# Optimization of transient expression of *uidA* gene in androgenic embryos of wheat (*Triticum aestivum* L. cv. Falat) via particle bombardment

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***Abstract***
Haploid microspore-derived embryos (MDEs) of wheat were obtained by *in vitro* androgenesis. These embryos were employed to evaluate the transient expression of GUS gene (*uidA*) following particle bombardment. Using the Bio-Rad PDS-1000/He system, the physical parameters including rupture disk pressure (900, 1100 and 1350 psi); microprojectile travel distance (6 and 9 cm); gold particles size (0.6 μm, 1 μm and 1.6 μm), DNA and microcarrier concentrations (0.5 μg of DNA with 150 μg of gold particles or 1.0 μg of DNA with 300 μg of gold particles/bombardment) and bombardment numbers (1x (single) and 2x (double)) were assessed. The effect of high osmoticum in the bombardment medium (0.3 M mannitol and 0.4 M maltose) and the age of embryos were also evaluated. Optimal expression in MDEs was obtained using the following conditions of double bombardment at 1350 psi, 9 cm target distance, a 1 μm gold particle size, 1.0 μg of DNA with 300 μg of gold particles/bombardment, and osmotic pretreatment of 4-6 weeks old embryos using 0.4 M maltose for 6 h before and 16 h after bombardment. The optimized transformation protocol presented in this study is expected to improve devalopment of commercial transgenic wheat lines expressing desirable agronomic traits.

***Keywords:*** Hexaploid wheat; Transient gene expression; GUS; Particle bombardment; Androgenic embryos

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## INTRODUCTION

Wheat is a worldwide cereal crop and a staple food source for billions of people. It is understandable that wheat has been the prime target for improvement of agronomic characteristics via genetic engineering (Pastori *et al*., 2001). Genetic transformation offers an attractive alternative to conventional breeding because it can allow specific traits to be transferred into selected genotypes without adversely affecting their desirable genetic background (Bhalla *et al*., 2006). Several different direct DNA transfer methods have been described, amongst which the particle bombardment method has been the most widely used for generating commercial transgenic crops (James, 2003). The method involves high velocity delivery of gold or tungsten mircocarriers coated with DNA into the target cells followed by regeneration of green plants in a selective medium (Bhalla *et al*., 2006).

Several agronomically important genes have been incorporated into wheat using particle bombardment, and the stable expression of transgenes has been achieved by several groups (Chugh and Khurana, 2003; Patnaik and Khurana, 2003; Permingeat *et al*., 2003; Altpeter *et al*., 1999, 1996; Bliffeld *et al*.,1999; Chen *et al*., 1998; Barro *et al*., 1997; Blechl and Anderson, 1996; Karunaratne *et al*., 1996; Ortiz *et al*., 1996).

Different plant tissues could be used as explant for

microprojectile bombardment. Microspores and microspore-derived embryos (MDEs) are attractive targets for genetic transformation due to the high frequency of plant regeneration and homozygotic diploid transformants that can be produced after chromosome doubling.

Several factors have been described to influence the applicability and efficiency of biolistic gene transfer. Alterations in the standard transformation protocol, such as preculture of the explant's material, use of smaller sized microprojectile particles and osmotic pretreatment of the target tissue have yielded improvement in the transformation efficiency (Finer *et al*., 1999). Effective factors such as alternative DNA/microcarrier coating procedures, microparticle type and size and tissue culture variables during transformation and regeneration steps were also extensively studied using the diploid explants (Janna *et al*., 2006; Rasco-Gaunt *et al*., 1999).

In general, the bombardment parameters are species and tissue-specific and need to be optimized according to the nature of the explants. The objective of this study was optimization of certain physical and biological parameters, on the basis of of *GUS* gene (*uidA*) transient expression experiments in the haploid microspore-derived embryos (MDEs) of an Iranian wheat cultivar using particle bombardment. Hence, a range of bombardment parameters was investigated with the aim of not only optimizing DNA delivery conditions but also identifying conditions which minimize damage to the target tissues.

## MATERIALS AND METHODS

**Plant material and microspore culture:** *Triticum aestivum* L. cv. 'Falat' was used in all experiments. Donor plants were grown in a controlled growth room at 25±2ºC (day) and 15±2°C (night) under a 18/6 h (day/night) photoperiod. Isolated microspores were cultured according to the protocol described by Liu *et al*. (2002). Spikes were aseptically pretreated in sterile inducer chemical formulation [100 mg/l of 2-hydroxynicotinic acid, 10 mg/l of 2,4-D and 2 mg/l of Benzyl Aminopurin (BAP)] and placed in an incubator at 33ºC for approximately 48 h. Microspores were isolated through blending of spikes in microspore isolation solution (0.3 M mannitol). Isolated microspores were co-cultured with wheat immature ovaries in NPB99 liquid medium (Konzak *et al*., 1999), at a density of approximately $4\times10^3$ microspores m/l and incubated at 28°C in the dark. Embryos (1-2 mm) were collected by random selection from 4-8 weeks old culture plates (unless otherwise noted) and used in the experiments.

**Osmotic treatment:** The influence of osmotic treatment of embryos on transient expression of the *UidA* gene was tested by incorporating 0.3 M mannitol or 0.4 M maltose in the solidified 190-2 medium (Wang and Hu, 1984) used in the pre- and post bombardment cultures of the explants. The osmotic treatment consisted of two steps: a 7 h treatment prior to bombardment and a 16 h treatment after bombardment.

**Plasmid construct:** The plasmid pCAMBIA 3301, containing the modified *uidA* gene (intron-containing) (Jefferson, 1987) encoding β-glucuronidase (GUS) and the *bar* gene encoding phosphinothricin-N-acetyltransferase (PAT), with both being under the control of the CaMV 35S promoter, was used in all experiments.

**Microprojectile bombardment and β-glucuronidase (GUS) assay:** Preparation of gold particles and precipitation of plasmid DNA onto the gold particles were carried out using the Biolistic PDS/1000 Helium System, according to supplier's instructions (Bio-Rad, USA). The biolistic device parameters analyzed were as follows: rupture disk pressure (helium pressure of 900, 1100 and 1350, psi); stopping plate to target tissue distance (6 and 9 cm) and gold microparticles sizes (0.6, 1.0 and 1.6 µm). Other parameters studied were the type of osmotic treatment (0.4 M maltose and 0.3 M mannitol); number of bombardments (1X and 2X); DNA and microcarrier concentration (0.5 µg of DNA with 150 µg of gold particles or 1.0 µg of DNA with 300 µg of gold particles/bombardment) and age of embryos (4-6 weeks old and 8-10 weeks old microspore cultures). The GUS assay was performed according to Jefferson (1987) with the addition of 20% (v/v) methanol in the reaction buffer to eliminate the influence of endogenous GUS activity. In all experiments, the following conditions were used unless mentioned otherwise: 4-6 weeks old embryos, osmotic pretreatment with 0.4 M maltose, double bombardment (2X) at 1350 psi, a 9 cm target distance, gold microcarrier size of 1 µm and 1.0 µg of DNA with 300 µg of gold particles/bombardmenet. In the osmotic pretreatment experiment, a single bombardment (1X) was used exceptionally.

**Data analysis:** The complete randomized design (CRD) or factorial experiment based on the complete randomized design was used in this study. Each bombardment treatment was carried out in four replica-

tions. Each replication consisted of a Petri dish (60×15 mm) containing 30 embryos. The number of GUS positive foci on embryos in each bombardment was counted using a stereomicroscope (Olympus, SZX12, Japan). Data were analyzed using the SPSS statistical package.

## RESULTS

**Effect of helium pressure and sample plate distance:** The results showed significant differences between the two sample plate distances, helium pressures and their interactive effects ($p \leq 0.01$) for the mean number of foci per bombardment. The helium pressure of 1350 psi produced the highest number of foci per bombardment (593.33 ± 43.96) at a distance of 9 cm from the target tissues (Figs. 1, 2). For a helium pressure of 1100 psi, the highest level of gene expression was observed at 9 cm, although a significant difference was not observed at the distance of 6 cm (Figs.1, 2).

**Effect of gold particles size**: The different gold microparticles size (0.6, 1.0 and 1.6 µm) in combination with two helium pressures (1100 and 1350 psi.) were compared for their efficiencies in delivering DNA into the target tissues. The results showed significant differences between the gold particles sizes, helium pressures and their interactive effects ($p \leq 0.01$) for mean number of foci per bombardment. The interaction between particle size (1 µm and 1.6 µm) and a pressure of 1350 psi produced the highest GUS foci per bombardment (Fig. 3).

**Effect of number of bombardments:** There were significant differences in GUS expression when single (1X) or double (2X) bombardments were carried out at different sample plate distances on wheat MDEs (Fig. 4). The highest number of foci per bombardment (615 ±48.56) was obtained when double bombardments (2X) were employed at a 9 cm distance from the target tissues. However, increasing the number of bombardments at the 6 cm sample plate distance significantly decreased the number of blue spots (93.33 ± 25) in the wheat MDEs.

**Effect of osmotic treatment:** In this study, no significant differences in GUS expression were observed between the two osmotic treatments (0.4 M maltose and 0.3 M mannitol) (Fig. 5). However, the use of 0.4 M maltose in the solidified medium as an osmotic pre-

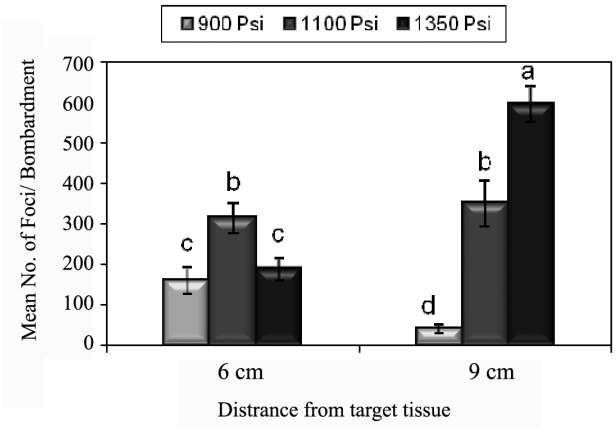


**Figure 1.** Interactive effects of helium pressure and distance from the stopping screen to the target tissues on transient GUS expression in wheat MDEs. Means with the same letter are not significant at p = 0.05 using Duncan's multiple range test.

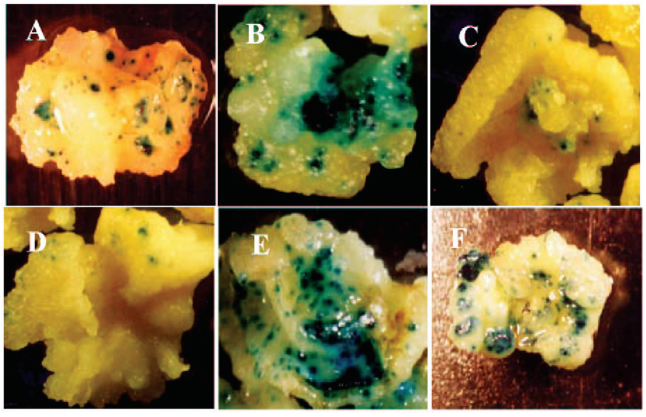


**Figure 2.** Comparison of transient GUS expression (blue dots) in wheat microspore-derived embryos bombarded under 1100 psi at 6.0 cm (A), 1350 psi at 6.0 cm (B), 900 psi at 6.0 cm (C), 900 psi at 9.0 cm (D), 1350 psi at 9.0 cm (E), and 1100 psi at 9.0 cm (F).

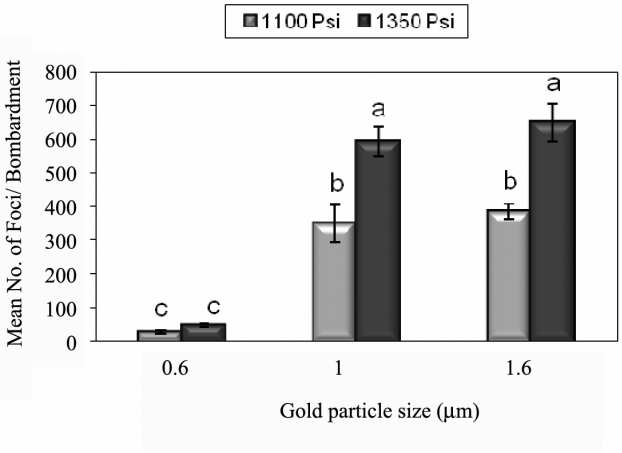


**Figure 3.** Interactive effects of the gold particles' size and rupture pressure on transient GUS expression in wheat MDEs. Means with the same letter are not significant at p = 0.05.

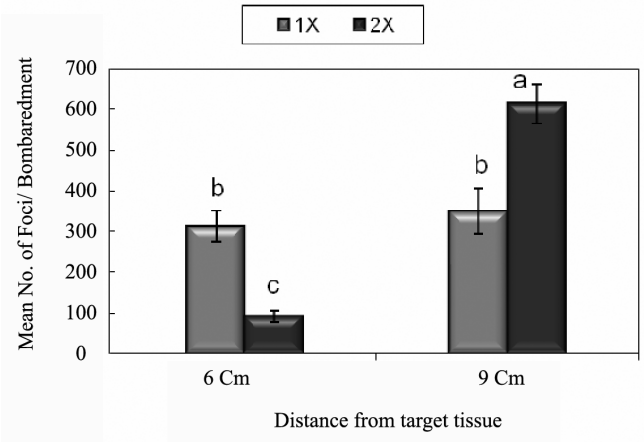


**Figure 4.** Interactive effects of the number of bombardments and distance from target tissue on transient GUS expression in wheat MDEs. Means with the same letter are not significant at p = 0.05.

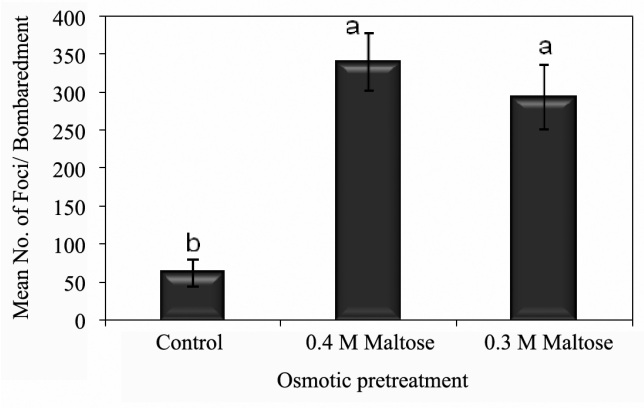


**Figure 5.** Effect of the osmotic treatment on transient GUS expression in wheat MDEs. Means with the same letter are not significant at p = 0.05.

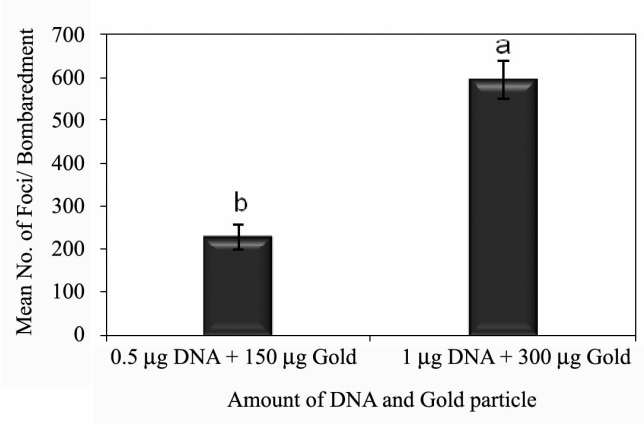


**Figure 6.** Effect of DNA and microcarrier concentrations on transient GUS expression in wheat MDEs.

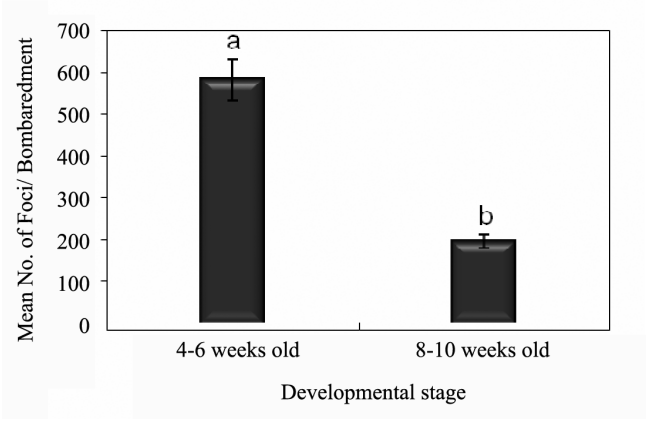


**Figure 7.** Effect of embryos age on transient GUS expression in wheat MDEs.

treatment for 6 h before and 16 h after bombardment slightly enhanced the number of blue spots (340 ± 37.64) in the wheat MDEs.

**Effect of the microcarrier amount and DNA concentration:** With respect to the amount of DNA actually loaded onto the particles, the best significant results were obtained using 1 µg of DNA with 300µg of gold particles per bombardment (593.33±43.96), whilst 0.5 µg of DNA with 150 µg of gold particles resulted in a lower number of blue spots per bombardment (228.33 ± 30.6) (Fig. 6).

**Effect of the age of microspore-derived embryos:** The results surprisingly showed that 8-10 weeks old embryos exhibited significant reductions in the number of foci as compared with 4-6 weeks old embryos (Fig. 7). In this experiment, the highest number of foci per bombardment (584.5 ± 49.13) was obtained when embryos were selected from 4-6 weeks old microspore cultures as target tissues.

## DISCUSSION

Particle bombardment has been widely exploited to produce tissues and plants expressing traits with agronomic value and has had a major impact on basic plant science research and biotechnology (Altpeter *et al*., 2005; Taylor and Fauquet, 2002).

The results obtained following the histochemical GUS assays revealed that transient gene expression events displayed a high level of variation when the same sample plate distances but a different rupture

disk pressure were used. A higher rupture disk pressure at a shorter sample plate distance reduced the transient GUS expression events. This was probably due to either extreme deep penetration of the gold particles leading to the inability of the assay solution to diffuse to such cell layers, or to possible tissue damage, since tissue dislocation has been observed at a pressure of 1350 psi at 6 cm. The optimal distance from the macrocarrier to the target tissue has been reported to be helium pressure dependent (Janna *et al*., 2006). The results of this research are consistent with observations obtained from previous studies on other plant species (Janna *et al*., 2006; Schopke *et al*., 1997).

Another noticeable effect of sample plate distance on GUS expression was the particles' distribution pattern following the bombardment. Under short distance bombardment conditions, only one or two explants received most of the gold particles, culminating in an extreme number of GUS expressions (Fig. 2), while longer distance bombardments revealed homogenous GUS expression in majority of the explants. This demonstrated the importance of the sample plate distance on the optimization process.

Choice of microparticle type and size is important, as this will determine the mass, and thus depth of penetration, of the accelerated microcarrier (Taylor and Fauquet, 2002). Moreover, particle size plays an important role in determining the optimum helium pressure. Larger particles will need a higher force to travel and penetrate the cells while the smaller particles need a lower force. Increasing the force for the small particles will result in a decrease of gene expression as a consequence of tissue damage (Parveez *et al*., 1997). Moreover, it seems that a bigger particle size especially in combination with high helium pressures, will lead to an increase in tissue damage and subsequently affect shoot regeneration. Folling and Olsen (2002) have also reported higher damaging effects with larger microparticle sizes during wheat transformation.

In this study, the optimal number of bombardments has been found to be dependent on the sample plate distance from target tissues. The highest GUS expression was observed when double (2X) bombardments were employed at a 9 cm distance. Double bombardments are sometimes considered as a useful alternative to cover a larger area of bombarded tissues especially or when longer distances are used during unsuccessful particle deliveries (King and Kasha, 1994). In agreement with the results of this research, Sreeramanan *et al*. (2005) found that two consecutive bombardments showed an increase in transient GUS and GFP expression in single banana buds as compared with those obtained from single or triple bombardments. Advantages of double bombardment in increasing transient GUS expression have been reported in rice and wheat suspension cultures (Wang *et al*., 1996); in *Pinus taeda* (Stomp *et al*., 1991) and cotton (Rajasekaran *et al*., 2000). Parveez *et al*. (1997), however, observed no significant differences between single and double bombardments in oil palm although double bombardments produced a higher transient GUS value. Similarly, Rasco-Gaunt *et al*. (1999) found no significant differences in GUS expression obtained from single or multiple (2 or 3X) bombardments on wheat tissues, although double bombardment also showed a higher transient GUS expression.

Increasing the number of shots would undoubtedly increase the injuring capacities of the targets, thereby decreasing the number of surviving cells capable of expressing the transgene. It is suggested that the number of bombardments in a specific tissue is dependent on other factors such as tissue type, particle velocity and particle size (Clemente *et al*., 1992).

The results of the current study clearly indicate the importance of the osmotic treatment of embryos on the transformation efficiency (Fig. 5). Short-term osmotic treatments, typically for a few hours before or after bombardment, are thought to minimize cytoplasmic leakage from the target cells. Supporting this, Ingram *et al*. (1999) reported that by using the maltose treatment, the mean number of blue GUS foci/bombarded MDEs, was 3-fold higher as compared to the number for MDEs treated with medium lacking an osmoticum treatment. A high osmoticum medium is thought to protect tissues during bombardment by reducing cell turgor, causing plasmolysis. This leads to reduced leakage of cell contents following bombardment (Vain *et al*., 1993a). This kind of medium may also induce membrane changes, leading to increased cell tolerance to microprojectile impact (Clapham *et al*., 1995).

The positive effects of a short-term osmotic preconditioning (plasmolysis) of target cells or tissues on transient and stable transformation have been reported in several other studies (Ingram *et al*., 1999; Altpeter *et al*., 1996; Perl *et al*., 1992). Moreover, the explants pre-cultured in osmotic medium prior to bombardment have a significantly higher plant regeneration capacity than explants bombarded with no or a short pre-culture treatment, indicating that pre-cultured explants are less

sensitive to tissue damage (Shimada *et al*., 1991).

In the present study, the amount of gold particles and DNA concentration significantly affected the frequency of transient GUS expression in wheat MDEs. Although, it was observed that embryos bombarded with lower amounts of gold particles, at lower pressures and especially at longer distances showed better shoot regeneration than those bombarded with high particle loads at higher pressure and shorter distances (data not shown). In agreement with this observation, Popelka *et al*. (2003) reported that the transient expression frequency is positively correlated to the amount of particles per bombardment but higher particle densities reduce the regeneration response of the cultures, most probably due to tissue damage caused by the microprojectiles.

For determining the effect of embryo growth stage on GUS transient expression, two groups of embryos were evaluated: embryoids selected from 4-6 weeks and 8-10 weeks old microspore cultures. The frequency of blue spots observed in 4-6 weeks old embryos was approximately 3 times higher than those of 8-10 weeks old embryos (Fig. 7). Similar to these results, Loeb and Reynolds (1994) observed that embryoids taken from a 7 weeks old anther culture were 13 times more likely to have embryos expressing foci than those of the 16 weeks cultures, while 8 weeks old cultures were 11 times more likely (P= 0.0020). It is known that the concentrations of endogenous hormones during wheat embryogenesis fluctuate markedly and the relative levels of these hormones at the time of bombardment may be crucial in determining the transformation frequency (Pastori *et al*., 2001).

In summary, the results of this study showed that the parameters of the delivery device and other physical and biological factors involved in particle bombardment, significantly affect DNA delivery into microspore-derived haploid embryos of hexaploid wheat. The optimized parameters obtained on the basis of transient GUS expression levels, could be used to provide efficient and stable transformation procedures involving the use of haploid embryos and thus leading to the rapid production of transformed homozygous wheat plants.

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