivars Giza 163 and Giza 164 could be detected histochemically in roots, shoots and leaves, shoots primordial and plantlets (Fig. 8a, b, c, d and e, respectively). The intensive blue color appeared along the veins in leaves (Fig. 8f). These results are in agreement with other findings reported by different researchers [16-21]. Transformed wheat of Bobwhite cultivar was identified by β-glucuronidase as described by Weeks et al. [22] and Haliloglu and Baenziger [23] who detected transient expression of the GUS gene in wheat (*Triticum aestivum* L.).

**Polymerase chain reaction (PCR):** Amplified DNA from putatively embryogenic plants resulting from transformation of immature embryo-derived calli revealed the presence of GUS gene fragment and bar gene fragment with expected molecular weights (750 and 484 bp, respectively). As shown in Fig. 9, the amplified fragments for GUS in lanes (3-7) have the expected size of coding region of GUS gene (750 bp), lane (2) positive control resulted from amplified plasmid pAB6 and lane (1) negative control resulted from non-transgenic. Figure 10 shows the presence of bar-DNA fragment at expected molecular weight (484 bp) in lanes (1-5), lane (6) positive control and Polymerase Chain Reaction (PCR) has been used in the detection of inserted foreign genes into the genome of the recipient cells by many investigators, these results are in agreement with those reported by different workers. Xing et al. [24] used the PCR analysis to detect GUS gene from genomic DNA of transformed petunia (*Petunia hybrida* C.v. Mitchell), Hamill et al. [25] used PCR as a routine analytical tools for quickly analyzing plant transformants for the presence of foreign genes for both GUS and kanamycin in Nicotina species. Marnerlof and Paul [26] used PCR analysis for rapid screening of a large number of putative transgenic shoots of many plants such as sugar beet, maize, potato, squash and tobacco. Vickers et al. [27] used a sensitive and reliable PCR assay for the presence of bar for rapid screening of putatively transgenic plants of barley transformed by bar gene Does et al. [28] used PCR as a quick method for detection of T-DNA copy number of young transgenic plants. Schrommeier et al. [29] confirmed the integration of GUS and NPTII genes into sunflower transgenic plant using PCR and Lee et al. [30] used PCR analysis to detect bar gene in the transgenic rice plant lane 7 negative control resulted from non-transgenic plant. Two commercial wheat cultivars, AC Karma and Hy417, were transformed by the bombardment of isolated scutella with two gene constructs. Li et al. [31] obtained three plants carrying plasmid pRC62 containing a gus::npt fusion gene and one plant carrying plasmid pBARGUS containing a bar gene and a GUS gene were recovered and characterized and the presence of transgenes in transgenic plants were confirmed by PCR.

**Southern blot analysis:** Results of southern blotting of the PCR amplified DNA confirmed the presence of GUS (750 bp) (Fig. 11) and bar (484 bp) (Fig. 12). Similarly, Zhong et al. [32] analyzed putatively transgenic turfgrass plant by PCR analysis using primers specific for the coding region of GUS genes, amplified DNA from four of these samples showed the presence of a (750 bp) GUS DNA fragment which was further confirmed by southern blot hybridization of the amplified DNA using the GUS coding region as a probe. Moreover, to confirm the integration of GUS gene, PCR product of putatively transformed plants were subjected to southern blot hybridization using probe prepared by digestion of recombinant plasmid pAB6 with Bam HI and SacI and labeled with the Digoxigenin non-radioactive labeling and detection kit. This result indicated that GUS gene is integrated into GUS positive samples. To confirm the integration of bar gene, the five PCR products positive for bar form same transformed plants tested for bar gene integration were subjected to southern blot hybridization using probe prepared by digesting the recombinant plasmid pAB6 with EcoRI and Bam HI to release the bar gene fragment (0.6 kb) and labeled with the Digoxigenin non-radioactive labeling and detection Kit. DNA hybridization analysis revealed that the PCR products lane 1-5 hybridized with bar probe. These results indicated that bar gene integrated into bar positive
samples. Southern blot hybridization analysis was used by many authors to confirm the integration of transgenes at the DNA molecular level. Barcelo et al. [33] used southern blot analysis to confirm GUS and NPTII genes in transgenic plant of Hordeum and Triticum, also Chen et al. [4] confirmed the integration of chill and bar genes in wheat plants cv. Bobwhite Cultivar. Tang et al. [21] confirmed the integration of the Xa21 gene in the transgenic plants of rice using southern blot analysis. Weeks et al. [22] confirmed the bar and GUS genes in transgenic wheat plant cv. Bobwhite cultivar using southern blot analysis. Gayne et al. [34] confirmed the GUS gene in four GUS positive plants of wheat cultivars by using Southern blot analysis. Archana et al. [35] stated that highly regenerable basal segment calluses of wheat (Triticum aestivum) cv (CPAN1676 and PBW343) were used as a target tissue for genetic transformation. The bar gene conferring herbicide resistance was introduced in calluses via particle bombardment transformation. Transgenic callus were selected on phosphinothricin-containing regeneration medium and presence of the transgene (bar) was confirmed by Southern hybridization.

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REFERENCES


