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Technical Report

Protoplast isolation and transient gene expression in switchgrass, *Panicum virgatum* L.

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Transient assay systems using protoplasts have been utilized in several plant species and are a powerful tool for rapid functional gene analysis and biochemical manipulations. A protoplast system has not been used in switchgrass (*Panicum virgatum* L.), even though it is a bioenergy crop that has received considerable attention. Here we report the first protocol to isolate large numbers of viable protoplasts from both leaves and roots of two switchgrass genotypes. Furthermore, we demonstrate transient expression of PEG-mediated DNA uptake in the isolated protoplasts by measuring the activity of β-glucuronidase (GUS) reporter gene driven by either the *Cauliflower mosaic virus* (CaMV) 35S promoter or the maize ubiquitin 1 promoter. Protoplast transformation with either the 35S or the ubiquitin promoter resulted in an increase in GUS activity compared to the untransformed controls; however, the extent of GUS activity was considerably higher for the ubiquitin promoter than for the 35S promoter. Collectively, our results indicate an efficient protoplast isolation and transient assay system that can be used to facilitate gene expression studies in switchgrass.

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1 Introduction

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass that is a major component of the prairies of North America. It is grown in monoculture for hay, grazing, and erosion control [1]. Also, switchgrass has a high biomass production potential as a feedstock for biofuel production [2, 3]. Genetic manipulation of the growth and development of switchgrass is needed for better cellulosic ethanol production, especially to improve cellulose-to-lignin ratios. The latest genomic and biotechnology tools can be used for the production of designer plants for this purpose, which is imminently feasible. Several genes can make significant improvements in agronomic and feedstock traits of switchgrass. These domestication, cell wall, and biocontainment traits might include altered cellulose levels, dwarfism, drought resistance, and pollen alterations that can be introduced *via* transgenesis. Although there is no substitute for stable transformation, current procedures are time-consuming, laborious, inefficient, and not suited for high-throughput assays. Conversely, the use of transient gene expression assays offers an opportunity to study large number of genes quickly, which would be advantageous for evaluating the transcriptional activity of different promoters, and might be especially useful for assaying cell biology and cell wall traits.

Protoplast-based transient assay systems provide powerful tools for many types of assays in plants. They have proven very useful for dissecting a broad range of plant signal transduction pathways, transcriptional regulatory networks, and

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Abbreviations: 4-MU, 4-methyllumbelliferone; **CaMV,** Cauliflower mosaic virus; **GUS,** β-glucuronidase

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evaluation of reporter gene expression [4–12]. However, most protoplast transient assay systems have been developed for dicots [4, 11], but protoplasts have been produced in rice [6, 7], maize [11, 13], and red fescue [14]. Establishment of protoplast techniques for switchgrass is of crucial importance to bioenergy biotechnology.

Here we report the first protocol for protoplasts isolation and transgene expression based on leaf- or root-derived protoplasts from switchgrass. Our efficient protoplasts isolation and transient gene expression system provide insight into the versatility of the transient assay system in studies of gene expression in switchgrass.

2 Materials and methods

2.1 Plant material

Switchgrass seeds (cv. Alamo) (Bamert Seed, Muleshoe, TX) were soaked overnight in deionized water, planted in potting media, and grown in a greenhouse for 2 wk. Leaves of 2-wk-old plants were used for protoplast isolation. For establishing the root tissues, 2-wk-old plants grown in potting media were transferred to hydroponic conditions [15] following gentle washing of the roots with deionized water. Plants were grown with aeration in opaque containers containing 600 mL of 25% Hoagland solution [15]. After 1 wk of growing in hydroponics, the initial fibrous roots were trimmed to allow the formation of straight primary roots, which were used for protoplast isolation.

Protoplasts were also isolated from leaf and root tissues of the Alamo 2 clone. This genotype was established in previous research [16], and it is highly embryogenic in tissue culture, which facilitates its efficient laboratory micropropagation *via* somatic embryogenesis [17]. Young Alamo 2 plants were produced under aseptic conditions *via* node culture of switchgrass as described in Alexandrova *et al*. [16]. Leaves from 3-month-old regenerated plants, growing in tissue culture, were used for protoplasts isolation. For root tissues, 3-month-old regenerated plants were transferred to hydroponic conditions and treated as described above.

2.2 Isolation of protoplasts

The basic procedure for protoplast isolation was adopted from that described for rice [6] but with several modifications. For leaf protoplasts, about 16 young, healthy, 4–5 cm long green leaves were used. For root protoplasts, about 1.5 g of 2–3 cm long straight primary roots were used. Leaf or root tissues were sliced into 0.5–1 mm long segments on a paper sheet using a sharp razor blade and immediately transferred into a 15 mL enzyme solution (0.6 M mannitol, 10 mM MES (pH 5.7), 1.5% cellulase (Onozuka R-10) (PlantMedia, Dublin, OH) for leaf tissues or cellulase (Onozuka RS) (Serva/Crescent Chemicals, Islandia, NY) for root tissues, 0.75% macerozyme R-10 (PlantMedia), 0.1% BSA, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol) filtered through a 0.45 μm microfilter (Millipore, Billerica, MA). The digestion was carried out in the dark under gentle shaking at 40 rpm for 3 h at room temperature, then for another 10 min at 60 rpm to release protoplasts. For leaf tissues, vacuum pressure was applied during the first hour of the incubation period to enhance infiltration of the enzyme solution in the leaf segments. After incubation, the solution was filtered through a 40 μm Falcon cell strainer (BD Biosciences, Bedford, MA) placed in a Petri-dish. One volume of W5 washing solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7)) was added and protoplasts were collected by centrifugation at 239 × *g* for 5 min and resuspended in 500 μL of Mmg solution (0.6 M mannitol, 15 mM $MgCl_2$, 4 mM MES (pH 5.7)). Protoplasts were observed under bright field light using an Olympus BX51 microscope with a 40× objective and quantified by a hemocytometer.

2.3 Protoplast viability assay

Viability of the protoplasts was examined by Evans blue staining assay [18, 19]. Evans blue dye (Sigma, St. Louis, MO) was dissolved in Mmg solution and added to aliquot of the protoplasts to a final concentration of 0.04% and incubated for 10 min at room temperature. Evans blue is excluded by living protoplasts whereas dead protoplasts and cell debris are stained a deep blue color. The number of unstained cells (viable protoplasts) was determined using a hemocytometer and the fraction of surviving cells was calculated by dividing the number of viable cells by the total cells count.

2.4 Plasmids

The 5.6 kb pBI221 plasmid DNA construct [20] contains the β-glucuronidase (GUS) reporter gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S constitutive promoter. The 9.6 kb pAHC25 plasmid [21] contains the GUS reporter gene under the control of the maize ubiquitin 1 constitutive promoter. The plasmid DNA constructs were propagated in *Escherichia coli* and purified using the Qiagen Maxi Plasmid kit (Qiagen, Valencia, CA, USA).

2.5 Protoplast transfection

PEG-mediated DNA transfection was performed essentially as described online by J. Sheen's laboratory http://genetics.mgh.harvard.edu/sheenweb with several modifications. The collected protoplasts were resuspended in an appropriate volume of Mmg solution. Plasmid DNA (about 40 μg) was mixed with 200 μL of protoplasts (approximately 2×10^5 cells). Then, 40% PEG solution (0.6 M mannitol, 100 mM $CaCl_2$, 40% PEG 4000 (Sigma/Fluka)) was added to the protoplasts in droplets and the solution was carefully mixed by hand to a final PEG concentration of 20%. After incubation for 20 min at room temperature, cells were washed with 5 mL of W5 solution and collected by centrifugation at $100 \times g$ for 5 min. The cells were resuspended in 1 mL of incubation solution (0.6 mM mannitol, 4 mM KCl, 4 mM MES (pH 5.7)), transferred to six-well Falcon culture plates (BD Labware, Franklin Lakes, NJ) and incubated at 25°C in the dark for 24 h.

2.6 GUS assay

Transfected protoplasts were harvested by centrifugation at $100 \times g$ for 5 min. One hundred fifty microliters of GUS extraction buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sacrosine, 0.1% Triton X-100) were added to the protoplasts before they were vortexed for 1 min to lyse the cells. Cellular debris was spun down by centrifugation at $10\,000 \times g$ at 4°C for 10 min and the supernatant was removed. Quantitative determination of the GUS activity was performed with 4-methylumbelliferyl-β-D-glucuronide (4-MUG) (Sigma), as substrate according to the procedure of Jefferson [22], adapted for use with microtiter plates and Synergy HT multidetection microplate reader (Bio-Tek Instruments, Winooski, Vermont). A standard curve was prepared using 4-methyllumbelliferone (4-MU) (Sigma). GUS activity is expressed as picomole of 4-MU *per* milligram of protein *per* minute. Protein concentration was determined using a Bradford assay reagent kit (Pierce, Rockford, IL).

3 Results and discussion

3.1 Protoplasts isolation from switchgrass

Protoplasts were successfully isolated from leaves and roots of two switchgrass genotypes: Alamo and Alamo 2. Leaves of 2-wk-old plants grown either in potting media (Alamo, Fig. 1A) or 3-month-old regenerated plants in tissue culture (Alamo 2, Fig. 1B) were used for protoplasts isolation. Root protoplasts were isolated from primary roots of Alamo or Alamo 2 plants after 2 wk of cultivation in the hydroponic conditions (Fig. 1C). The isolation procedure was optimized for the leaf and root tissues. After several trials, the time period for the incubation of the tissues in the enzyme solution was adjusted to 3 h with gentle shaking at 40 rpm. It was found that an extra incubation of 10 min at 60 rpm released protoplasts from the leaf tissues more efficiently. Longer incubation time or vigorous shaking (*e.g.*, 80 rpm), in particular for the root tissue, were found to decrease protoplast quality by breaking protoplasts and increasing cell debris, which resulted in a dramatic reduction in yield of competent protoplasts. Leaf protoplasts were uniformly spherical, containing green chloroplasts (Fig. 2A), whereas the root protoplasts were colorless and mostly spherical with well-organized intervacuolar strands and active cytoplasmic streaming (Fig. 2B).

Figure 1. Switchgrass leaf and root tissues used for isolation of protoplasts. (A) Leaves from 2-wk-old Alamo genotype grown in soil were used for protoplast isolation. (B) Leaves from 3-month-old regenerated Alamo 2 genotype grown in tissue culture were used for protoplast isolation. (C) Roots of Alamo and Alamo 2 plants grown under hydroponic conditions were used for protoplast isolation.

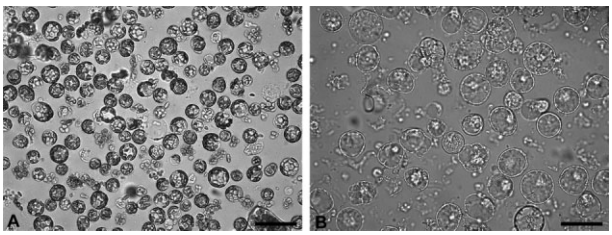

Figure 2. Isolation of protoplasts from switchgrass. Protoplasts isolated from leaf (A) and root (B) tissues. Pictures were taken under bright field light using an Olympus BX51 microscope with a 40× objective. Scale bar = 50 µm. Color figure can be seen under Supporting information.

Leaf protoplasts were relatively smaller in size compared to root protoplasts, but a variety of sizes were observed for root protoplasts (Fig. 2B). Total number of protoplasts isolated from leaf or root tissues was in the range of 6–8 × 10^5 cells *per* isolation with a viability of 88–92% (Table 1). Protoplasts from green leaf tissue might be necessary for those cellular processes that are light sensitive including disease resistance [23], while etiolated-derived protoplasts are useful for many other types of assays [11]. We attempted to isolate protoplasts from the mesocotyl of etiolated switchgrass plants and from cell suspension culture generated from the Alamo 2 genotype [17]. Our attempts to do so were inefficient and we deemed them as not successful. Further optimization needs to be performed if these explants are necessary to answer a specific biological question.

3.2 Transient expression in switchgrass protoplasts

To examine the suitability of the protoplasts system for transgene expression in switchgrass, quantitative GUS expression was assessed using CaMV 35S- and maize ubiquitin 1 promoters. In order to optimize transformation efficiency for our system, we experimented with different transformation conditions. We found that 2 × 10^5 protoplasts for each transformation event gave the highest level of GUS expression. We also tested different concentrations of DNA (20, 40, and 80 µg) and found that 40 µg was the optimum concentration for transformation (data not shown). As shown in Fig. 3, transfection of protoplasts isolated from leaf or root tissues with either 35S or ubi1 promoter construct resulted in an increase in GUS activity compared to untransformed controls. Similar results were obtained when 35S or ubi1 promoter constructs were compared to transformed empty vector plasmid pUC19 controls (data not shown). GUS under the control of the ubi1 promoter had much higher gene expression than for the similar 35S promoter cassette (Table 2). GUS activity for the 35S promoter was 17% (in leaf protoplasts) and 14% (in root protoplasts) relative to that of the ubiquitin promoter in Alamo. For the Alamo 2 clone, GUS activity for the 35S promoter was 10% (in leaf protoplasts) and 8% (in root protoplasts) relative to that of the ubi1 promoter (Table 2). The ubi1 promoter has been shown to be highly active in monocots [21], and to a lesser extent the 35S promoter is transcriptionally active as a semiconstitutive promoter in monocots [6, 7]. Our findings suggest that ubi1-regulated gene expression is 5–10-fold higher than the 35S in switchgrass leaf and root cells, giving researchers guidelines for choosing a promoter based upon expected transcriptional activity. Comparison of the transfected leaf- and root-derived protoplasts also indicated a higher level of GUS expression in root protoplasts for both the ubiquitin or 35S promoters (Fig. 3). This higher competency for root protoplasts may result from experimental conditions used for generating the root materials. Nevertheless, our results show an efficient GUS expression in both leaf and root protoplasts from two switchgrass genotypes.

Table 1. Total number of protoplasts isolated from leaf or root tissues of two switchgrass genotypes with percentage of viability

Plant tissue (genotype)	Number of cells × 10^5	Protoplasts viability (%)
Leaf (Alamo)	6 ± 4.7[a)]	91.3
Root (Alamo)	6 ± 11.8	88.6
Leaf (Alamo 2)	8 ± 11.9	92.1
Root (Alamo 2)	6 ± 4.8	89.5

a) Each value represents the mean of four independent isolations ± standard error.

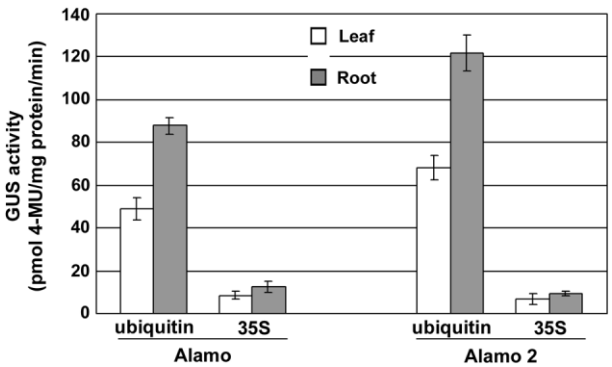

Figure 3. Transient GUS expression in protoplasts of switchgrass. Fluorometric determination of GUS activity in protoplasts isolated from leaf or root tissues of genotypes Alamo and Alamo 2 transformed with ubiquitin::GUS or 35S::GUS reporter construct. GUS activity is shown relative to the corresponding untransformed control (set to 1.0). Each bar represents the mean value of GUS activity obtained from four independent experiments ± standard error.

Table 2. A comparison of the GUS activity between 35S and ubiquitin promoters in the switchgrass protoplasts

Plant tissue (genotype)	35S		Ubiquitin
Leaf (Alamo)	8.4 ± 1.87[a)]	(17%)[b)]	49.14 ± 5.18 (100%)
Root (Alamo)	12.5 ± 2.69	(14%)	87.87 ± 3.96 (100%)
Leaf (Alamo 2)	6.86 ± 2.50	(10%)	68.18 ± 5.65 (100%)
Root (Alamo 2)	9.43 ± 0.99	(8%)	121.73 ± 8.18 (100%)

a) Each value represents the mean of four independent experiments ± standard error. GUS activity is expressed as picomole of 4-MU *per* milligram of protein *per* minute.
b) Percentage of GUS activity for 35S with that for ubiquitin.

Transient gene expression based on *Agrobacterium*-mediated transformation has been reported in switchgrass leaves [24]. The highest activity of transgene expression was achieved after vortexing the leaf segments with carborundum before cocultivation with *Agrobacterium* [24]. Compared with the *Agrobacterium* cocultivation transient expression assay, recent development of protoplast transformation methods with plasmid DNA by PEG, electroporation, and microinjection offers distinctive advantages facilitating the application of transient gene expression assays in switchgrass cells [11]. Furthermore, protoplast-based transient expression systems offer useful tool for investigating cell wall regeneration, cell division, embryogenesis, and differentiation, among others [11]. Our protoplasts transient assay system provides insights into the usefulness of the system in future studies of gene expression in switchgrass cells.

4 Concluding remarks

We have developed protocols for rapid protoplast isolation and transient assays from switchgrass, which utilizes leaf- or root-derived protoplasts. The possible versatility of the facile system for assays of gene overexpression or gene suppression, analysis of signaling pathways and detection of protein–protein interactions will facilitate large-scale functional analysis of genes in switchgrass.

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