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Factors affecting transient gene expression in protoplasts isolated from very slowly growing embryogenic callus cultures of wheat (*Triticum aestivum* L.)

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Abstract. Protoplasts isolated from embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') calli of wheat (*Triticum aestivum* L.) genotypes transiently expressed β-glucuronidase (GUS) activity when electroporated with a plasmid containing the GUS gene and driven by an enhanced 35S promoter and a TMV leader sequence. Conditions for the maximum expression of GUS activity were: electroporation of the freshly isolated protoplasts at 250 Vcm^{-1} and 250 μF for 2 s using 50 μg/ml of plasmid DNA; incubation of the protoplasts with the plasmid before the pulse for 2 h; and a 15-min recovery period on ice after the pulse. In general, a higher GUS activity was obtained in protoplasts of non-embryogenic (NE) callus origin than in those of embryogenic (E) callus origin. Only GUS constructs containing a duplicate 35S promoter derivative resulted in a significant level of GUS expression. The presence of the TMV viral leader sequence in the pAGUS1-TN2 plasmid construct resulted in a significant increase of GUS activity in the electroporated protoplasts of both callus types. On the other hand, protoplasts electroporated with the *Adh1* promoter and intron showed a threefold less GUS activity than those electroporated with pAGUS1-TN2. Optimized conditions for DNA uptake and expression were very similar for protoplasts of both callus types. The importance of these findings for the successful regeneration of transgenic and fertile wheat plants is discussed.

Key words: Wheat – Protoplast – Electroporation – GUS activity – Transient expression

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Introduction

The application of transformation techniques to crop improvement depends on the ability to regenerate fertile plants from transformed tissues. In cereals, embryogenic suspension culture has been widely used in transformation studies (Harris et al. 1988; Rhodes et al. 1988; Potrykus 1989; Shimamoto et al. 1989; Datta et al. 1990; Gordon-Kamm et al. 1990; Oard et al. 1990; Vasil et al. 1991). The use of fast-growing embryogenic suspension cultures offers many advantages, such as rapidity of growth, ease of protoplast isolation, and high rate of plating efficiency (Vasil et al. 1990). On the other hand, the establishment of a fast-growing embryogenic suspension cell culture is a tedious, lengthy, and time-consuming process and, in addition, most of the plants regenerated from these cultures are sterile (Vasil et al. 1990). While embryogenic suspension cultures lose their fertility competence at a much faster rate than callus tissues (Potrykus 1989; Vasil et al. 1990), callus tissues are not as responsive to cultural and electroporation conditions and are more resistant to protoplast release. The use of callus tissue in transformation studies requires a reliable system for the introduction of exogenous plasmid DNA and for the regeneration of fertile plants. Gene transfer methods used for cereals are limited to electroporation (Dekeyser et al. 1990; Hauptman et al. 1988), microprojectile (Fromm et al. 1985; Klein et al. 1987; Wang et al. 1988; Klein et al. 1989; Lonsdale et al. 1990; Chibbar et al. 1991; Vasil et al. 1991), polyethylene glycol (PEG) (Potrykus et al. 1985; Shillito et al. 1985; Oard et al. 1990), and in a few instances *Agrobacterium tumefaciens* (Potrykus 1989; Rainieri et al. 1990). Microprojectile (particle bombardment) has been used to genetically transform corn (Gordon-Kamm et al.

1990) and wheat (Vasil et al. 1991), but this technique is not as efficient as originally thought. This inefficiency can be attributed to the inability to efficiently bombard all of the competent cells in the culture to ensure a high rate of integration and plant regeneration. Although this problem can be avoided by mediating DNA uptake into protoplasts with either PEG or electroporation, the development of an efficient system for protoplast isolation, culture, and plant regeneration is necessary. In addiation, protoplast viability and mitotic capacity will be greatly reduced as a result of either PEG or high voltage exposure (electroporation) (Potrykus 1989). Consequently, efficient procedures for the regeneration of transgenic fertile plants have been extremely difficult to develop.

Vasil et al. (1992) have recently reported the regeneration of transgenic fertile wheat plants. Unfortunately, the frequency of stably transformed fertile plants was very low. Prior to that report, there was no mention of transgenic fertile plants having been regenerated from transformed tissues in wheat. To circumvent many of the problems encountered in wheat transformation, our research was initiated with the following objectives: (1) to optimize the conditions for the isolation of large numbers of viable protoplasts from slow-growing, compact embryogenic callus with complete sets of chromosomes; (2) to determine if transient GUS activity can be detected in protoplasts originating from very slow-growing embryogenic callus tissues; (3) to develop and optimize the conditions for electropermeability and expression to the introduced foreign plasmid DNA in the protoplasts of an embryogenic (E cell type) and non-embryogenic (NE cell type) callus tissue origin; (4) to determine the rate of transient GUS activity as influenced by the promoter and the embryogenic nature of the callus tissue (E versus NE); (5) to determine if higher rates of exogenous DNA uptake and expression are enhanced if electroporation is used in combination with low concentrations of PEG; and (6) to compare the rate of transient gene activity in protoplasts isolated from calli of wheat (monocot) and grape (dicot) electroporated with pGUS1-TN2 [enhanced 35S promoter (Odell et al. 1985) + TMV leader sequence (Skuzeki et al. 1990)]. In this report, we demonstrate the utility of electroporation as an efficient method to transform protoplasts from a very slowly growing embryogenic callus tissue with plasmid DNA. The standardized conditions developed in this study have enabled us to use callus as a protoplast source in stable wheat transformation studies.

Materials and methods

Callus induction and maintenance

The procedures followed for callus induction from immature embryos of cv 'Chinese Spring' are those of Searl and Deckard (1982). Mature embryos of other wheat genotypes, 'Mit' and 'Mustang' (Kindly provided by E. Mellonlly and J. Burk, respectively), were placed on modified Murashige and Skoog (MS) basal salt medium (Murashige and Skoog 1962), which was supplemented with 0.45 μM 2,4-D and 1.14 mM asparagine. Calli of all genotypes were subcultured on fresh medium of the same components mentioned above every 4–6 weeks. Callus cultures were grown in the dark at 24°–28 °C.

Protoplast isolation

Approximately 4 weeks prior to isolation, calli of all the genotypes used were subcultured on modified MS medium supplemented with 9.0 μM 2,4-D to facilitate protoplast isolation. For protoplast isolation, calli were gently teased into small sections and mixed with an enzyme mixture consisting of 1.25% (w/v) cellulysin (Calbiochem, Calif.), 1.25% (w/v) driselase (Kyowa Hakko, Japan), 0.025% (w/v) pectolyase Y-23 (Sigma, Mo.), cellulase types VII (Sigma, Mo.), 0.318 M sucrose, 0.109 M mannitol, 0.266 M glycine, 120 mM sodium thiosulfate, and 3 mM MES [2-(N-morpholine)-ethanesulfonic acid]. The ratio of calli to the enzyme solution was 1 g callus fresh weight to 5 ml of isolation enzyme mixture. The mixture was incubated on a gyratory shaker at 24°–28 °C for 8–10 h with very slow agitation. Thereafter, the protoplast mixtures from all genotypes were filtered through a 30-μm metal mesh and collected in 15-ml Falcon tubes that were then centrifuged (3 times, 3 min, 100 g). The supernatant was discarded, and the pellet was resuspended with the protoplast isolation buffer (enzyme isolation mixture minus the enzyme). After the final wash, the pellet was resuspended in protoplast culture medium (medium 1), which consisted of full strength MS basal salts, vitamins and carbohydrates (Kao and Michayluk 1975), 9.0 μM 2,4-D, 0.318 M sucrose, and 0.109 M mannitol (pH 5.7). The viability of the isolated protoplasts was estimated 3 times with florescein diacetate (0.1% final), and the number of viable protoplasts per milliliter was adjusted to 7×10^5 per milliliter with medium 1.

Plasmid DNA contructs

Plasmids containing the β-glucuronidase (GUS) gene were used in this study. The plasmids pAGUS1-TN2 and pAGUS1 (kindly provided by J. M. Skuzeski, University of Utah) have the structural sequence encoding the GUS gene cloned in the vector pBI221. The GUS gene in both constructs was driven by the cauliflower mosaic virus (CaMV)-enhanced "35S" promoter (Odell et al. 1985; Kay et al. 1987). In addition, the pAGUS1-TN2 plasmid contains the TMV leader sequence, which was made by replacing the 5′ untranslated leader region in the pAGUS1 after digestion with *Bam*HI (Skuzeski et al. 1990). This viral leader region was inserted to direct greater GUS expression. The pNG1 (kindly provided by M. Fromm, Monsanto Colo.) contains GUS controlled by the *Adh1* promoter and intron (Fromm et al. 1985). This construct contains scorable (GUS gene driven by the *Adh1* promoter and intron) and screenable (neomycine phosphotransferase II gene driven by 35S) markers.

Electroporation

The insertion of plasmid DNA into target protoplasts was by a Bio-Rad pulsar apparatus that produces controlled exponential

pulses for protoplast electroporation. The electrical field strength parameters (voltage, pulse amplitude, and duration) can be independently adjusted. The cuvette electrodes (0.4 cm apart) were placed in the culture medium containing protoplasts. After electroporation the sample was placed on ice for 15 min, followed by 15 min at room temperature before the addition of 200 µl of medium 1. The contents were then poured into Falcon plastic plates (35 × 10 mm) that were sealed with parafilm and incubated in the dark for 24 h. Plasmid DNA from all of the contructs used were isolated and purified with cesium chloride (Maniatus et al. 1982).

Measurement of GUS enzymatic transient activity

GUS activity measured essentially as described by Jefferson et al. (1987) unless otherwise noted. Protoplasts were collected for assay after 24 h of incubation by centrifugation at 2200 *g* for 5 min. Pellets from each plate were resuspended in 1 ml GUS extraction buffer (50 m*M* $NaPO_4$, pH 7.0; 10 m*M* β-mercaptoethanol; 10 m*M* Na_2 EDTA; 0.1% sodium laurly sarcosinate; 0.1% Triton X-100). The mixture was then disrupted by vortexing. To clarify the supernatant from debris, tubes were centrifuged for 10 min at 12 000 *g*. For the assay, 50 µl of sample supernatant was mixed with 150 µl of the GUS assay buffer (GUS extraction buffer with 2 m*M* 4-methylumbelliferyl β-D-glucuronide (MUG), mixed, and incubated at 37 °C for 2 h. The reaction was stopped by the addition of 1 ml 0.2 *M* sodium carbonate. The fluorescence emission was measured with a TKO 100 fluorometer (Hoefer Scientific Instruments), with an excitation wavelength of 365 nm and an emission filter of 460 nm. Data were extrapolated from a standard curve generated from a dilution series of 4-methylumbelliferone (MU). The GUS enzyme activity is reported in picomole (pmole) of MU/min per 10^5 viable protoplasts.

Data analysis

Three types of controls were used. The first consisted of protoplasts (not electroporated) without plasmid DNA; the second, electroporated protoplasts without plasmid DNA; and the third, protoplasts not electroporated with plasmid DNA. Their means were calculated and subtracted from the means of the treated samples. The reported data are the means of three independent experiments with ten replicates. Duncan's multiple range test was used to measure the level of significance among treatments. The percentage of protoplasts showing GUS activity was not collected due to the endogenous GUS activity present in the non-transformed wheat protoplasts. In many instances, it was hard to distinguish between the non-transformed and transformed protoplasts unless the activity in the latter was very high.

Results

Effect of protoplast source on transient GUS activity

The most critical factor affecting transient GUS activity appeared to be the protoplast source. Transient GUS activity in wheat calli of the three genotypes electroporated at 250 Vcm^{-1}, 250 µF, one pulse (2 s) in the presence of three GUS-constructs driven by a variety of promoters is shown in Table 1. In general, protoplasts of both NE or E cell types electroporated with pNG1 showed a greater decrease in GUS activity than those electroporated with either pAGUS1-TN2 and pAGUS1. Transient GUS activity was higher in protoplasts isolated from E callus of 'Mustang' than in those of 'Chinese Spring'.

Effect of the promoter on transient GUS activity

In transformation studies, the strength of the promoter is very critical in determining the rate of expression of the introduced gene. Therefore, protoplasts isolated

Table 1. Relative transient GUS activity in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Construct	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	pAGUS1-TN2	4.9 (87%)[a]	4.1 (85%)	44.4a[b]
	pAGUS-1	5.0 (90%)	4.4 (88%)	30.9b
	pNG1	5.3 (89%)	4.3 (89%)	9.5c
Mustang				
	pAGUS1-TN2	5.2 (86%)	4.1 (88%)	37.4a
	pAGUS-1	4.3 (92%)	4.1 (89%)	25.7a
	pNG1	5.2 (89%)	4.2 (90%)	8.9b
Chinese Spring				
	pAGUS1-TN2	5.2 (92%)	4.2 (89%)	23.8a
	pAGUS-1	4.9 (95%)	4.0 (89%)	15.8b
	pNG1	5.3 (89%)	3.8 (89%)	10.1c

[a] Numbers in parentheses represent the percentage of viable protoplasts

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

Table 2. The effect of voltage ($V\,cm^{-1}$) on transient GUS activity in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. The electroporation conditions were as described in the Materials and methods. Protoplast density prior to electoporation 7×10^5 per milliliter

Genotype	Voltage ($V\,cm^{-1}$)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	250	4.9 (90%)[a]	3.4 (85%)	30.2a[b]
	500	3.4 (89%)	1.7 (61%)	8.7b
	750	1.5 (32%)	0.2 (0.1%)	1.4c
Mustang				
	250	5.0 (88%)	3.4 (90%)	28.3a
	500	1.8 (86%)	0.8 (33%)	4.7b
	750	0.7 (77%)	0.01 (0.0%)	0.0b
Chinese Spring				
	250	4.2 (89%)	13.1 (89%)	23.5a
	500	3.4 (84%)	1.1 (21%)	9.0b
	750	2.0 (73%)	0.0 (0.0%)	0.0c

[a] Numbers in parentheses represent the percentage of viable protoplasts
[b] Means within a column of each genotype not sharing the same letter are significantly different at the 0.05 probability level according to Duncan's multiple range test

Table 3. The effect of capacitance (μF) on transient GUS activity in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Capacitance (μF)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	250	5.5 (92%)[a]	4.1 (89%)	24.8a[b]
	500	4.5 (92%)	1.2 (89%)	5.6b
	700	3.3 (84%)	1.0 (61%)	4.6b
Mustang				
	250	4.7 (92%)	3.5 (91%)	33.4a
	500	4.4 (87%)	3.9 (83%)	27.7a
	750	3.6 (79%)	2.1 (51%)	11.3b
Chinese Spring				
	250	4.2 (92%)	3.5 (86%)	26.2a
	500	4.2 (91%)	3.2 (79%)	31.2a
	750	3.3 (68%)	0.9 (79%)	16.3b

[a] Numbers in parentheses represent the percentage of viable protoplasts
[b] Means within a column of each genotype not sharing the same letter are significantly different at the 0.05 probability level according to Duncan's multiple range test

from calli of the three wheat genotypes were electroporated with plasmids containing the GUS gene driven by a variety of promoters (Table 1). These constructs were pAGUS1-TN2 (enhanced 35S + TMV leader sequence), pAGUS-1 (enhanced 35S), and pNG1 (*Adh1* promoter + intron). The presence of the TMV viral leader sequence in pAGUS1-TN2 resulted in a twofold increase in transient GUS activity in protoplasts of both 'Mit' and 'Mustang' calli tissue. As a result of the significant increase shown from electroporation with pAGUS1-TN2, this construct was in subsequent experiments.

Effects of electrical field strength on protoplast viability and transient GUS activity

In an effort to maximize the expression of transient GUS activity in protoplasts of E and NE callus cell types, electroporation was done with pAGUS1-TN2 under a wide range of electrical voltage, pulse ampli-

tudes, and durations. A higher rate of protoplast mortality was observed as electrical voltage increased above 250 V cm^{-1} (Table 2).

The effect of pulse amplitude and duration on transient GUS activity was also assessed (Table 3 and 4). Maximum activity was obtained by increasing the capacitance and duration to 250 μF and 9 s, respectively. When the pulse length and the capacitance values were increased still further, GUS activity declined drastically. This decline is due to a decrease in protoplast viability. To attain a maximum rate of GUS activity and sufficient numbers of viable protoplast, we used conditions of 250 V cm^{-1}, 250 μF, and 2 s in subsequent electroporation studies.

Effects of incubation of protoplasts with plasmid before the pulse and recovery period on ice after the pulse

Preincubation methods that would facilitate plasmid DNA adsorption to the protoplast membrane and entry into the nucleus were investigated. Data in Table 5 indicate that the preincubation of protoplasts from both NE callus of 'Mit' and E callus of 'Mustang' with the pAGUS1-TN2 plasmid DNA for 2 to 3 h significantly

Table 4. The effect of pulse duration on transient GUS activity in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Pulse duration (s)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	2	4.5 (89%)[a]	4.1 (87%)	40.5a[b]
	4	2.5 (87%)	1.9 (39%)	3.4b
	6	1.3 (86%)	0.75 (0.0%)	0.0b
Mustang				
	2	4.3 (90%)	3.9 (87%)	40.4a
	4	1.9 (90%)	0.8 (75%)	3.0b
	6	1.4 (89%)	0.3 (42%)	0.1b
Chinese Spring				
	2	5.1 (89%)	4.4 (86%)	25.1a
	4	2.0 (89%)	0.5 (24%)	2.1b
	6	1.0 (89%)	0.1 (0.6%)	0.0b

[a] Numbers in parentheses represent the percentage of viable protoplasts

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

Table 5. The relationship between transient GUS activity and the incubation time with the plasmid prior to pulse delivery in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Incubation time (h)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	1	5.3 (89%)[a]	4.1 (83%)	23.7b[b]
	2	4.9 (87%)	4.6 (88%)	44.3a
	3	5.6 (89%)	4.3 (84%)	33.2a
Mustang				
	1	4.8 (86%)	4.2 (83%)	21.3b
	2	5.0 (86%)	4.3 (87%)	37.7a
	3	5.4 (87%)	4.7 (83%)	36.5a
Chinese Spring				
	1	5.5 (90%)	4.6 (85%)	26.6a
	2	5.4 (87%)	4.7 (83%)	31.7a
	3	5.5 (84%)	4.6 (87%)	21.9b

[a] Numbers in parentheses represent the percentage of viable protoplasts

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

Table 6. The relationship between transient GUS activity and ice incubation period following the pulse delivery in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Incubation period (min)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	15	5.3 (89%)[a]	4.9 (89%)	39.5a[b]
	30	5.5 (87%)	5.1 (79%)	38.8a
	60	5.3 (89%)	4.8 (83%)	33.7a
Mustang				
	15	4.5 (91%)	4.2 (86%)	27.3a
	30	4.6 (90%)	4.3 (82%)	31.6a
	60	4.5 (91%)	4.1 (82%)	23.2a
Chinese Spring				
	15	5.2 (91%)	5.1 (83%)	20.4a
	30	5.1 (85%)	4.7 (84%)	24.8a
	60	5.3 (91%)	4.6 (89%)	25.2a

[a] Numbers in parentheses represent the percentage of viable protoplasts
[b] All means within a column of each genotype did not differ significantly at the 0.05 probability level according to Duncan's multiple range test

Table 7. The relationship between transient GUS activity and the concentration of the PAGUS1-TN2 in electroporation protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Plasmid concentration (μg/ml)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	20	5.3 (92%)[a]	3.6 (91%)	3.4d[b]
	40	5.0 (85%)	4.5 (87%)	13.2c
	50	5.3 (90%)	5.0 (87%)	21.0b
	60	5.4 (91%)	4.9 (86%)	29.0a
	80	5.2 (93%)	4.6 (87%)	30.3a
	100	5.2 (90%)	4.2 (81%)	33.0a
Mustang				
	20	4.9 (89%)	4.4 (87%)	11.9d
	40	5.0 (89%)	4.4 (89%)	23.2c
	50	5.2 (91%)	4.5 (85%)	37.3b
	60	4.9 (88%)	4.4 (83%)	36.9b
	80	4.3 (87%)	4.1 (84%)	39.8b
	100	5.2 (89%)	4.5 (84%)	48.3a
Chinese Spring				
	20	5.5 (88%)	4.9 (87%)	4.7c
	40	4.9 (86%)	4.1 (84%)	19.7b
	50	5.1 (88%)	4.3 (87%)	21.6b
	60	4.9 (92%)	4.1 (87%)	26.2b
	80	5.2 (90%)	4.6 (87%)	27.5b
	100	5.4 (86%)	3.6 (86%)	36.8a

[a] Numbers in parentheses represent the percentage of viable protoplasts
[b] Means within a column of each genotype not sharing the same letter are significantly different at the 0.05 probability level according to Duncan's multiple range test

increased the GUS activity rate at least twofold. In 'Chinese Spring', 1–2 h of preincubation resulted in a higher of GUS activity than a 3-h preincubation period. Similar results have been reported with other plant species where it was suggested that plasmid DNAs bind to the protoplasts prior to entry (Fromm et al. 1985).

The ice recovery period after electroporation (0–60 min) resulted in approximately a twofold increase in GUS activity with no significant difference among protoplasts incubated for 15, 30, or 60 min (Table 6). Therefore, a 15-min recovery time on ice was chosen for further studies.

Transient GUS activity varied with DNA concentration

Varying the amount of the plasmid DNA affected the rate of GUS activity (Table 7) and increasing the concentration elevated transient GUS activity. However, protoplasts electroporated with 60–100 μg/ml plasmid DNA did not differ in their expression of GUS activities from those electroporated with 50 μg/ml plasmid DNA (Table 7). This lack of increased GUS activity in protoplasts electroporated with more than 50 μg/ml plasmid DNA might have resulted from limited numbers and sizes of the pores generated: either the numbers and sizes of the pores were not sufficient or the pores were healed so quickly that no more DNA was allowed to pass through. To test the latter possibility, electroporated protoplasts were placed on ice in order to keep the pores that were generated open for a longer time. This treatment did not result in a significant increase in GUS expression if 100 μg/ml as opposed to 50 μg/ml was electroprated. Hence, 50 μg/l was used in subsequent transformation studies for the assessment of other constructs containing a variety of promoters.

Effect of PEG on transient GUS enzymatic activity

The effect of PEG on transient GUS activity in protoplasts of the three genotypes is shown in Table 8. Protoplasts were electroporated with pAGUS1-TN2 (50 μg/ml) in medium containing either 0.0 (control), 5, 10, or 15% (w/v) PEG 6000. PEG at all concentrations increased the level of transient GUS activity over that of the control for all three genotypes (Table 8). In protoplasts of both cell types, the addition of 10% PEG resulted in the largest increase in GUS activity. Further increases in PEG concentration resulted in a reduction in both protoplast viability and GUS activity (Table 8).

Comparison of GUS activity in protoplasts of wheat (monocot) and grape (dicot) as measured by transient GUS activity

In an earlier experiment in which protoplasts from soybean NE callus type were electroporated with 50 μg/ml pAGUS1-TN2 plasmid, the level of transient GUS activity was at least 100- to 150-fold greater than that of the control (data not shown). Our wheat protoplasts that were electroporated with plasmid under the same conditions had a much lower GUS activity than those of soybean. This difference is unlikely to have been caused by electroporation efficiency (DNA uptake). In most cases and within the same experiment, this variation did not exceed 50% of the mean. Therefore, it might be possible that either the 35S promoter was more active in GUS in soybean protoplasts than in wheat callus protoplasts or that the presence of RNA polymerase II inhibitors in wheat protoplasts hindered the essential binding step for the transcription to proceed. To test the validity of the assumption that protoplasts of a dicot source are more active in processing

Table 8. Effect of PEG on transient GUS activity in electroporated protoplasts of embryogenic ('Mustang' and 'Chinese Spring') and non-embryogenic ('Mit') wheat calli. Electroporation conditions were as described in the Materials and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	PEG concentration (%)	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Mit				
	0	5.3 (92%)[a]	4.8 (89%)	30.1c[b]
	5	4.1 (86%)	3.7 (85%)	76.4ab
	10	2.3 (83%)	2.0 (87%)	87.3a
	15	2.4 (83%)	2.2 (78%)	66.2b
Mustang				
	0	5.3 (90%)	4.8 (84%)	31.2c
	5	4.5 (90%)	4.1 (87%)	53.4a
	10	3.4 (87%)	2.6 (78%)	60.0a
	15	3.4 (84%)	2.7 (78%)	60.0a
Chinese Spring				
	0	5.2 (90%)	4.2 (84%)	29.5b
	5	4.5 (90%)	3.7 (88%)	45.2a
	10	3.4 (86%)	2.5 (84%)	38.42a
	15	2.3 (79%)	1.7 (53%)	27.0b

[a] Numbers in parentheses represent the percentage of viable protoplasts

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

Table 9. Transient GUS activity in protoplasts of wheat and grape electroporated at 250 V cm^{-1}, 150 μF and one pluse (2 s) in the presence of PAGUSTN2 plasmid (50 μg/ml). Other conditions were as described in the Material and methods. Protoplast density prior to electroporation was 7×10^5 per milliliter

Genotype	Protoplasts after electroporation ($\times 10^5$)	Protoplasts prior to assay ($\times 10^5$)	Total GUS activity in protoplasts (pmole MU/min)
Wheat (cv Mustang)	5.3 (92%)[a]	4.9 (87%)	30.6b[b]
Grape (cv WWG)	5.7 (85%)	5.1 (82%)	111.2a

[a] Numbers in parentheses represent the percentage of viable protoplasts
[b] Means of the two genotypes were significantly different at the 0.05 probability level

gene products than those of a monocot when electroporated with similar 35S-promoter-GUS gene contructs, protoplasts of wheat (monocot) and grape (dicot) were electroporated in the presence of pAGUS1-TN2. Previously optimized electroporation and culture conditions were used. The promoter 35S was more active in driving GUS expression in grape protoplasts that in wheat protoplasts (Table 9). This was evident from the approximately three-fold increase in transient GUS activity in grape relative to that found in wheat protoplasts (Table 9).

Discussion

The results presented here show that protoplasts of E and NE cell types can be efficiently transformed by electroporation. The method we used for protoplast isolation from callus tissue is rapid, reliable and yields enough protoplasts for studies involving transient transformation in wheat. The optimization of crucial parameters such as selection of promoter type, plasmid concentration, electroporation conditions, and PEG concentration may explain our success in transiently transforming protoplasts of the very slow-growing embryogenic calli of either 'Mustang' or 'Chinese Spring' (Tables 1–8). The optimized conditions of GUS expression were very similar in protoplasts of the E and NE callus type. In future transient expression studies, this finding will have practical value in reducing the subculture cycle to a minimum for the E callus type. The relationship between number of subcultures (age of culture) and fertility of the regenerant is well documented in cereals (Haris et al. 1988; Potrykus 1989). Wheat cultures have been reported to lose their ability to produce fertile plants as early as 1–2 years following initiation (Vasil et al. 1990, 1991). In general, there is a better chance of regenerating fertile plants from cultures that have been subcultured only for a short time. Therefore, this practice will improve our chances of regenerating fertile and transgenic plants.

In this study the suitability of a specific promoter type construct to provide a reliable and efficient transient GUS activity in the target protoplast was tested. Undoubtedly, the use of a suitable construct will result in an increase in the efficiency of selecting transformants and in a reduction in the number of the escapees. This is more important in wheat where untransformed cells have been reported to contain high levels of endogenous resistance to selectable markers like kanamycin (Dekeyser et al. 1090; Hauptman et al. 1988) that result in time wasted on screening the untransformed cells.

In general, higher levels of GUS transient activity resulted from electroporation with either pAGUS1-TN2 (enhanced 35S + TMV leader sequence) or pAGUS-1 (enhanced 35S) than with pNG1 (*Adh1* promoter + intron) (Table 1). In dicot transformation studies, the 35S promoter has been shown to be very effective in driving the expression of a variety of genes (Last et al. 1991). Last et al. (1991) have shown that genes in constructs driven by the 35S promoter were not as efficiently expressed in monocots and particularly in cereals as in dicot tissues. The data from our investigation provided another line of evidence in this regard. The *Adh1* promoter and intron have been reported to be very effective in inducing gene activity in cereal tissues (Callis et al. 1987; Kyozuka et al. 1990). However, our data shows that protoplasts electroporated with the construct containing the *Adh1* promoter and intron (pNG1) had a much lower GUS activity than those in which the GUS gene was driven by enhanced 35S promoter (Table 1). The poor performance and greatly decreased activity of the *Adh1* promoter and intron relative to the 35S promoter in protoplasts of wheat calli might be attributable to several factors: (1) limited amounts of *trans*-acting transcription factors essential for the newly introduced promoter to initiate maximum levels of expression, which might result from the interaction between the intragenic sequence and the reporter gene driven by these promoters or to the recognition of the promoter and (2) the intron sequences in the recipient tissues.

Although PEG is not essential for the generation of pores and plasmid DNA uptake in wheat protoplast electroporation, it significantly increases the rate of

GUS transient activity (Table 8). PEG has been used in transient and transformation studies (Potrykus et al. 1985; Potrykus 1991). In rice (Hayashimoto et al. 1990), the optimal concentration for a maximum rate of transformation was found to be 30–40% (w/v). As this concentration is likely to increase the rate of protoplast fusion, genetic abnormality, and protoplast mortality, plant regeneration ability may be greatly affected. On the other hand, at a PEG concentration of 5–10% the number of fusants and transformants will be reduced dramatically (Table 8). The enhancement effect of PEG might have been caused by either an increase in the contact between the added plasmid DNA and the clustered protoplasts or the result of an alteration in the integrity of the plasma membrane that allowed the added plasmid DNA to reach the nucleus more efficiently and less damaged. Moreover, in the presence of PEG, the diameter to the protoplast is reduced and, consequently, the introduced plasmid DNA has a smaller distance to travel to the nucleus. Therefore, the effect of the cytoplasmic nucleases will be reduced. An additional advantage to obtaining a significant increase in GUS expression in the presence of PEG is that less plasmid DNA is needed for the transient expression optimization studies.

In conclusion, conditions for obtaining a singnificant increase in GUS transient gene expression in electroporated protoplasts of wheat calli have been developed. These appear to be greatly dependent on the protoplast source, type of promoter construct, PEG concentration, and plant species. The data indicate that a significant level of GUS transient activity can be expressed in protoplasts of the very slow-growing embryogenic wheat calli. Our experiments were directed toward increasing the frequency of stable transformation in very slow-growing embryogenic callus. However, it remains to be seen if the embryogenic calli resulting from these protoplast transformation studies will result in transgenic fertile wheat plants.

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References

Callis J, Fromm M, Walbot V (1987) Introns increase gene expression in cultured maize cells. Genes Dev 1:1183–1200

Chibbar RN, Karatha KK, Leung NJ, Qureshi J, Caswell K (1991) Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum*) through microprojectile bombardment. Genome 34:453–460

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile indica rice recovered from protoplasts. Bio/Technology 8:736–740

Dekeyser RA, Claes B, De Rycke RMU, Habets ME, Van Montagu MC, Caplan AB (1990) Transient gene expression in intact and organized rice tissues. Plant Cell 2:591–602

Fromm M, Taylor LP, Walbot V (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82:5824–5828

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willets NG, Rice TB, Mackey CJ, Kruger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Harris R, Wright M, Byrne M, Varnum J, Brightwell B, Schubert K (1989) Callus formation and plant regeneration from protoplasts derived form suspension cultures of wheat (*Triticum aestivum* L.). Plant Cell Rep 7:337–340

Hauptman RM, Vasil V, Ozias-Akins P, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB, Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol 86:602–606

Hayashimoto A, Li Z, Murai N (1990) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol 93:857–863

Jefferson RA, Kavanage TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and verstile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Plants 126:105–110

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of the CaMV 35S promotor sequence creates a strong enhancer for plants. Science 236:1299–1302

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Klein TM, Kornstein L, Stanford JC, Fromm ME (1989) Genetic transformation of maize cells by particle bombardment. Plant Physiol 91:440–444

Kyozuka J, Izawa T, Nakajima M, Shimamoto K (1990) Effect of the promoter and the first intron of maize *adh1* on the foreign gene expression in rice. Maydica 35:353–357

Last DI, Brettell RIS, Chamberlain DA, Chauhury AM, Larkin PJ, Marsh EL, Peacock WJ, Dennis ES (1991) pEmu: an improved promoter for gene expression in cereal cells. Theor Appl Genet 81:581–588

Lonsdale D, Onde S, Cumming A (1990) Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. J Exp Bot 41:1161–1165

Maniatus T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plant 15:473–493

Oard JH, Paige DF, Simmonds JA, Gradziel TM (1990) Transient gene expression in maize, rice and wheat cells using airgun apparatus. Plant Physiol 92:334–339

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812

Potrykus I (1989) Gene transfer to cereals: an assessment. Trends Biotechnol 7:269–273

Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. Annu Rev Plant Physiol 42:205–225

Potrykus I, Saul MW, Petruska J, Paszkoski J, Shillito RD (1985) Direct gene transfer to cells of a graminaceous monocot. Mol Gen Genet 199:183–188

Rainieri DM, Bottino P, Gordon MP, Nester EW (1990) *Agrobacterium*-mediated transformation of rice *Oryza sativa* L. Bio/Technology 8:33–38

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer J (1988) Genetically transformed maize plants from protoplasts. Science 240:204–207

Sears RG, Deckard EL (1982) Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci 22: 546–550

Shillito RD, Saul MW, Paszkowski J, Muller M, Potrykus I (1985) High efficiency direct gene transfer to plants. Bio/Technology 3:1099–1103

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:736–740

Skuzeski JM, Nicholes LM, Gestland RF (1990) Analysis of leaky viral translation termination codons in vitro by transient expression of improved β-glucuronidase. Plant Mol Biol 15:65–79

Vasil V, Redway F, Vasil IK (1990) Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.) Bio/Technology 8:429–433

Vasil V, Brown SM, Re D, Fromm ME, Vasil IK (1991) Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio/Technology 9: 743–747

Vasil V, Casillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10:667–674

Wang YC, Klein TM, Fromm ME, Cao J, Stanford JC, Wu R (1988) Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Mol Biol 11:433–439