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Exogenous DNA Uptake via Cellular Permeabilization and Expression of Foreign Gene in Wheat Zygotic Embryos

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Abstract

Exogenous DNA uptake and transient expression of the GUS reporter gene was studied in wheat (*Triticum aestivum* L.) zygotic embryos employing a simple procedure of cellular permeabilization by membrane interactive agents like saponin and toluene. Uptake of expression vectors with different promoters was detected in mature and immature embryos. The frequency of GUS expression was detected to be higher in mature embryos than immature embryos probably due to a certain degree of disorganization of the plasmalemma during the dehydrated state of the embryos. Of the various permeabilizing agents employed, saponin and toluene were detected as potential membrane permeabilizers without adversely affecting embryo viability. Variations in GUS gene expression were not significant among different genotypes of *T. aestivum* CPAN1676, PBW343 and HD2329, thus indicative of the relative genotype independence of this process. The results support the proposed idea of direct DNA uptake as an alternate and simple method for inserting foreign DNA into plant cells and its use for transformation of higher plants.

1. Introduction

Genetic engineering of crop plants rely resolutely on efficient protocols of regeneration and transformation. The notorious recalcitrance exhibited by members of the Graminae to regenerate plants *in vitro* (Vasil *et al.*, 1994) and the lack of affinity of *Agrobacterium* for monocots (Mahalakshmi and Khurana, 1995; 1997), has severely hampered the progress of cereal transformation in general, and wheat in particular. A range of plant transformation techniques based on direct DNA transfer has thus been exploited for genetic transformation of these crop species. DNA transfer to protoplasts (Paszkowski *et al.*, 1984; Fromm *et al.*, 1985), agroinfection (Grimsley *et al.*, 1986; Dale *et al.*, 1989), microinjection (Crossway *et al.*, 1987) and microprojectile bombardment (Vasil *et al.*, 1991) are the major transformation strategies utilized for transforming cereals. Amongst these microprojectile bombardment has achieved wider popularity due to its greater range of applicability and relative ease of use in organized tissues (Hansen and Wright, 1999). However, in many plant species the transformation efficiency of bombardment is very low and requires a labour-intensive procedure (Christou, 1994). Thus the other simpler procedures for DNA delivery into plant cells attain importance (Weissenger, 1992). Exogenous DNA uptake came into limelight in early 1970s, employing dry tissues such as mature embryos and seeds as the target material and exploited the physico-chemical and structural properties of plasmalemma of the cell (Hess *et al.*, 1970; Ledoux *et al.*, 1971). The simplicity of the technique and elimination of skill and labour involved in tissue culture continued to allure scientists to explore its mechanism for the field of plant transformation. Later efforts were successful in establishing the expression of exogenous DNA in plant cells by imbibition and its subsequent integration in the plant genome (Töpfer *et al.*, 1989; Senaratna *et al.*, 1991; Yoo and Jung 1995; Luan *et al.*, 1996).

Axiomatically, plant cells unlike animal cells possess a double protection system against foreign molecules, i.e. the molecular sieving-properties of the cell wall in conjunction with the impermeant lipid bilayer of plasmalemma offer resistance to the entry of exogenous macromolecules. Nevertheless, by challenging such barriers through effective membrane permeabilizers such as DMSO, toluene and saponin, the permeation of exogenous DNA molecules in the target plant tissue has been achieved (Töpfer *et al.*, 1989; Parihar *et al.*, 1994;

Yoo and Jung 1995; Luan *et al.*, 1996). Based on the supportive data obtained in cellular permeabilization of *Brassica*, an important oil crop (Parihar *et al.*, 1993; 1994), the present investigation was directed towards exploring the scope of the technique as an alternate and simple procedure for wheat transformation using zygotic embryos as the target tissue.

2. Materials and Methods

2.1 Plant material

Seeds of the three varieties of *T. aestivum*, CPAN1676, PBW343, HD2329 were obtained from Directorate of Wheat Research, Karnal, India. The mature embryos were isolated from the dry seeds using a waring blender, hand picked, and surface sterilized using 4% hypochlorite (1:1 v/v dilution with sterile water) for one minute. The embryos were then washed four to five times with sterile distilled water and allowed to air dry in the laminar flow for 30-45 minutes prior to use. For immature embryos, seeds were harvested at milky stage and embryos hand dissected. These were surface sterilized with 4% hypochlorite (1:1 v/v dilution with sterile water) for thirty seconds followed by four to five washings with sterile water.

2.2 Cellular Permeabilization

Plasmids pBI121 and pActGUS were employed for DNA uptake studies. Both the vectors carry a GUS reporter gene but are driven by different constitutive promoters; 35S from cauliflower mosaic virus (CaMV35S) and Act-1 from rice, respectively. Approximately 60-70 mature and immature embryos were imbibed in 500 μl of 15mM NaCl, 1.5mM sodium citrate buffer, pH7.1 containing 100 μg plasmid DNA and one of the permeabilizing agents - DMSO (3%-30%), Triton X-100 (0.01%-1.0%), saponin (0.5-10 mg ml^{-1}), toluene-ethanol mixture; 1:4 v/v (1:20 or 1:50 dilution with the permeabilization buffer). Embryos were then washed thrice with buffer to remove excess DNA and permeabilizing agents. These were then placed on MS medium (pH 5.8) supplemented with Cefotaxime (250 mg l^{-1}) for 3-5 days for germination in dark at 26-28 ℃. GUS histochemical assay was performed according to Jefferson (1987). Percentage GUS expression was calculated as the number of embryos exhibiting GUS expression/ total number of treated embryos x100 and the mean value plotted.

2.3 Transmission Electron Microscopy

Following treatment with cell permeabilizing agent the tissue samples were fixed for 30 min in 3% gluteraldehyde (v/v) in 0.1M phosphate buffer (pH 6.8) by vacuum infilteration for 5 min, followed by several washings with 0.1M phosphate buffer at 4 ℃. The tissue was postfixed in 1% osmium tetraoxide followed by dehydration, infiltration and embedding (Fowke, 1984).

3. Results and Discussion

Macromolecules can be incorporated into living cells by employing various permeabilizing agents (Meiners *et al.*, 1991) and these methods have been extended for direct DNA uptake as well (Töpfer *et al.*, 1989; Parihar *et al.*, 1994). Zygotic embryos serve as an excellent receipient tissue for direct DNA uptake studies by circumventing tissue culture procedures. Thus, in the present investigation the wheat zygotic embryos were employed to investigate the influence of various factors on DNA uptake by cellular permeabilization. Evidence has been provided for the comparative reporter gene expression in mature and immature embryos, utility of permeabilizing agents, and efficacy of various host genotypes.

The optimum temperature for DNA uptake was found to be 26-28 ℃. The process of DNA uptake was not favored when embryos were treated at low temperature (4 ℃) or higher temperature (37 ℃) though Yoo and Jung (1995) reported increase in the GUS expression at low temperature in rice. The treatment involving alternate heat shock (37 ℃) and cold shock (4 ℃) to the wheat embryos during the incubation period also proved to be futile.

3.1 Influence of plant material and tissue organization

In the present investigation, mature embryos were observed to be more suitable than the immature embryos for direct DNA uptake: 25-30% increase in GUS expression (**Fig. 1**). This could partially be due to greater water potential gradient created in the dry embryos during imbibition leading to an enhanced release of cytoplasmic electrolytes resulting in facilitated uptake of exogenous DNA (Simon 1974, Senaratna *et al.*, 1983; 1991). Additionally, the dehydrated state also leads to a higher degree of membrane discontinuity and disorganization aiding in uptake of exogenous macromolecules (Töpfer *et al.*, 1989). On electron microscopic examination of the wheat zygotic embryos, abnormalities in terms of reptated cell wall were also noticed in the dry embryos (**Fig. 2A**) which get relatively normalized by a period of imbibition in water (**Fig. 2B**). Thus cellular organization of the cell wall and the plasma

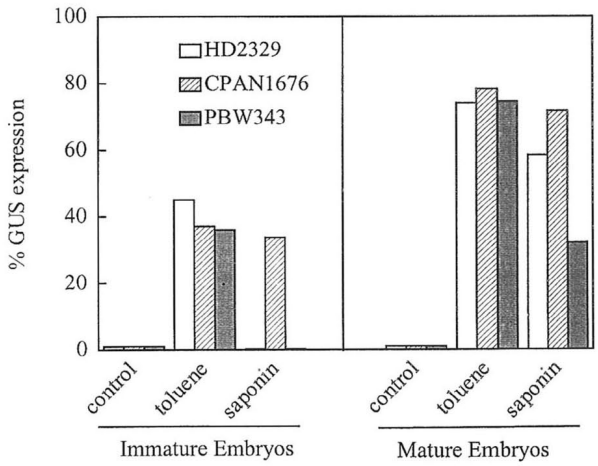

Fig. 1. Transient GUS expression in immature and mature embryos of the three varieties of *T. aestivum* treated with various permeabilizing agents and plasmid DNA pActGUS for 45 min. After treatment embryos were washed thrice with the permeabilization buffer and placed on MS medium supplemented with Cefotaxime (250 mg l^{-1}) in dark. Histochemical GUS assay of embryos was carried out after 3-5 days.

membrane appear to play a vital role in DNA imbibition.

3.2 Influence of permeabilizing agents

In animal systems, cellular permeabilization has been commonly used for introducing foreign macromolecules into living cells in a non-invasive manner (Kawai and Nishizawa, 1984). The same approach has also been adapted to permeabilize plant cells for various cellular metabolites and has been extrapolated for exogenous DNA uptake (Delmer, 1979; Meiners *et al.*, 1991; respectively).

In the present investigation, GUS expression was monitored histochemically to investigate the relative suitability of various permeabilizing agents in aiding DNA uptake. The embryos treated with common membrane destabiliser DMSO (20%) failed to exhibit histochemical GUS expression and also lowered the percentage of embryo germination (**Table 1**), in contrast to that reported by Töpfer *et al.* (1989). Similarly, Triton X-100 (0.1%), a popular membrane surfactant involved in selective delipidation of membranes and also used to extract

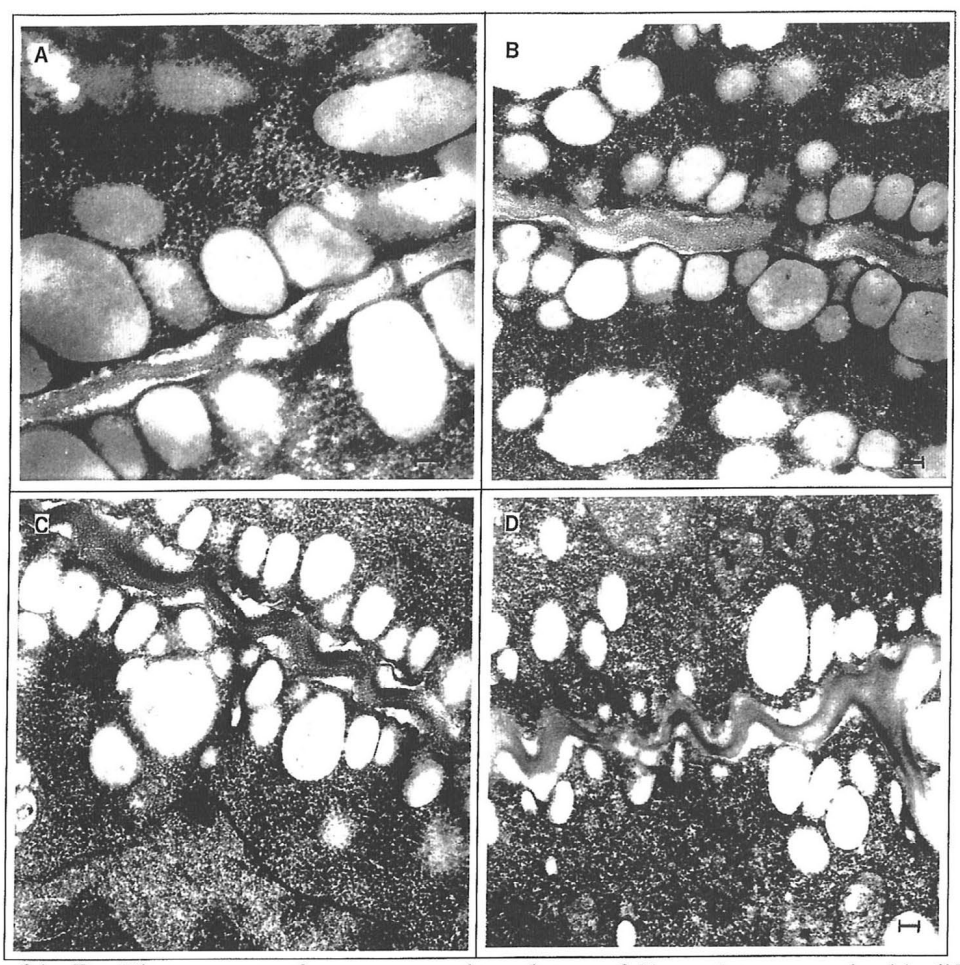

Fig. 2A-D Ultrastructure of mature zygotic embryos of *T. aestivum* treated with different permeabilizing agents. **A.** control, dry mature embryo; **B.** control, embryos imbibed in buffer alone for 45 min; **C.** embryos imbibed in buffer containing 1.0 mg ml^{-1} saponin for 45 min; **D.** embryos imbibed in buffer containing 1:20 v/v dilution with toluene-ethanol mix for 45 min. Bar represents 1 μm.

Table 1. Effect of various permeabilizing agents on germination of immature and mature embryos of the three varieties of *T. aestivum*. The sterilized embryos were incubated for 45 min. with the permeabilizing agent, washed thrice with the permeabilization buffer, and placed on MS medium for germination in dark after 3 - 5 days.

Permeabilization treatment	HD2329	CPAN1676	PBW343
A. IMMATURE EMBRYOS			
Control	91.0 ± 1.0	93.0 ± 1.0	96.0 ± 1.0
Triton X - 100	3.7 ± 1.8	4.0 ± 2.0	—
DMSO	76.5 ± 3.5	84.5 ± 1.5	49.5 ± 21.5
Saponin	93.2 ± 0.2	92.5 ± 1.5	91.5 ± 1.5
Toluene	96.0 ± 4.0	87.3 ± 6.3	91.5 ± 1.5
B. MATURE EMBRYOS			
Control	88.0 ± 4.0	74.0 ± 2.0	88.0 ± 4.0
Triton X - 100	4.9 ± 0.3	5.1 ± 0.1	4.9 ± 1.6
DMSO	74.3 ± 10.3	65.1 ± 13.1	69.1 ± 2.3
Saponin	87.8 ± 5.2	79.5 ± 3.5	86.3 ± 3.0
Toluene	90.3 ± 5.0	88.3 ± 8.3	89.6 ± 0.3

membrane proteins (Sandstorm and Cleland, 1989), was found inefficient for DNA uptake. Triton X-100 also had a detrimental effect on embryo germination greater than any other membrane permeabilizer. The effect on germination could be the principal reason for the ineffectiveness of both DMSO and Triton X-100 in the wheat system, which was not so in the case of *Brassica* spp. (Parihar *et al.*, 1994). Interestingly, the embryos treated with toluene, a widely known inducer of transient 'pore' formation of specific sizes (Lerner *et al.*, 1978), exhibited significantly higher frequency of GUS expression (78%) at 1:20 v/v dilution with permeabilization buffer (**Fig. 1**). Saponin, a natural plant glucoside, was also found to be effective at 1.0mg ml^{-1} and a high frequency of GUS gene expression (71%) was obtained in treated embryos (**Fig. 1 & 3**). Toluene and saponin also do not significantly influence the embryo germination (**Table 1**) although, a greater degree of cell wall reptations is seen in both (**Fig. 2C & D**). Thus the successful permeabilization by saponin and toluene is linked to their ability to cause a greater degree of cell wall disorganization, indirectly aiding DNA uptake, without exhibiting an adverse effect on germination or cell viability.

3.3 Genotypic effect

Variations in response to tissue cultures by different genotypes is often exhibited in wheat

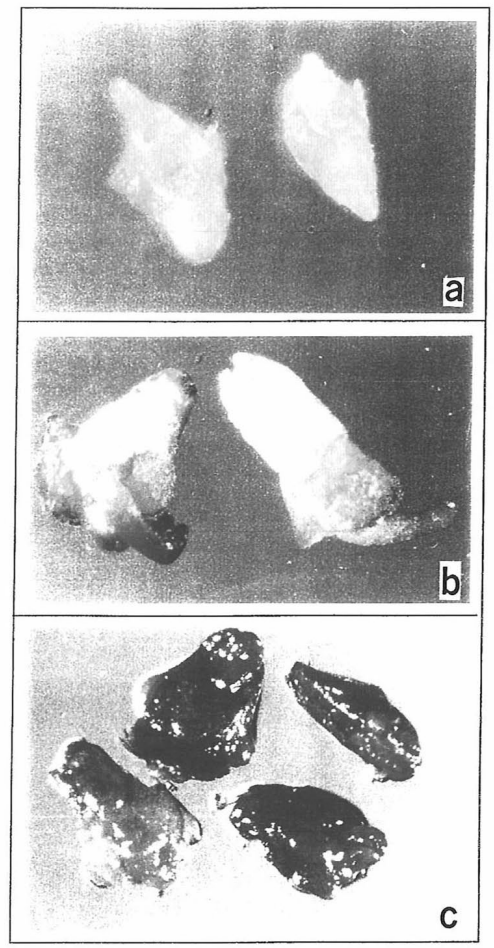

Fig. 3 a - c. Histochemical localization of GUS activity in dry embryos of *T. aestivum* two days following permeabilization with 1.0 mg ml^{-1} saponin for 45min a. control; b. dry embryos incubated with plasmid DNA alone; c. saponin permeabilized dry embryos incubated with plasmid DNA.

(Ahloowalia, 1982; Özgen *et al.*, 1998) which led us to investigate the genotypic influence in the present study. Amongst the three varieties of *T. aestivum* employed GUS expression was observed in all the genotypes though at varying levels (**Fig. 1**). This indicates that this method is almost independent of the genotypic influence (see also Parihar *et al.*, 1994) probably due to the underlying biochemical/biophysical principle involved during cellular permeabilization. It also offers a relative advantage over the other techniques, which have limited utility for recalcitrant genotypes (Tyagi *et al.*, 1989; DeBlock and DeBrouwer, 1991; Maheshwari *et al.*, 1995).

Although biolistics appear to be the method of choice for cereal transformation (Hansen and Wright, 1999), cellular permeabilization offers various advantages. Particle bombardment requires sophisticated equipment while transformation via

cellular permeabilization is an economical and a user-friendly procedure. The former is heavily tissue culture dependent while latter is relatively independent of this technique. Though extremely desirable, it is too early to comment upon the transformation efficiency at stable level by cellular permeabilization as there are scanty reports exihibiting the integeration of the foreign genes (Töpfer *et al.*, 1989; Parihar *et al.*, 1993; Yoo and Jung, 1995). Nonetheless, cellular permeabilization does have the potential to be a non tissue culture based, genotype independent, and a relatively cost effective method of gene delivery in plants.

To conclude, a novel technique of cellular permeabilization has been developed for direct exogenous DNA uptake in wheat zygotic embryos, without compromising cell viability and proliferative ability. The technique is simplistic in approach and is relatively independent of the skill, labour and intricacies involved in tissue culture procedures. By and large, it is also applicable to various genotypes. Although, the incorporation of exogenous DNA in seed embryos and its transient expression affirms its potential as an effective and efficient technique, further attempts are required to understand the exact mechanism of foreign DNA uptake by the living cells. The technique though in its infancy, possesses tremendous scope for routine application in a wide range of crop species.

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