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Transformation of protoplasts and intact cells from slowly growing embryogenic callus of wheat (*Triticum aestivum* L.)

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Abstract A procedure for culturing protoplasts from slowly growing embryogenic calli of wheat was developed. The procedure was dependent on the ability to isolate large numbers of culturable protoplasts from slowly growing embryogenic callus. Approximately 68% of the isolated protoplasts divided, and 22% formed colonies; of the latter, 67% continued to proliferate. Plating efficiency was reduced when protoplasts were transformed by polythylene glycol, electroporation, and/or *Agrobacterium*. Intact cells were also directly transformed by electroporation. Direct electroporation of the *Agrobacterium* binary vector into intact cells resulted in a significant increase of GUS activity over the control.

Key words Wheat · Transformation · *Agrobacterium* · Electroporation · Polyethylene glycol

Introduction

The production of transgenic fertile cereal (e.g., wheat) plants has been hampered by factors such as the absence of reliable and efficient methods for maintaining embryogenic cultures, culturing protoplasts, and delivering and expressing genes (Harris et al. 1989; Vasil et al. 1990–1992; Potrykus 1991). Rice and corn are much more responsive to regeneration and transformation (Rhodes et al. 1988; Shimamoto et al. 1989; Gordon-Kamm et al. 1990; Hayashimoto et al. 1990; Rainieri et al. 1990). A few fertile transgenic plants have been regenerated from wheat cultures transformed by microprojectiles (Vasil et al. 1992). However, mork work still lies ahead to make the transformation and regeneration of fertile plants from wheat less sporadic and more reliable.

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Because there are many problems with the protoplast system, recent attempts to transform wheat have favored particle bombardment of organized plant parts (Chibbar et al. 1991; Vasil et al. 1992). Unfortunately, the efficiency is low due to the inability of the randomly delivered DNA to enter embryogenic (regenerable) cells that are deeply embedded among the non-embryogenic (non-regenerable) cells. Other methods, like electroporation, have been used to transform protoplasts of rapidly dividing embryogenic suspension cells, but only sterile plants were regenerated, and these are not useful in breeding programs (Rhodes et al. 1988; Vasil et al. 1990, 1991; Potrykus 1991). In wheat, suspension cells have been shown to lose fertility shortly after culture (Vasil et al. 1990, 1991; Potrykus 1991). For this reason, embryogenic callus was used in the investigation reported here. Furthermore, to increase the transformation frequency and possibly the number of fertile plants regenerated, protoplasts were isolated only from embryogenic callus and transformed by polyethylene glycol, electroporation, and *Agrobacterium tumefaciens*. Protoplasts isolated from embryogenic calli of wheat cv 'Chinese Spring' were able to divide and form calli from transformed and non-transformed tissues. This article is the first to report the success of this method in cereals.

Experiments were conducted to optimize culturing conditions for protoplasts isolated from slowly growing embryogenic callus; to determine conditions under which a reporter gene (GUS) driven by an enhanced 35S promoter in the presence of TMV leader sequence can be expressed in polyethylene glycol (PEG)-treated protoplasts and intact cells; to compare viability and GUS activity of protoplasts and intact cells after PEG, electroporation, and *Agrobacterium*-mediated transformation; and to determine if different plasmid DNA isolation methods (cesium chloride (CsCl) vs minipreparation) will significantly affect the rate of transformation and transient GUS activity in wheat protoplasts.

Materials and methods

Tissue culture

Callus induction and maintenance, protoplast isolation, washing, and viability assessment have been described elsewhere (Zaghmout and Trolinder 1993a). In this study, protoplasts isolated from embryogenic callus 'Mustang' and 'Chinese Spring' were plated in medium 1, which contained full-strength MS basal salts (Murashige and Skoog 1962), KM vitamins and carbohydrates (Kao and Michayluk 1975), 9.0 μM 2,4- dichlorophenoxyacetic acid (2,4-D), 0.318 M sucrose, 0.109 M mannitol, and 10 g/l agarose (Sea Plaque, FMC Bioproduct, Rockland, Me.). All media components other than agarose were filter sterilized. One milliliter of medium 1 containing 7×10^5 protoplasts was first mixed with 1 ml of medium 1 supplemented with agarose (10 g/l), then poured into 30 × 10 mm Falcon petri plates. The plates were kept in the dark at 24 °–26 °C. Every 3–5 days, 1 ml of medium 1 (no agarose) was gently added. When colonies became visible to the naked eye, they were counted and then subcultured on media containing the same components, except that gelrite (2 g/l, w/v) (Scott Laboratories, Carson, Calif.) was substituted for Sea Plaque agarose.

Transformation experiments

Protoplasts and intact cells from callus tissues were subjected to plasmid DNA delivery by three transformation methods (PEG, electroporation, and *Agrobacterium*). Methods for plasmid DNA delivery by electroporation have been described earlier (Zaghmout and Trolinder 1993a). In this study, PEG concentrations ranged up to 40% (w/v). Plasmid pAGUS1-TN2 contains the GUS gene, while pTRA 132 contains the hygromycin gene. The GUS gene is driven by an enhanced 35S promoter with a TMV leader sequence (Skuzeski et al. 1990), while plasmid pTRA 132 containing the hygromycin gene is driven by 35S only. Both plasmids were isolated by minipreparation and CsCl methods according to Maniatis et al. (1982). The binary vector used in this study (pKIWI 105) contained the GUS and neomycin phosphotransferase genes driven by the 35S and manopine synthase promoters, respectively. Cultures of *Agrobacterium* strain A281 harboring the binary vector were grown overnight on Luria Broth (LB) supplemented with 50 µg/ml kanamycin sulfate. Cocultivation experiments were initiated by mixing the pellet resulting from a 30 µl bacterial suspension (1000 g, 5 min) with 1 ml of protoplast culture medium containing 7×10^5 protoplasts or intact cells. This mixture was placed in the dark under static conditions at 24–28 °C and allowed to incubate for not more than 24 h. The electroporation technique of the *Agrobacterium* binary vector into intact cells, the electroporation buffer, and the measurement of GUS activity have been described earlier (Zaghmout and Trolinder 1993b). Protoplasts and intact cells resulting from these transformation methods were grown by being plated in a manner similar to that of non-transformed protoplasts except that the medium of the former contained no antibiotics.

Results

Protoplast isolation and culture

High yields of protoplasts ($6–7 \times 10^6$ protoplasts/g fresh weight of embryogenic callus) were obtained 6–10 h after incubation with the enzyme solution at slow agitation (20–30 strokes/min). The protoplasts were highly cytoplasmic and contained small vacuoles having an average diameter of 10–15 µm. All efforts to culture these protoplasts in liquid media failed. In most cases, the protoplasts were viable for only 24–48 h. Increases in the percentage of protoplasts that divided and formed growing colonies were only possible when the protoplasts were mixed with Sea Plaque agarose before plating [final agarose concentration 0.5% (w/v)] (Table 1). Under such conditions, the percentage of dividing protoplasts and proliferating colonies increased, even in the absence of nurse cells (Fig. 1A–C). On the basis of comparable results from several repetitions, this is a reliable method for culturing protoplasts. When microcalli were transferred to a regeneration medium supplemented with low concentrations of 2,4-D (0.45 μM), root overproduction became problematic. This was avoided by including indolacetic acid-degrading compounds such as citric acid (0.3 mM) in the media.

Transient GUS activity in protoplasts and intact cells transformed by PEG

An increase in GUS activity was observed in protoplasts incubated in the presence of 15–20% PEG (w/v) and pAGUS-TN2 (Table 2). However, GUS activity was higher in the protoplasts of 'Mustang' than in those of 'Chinese Spring'. A decline in GUS activity at higher concentrations of PEG [> 20% (w/v)] was probably caused by a decline in protoplast viability (Table 2). No detectable increase in GUS activity was observed in PEG-treated intact cells.

Effect of plasmid DNA isolation methods on transient GUS activity

Previous reports have emphasized the use of CsCl-purified plasmids in transformation experiments (Po-

Table 1 Division of protoplasts, formation of colonies, and proliferation of colonies from 'Chinese Spring' protoplasts. Protoplast density prior to plating was 5×10^6 protoplasts/ml. Data represent the average of three replicates. Data on protoplasts dividing, colony formation, and proliferating colonies were taken 14, 45 and 75 days after plating, respectively. The diameter of microcalli used to calculate the percentage of colony formation ranged from 0.1 to 0.5 mm. Larger colonies were categorized as proliferating ones

Final agarose concentration (%,w/v)	% Protoplasts dividing (visual estimate)	% Colony formation (visual estimate)	% Proliferating colonies
0 (no agarose)	3	0	0
0.25	33	5	0.1
0.5	68	22	14.7
1.0	45	19	4.5
1.5	48	4	0.4
2.0	40	0	0

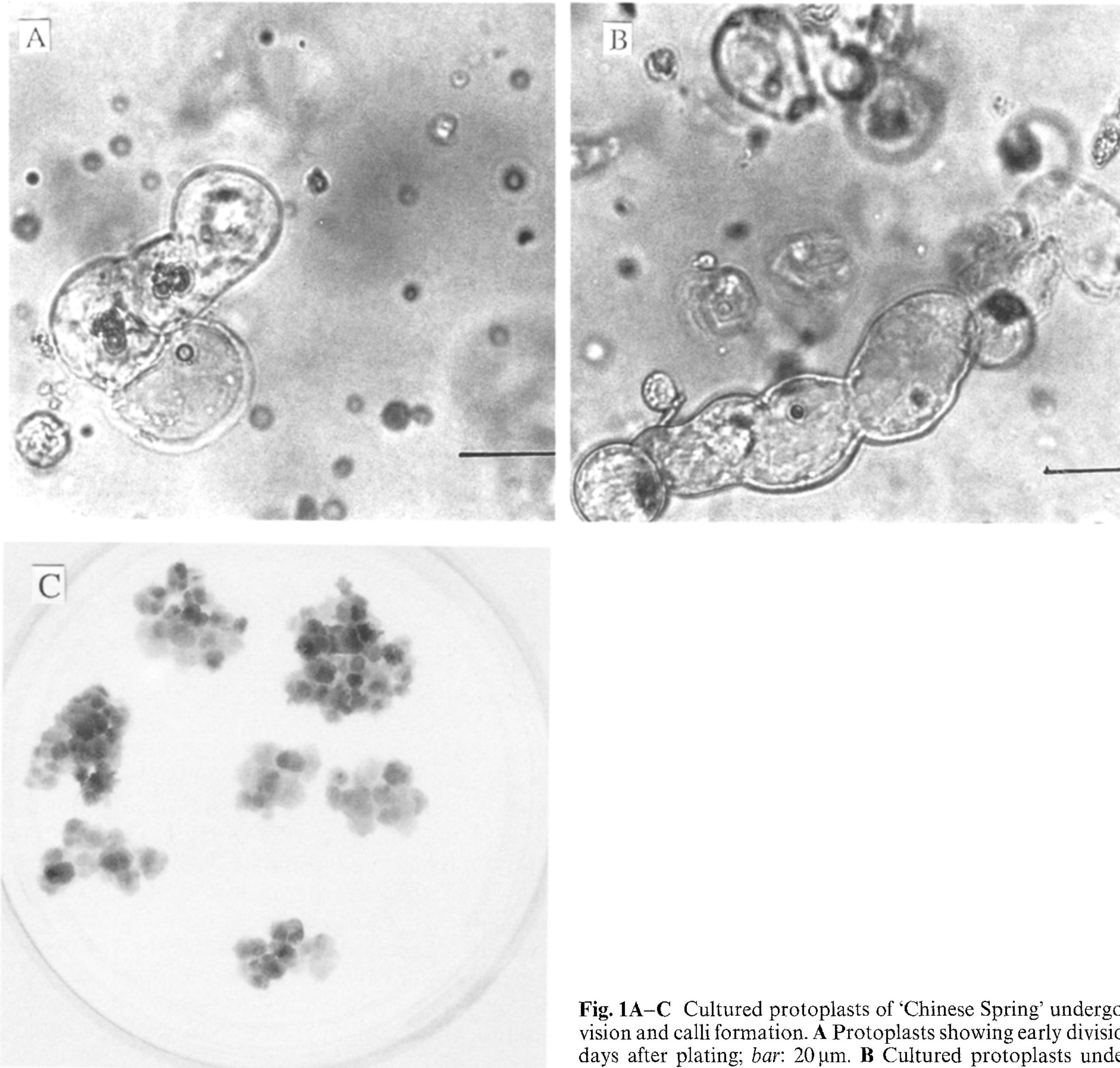

Fig. 1A–C Cultured protoplasts of 'Chinese Spring' undergoing division and calli formation. **A** Protoplasts showing early divisions 1–2 days after plating; *bar*: 20 μm. **B** Cultured protoplasts undergoing division, 5 days after plating; *bar*: 20 μm. **C** Calli formation from protoplasts

trykus et al. 1985; Potrykus 1991). It has been suggested that CsCl purification is essential for ensuring a high transformation frequency. In Table 3 it can be seen that there was no difference in GUS activity resulting from the electroporation of a plasmid DNA obtained by two different isolation techniques (CsCl vs. minipreparation). The rate of transient GUS expression in PEG-transformed protoplasts was also similar to that of plasmids isolated by these two methods (data not shown). However it was essential to clean plasmid DNA isolated by minipreparation at least twice with one-half volume 7.5 *M* ammonium acetate and two volumes 95% ethanol.

Effect of DNA delivery method on protoplast viability

Agrobacterium- transformed protoplasts had the highest viability and the highest GUS activity, followed by those transformed by electroporation (Table 4); viability and GUS activity were low in those protoplasts that underwent PEG treatment (Table 4). The number of protoplasts undergoing cell division was approximately equal following electroporation or *Agrobacterium*-mediated transformation (Table 5); this frequency was reduced following PRG-mediated transformation.

Transformation of intact cells

Experiments were initiated to transform intact cells by *Agrobacterium*, electroporation, and PEG. As expected, no GUS activity was obtained in intact cells transformed by either *Agrobacterium* or PEG. With electroporation, the application of a high voltage (exceeding 2000 volts) was essential prior to the detection of an increase of GUS activity over the control. As tissue culture medium has a low electrical resistance (ca.

Table 2 Effect of PEG on transient GUS activity in protoplasts of embryogenic calli of 'Mustang' and 'Chinese Spring'

Cultivar	% PEG concentration	Protoplasts prior to assay ($\times 10^5$)	% Viable protoplasts	Total GUS activity[a] in protoplasts (picomole MU/min)
Mustang	0	6.3	99	0.0c
Mustang	5	4.3	89	2.3c
Mustang	10	3.4	92	5.8bc
Mustang	15	3.1	79	12.5ab
Mustang	20	2.9	80	5.4a
Mustang	30	1.8	52	7.9b
Mustang	40	0.3	22	2.9c
Chinese Spring	0	5.8	96	0.0b
Chinese Spring	5	4.3	88	1.9b
Chinese Spring	10	3.9	84	3.2b
Chinese Spring	15	3.2	71	5.4ab
Chinese Spring	20	2.7	63	8.3a
Chinese Spring	30	1.5	39	3.1b
Chinese Spring	40	0.2	33	0.5b

[a] Means within a column of each cultivar not sharing the same letter are significantly different at the 0.05 probability level according to Duncan's multiple range test

Table 3 GUS activity in electroporated protoplasts of wheat callus as affected by the plasmid DNA isolation method. Protoplast density prior to electroporation was 7×10^5/ml

Cultivar	Plasmid isolation method	Total GUS activity in protoplasts (picomole MU/min)
Mustang	Cesium chloride	35.5
	Minipreparation	29.1
Chinese Spring	Cesium chloride	17.2
	Minipreparation	15.0

20 Ohms), the application of an electrical charge of 2000–6000 volts can cause arcing, a loss of energy, a shattering of the cuvette, and an ejection of the cuvette contents. To avoid all of these problems, a special electroporation buffer solution was used (Zaghmout and Trolinder 1993b). When the *Agrobacterium* binary vector (pKIWI 105) was electroporated directly into 'Mustang' intact cells, a significant increase in GUS activity was obtained (159.5 picomole MU/min vs. 8.6 with electroporation or 0.0 with PEG). A similar trend was observed in intact cells of 'Chinese Spring' transformed under similar conditions. GUS activity originating from *Agrobacterium* was found to be negligible, since the vector's prokaryotic translation initiation site was disrupted (Rainieri et al. 1990); GUS activity was also found to be negligible in PEG-treated intact cells. PEG added to the electroporation mixture (5–10%, w/v) immediately prior to electroporation resulted in at least a one-fold increase in GUS activity (data not shown).

To allow the transformed protoplasts and intact cells to recuperate from transformation-related stress, the addition of antibiotics to the culture media was delayed

Table 4 GUS activity in protoplasts of embryogenic 'Mustang' and 'Chinese Spring' calli as affected by the DNA delivery methods. Protoplasts density prior to DNA delivery was 7×10^5/ml

Cultivar	DNA delivery method	Promoter type	Protoplasts prior to assay ($\times 10^5$)	% Protoplast viability	Total GUS activity in protoplasts (picomole MU/min)[a]
Mustang					
	PEG	35S + 35S + TMV leader sequnece	3.2	69	17.2c
	Electroporation	35S + 35S + TMV leader sequence	4.9	92	31.7b
	Agrobacterium (no electroporation)	35S	6.3	94	310.5a
Chinese Spring					
	PEG	35S + 35S + TMV leader sequence	2.4	59	9.2c
	Electroporation	35S + 35S + TMV leader sequence	4.8	88	23.6b
	Agrobacterium (no electroporation)	35S	6.5	93	178.5a

[a] Means within a column of each cultivar not sharing the same letter are significantly different at the 0.05 probability level according to Duncan's multiple range test

Table 5 The number of proliferating colonies from transformed 'Chinese Spring' tissues. Protoplast and cell density prior to transformation was 5×10^6/ml

Method of transformation	Tissue type	Number of colonies proliferating[a]
PEG		
	Protoplasts	5
	Intact cells	0
Electroporation		
	Protoplasts	13
	Intact cells	20
Agrobacterium		
	Protoplasts	8
	Intact cells (without electroporation)	0
	Intact cells (with electroporation)	60

[a] Average of two experiments. Each experiment had at least five plates. Proliferating colonies were observed 70–80 days after plating. Transformed cultures from PEG and electroporation experiments were plated on media supplemented with 60 μg/ml hygromycin, whereas *Agrobacterium*-transformed cultures were plated on the same type of media but with 80 μg/ml kanamycin

for 7–10 days. This delay was essential to an increase in plating efficiency, which otherwise would have been drastically reduced. A larger number of proliferating colonies were obtained from transformation experiments when intact cells instead of protoplasts were used (Table 5).

Discussion

The aim of this study was to develop a system for the enhancement of cell division and cellus formation from protoplasts isolated from slowly growing embryogenic wheat callus. Because of the slow-growing nature of the embryogenic callus, regenerants of protoplasts isolated from these tissues might have a good chance of being fertile. Suspension cells are more likely to have chromosomal instabilities. (Vasil et al. 1990, 1991).

The plasmid DNA delivery method is critical in determining the level of gene expression. This was clear from the amount of GUS activity obtained in protoplasts transformed by *Agrobacterium* harboring the binary vector (pKIWI 105). Far less activity was obtained when the same binary vector was isolated and delivered into protoplasts and intact cells by either PEG or electroporation. These results show that *Agrobacterium* is more efficient in delivering the plasmid DNA into cells than electroporation and/or PEG. The increase in GUS activity in *Agrobacterium*-mediated transformation might be attributed either to the increase in the number of transformed protoplasts and/or the high gene copy number per protoplast resulting from more than one transformation event. The comparatively low GUS activity in protoplasts transformed with either electroporation or PEG might be because the plasmid DNA is freely and randomly suspended in the transformation mixture. As such, the chances of delivering them into protoplasts and intact cells are low. The importance of the transformation (gene delivery) method is shown by the increased GUS expression in protoplasts transformed with PKIWI 105 contained in *Agrobacterium* than in those transformed by either electroporation and/or PEG containing a stronger construct where the GUS gene was driven by a tandem repeat 35S and a TMV leader sequence. Protoplast viability did not decline significantly with *Agrobacterium* compared with the electroporation or PEG transformation methods. The significant increase in GUS activity in protoplasts transformed with *Agrobacterium* cannot be attributed, however, solely to this factor. This assumption was based on the fact that the increase was in the six- to tenfold range and not the two- to threefold range. Another advantage of *Agrobacterium*- mediated transformation over the other DNA delivery methods used in this report is the ability to obtain a significant increase in GUS gene expression in a short time (12 h). A longer time (24–48 h) is required for GUS expression after transformation by electroporation or PEG. In fact, transient GUS activity has been detected in protoplasts co-cultivated with *Agrobacterium* for 5 min (O.M.-F. Zaghmout, unpublished).

The purity of the plasmid DNA has been shown to be critical in transient and stable transformation studies (Potrykus et al. 1985; Potrykus 1991). In most of the reports on transformation, CsCl-purified DNA was used (Potrykus et al. 1985; Potrykus 1991). The CsCl method requires the availability of an ultra-centrifuge, is time-consuming, entails the use of hazardous materials (e.g., ethidium bromide), and is expensive. No significant difference in GUS activity was observed in cells transformed with plasmids isolated by CsCl and those by minipreparation methods. The question arises whether DNA used in gene expression studies have to be extremely clean (e.g., purification with CsCl). It is likely that many of the contaminants present in plasmid DNA isolated by minipreparation methods do not affect transient gene expression in wheat protoplasts and intact cells. This elimination of the CsCl step clearly would reduce the time and cost of isolating the large batches of plasmid required by transformation methods other than *Agrobacterium*.

This report has shown for the first time that sustained cell division can be obtained in protoplasts isolated from slowly growing wheat embryogenic callus and clearly opens the possibility of using these cells in transformation studies. Furthermore, the data showed that intact cells of these calli can be efficiently transformed by electroporation. More importantly, *Agrobacterium* has been introduced as a possible vector for transformation in wheat. More work on *Agrobacterium*-mediated transformation in cereals is needed prior to its full utilization. Efforts to regenerate these cultures are underway; it is yet to be seen if fertile, transgenic plants can be regenerated from these cultures.

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