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Optimization of Wheat (*Triticum Aestivum* L.) Anther Culture-Derived Embryos Transformation by Microprojectile Bombardment

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ABSTRACT: Wheat transformation method is being developed using microprojectile bombardment of anther culture derived embryos. Anther culture derived embryos were initiated from the spring cultivar 'Pavon 76'. Transient expression of the *gus* gene was detected in which anther culture derived embryos were treated via microprojectile bombardment. N6 osmatic medium and washing embryos with Liang's 85D12 salt and organics prior to bombardment increased the transient expression of the *gus* gene.

Key Words: Anther culture, transformation, biolistic method.

Partikül Bombardımanı ile Buğday (*Triticum Aestivum* L.) Anter Kültürü kaynaklı Embriyolara Gen Aktarımının Optimizasyonu

ÖZET: Partikül bombardıman yöntemi, buğday anter kültürü kaynaklı embriyolara gen aktarım metodu ile geliştirilmeye çalışılmıştır. Bu araştırmada, "Pavon 76" çeşiti anter kültürü kaynaklı embriyoların eldesi için kullanılmıştır. Mikroprojektil bombardımanı ile aktarılan Gus geninin geçici ifadesi anter kültürü kaynaklı embriyolarda tespit edilmiştir. Bombardımandan once, N_6 ozmotik ortam ve embriyoların Liang's 85D12 tuz ve organikleri ile yıkanması Gus geninin geçici ifadesini önemli derecede artırmıştır.

Anahtar Kelimeler: Anter kültürü, gen aktarımı, biyolistik metod.

INTRODUCTION

The ability to transform plants through genetic engineering has been of great value in exploring fundamental questions in biology and enabling crop improvement. Current wheat transformation attempts have focused on microprojectile bombardment (Vasil et al., 1991; 1992; 1993; Weeks et al., 1993). The first report of transformed wheat plants was achieved by Vasil et al. (1992). Embryogenic cell cultures were transformed by microprojectile bombardment and fertile plants were regenerated. The type C embryogenic callus tissue used by Vasil et al. (1991; 1992) for bombardment occurs at very low frequency, and is difficult to identify and maintain. Microprojectile bombardment has now successfully been used to produce fertile transgenic wheat plants from immature embryo callus (Vasil et al., 1993; Weeks et al., 1993).

Wheat anther culture and immature embryo tissue culture systems are well defined and plants are routinely regenerated from both culture methods (Schaeffer et al., 1979; Yuan et al., 1990; Simonson and Baenziger, 1992). The culture of wheat anther produces embryos (Rybczynski et al., 1991), which provide a source of embryogenic tissue for microprojectile bombardment studies. Our goal was to develope a transformation procedure, which avoids the difficulties of protoplast culture and labor-intensive culture of specific callus types.

MATERIAL AND METHODS

Plant material and culture conditions:

Spring wheat cultivar, Pavon 76, was grown and anthers were excised as described by Navarro-Alvarez et al., (1994). Thirty anthers from each spike were plated on 15x60 mm petri dishes containing anther initiation (AI) medium. The AI medium contained Liangs's 85D12 salts and organics (Liang et al., 1987) with 94 g l^{-1} maltose, 254 mg l^{-1} glutamine, 2 mg l^{-1} 2,4-D and 1 mg l^{-1} NAA, pH 5.8 (prior to addition of wheat starch), filter sterilized, and thickened with 5% (w/v) autoclaved wheat starch (Navorro-Alvarez et al., 1994). Plates were incubated at 25^{o}C in the dark. Embryos were visible after four weeks of incubation. Four to six-week old embryos were used for microprojectile bombardment. After microprojectile bombardment, the embryos were transferred to 15x60 mm petri dishes containing regeneration media (AR). Regeneration medium was AI medium with following modifications; no plant growth regulators, 21.6 g l^{-1} maltose, 146 mg l^{-1} glutamine and 7% (w/v) wheat starch (Navorro-Alvarez et al., 1994). Regenerated plantlets in AR medium were transferred to phytocons with rooting medium (MSO). MSO medium consisted of MS salts (Murashige and Skoog, 1962) with 20 g l^{-1} sucrose, and 8 g l^{-1} agar. The PH was adjusted to 5.8 prior to autoclaving.

Microprojectile Bombardment:

Gold particles were sterilized and coated with plasmid DNA according to Kikkert (1993). A total of 40 mg gold particles measuring 1.0 µm in diameter (Bio-Rad Laboratories, Hercules, CA) were sterilized in 0.5 ml 70% ethanol for 15 minutes. After microcentrifugation and washing with sterile water, particles were resuspended in 50 µl sterile glycerol. Particles (50 µl) were coated with 5 µl (1 µg/µl) plasmid DNA using CaCl/spermidine free base precipitation according to Kikkert's (1993) procedure. After precipitation microcarriers were vortexed for 10 minutes at room temperature. After microcentrifugation and washing of the pellet with 70% ethanol and then 100% ethanol, DNA-coated particles were resuspended in 50 µl absolute ethanol. Macrocarriers were loaded with 10 µl of the particle/DNA mixture. Loaded macrocarriers were promptly placed in a sterile desiccator to dry. Gold particles prepared without DNA were included in each experiment as control.

Bombardments were performed using the Bio-Rad Biolostic PDS-1000/He particle delivery system (Bio-Rad Laboratories, Hercules, CA). The distance between the rapture disk and launch point was 1 cm. The target material was placed at 9 cm from the stopping screen. The burst pressure of the rapture disks for all the experiments was 8970-kPa helium pressure. The microprojectiles were lunched when the chamber reached a vacuum of 66.0 cm Hg. Maize callus tissue was used as an internal control to be sure the system worked properly.

Transient gene expression was assayed 2 days after bombardment by incubating the embryos in 5 ml of X-Gluc (5-bromo-3-chloro-3indolyl- -D-glucuronic acid) staining solution as described by Stomp (1992). After 4 hr incubation at 37^{O}C, blue spots were counted using a dissecting microscope. In the first microprojectile bombardment experiment, maize callus tissue was used as internal control. Three plates containing maize callus tissue were bombarded with pAHC25 plasmid DNA containing *bar* and *gus* genes. In the second experiment, 9 plates containing wheat anther culture derived embryos were bombarded. Three plates were shot with pAM2100 (Figure 1A) plasmid DNA, three plates were shot with pAHC25 (Figure 1B) plasmid DNA and three plates were shot without plasmid DNA. In first two experiments, N6 osmatic medium was used and embryos were not washed with the Liang's 85D12 salt and organics.

In the third experiment, 8 plates were bombarded. Embryos in two of the plates were treated with N6 osmotic medium and embryos were washed with Liang's 85D12 salt and organics. Embryos in another two plates were treated N6 medium no osmoticum and embryos were washed Liang's salt and organics. Embryos on another two plates were treated N6 osmatic medium and embryos were not washed Liang's 85D12 salt and organics. Embryos on remaining two plates were treated N6 medium no osmoticum and embryos were not washed with Liang's 85D12 salt and organics.

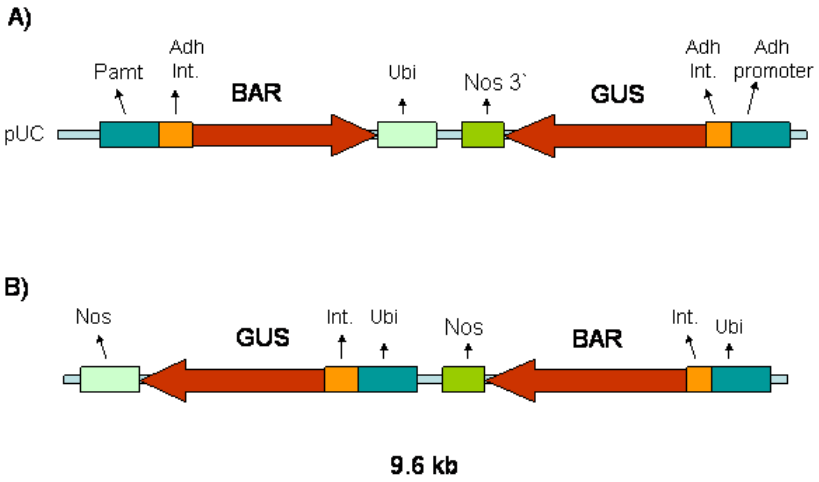


Figure 1: Plasmids used for transformation. A) pAM2100 B) pAHC25

RESULTS AND DISCUSSION

In the first microprojectile bombardment experiment, the transient GUS assay for treated maize cells showed that the average numbers of blue cells or groups of adjacent blue cells of three plates was 827 blue spots. This result indicated that the gene gun parameters and plasmid DNA preparation were properly set and prepared. This research was completed to ensure the procedure worked before shooting the wheat anther culture derived embryoids.

In the second experiment, nine plates containing wheat anther culture derived embryoids were bombarded. According to the results of the GUS assays, three plates that were bombarded without plasmid DNA were negative as expected. The three plates that were bombarded with pAM2100 plasmid DNA had a total 12 blue spots. The remaining 3 plates that were bombarded with pAHC25 had 52 blue spots. There are two possible interpretations of this result. The first would be that pAM2100 plasmid DNA had started degrading. The second would be that promoter of pAHC25 plasmid DNA, maize ubiquitin (Ubi), works better in monocots than the promoter of pAM2100. Weeks et al (1993) reported that the ubi promoter conferred high levels of expression of the marker genes, *bar* and *gus*. In a detailed study, Taylor et al (1993) showed enhanced GUS expression with a ubiquitin-based plasmid construct, pAHC25, in a number of cereals and grasses, including wheat.

The analysis of variance of the third experiment determined that there were significant differences among treatments. The highest transient expression of *gus* gene was obtained from the plates which were treated with N6 osmotic medium and embryoids that were washed with Liang's 85D12 salt and organics (148±7.45) (Table 1, Figure 3A). The fewest blue spots were found on the embryoids that were placed on N6 osmotic medium and not washed with Liang's 85D12 salts and organics and on embryoids that were placed on N6 medium (no osmoticum) and not washed with Liang's 85D12 salt and organics. An intermediate level of blue spots were found on embryoids treated with N6 medium and embryoids that were washed with Liang's 85D12 salt and organics (Figure 3B).

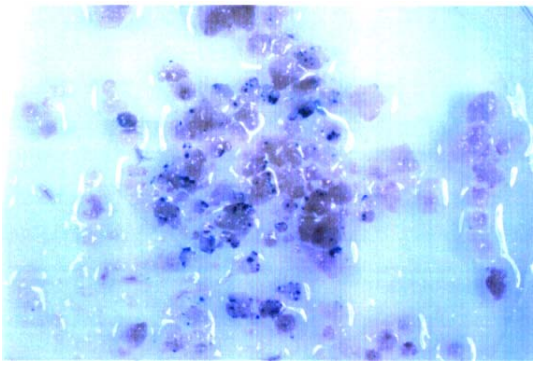
Table 1: Means of the transient expression of the *gus* gene on embryos bombarded after osmotic and washing treatments.

Treatments	Mean	Walter Grouping *
N_6-Os and washed	148	A
N_6 and washed	63.5	B
N_6 and unwashed	20.5	C
N_6-Os and unwashed	10	C

*Means with the same letter are not significantly different

The results of the third microprojectile bombardment experiment can be explained that the semi-liquid initiation medium surrounding the embryoids blocked the effect of the microprojectile bombardment. Washing embryoids with Liang's salt and organics removed the semi-liquid medium around the embryoids. Therefore, microprojectile bombardment became more efficient and the micro-gold particles penetrated better into the embryoid tissue. Washing embryoids and removing medium that was attached to them would explain the increase of transient expression of *gus* gene.

A)



B)

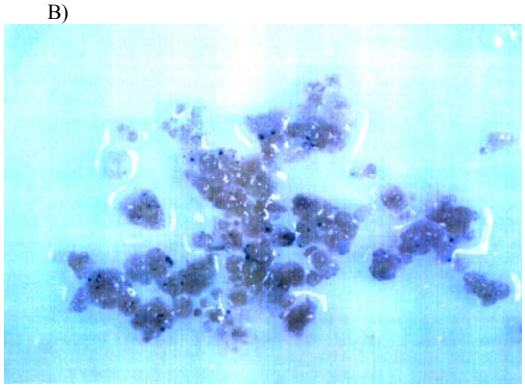


Figure 3: Transient expression of *gus* gene on anther culture derived embryos.
A) Treatment of N_6 osmotic medium and washing with Liang`s 85D12 salt and organic
B) Treatment of N_6 medium and washing with Liang`s 85D12 salt and organic.

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