Factors influencing transient gene expression in electroporated

tall fescue (*Festuca arundinacea* Schreb.) protoplasts

by

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(ABSTRACT)

For the rapid establishment of optimal conditions for a genetic transformation system for tall fescue, several factors influencing transient gene expression were studied in protoplasts, after the reporter β-glucuronidase (GUS) gene was introduced by electroporation. In a time-course study of transient gene expression, GUS activity peaked at 24 h after electroporation. Among the different field strength conditions tested, maximum GUS activity was observed at 750 V/cm. Increases in the amount of plasmid DNA to 80 μg/ml led to increased GUS activity. GUS activities increased in linear fashion with increasing protoplast densities up to 2 x 10^6/ml. Age of suspension cells from which protoplasts were derived influenced transient expression with maximum GUS activity obtained in 3 and 5 day old suspensions. These results show that transient expression studies can be used to optimize electroporation parameters rapidly. Results of such rapid assays can be used as a basis for further studies on stable transformation of this important turf-grass species.
Dedicated to my parents:

P. V. Satyanarayana Raju and P. Janaki
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INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.) is the predominant cool season grass species that is grown on about 35 million acres in the United States (Barnes, 1990). Forage-type and turf-type of tall fescue are extensively cultivated on pastures, parks, lawns, highway medians, athletic stadia and golf course fairways. Such prominence in the uses of tall fescue has meant a continuous demand for new improved cultivars.

The genetic improvement of tall fescue has, so far, been possible only through conventional plant breeding and selection procedures. In general, these procedures involve identification of plants that are superior for characteristics such as yield, disease resistance, geographic adaptation and composition of seeds or leaves. Superior plants are then interbred to concentrate the desirable genes in a single plant or population. In cross pollinated species that are self-fertile, repeated selfing of plants having desirable characteristics can be undertaken to generate a series of inbred lines each containing a superior combination of desirable genes. These inbred lines can then be crossed with one another to obtain hybrids where the sets of desired genes have been concentrated. Tall fescue is a wind-pollinated hexaploid species and exhibits severe sterility on selfing which prevents the exploitation of hybrid vigor. Besides such a limitation, few efforts have been devoted to the genetic study of agronomically important traits in this species. Such limitations have meant, until the
recent advent of biotechnology, that the genetic improvement of this important grass species would proceed at a slow pace.

Biotechnology offers solutions to circumvent many of the constraints that have so far limited the genetic improvement of tall fescue. The extent of variation currently available in tall fescue can be vastly enhanced by the use of biotechnology. Potential biotechnological approaches include mass clonal propagation of elite genotypes, selection at the cell or tissue level, generation of haploid and double haploids to obtain near homozygous genotypes for evaluation of superior gene and allele combinations, somatic hybridization and genetic engineering.

Genetic engineering provides the ability to introduce novel genes (those not present in natural germplasm) directly into tall fescue. Also, once genetic engineering procedures are developed for tall fescue, novel genes from any source or desirable genes isolated from tall fescue germplasm can be rapidly incorporated into agronomically superior lines or cultivars of tall fescue. This could circumvent time-consuming backcrossing procedures required in conventional breeding programs, since genetic engineering allows the introduction of only the specific gene of interest. Thus, genetic engineering can improve the power, speed and precision of crop improvement of tall fescue.

Plant genetic engineering has been successful largely through the exploitation of *Agrobacterium* as a vector for gene delivery (Potrykus, 1991). Unfortunately,
Agrobacterium-mediated transformation has been generally successful for
dicotyledonous plants whereas monocotyledonous crop species to which tall fescue
and other important cereal crops belong prove largely refractile to Agrobacterium-
mediated transformation (Potrykus, 1990). Although recent advances in the
molecular biology of Agrobacterium have improved the potential use of
Agrobacterium to transform monocotyledonous crops such as corn (Grimsley et al.,
1987) and rice (Raineri et al., 1990), it is far from a routinely applicable procedure.
In the absence of a routine Agrobacterium-mediated method for monocots, various
direct gene transfer methods have been developed: introduction of foreign DNA into
protoplasts by electroporation or chemical treatment, bombardment of cultured cells
or tissues with particles coated with foreign DNA, incubation in DNA of seeds or
embryos (Topfer et al., 1989), fusion of DNA-loaded liposomes with protoplasts or
tissues (Gad et al., 1990; Ahokas, 1987), microinjection (Miki et al., 1987; Neuhaus
et al., 1987), macroinjection (De la Pena et al., 1987) and introduction of foreign
DNA via the pollen-tube exposed by the decapitation of floral styles after pollen
germination (Luo and Wu, 1988). Of these, two methods, introduction of foreign
DNA into protoplasts by electroporation or chemical (e.g, polyethylene glycol)
treatment and bombardment of tissues with particles coated with foreign DNA have
proven to be most reliable and reproducible for a range of plant species and have
been successfully used to genetically transform rice (Shimamoto et al., 1989; Christou
et al., 1991), maize (Fromm et al., 1985; Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992) and tall fescue (Wang et al., 1992; Ha et al., 1992).

Although transgenic turf-type tall fescue plants have been recently developed from electroporated protoplasts (Ha et al., 1992), the transformation frequency needs to be further improved. An optimized procedure for the routine and efficient electroporation-mediated transformation of turf-type tall fescue is therefore to be established.

One way of establishing an optimized electroporation procedure for tall fescue is by studying electroporation parameters using stable transformation assays alone. Stable transformation assays involve the detection of the introduced gene in tissues regenerated from electroporated protoplasts. Since stable transformation involves the formation of calli from protoplasts, a process that requires several weeks in tall fescue, use of stable transformation assays alone becomes a time-consuming strategy of optimizing the electroporation protocol for tall fescue. An alternative strategy is to use transient expression assays prior to using stable transformation assays: an approach that would save both time and effort needed to establish a stable transformation procedure. Transient expression assays involve the measurement of gene expression shortly (usually within 2-3 days) after introduction of foreign DNA into protoplasts or target tissue and can therefore be used to optimize electroporation parameters for efficient gene delivery into protoplasts. This would minimize both
time and effort needed to establish an efficient stable transformation protocol for tall fescue. Also, there are evidences in the literature demonstrating the usefulness of transient assays for the rapid optimization of stable transformation conditions. Guerche et al. (1987b) demonstrated that results of transient assays, subject to some constraints, directly reflect optimal conditions for stable transformation. While other workers (Negrutiu et al., 1987; 1990; Larkin et al., 1990) urged caution in directly extending results of transient assays to stable transformation protocols, results of transient assays can serve as a basis for the development of efficient stable transformation procedures.

Many factors influence the efficiency of foreign gene introduction by electroporation (Dhir et al., 1991; Hauptmann et al., 1987; Okada et al., 1986; Bower and Birch, 1990). Such factors include voltage, type of electric pulse, age of suspension cells, density of protoplasts, buffer composition and amount of DNA. The optimal conditions for these factors are highly species-dependent and hence need to be determined for each given species.

As the optimal electroporation conditions were not established for tall fescue, this study was conducted to determine the optimal conditions for several factors influencing transient gene expression by electroporation in tall fescue.
REVIEW OF LITERATURE

1. Direct gene transfer into protoplasts

Direct gene transfer into protoplasts has been effective in obtaining stably transformed cereal crops. Direct uptake of foreign DNA can be effected by either chemical induction or electroporation. Direct gene transfer has allowed efficient integrative transformation (Negrutiu et al., 1987). Since these gene transfer methods accomplish DNA delivery by a physical process, a barrier for gene delivery into protoplasts has not yet been detected (Potrykus, 1991).

The major limitation in the application of direct gene transfer into cereal protoplasts has been the inability to regenerate transformed cereal protoplasts into whole plants. Regeneration of plants from protoplasts depends on parameters such as species and genotype dependence of competence and regeneration which are not under experimental control (Potrykus and Shillito, 1986). Although exciting progress has been made in the area of cereal protoplast regeneration (Vasil, 1987; Roest and Gilissen, 1989), plant regeneration from protoplasts, especially of cereals, is likely to continue to be a delicate process (Potrykus, 1991).

1.1. Chemical-induced DNA uptake

Many chemicals are known to protect and stimulate the uptake of DNA into
protoplasts. Such chemicals are generally either long chain polyacations (poly-L-ornithine, poly-L-lysine, polyethylene glycol (PEG) or charged carbohydrate polymers (e.g., dextran sulphate). These compounds form more neutral complexes upon tight binding with the strongly negatively charged nucleic acids. Such binding reduces the negative charge of the DNA-chemical complex which decreases the forces of repulsion between the complex and the negatively charged plasma membrane. Of the several chemicals known to stimulate the uptake of DNA into protoplasts, PEG has been the most successful, especially when used in the presence of calcium ions (Paszkowski et al., 1984). Use of PEG has been reported to enhance transformation efficiencies of the electroporation method (Shillito et al., 1985). PEG-induced uptake method has been used to obtain transgenic orchard grass (Horn et al., 1988) and tall fescue (Wang et al., 1992).

1.2. Electroporation

Electroporation involves subjecting protoplasts to a brief (in the range of a few hundred microseconds to a few hundred milliseconds) electric pulse. In an electric field, temporary pores are created in the plasma membrane through which DNA enters into the cytoplasm. Electroporation has been used to introduce foreign genes stably into dicots such as tobacco (Riggs and Baoes, 1986), brassica (Guerche et al., 1987a) and several cereal species including rice (Shimamoto et al., 1989), maize
(Fromm et al., 1986) and tall fescue (Ha et al., 1992).

Besides its use for stable transformation of plants, electroporation is also widely used in transient expression assays. Transient gene expression is a result of transcription and translation of introduced genes in host cells. If correct regulatory sequences are present, coding sequences of introduced genes are transcribed and their mRNAs translated into protein products. Since transient assays measure gene expression shortly after gene transfer, they are a rapid and convenient tool to study the properties of promoters or the effect of alterations in the foreign genes. Transient assays have been used for the rapid testing of new gene reconstructions such as deletion analysis of gene promoters (Howard et al., 1987). Transient expression assays have also been very useful for the rapid optimization of transformation conditions (Negrutiu et al., 1987; 1990; Larkin et al., 1990)

1.2.1. Factors influencing transient gene expression

Research in other crops (Dhir et al., 1991; Hauptmann et al., 1987; Okada et al., 1986; Bower and Birch, 1990) indicates that the efficiency of foreign gene introduction, as measured by transient assays, is dependent on several factors. Such factors include voltage, type of electric pulse, age of tissue from which protoplasts are derived, density of protoplasts in electroporation buffer during electroporation, buffer composition and amount of DNA. Such conditions are highly species-dependent and
need to be determined for each species.

1.2.1.1. Time course of gene expression

The kinetics of transient gene expression have been reported for all suspension-derived protoplasts of carrot (Hauptmann et al., 1987), tobacco (Okada et al., 1986), maize (Fromm et al., 1986) and petunia (Ballas et al., 1987). In these studies, reporter gene activities were measured for periods of between 12-132 h, 3-72 h, 3-96 h and 3-60 h, respectively. Peak expression has been observed between 24-36 h for carrot (Hauptmann et al., 1987), 24-48 h for maize (Fromm et al., 1986), 18-36 h for tobacco (Okada et al., 1986) and 15-24 h for petunia (Ballas et al., 1987). Results obtained in these suspension cell protoplast systems are even comparable with results obtained using protoplasts derived from tissues such as tobacco mesophyll (Proks et al., 1988) and soybean cotyledons (Lin et al., 1987; Dhir et al., 1991) where maximal transient expression was observed between 4-24 h for tobacco and 48-72 h for soybean.

1.2.1.2. Influence of field strength

There are two principal types of electroporation devices: high-voltage rectangular wave pulse type and low-voltage exponential decay type. As the name suggests, in high-voltage rectangular wave type electroporators, field strengths
generated are high (2,000-10,000 V/cm) while the duration of each wave is very short (in range of microseconds). In low-voltage exponential decay type machines, electroporation is achieved by using a combination of lower field strength (250-2,000 V/cm) with pulse half-lives (defined as time required for field strength of a pulse to drop to half the initial field strength) in the range of a few to several hundred milliseconds (Larkin et al., 1990). Both types of electroporators have been used to accomplish gene transfer to plants. Results obtained using either type of electroporator are not directly comparable. The optimal field strength in either type of machine varies among species or types of protoplasts.

Field strength has been observed to influence the viability of protoplasts inversely in carrot (Hauptmann et al., 1987; Bower and Birch, 1990), sugar beet (Lindsey and Jones, 1987), tobacco (Guerche et al., 1987b) and soybean (Dhir et al., 1991).

1.2.1.3. Amount of Plasmid DNA

The amount of plasmid DNA used for electroporation affects the level of transient gene expression in carrot (Bower and Birch, 1990), tobacco (Okada et al., 1986; Shillito et al., 1985) and soybean (Dhir et al., 1991). This could be due to either more protoplasts taking up foreign DNA (Dhir et al., 1991) or more copies of plasmids entering protoplasts (Bower and Birch, 1990).
In these systems, transient gene expression was found to increase in response to increases in the amount of plasmid DNA up to a certain level (plateau level). Increasing the amount of plasmid DNA beyond such a plateau level results in little or no increase in transient gene expression. The actual amount of plasmid DNA that is adequate to reach a plateau level differs from one protoplast system to another, varying from 5-10 ug/ml for Nicotiana plumbaginifolia (Negrutiu et al., 1987), 15-20 ug/ml for tobacco (Okada et al., 1986), 30 ug/ml for carrot (Bower and Birch, 1990) to 50 ug/ml for soybean protoplasts (Dhir et al., 1991).

1.2.1.4. Heat shock

Subjecting protoplasts to heat-shock (5 minutes at 45°C) prior to electroporation affects transient gene expression, though the effect varies depending upon different species, genotypes or the gene introduction method employed. Transient gene expression is enhanced by heat shock in carrot (Boston et al., 1987), and soybean (Dhir et al., 1991). In Nicotiana plumbaginifolia, heat-shock treatment has a neutral effect (Negrutiu et al., 1987; Guerche et al., 1987b). The influence of heat-shock on transient gene expression in tobacco is varied, being detrimental in leaf protoplasts of cv. W38 electroporated using a square-wave electroporator (Larkin et al., 1990) and stimulatory in leaf protoplasts of cv. Petit Havana SR1 electroporated using a exponential-wave electroporator (Shillito et al., 1985). Whether this variation
is due to differences in genotype or to differences in electroporation conditions used is unclear.

1.2.1.5. Protoplast density

The density of protoplasts in electroporation buffer during electroporation has been shown to influence transient expression in carrot suspension protoplasts (Lindsey and Jones, 1987). A roughly linear relationship exists between transient expression and protoplast density over densities of $10^4$-$10^6$ protoplasts/ml with a breakdown in linearity at densities over $10^6$ protoplasts/ml. Since protoplast density during electroporation is an easily controlled factor, determining its influence on gene delivery by electroporation is important. Also, determining the optimal protoplast density for electroporation can lead to the minimization of protoplasts that need to be isolated since obtaining large numbers of protoplasts is often a problem.

1.2.1.6. Age of suspension cells

Protoplasts isolated from exponentially dividing cultured cells are best suited for uptake of foreign DNA in sugar beet (Lindsey and Jones, 1987). The age of a suspension culture determines the distribution of protoplasts over different stages of the cell cycle. Different stages of the cell cycle vary in their abilities to take up and express foreign genes. For example, in synchronized tobacco cell suspensions,
transient gene expression is 3-4 fold higher in protoplasts electroporated during the mitotic (M) phase of the cell cycle compared to protoplasts in other phases (Okada et al., 1986). This result suggests that the lack of a nuclear membrane during the M phase of the cell cycle favors the delivery of foreign DNA to the proximity of the nucleus. Gallie et al. (1989) found that upon electroporation, almost all tobacco protoplasts were capable of uptake and expression of RNA and Nishiguchi et al. (1987) could infect 80-90% of tobacco protoplasts with TMV by electroporation. These results suggest that the entry of nucleic acids into the cytoplasm is not the limiting factor. Rather, some other factor(s) such as stage of cell cycle may be critical in determining the efficiency of gene transfer.
MATERIALS AND METHODS

1. Preparation of carrier DNA

Carrier DNA was prepared by digesting calf thymus DNA (Sigma Chemical Co., St. Louis, MO) with restriction enzyme Eco RI to completion. Calf thymus DNA (10mg dissolved in 4.5 ml distilled water by thorough vortexing) was digested with 500 units of restriction enzyme Eco RI in 5 ml of high salt buffer. The digested DNA was filtered through a sterile 0.2 um nylon filter into sterile 1.5 ml eppendorf tubes. The eppendorf tubes were then heated at 65°C for 10 min on a heating block to inactivate the restriction enzyme. An aliquot was removed from the filtered DNA preparation and DNA concentration determined using a spectrophotometer (‘Spectronic 1001’, Milton Roy Co., Rochester, NY).

2. Plasmid DNA

The plasmid pZO-1052 (Ha et al., 1992) was used for all experiments. This plasmid contains a reporter gene, uidA, encoding β-glucuronidase (GUS). The transcription of uidA coding sequence is driven by the cauliflower mosaic virus (CaMV) 35S promoter. The intron 6 of maize alcohol dehydrogenase (adh1) gene is inserted between CaMV 35S promoter and uidA coding sequence to enhance the GUS expression.
3. Preparation of plasmid DNA

Plasmid DNA was isolated from the host strain by alkaline lysis according to Birnboim and Doly (1979) and purified by CsCl density gradient centrifugation in the presence of ethidium bromide. DNA concentration was determined by measuring absorbance at 260 nm on a spectrophotometer.

4. Establishment of rapidly dividing fine cell suspensions

Rapidly growing cell suspensions are a good source for the convenient and rapid isolation of large numbers of protoplasts. The initiation and maintenance of rapidly dividing cell suspensions of tall fescue was carried out according to Dalton (1988) with minor modifications. Seeds of cultivar ‘Silverado’ were surface sterilized by the method described below. Seeds were placed in a sterile 100 mm petri-dish. 70% ethanol was added and the plates placed for 2 minutes on a rotary shaker set at 40 rpm. The ethanol was then removed using a sterile pipette. The seeds were then soaked in 100% bleach for 20 minutes on a rotary shaker. The bleach was washed out by rinsing seeds four times in sterile distilled water. The seeds were soaked in sterile water and kept for 4-7 days at 4°C. After this period of cold treatment, the seeds were again sterilized with 100% bleach for 10 minutes followed by 4 rinses with sterile distilled water. From these seeds, mature embryos were excised under a dissecting microscope. Groups of about 30 embryos were transferred
to 125 ml flasks containing 10 ml of suspension induction (SI) medium. After 10 days, the concentration of 2,4-D in the medium was reduced to 3 mg/l by replacing SI medium with suspension maintenance (SM) medium. Six to eight weeks after suspension induction, cell suspensions were transferred to a larger (250 ml) flask containing about 50 ml suspension maintenance medium and subcultured at weekly intervals for 12 weeks. During the period when weekly subcultures were performed, fine cell aggregates were preferentially selected for subculture. This was done by allowing stirred cell aggregates to settle down partially when aliquots from the upper layers of the suspension were removed for subculture. As fine cell aggregates divide more rapidly than larger aggregates, the selection of fine cell aggregates for subculture quickened the establishment of rapidly growing cell suspensions. During all the experiments, suspension cells were grown on a rotary shaker set at 150 rpm, 25°C.

5. Protoplast isolation

Protoplasts were isolated by resuspending suspension colonies, 3-4 days after subculture, in protoplast isolation (PI) medium. About 10 ml of PI medium was used per gram of fresh cells. This mixture of cell suspension and PI medium was placed in a 100 mm diam petri dish and placed in a incubator rotary shaker set at 28°C, 40-50 rpm. After 1-1.5 hours, to separate protoplasts from undigested cell aggregates,
the digestion mixture was filtered through a sterile 50 um nylon mesh and the filtrate transferred to 10 ml screw cap tubes. The screw cap tubes were centrifuged for 6 min about 250 rpm. Protoplasts packed at the bottom of the tubes were resuspended twice in PC4 medium. From the second resuspension in PC4 medium, an aliquot was removed and protoplast yield estimated. Protoplasts were then suspended at the desired density in electroporation buffer.

6. Electroporation

Aliquots of 0.5ml of protoplast suspension (4x10⁶/ml unless otherwise mentioned) were pipetted into sterile 1.25 ml cuvettes. Unless otherwise mentioned, 200 ug/ml of carrier DNA and 80 ug/ml of plasmid DNA were added and mixed gently. The cuvettes were then placed on ice for 5 min, protoplasts were resuspended by gentle pipetting and subjected to electroporation at desired settings of field strength and capacitance, using an exponential-wave electroporation apparatus (‘BTX-300 Tranfactor’, Biotechnologies and Experimental Research Inc., San Deigo, CA). This electroporator consisted of parallel platinum-plated electrodes set 0.35 cm apart. At the conditions usually used (400 uF capacitance, 750 V/cm) for electroporation, the electroporator delivered pulses of half-life of 200 milliseconds. After electroporation, the cuvettes were placed back on ice for 15 min when the protoplasts were transferred to 35 mm diam petri plates containing 1 ml PC4 culture
medium. The plates were then wrapped in saran wrap and incubated in dark at 26°C until the time of harvest of total protein.

7. Protoplast viability

Protoplast viability was determined using fluorescein diacetate (FDA) according to Widholm (1972). Briefly, 20 ul of stock FDA solution (0.5% w/v in acetone) stored at 0°C was diluted in 1 ml PC4 culture medium and kept on ice. One drop of protoplast suspension was placed on a glass slide to which 10 ul of diluted FDA solution in PC4 was added. The protoplasts were mixed by very gentle swirling of the slides before coverslips were placed over them. A period of 5 min was allowed for the uptake of the dye. Viability counts were made within 20 min after addition of dye to minimize dye leaching which produces a fairly strong background fluorescence. When viewed under blue light (excitation 490 nm; emission 515 nm and up), viable cells fluoresced a bright green color whereas nonviable cells were invisible. Using an inverted microscope, a total of about 100 cells were counted under normal (white) light and then viewed in blue light to obtain the count of viable protoplasts. Percent viability was calculated as: 100 x the number of fluorescing (viable) cells / total number of cells.
8. Protein extraction and quantification

Unless otherwise mentioned, protoplasts were harvested 24 h after electroporation. The procedure used is described below. Cultured protoplasts were transferred to 1.5 ml eppendorf tubes and spun down at 9,000 rpm for 1 min. The supernatant was discarded and the pelleted protoplasts resuspended in 100 ul of GUS extraction buffer. Protoplasts were disrupted by sonication (8 seconds-10 second interval-8 seconds) on ice. After sonication, tubes containing disrupted protoplasts were spun at 14,000 rpm for 4 min and the supernatant (containing the total protein fraction) transferred to a fresh set of tubes. Protein extracts were diluted with 150-200 ul of GUS extraction buffer and analyzed immediately or stored overnight at -70°C. Protein concentration was determined according to the Bradford spectrometric assay (Bradford 1976).

9. GUS quantitative assay

Determination of GUS enzyme activities was carried out according to Jefferson (1987). Equal amounts of total protein were added to 100 ul of GUS assay buffer (1 mM 4-methyl umbelliferyl β-D-glucuronide) and incubated at 37°C. At two time intervals, usually 15 and 30 min after initiation of incubation, 40-50 ul of the assay mixtures were pipetted to 500 ul of stop buffer (0.2 M Na₂CO₃) to obtain two sets of reaction points. 100 ul aliquots of each stopped reaction was measured on a
fluorometer ('TKO-100', Hoefer Scientific, San Francisco, CA) calibrated with 1 mM 4-methyl umbelliferone as the standard. GUS activity for each sample was then calculated from the slope obtained from the two sets of time point data.

10. Establishment of suspension cultures of different ages

For this experiment, a series of suspension cultures that were identical except with respect to age on the day protoplasts were isolated from them, were needed. The series of flasks were established in duplicate so that each suspension age was represented by two flasks. The procedure followed to establish one (of the two identical) series of flasks is described below. A flask ('initiator flask') containing culture medium saturated with suspension cells was selected (on day '0') for use as the source of inoculum. On that day (0) and 3, 5, 7 and 9 days later, aliquots of 2 ml of medium from the initiator flask were transferred to 125 ml flasks (in Fig. 1, flasks A, B, C, D and E, respectively) containing 20 ml suspension culture medium. To prevent cell death while retaining the saturation of cells in the initiator flask, 2 ml of fresh culture medium was added to the initiator flask upon removal of the 2 ml aliquots. Such a replacement of culture medium in the initiator flask may affect the uniformity of suspension cells over time. To minimize variation that might have resulted from differences in the suspension cells at the time of the first subculture, each flask representing a particular age of suspension was subcultured weekly for
4 weeks. Thus, 38 days after the day initiator flasks were selected, 10, 7, 5, 3 and 1-day-old suspensions (in Fig. 1, flasks A’, B’, C’, D’, and E’, respectively), were established and protoplasts isolated from these suspensions.

For characterization of growth curve, 5 ml aliquots each from two separate flasks were transferred to two preweighed \((W_i)\) sterile 60 mm diam petri dishes. The culture medium was pipetted and the plates weighed \((W_t)\). The plates were dried in a 70°C oven for 60 h and weights \((W_d)\) of the plates recorded. Fresh and dry weights were calculated by deducting the tare weight of each petri plate from the weight of petri plates before \((W_r-W_i)\) and after \((W_r-W_d)\) drying.

11. Experimental design

Variations in transient assays are generally greater in protoplast populations prepared independently from a common source than populations prepared simultaneously. To minimize experimental error caused by differences in experimental material, each experiment was done using protoplasts prepared simultaneously. Each treatment tested was replicated 3 times to obtain reliable measurements of treatment effects. Each electroporation factor was tested independently using at least two independently isolated protoplast populations and the results of representative experiments have been presented.
RESULTS

1. Time course of GUS expression

The kinetics of transient gene expression were studied after the plasmid pZO 1052 containing the β-glucuronidase gene was introduced into tall fescue protoplasts by electroporation and transient GUS activities were measured at 6, 12, 24, 48 and 72 h after electroporation. Significant transient GUS expression was detected as early as 6 h after electroporation (Fig. 2). GUS expression increased rapidly from the 6 h treatment until it peaked at 24 h after electroporation. After 24 h, GUS expression decreased gradually with significant activity remaining at 72 h after electroporation. Since transient GUS expression in tall fescue protoplasts peaked at 24 hours after electroporation, GUS activity was measured after electroporated protoplasts were incubated for 24 hours in subsequent experiments.

2. Field Strength

Field strength significantly affects transient gene expression by influencing both permeation of foreign DNA and the survival of the electroporated protoplasts. In order to determine the influence of field strength on viability and transient gene expression in tall fescue protoplasts, transient GUS activity and viability were measured at field strengths of 300, 450, 600, 750, 900 and 1050 V/cm (Fig. 3).
Viability of unpulsed protoplasts was 81% 2 h after preparation and about 78% 24 h later. When measured two hours after electroporation, protoplast viability was 66.4% in the lowest field strength tested (300 V/cm) and further decreased steadily to 46.1%, 34.7%, 25.6%, 19.5% and 13.5% with increasing field strengths of 450, 600, 750, 900 and 1050 V/cm, respectively. For each field strength tested, viability at 24 h was slightly lower than that measured 2 h after electroporation.

As for gene expression, a minimal field strength of 300 V/cm was sufficient to provide detectable transient GUS expression. Increases in field strength through 450, 600 and 750 V/cm resulted in progressively higher transient GUS activity with maximal activity at 750 V/cm. Further increases in the field strength to 900 and 1050 V/cm led to a gradual reduction in the reporter gene expression. These decreases were probably due to the further decreases in viabilities of protoplasts under these field strength conditions. A field strength of 750 V/cm was therefore used in all other experiments.

3. Plasmid DNA quantity

The effect of concentration of plasmid DNA on transient gene expression was studied after protoplasts were electroporated in the presence of different concentrations (2, 8, 20, 80, 140 and 200 ug/ml) of plasmid DNA and a constant plasmid amount of 200 ug/ml carrier DNA. When GUS activities were measured, 2
ug/ml of plasmid DNA was sufficient to lead to detectable amounts of GUS activity (Fig. 4). Increases in the amount of plasmid DNA to 8, 20, and 80 ug/ml lead to a sharp increase in GUS activity. Only a small increase in gene expression occurred when plasmid DNA amounts were further increased to 140 or 200 ug/ml, but the increase was not statistically significant. Therefore, 80 ug/ml of plasmid DNA was used in all other experiments.

4. Heat shock

Subjecting protoplasts to ‘heat shock’ (a brief exposure of 5 min at 45°C) has been shown to stimulate transient gene expression in tobacco (Shillito et al., 1985) and soybean (Dhir et al., 1991). In order to study the effect of heat shock on transient GUS expression in tall fescue, protoplasts were incubated at 45°C for 2 and 5 min just before electroporation. Compared to protoplasts electroporated without heat shock, heat shock treatment proved to be detrimental to transient gene expression in tall fescue protoplasts (Fig. 5). The longer the heat shock given, the greater the decrease in GUS expression.

5. Protoplast density

The density of protoplasts in suspension at the time of electroporation influences transient gene expression by influencing the availability of DNA for each
protoplast and/or by influencing the distribution/alignment of protoplasts between the electrodes at the time of electroporation (Lindsey and Jones, 1987). The effect of protoplast density on transient GUS expression was tested using different protoplast densities of 0.5, 1, 2, 4 and 6 x 10^6/ml. GUS activity increased with increases in the density of protoplasts up to a density of 2 x 10^6/ml (Fig. 6). At higher densities (4 and 6 x 10^6/ml) GUS activity was not significantly greater than that of the 2 x 10^6/ml treatment. A linear relationship was found between protoplast density and transient GUS activity over the density range 0.5-2 x 10^6/ml, with coefficient of determination (r^2) values in two independent experiments varying from 0.98 to 0.99. Although a density of 2 x 10^6/ml was found to be optimal, a protoplast density of 4 x 10^6/ml was used in all other experiments to compensate for low viability (20-30%) that occurred due to electroporation.

6. Age of suspension cell cultures

For the study of the effect of age of the suspension cells on transient gene expression, fresh and dry weights, and GUS activities in electroporated protoplasts were measured in 1, 3, 5, 7, and 10 day old suspensions. Tall fescue cell suspensions follow a typical sigmoid type of growth pattern (Fig. 7). Dry weight increased relatively slowly (lag phase) one day after subculture after which it increased rapidly (exponential/linear phase) over 3 and 5 days. Beyond 5 days, suspension growth was
stationary, with little (5-7 days) or no increase in dry weight (7-10 days). Fresh weight increases were generally similar to dry weight increases except that the stationary phase was reached in 5 days in dry weight compared to 7 days needed for fresh weights. Transient GUS activity was highest in protoplasts derived from rapidly growing (3-5 day old) cell suspensions and gradually decreased in protoplasts derived from slower growing (5-7 days) and stationary cell suspensions (7-10 days).
DISCUSSION

This is the first study of factors influencing transient gene expression in tall fescue protoplasts. The influence of field strength, plasmid DNA amount, heat-shock, protoplast density, and age of subculture was studied using transient expression assays of the GUS reporter gene system.

The kinetics of transient gene expression in tall fescue are in general comparable to those reported for carrot (Hauptmann et al., 1987), tobacco (Okada et al., 1986), maize (Fromm et al., 1986) and petunia (Ballas et al., 1987). Determining the time when maximal transient expression occurs allows the maximization of sensitivity of the transient assay. Although different chimeric gene constructs and different DNA delivery conditions were used among these protoplast systems, the overall time course of transient gene expression is comparable with expression being detectable within a few hours after gene delivery with maximal expression levels observed at about 24 to 48 h. The general similarity in expression kinetics among these species may be a consequence of the methods used to effect direct DNA transfer. Since direct gene transfer is a physical process, it may not discriminate protoplasts of different species. However, this argument would not explain the considerable variability among these systems. Part of such variation in expression kinetics could be due to the differences in species, physiological status of
protoplasts or transformation conditions used.

Field strength inversely influenced the viability of electroporated protoplasts of tall fescue, with a gradual reduction in viability with increasing field strength. These data are in agreement with the results obtained in other electroporation systems such as carrot (Hauptmann et al., 1987; Bower and Birch, 1990), sugarbeet (Lindsey and Jones, 1987), tobacco (Guerche et al., 1987b) and soybean (Dhir et al., 1991).

Viabilities of tall fescue protoplasts 2 h after electroporation were slightly higher than viabilities measured at 24 h. The effect of voltage on viability appears to manifest in two ways: a short-term effect, resulting in irreversible membrane damage and protoplast bursting, apparent within 1-2 h after the application of electric pulse, and a long-term effect observed after 1-2 days (Lindsey and Jones, 1987). At optimum voltage capacitance combinations of 200 V/1000 uF or 300 V/510 uF, viability of pulsed carrot protoplasts is about 90% immediately after electroporation but decreases to 20-30% in measurements made 48 h later (Hauptmann et al., 1987). Similarly, in sugar beet protoplasts, viability is about 90% 2 h after electroporation but decreases to under 60% when measured 45 h after electroporation. Although the viability of unpulsed tall fescue protoplasts (about 80%) is comparable to that of unpulsed carrot or sugar beet protoplasts, a long-term effect of electric pulse on the protoplast viability was much less in tall fescue than in carrot or sugar beet. This
difference may be due to different electroporation conditions used in each system or due to different physiological conditions of protoplasts of these species.

Optimal gene expression occurs under conditions that are a compromise between viability and electro-permeation of DNA. Under such conditions, larger pores, more pores or longer lasting pores that are reversible may be induced in the plasma membrane. At very low voltages, the field strength is likely to be insufficient for induction and/or maintenance of pores during DNA uptake. At supra-optimal voltages, larger or more pores are induced but the concomitant damage to the integrity of the plasma membrane would be too great for the protoplasts to remain viable. Although transient GUS expression clearly peaked at 750 V/cm in tall fescue, further study is needed to determine whether this field strength is optimal for stable transformation.

For tall fescue, 200 μg/ml of carrier DNA was routinely used as it appears to enhance expression of foreign genes in systems such as tobacco (Shillito et al., 1985) and soybean (Dhir et al., 1991). The presence of carrier DNA may act as a ‘sacrificial host’ for plant nucleases and thereby protect plasmid DNA. In the presence of carrier DNA, use of even 2 μg/ml of plasmid DNA led to detectable transient GUS activity. Although this appears to be a small amount when compared to the other treatments tested, it still represents an enormous number (about 1 x 10^5) in terms of copies of plasmid DNA per protoplast.
The amount of plasmid DNA used for electroporation affected transient gene expression in tall fescue as has been shown for carrot (Bower and Birch. 1990), tobacco (Okada et al., 1986) and soybean (Dhir et al., 1991). The differences in the optimal amount of plasmid DNA could be due to the form of DNA used (linear vs. circular), to differences in molar concentrations (resulting from size differences among plasmid constructs) used, or to the genotypic or physiological status of target protoplasts. Despite such a range in the optimal amount of plasmid DNA, there appears to be a common trend in the response of transient expression in protoplasts to the amount of plasmid DNA, with a rapid response at lower DNA amounts and a plateau at higher DNA amounts.

Shillito et al. (1985) suggested two possible mechanisms to explain such a plateau in the response of protoplasts to increased DNA amounts. They suggested that a saturation in the amount of DNA to be taken up by each cell occurs, or that a competence phenomenon exists whereby only a small percentage of cells is capable of being transformed. Two groups, Bower and Birch (1990) and Dhir et al. (1991) attempted to resolve the underlying mechanism by measuring both transient gene expression and estimating the proportion of cells expressing the introduced gene (using the histochemical GUS staining procedure). In their work on carrot suspension cells, Bower and Birch (1990) observed a plateau in the proportion of cells (about 20%) showing the GUS stain in response to increased plasmid DNA.
amounts over 20-50 ug/ml. Quantitative GUS expression exhibited no plateau over this range of DNA amounts, continuing to increase with increases in DNA amount (up to 100 ug/ml). These data suggest that a limited proportion of carrot protoplasts is competent for DNA uptake and gene expression. Dhir et al. (1991), in their work on electroporation of soybean protoplasts, did not detect a plateau in the response of either the proportion of protoplasts showing the GUS stain or the quantitative GUS activity. A possible explanation is that the differences between the two systems is due to some factors other than competence. In carrot, 20-25% of electroporated protoplasts stain positive whereas in soybean only 1.6% do so. In soybean, plasmid DNA amount may be the limiting factor rather than the proportion of competent protoplasts. Thus, when plasmid DNA amounts are increased, there are sufficient numbers of competent protoplasts to take up the additional DNA. Another explanation for such differences could be the species themselves, or gene transfer procedures used by these groups. Alternatively, some other yet unknown mechanism could be responsible for such effects.

As heat-shocking is a simple step with potential benefits in enhancing transient and stable transformation efficiencies, its use was evaluated in tall fescue. Although the effect of heat shock treatment on foreign gene expression is enhancement in carrot (Boston et al., 1987) and soybean (Dhir et al., 1991) or neutral in *Nicotiana plumbaginifolia* (Negrutiu et al., 1987), its effect is detrimental in tall fescue as in
tobacco cv. 'W38' (Larkin et al., 1990). Since tall fescue is a cool-season crop, the detrimental effect of heat-shock may be related to its adaptation to cool climates, whereby elevated temperatures are poorly tolerated. Why heat shock differently influences transient gene activity in different plant species and varieties is poorly understood. Heat-shock may exert its influence by affecting plasma membrane permeability in a species-dependent manner or by protecting introduced DNA by switching off normal protein synthesis and inducing the synthesis of heat shock responsive proteins whose distribution may be species-specific.

The increase of protoplast density during electroporation increased in a linear fashion transient gene expression in tall fescue upto a density of $2 \times 10^6$ protoplasts. At higher densities, a breakdown of linearity between protoplast density and transient expression occurred and GUS activities at the 4 and $6 \times 10^6$/ml densities were not significantly greater than that of the $2 \times 10^6$/ml treatment. Similar results were obtained for sugar beet by Lindsey and Jones (1987) who observed a roughly linear relationship between protoplast density and transient gene expression over densities of $10^4$-$10^6$ and a breakdown in the linear relationship at densities greater than $1 \times 10^6$/ml. The breakdown in linearity at the 4 and $6 \times 10^6$/ml densities in tall fescue may be because at these densities the amount of DNA becomes limiting or that at such high densities electroporation may be inhibited.

Transient gene expression in tall fescue is closely correlated with the growth
phase of cell suspensions, with highest transient expression corresponding to protoplasts isolated from the most rapidly growing (3-5 day old) cell suspensions. Similar results have been observed in electroporated sugar beet protoplasts (Lindsey and Jones, 1987), where peak transient expression observed in 4-6 day old suspension cells corresponds to rapid (log phase) growth stage of suspension culture. Compared to fresh weight measurements, dry weight is a better measure of growth because phenomenon such as vacuolation (that increase water content in cells without a corresponding dry weight increase) are known to occur (Lindsey and Jones, 1987). This may account for why, in tall fescue protoplasts, transient gene expression correlates better with dry weight increases rather than increases in fresh weights.

A majority of protoplasts seems capable of foreign DNA up-take (Gallie et al., 1989; Nishiguchi et al., 1987). Thus, levels of foreign gene expression seem to be determined by factor(s) other than the delivery of DNA into the cytoplasm. The access of introduced DNA to host transcriptional machinery within the nucleus appears to be critical. The age of suspension cells may influence transient gene expression by determining the proportion of protoplasts that are competent to express foreign genes. Besides transient expression, the efficiency of stable transformation is higher when rapidly dividing cells are used as shown for Agrobacterium-mediated transformation of tobacco cells (An, 1985). An absence of the nuclear membrane during a specific stage (M stage) of the cell cycle would
provide foreign DNA (delivered into the cytoplasm) access to host cell’s nuclear transcriptional apparatus. This has been shown in tobacco cell suspension derived protoplasts (Okada et al., 1986), where transient expression is 3-4 fold higher at M phase of the cell cycle compared to that of the other phases.

The effect of each factor studied on transient GUS expression was reproducible in independent experiments. Although the level of overall GUS expression varied among independent experiments, the variation among all experiments did not exceed a factor of two.

From this study, field strength, plasmid DNA amount, heat-shock, protoplast density and age of subculture were found to influence transient expression of the reporter (GUS) gene significantly in electroporated tall fescue protoplasts. These results demonstrate that the use of transient expression assays can be extended to optimize electroporation conditions for factors that were not tested in this study. Such factors include buffer composition, form of DNA (linear vs circular), use of PEG along with electroporation and capacitance during electroporation. The information obtained from transient assays in this study could be the basis for further studies on optimizing factors for stable transformation. Although there is evidence that optimized transformation conditions from transient assays can be directly extended to stable transformation (Guerche et al., 1987b), factors such as viability play a more important role in stable transformation than in transient assays. Further
studies are therefore needed before an efficient genetic transformation procedure for
tall fescue is established. Efficient genetic transformation procedures for turf-type
tall fescue will enable the rapid introduction of desirable genes into this species and
also permit studies on the genetics and biology of this important turf and forage
glass.
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Fig. 1. Scheme followed to establish cell suspensions differing in age.
Fig. 2. Time course of transient GUS expression. Bars represent standard errors of 3 replications.
Fig. 3. Effect of field strength on viability and transient GUS activity. Bars represent standard errors of 3 replications.
Fig. 4. Effect of concentration of plasmid DNA on transient gene expression. Bars represent standard errors of 3 replications.
Fig. 5. Effect of heat-shock on transient GUS expression. Bars represent standard errors of 3 replicates.
Fig. 6. Effect of protoplast density on transient GUS expression. Bars represent standard errors of 3 replications.
Fig. 7. Effect of cell suspension age on transient GUS expression. Bars represent standard errors of 3 replications.
APPENDIX

1. High Salt buffer

Tris-HCl (pH 8.0)  50 mM
Magnesium chloride  10 mM
Sodium chloride  100 mM
2. Suspension Induction medium

Murashige and Skoog (1962) salts mixture (Gibco Labs, NY, U.S.A) 4.3 g/l
Sucrose 3%
2,4-dichlorophenoxy acetic acid (2,4-D) 10 mg/l
pH 5.8
Autoclave
3. Suspension Maintenance medium

Murashige and Skoog (1962) salts mixture (Gibco Labs, NY, U.S.A) 4.3 g/l
Sucrose 3%
2,4-dichlorophenoxy acetic acid (2,4-D) 3 mg/l
pH 5.8
Autoclave
4. Protoplast Isolation medium

Cellulase Onozuka RS (Yakult Honsha Co. Ltd., Tokyo, Japan) 1%
Pectolyase Y23 (Seishin Pharmaceutical Co., Tokyo, Japan) 0.1%
Murashige and Skoog (1962) salts mixture (Gibco Labs, NY, U.S.A) 4.3 g/l
Sucrose 3%
Mannitol 8%
2,4-dichlorophenoxy acetic acid 2 mg/l
pH 6.0

Filter-sterilize using a 0.2 um filter and store at 0°C
5. **Protoplast Culture (PC4) medium**

Murashige and Skoog (1962) salts mixture (Gibco Labs, NY, U.S.A) 4.3 g/l

Glucose 6.84%

Mannitol 5%

2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg/l

Benzylaminopurine (BAP) 1 mg/l

pH 5.8

Filter sterilize with a 0.2 um filter and store at 4°C
6. Electroporation Buffer

HEPES  
Sodium chloride  
Calcium chloride  
Mannitol  
pH 5.8  
Auto clave

10 mM  
150 mM  
5 mM  
7.28%
7. GUS extraction buffer

NaPO₄ (pH 7.0)  50 mM
β-mercaptoethanol  7 mM
EDTA (pH 8.0)  10 mM
Sodium lauryl sarcosine  0.1%
Triton X-100  0.1%
Vita

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