
GENETIC TRANSFORMATION OF WHEAT (*TRITICUM AESTIVUM* L.) ANTHHER CULTURE- DERIVED EMBRYOS BY ELECTROPORATION

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ABSTRACT

This report describes the delivery of plasmid DNA into wheat anther culture derived embryos. Initial electroporation experiments were conducted using enzyme (NT-1) pretreatment, 500 μ F capacitance, and plasmid pAM2100, which carries the herbicide resistance gene, bar, and the screenable marker gene, Gus. Electroporated embryos were allowed to recover 10 days on regeneration medium and then transferred to regeneration medium containing 0, 10, 50, and 100 ppm phosphinotricin (PPT). Twenty eight albino and 13 green plants were regenerated on control (0 ppm PPT) selection medium from 640 embryos treated with pAM2100. The regeneration frequency was similar to embryos electroporated without pAM2100 demonstrating that electroporation was not deleterious for regeneration. Four albino plants were regenerated on selection medium (10, 50, and 100 ppm PPT). One plant was screened and shown to be transgenic by Southern blot analysis of a bar gene.

Introduction

The ability to transform plants through genetic engineering has been of great value in exploring fundamental questions in biology and enhancing crop improvement. Wheat is the most significant of the cereals in terms of being the main source of food for human being. There are number of DNA delivery techniques which have been developed for wheat transformation. They include direct gene transfer by microprojectile bombardment (1, 2) and *Agrobacterium*-mediated transformation (3, 4). Such successes have been achieved with model genotypes, but the methods are still not sufficiently reliable to be applied to a wide spectrum of commercial wheat varieties.

Electroporation has proved to be a broadly applicable gene transfer technique in animal,

plant, or microbial systems. It has the advantages of being simple, effective, and applicable to a wide range of plant species, higher DNA delivery rate and can be used to deliver DNA into large numbers of cells within a very short time.

The greatest electroporation success among cereal crops has been in transformation of maize (*Zea mays* L.; 5) and rice protoplasts (*Oriza sativa* L.; 6, 7, 8). The most severe drawback of electroporation-mediated gene transfer in plant research is only useful using protoplasts, which is strongly genotype dependent, and regeneration from protoplast has been difficult for most cereal genotypes. Thus, plant regeneration from wheat (*Triticum aestivum* L.) protoplasts has had very limited success (9, 10, 11). Transformation methods which do not use protoplasts are being investigated to avoid the drawbacks

of using protoplast-based transformation systems. There also have been reports of transformation by electroporation immature embryos (maize-12, 13; wheat- 14, 15), demonstrating that cells with intact cell walls also may be transformed.

Wheat anther culture system is well defined and plants are routinely regenerated (16, 17, 18). The culture of wheat anthers produces embryos (19) which provide a source of embryogenic tissue for electroporation studies. Our goal was to create a transformation procedure, which avoids the difficulties of protoplast culture and labor-intensive culture of specific callus types.

Materials 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., (20). 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 (21) 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 (20). Plates containing anthers were incubated at 25°C in the dark. Embryos were visible after four weeks of incubation. Four to six week old embryos were used for electroporation. After electroporation, 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 (20). Regenerated plantlets in AR medium were transferred to phytocons with rooting medium (MSO). MSO medium consisted of MS salts (22) with 20 g l⁻¹ sucrose, and 8 g l⁻¹

agar. The pH was adjusted to 5.8 prior to autoclaving. When the plantlets had well developed roots, they were transferred to vermiculite in the 12 hr light (18°C) and 12 hr dark (13°C) growth chamber. Plants were transferred to soil when they were approximately 20 cm tall.

Electroporation

Twenty anther embryos were kept in a 0.4 cm cuvette in NT-1 enzyme solution for 5 minutes at room temperature and then rinsed five times with electroporation buffer. NT-1 enzyme solution consisted of 0.1% pectolyase Y-23, 1% Cellulase RS, and 0.4 M mannitol, pH 5.5. (23). Electroporation buffer consisted of 10 mM HEPES buffer, 150 mM NaCl, 5 mM CaCl₂, and 0.4 M mannitol, pH 5.8. After the final rinse, the embryos, in 400 µl of electroporation buffer, were placed on ice for 10 minutes before electroporation. Electroporation was done at 0.4 kV with a Gene Pulser with a capacitance extender (Bio-Rad Laboratories, Hercules, CA). Each cuvette was considered an experimental unit. After electroporation, the embryos rested for 10 minutes on ice before being removed and plated on the regeneration medium.

A factorial treatment design was used in the first experiment. Two levels of plasmid DNA (presence or absence at time of electroporation), four levels of herbicidal compound phosphinothricin (PPT) (0-ppm-control, 10, 50, and 100 ppm PPT) in the regeneration medium provided eight treatment combinations.

For electroporation with the plasmid DNA treatment, 20 µl (1 ng µL⁻¹) plasmid was added per cuvette before the cuvettes were placed on ice for 10 minutes. The pAM2100 plasmid contained the *UidA* (GUS) gene encoding B-glucuronidase under the control of the *Adh* promoter, and *bar* gene encoding the enzyme PAT, which inactivates phos-

phosphinothricin (the active ingredient of the herbicide Basta) under the control of a viral adenine methyl transferase gene promoter (**Fig. 1A**). Embryos were allowed to recover on regeneration medium without PPT for 10 days and then transferred to AR media containing PPT (0, 10, 50, and 100 ppm). Embryos were left on the selection medium until the formation of plantlets or the death of the embryos. The number of regenerated plantlets were counted. The experiment was analyzed as a randomized complete block with 4 replications of 8 treatments. All data underwent arcsine square root transformation to improve the normality of data. Analyses of variance and Duncan's multiple range test (DMRT) were computed by SAS/PC statistical programs (24).

In the second experiment, two levels of two factors, specifically, enzyme pretreatment (enzyme treated versus non-enzyme treated embryos); and capacitance (500 or 960 mF) was undertaken in a factorial experiment with one replication. Enzyme pretreatment was done as described earlier. Twenty embryos were used for each treatment. The CAT marker gene encoding chloramphenicol acetyltransferase was used in this experiment. This bacterial enzyme catalyzes the transfer of the acyl group from acetyl-CoA (or any of several other acyl CoA cofactors) to chloramphenicol. The CAT assay measures the production of acetylated chloramphenicol when extract of transected with radioactive chloramphenicol and acetyl-CoA. After two days, 10 of the electroporated embryos were assayed for transient CAT activity. After 7 days, the remaining 10 embryos were used to measure stable CAT activity.

In the third experiment, a new plasmid and different PPT levels were examined. The treatment design was a factorial. Two levels of plasmid DNA (presence or absence at time

of electroporation), four levels of PPT (0-ppm-control, 10, 40, and 200 ppm) provided eight treatment combinations. For the plasmid DNA treatment, plasmid pAHC25 consisting of the *UidA* and *bar* genes, each under control of the maize ubiquitin *Ubi1* promoter was used. The *UidA* gene encodes the enzyme GUS (**Fig. 1B**). Twenty μl ($1 \mu\text{g} \mu\text{l}^{-1}$) was added per cuvette before the cuvettes were placed on ice for 10 minutes. Embryos were allowed to recover on the regeneration medium without the herbicidal compound phosphinothricin (PPT) for 10 days and then were transferred to selection medium containing PPT (0, 10, 40, and 200 ppm).

Plant Genomic DNA isolation and Southern hybridization

Genomic DNA was extracted from wheat transformants using the extraction procedure of Dellaporta *et al.* (25). Fifteen μg of genomic DNA, digested with restriction endonuclease, was separated on an agarose gel and analysed by Southern blotting as described by Gill *et al.* (26). A fragment containing *bar* gene was used as a probe and labeling was done by ^{32}P -dCTP using random primer labeling.

Results and Discussion

There is one previous report of electroporation of anther culture derived embryos in wheat. Gustafson (23) reported that capacitance was the only factor which affected the production of regenerable embryos. A capacitance of 500 μF produced greater amounts of regenerable embryos than the 960 μF . In our electroporation experiment, therefore, 500 μF capacitance was used. Enzyme pretreatment has been reported to be necessary for transformation (12). Also, Laursen *et al.* (27) reported that transient expression was not observed without the use of a cell wall degrading enzymes when elec-

propagating maize suspension culture cells. In the first experiment, 640 embryos were used. The analysis of variance of regenerated green plantlets determined there were significant differences among the different PPT levels used after transformation, and presence or absence of the plasmid, and there was an interaction between the two main effects. These results can be explained by our selection media being very efficient (Table 1). More plants were regenerated on the control medium (0 ppm PPT). The plasmid effect can be explained by only embryos transformed with the plasmid were able to regenerate green plants on selection medium. The interaction between PPT media and plasmid can be explained by having the same number of plants regenerate on 0 ppm PPT medium from embryos that were electroporated with or without the plasmid, while only embryos that were electroporated with the plasmid regenerated green plants on medium containing PPT. The analysis of variance of albino plantlets showed that the only significant difference was among the different PPT levels used after transformation. Gurel and Gözükırmızı (28) reported that higher concentration of selection pressure reduced germination and resulted in an albino phenotype for the few regenerated plants in tissue electroporation of barley. Absence

or presence of plasmid DNA was not significant. The interaction between two factors (plasmid DNA and PPT levels) was significant for albino plant regeneration. No plants were regenerated from PPT medium that were electroporated without plasmid containing the herbicide resistance gene, *bar*. This was the control treatment and ensured that our system was effective. Forty albino and 8 green plants (10%) were regenerated from electroporation treatments without the plasmid pAH2100 on control regeneration medium (0 ppm PPT). Thirty two albino and 13 green plants (16.3%) were regenerated from electroporation treatments with the plasmid pAH2100 on control regeneration medium (0 ppm PPT). These two results indicated that electroporation (with or without the plasmid) did not effect the ability of embryos to regenerate. Previous research on the electroporation of wheat anther culture derived embryos reported that 58 green plantlets (9.1%) were regenerated from 640 embryos (23). In our experiment, four albino plants regenerated from embryos electroporated with the plasmid pAH2100 on PPT medium (3 albino plantlets from 50 ppm PPT and 1 from 100 ppm PPT selection medium). One of the plants was screened and shown to be transgenic by Southern blot analysis (Fig. 2). This result indicated that

TABLE 1

Means of green plantlets from (0, 10, 50 and 100 ppm PPT regeneration medium) electroporation experiment 1.

PPT levels	Means
0	4.00 A
10	0.00 B
50	0.13 B
100	0.00 B

*The same letters within the same column are not significantly different.

TABLE 2

Means of green plantlets from (0, 10, 50 and 100 ppm PPT regeneration medium) electroporation experiment 3.

PPT levels	Means
0	2.63 A
10	0.00 B
40	0.25 B
200	0.13 B

*The same letters within the same column are not significantly different.

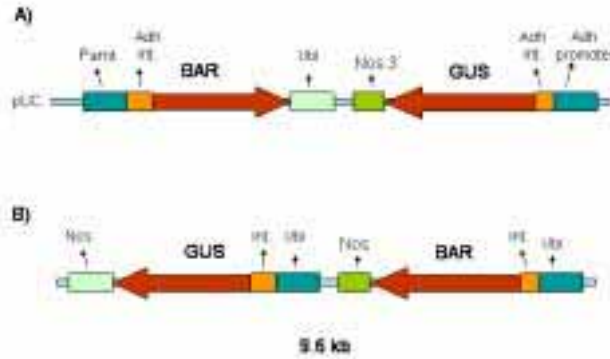


Fig. 1. Plasmids used for transformation. A) pAM2100 B) pAHC25.

the selection medium was able to select for transgenic plants from electroporation. This experiment has clearly demonstrated that DNA has been delivered into wheat anther culture derived embryos, although it should be noted that Gustafson et al., (23) reported the electroporation of wheat anther culture-derived embryos with plasmid pAM2100 containing the uidA and bar genes, and obtained 66 these plants, however, no molecular evidence was presented. The second experiment was conducted to determine if the electroporation procedure

effectively inserted the foreign gene into wheat anther culture derived embryo tissue. Two levels of capacitance were used because the higher level may puncture cell walls more efficiently, thus allowing more DNA to enter the cell. Not using an enzyme or using the lower capacitance may be “gentler” and allow better regeneration. For transient CAT assays, all treatments showed positive activity which indicated the DNA entered the cell (**Fig. 3**). Stable CAT expression, which is a necessary step for developing transgenic plants, was not found in any treatments. There are two possible explanations of this result. The first would be that the DNA inserted into the cell was degraded before it could be stably incorporated into the chromosomes. The second would be that some DNA was incorporated into plant chromosomes, but it was infrequent, hence without selection was below the level of detection in this experiment.

The third electroporation experiment was slightly different from the first experiment. In this experiment, plasmid pAHC25 was used and there were different PPT levels in the selection medium. The analysis of variance of green plantlets showed that the only significant differences were among the different PPT levels used after transformation (**Table 2**). This difference can be explained



Fig. 2. Presence of the BAR gene in transgenic albino wheat plant, where T1 & T2 are separately run extracted DNA from same albino plant. C is control plant (not electroporated with pAM2100).

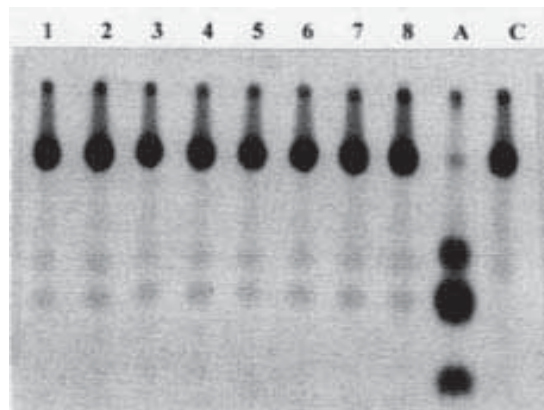


Fig. 3. Transient CAT assay of wheat anther culture derived embryos electroporated with enzyme pretreatment (presence or absence) and capacitance (500µF or 960µF).

Where 1 & 2 = embryos electroporated with enzyme and 500 µF
 3 & 4 = embryos electroporated with no-enzyme and 500 µF
 5 & 6 = embryos electroporated with enzyme and 960 µF
 7 & 8 = embryos electroporated with no-enzyme and 500 µF

by medium with no PPT regeneration more than media containing PPT. The effect of absence or presence of plasmid DNA was not significant, and the interaction between two factors (plasmid DNA and PPT levels) was not significantly different for green plant regeneration. These results suggest that stable transformation was rare (plasmid effects were unstable) and we could not show the expected interaction with PPT selection media. The analysis of variance of albino plantlets showed that the only significant difference was among the different PPT levels used after transformation (Table 2). Absence or presence of plasmid DNA was not significant, and the interaction between two factors (plasmid DNA and PPT levels) was not significantly different for albino plant regeneration. No plants were regenerated from PPT medium that were electroporated without plasmid pAHC25. Forty two albino and 17 green plants (21.3%) were regenerated from electroporation treatments without the plasmid on control regeneration me-

dium (0 ppm PPT). Forty five albino and 15 green plants (18.8%) were regenerated from electroporation treatments with the plasmid containing the herbicide resistance gene, bar, on control regeneration medium. These two results again indicated that electroporation (with or without the plasmid) did not affect the ability to regenerate in the absence of the herbicide and agreed with results of Gustafson (23). The third electroporation experiment had better results (green plant regeneration) than the earlier experiment because the earlier electroporation experiment was conducted before determination of the optimal embryo size and time in culture. Therefore, in earlier experiment, the embryo size and time in culture factors were not closely monitored. However, the third electroporation experiment was conducted based on determined the optimal size and time in culture for regeneration green plants (18). By using more regenerable embryos, the third electroporation experiment produced more regenerated plants (20.1%) than did the

earlier electroporation experiment (13.5%). Zhou et al. (11) electroporated protoplasts from anther-derived callus. They selected for transformation using 10 mg L⁻¹ phosphinothricin (PPT) in the medium and reported that transformed tissue survived on medium that contained up to 2560 mg L⁻¹ PPT. Sorokin et al., (15) produced of fertile transgenic wheat plants using via electroporation of immature embryos.

As a conclusion, selection medium was able to select for transgenic plants from electroporation. Electroporation (with or without plasmid) did not affect the ability to regenerate in the absence of the herbicide. These experiments have demonstrated that electroporation procedure effectively inserted the foreign DNA into wheat anther culture derived embryos. At least one transgenic plant was obtained from electroporation of wheat anther culture derived embryos and confirmed by Southern blot analysis. The efficiency of this technique still depends on a combination of factors including the characteristics of the recipient tissue, the culture efficiency and the electroporation conditions.

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