Comparison of mesophyll protoplast isolation and transformation between *Panicum virgatum* and *Panicum hallii*

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Abstract

Protoplasts are appropriate targets for genome editing, DNA functional screens and transgenesis. This project focuses on the comparison of inexpensive mesophyll protoplast isolation via the use of food-grade enzymes and transformation between diploid *Panicum hallii* Vasey (PAH) and polyploid cellulosic feedstock *Panicum virgatum* L (switchgrass), a relative of PAH. PAH has great potential as a C4 model species for crop and bioenergy research. Here an inexpensive switchgrass and PAH mesophyll protoplast isolation and transformation system was developed; the first protoplast system for PAH. Using low-cost commercial food-grade enzymes, a cost reduction of ~1000-fold was achieved compared to traditional protoplast isolating enzymes with a cost of $0.003 (USD) per reaction for switchgrass mesophyll protoplasts and $0.0018 (USD) per reaction for switchgrass cell-suspension culture-derived protoplasts. Additionally, PEG-mediated switchgrass mesophyll protoplast transformation was improved to a maximum 30.4 % over the previous transformation efficiency of 9.1 %, achieving higher transformation efficiency with a reduction in DNA quantity. In the first protoplast isolation system for PAH, an average fivefold increase in protoplast yield from PAH leaf tissue over the optimum switchgrass tissue protoplast isolation was shown. PAH yielded an average 7340 ± 1816 viable protoplasts per mg mesophyll tissue and switchgrass yielded 1468 ± 431 viable protoplasts per mg mesophyll tissue with both species having greater than 95 % viable protoplasts. With additional food-grade enzyme concentration optimization, an additional cost decrease to

$0.001 (USD) per reaction was shown. PAH mesophyll protoplasts have a diameter from 3.9- 28.1 µm [micrometer], with a mean of 13.5 µm, which are significantly smaller than switchgrass mesophyll protoplasts which range from 6.5- 39.4 µm with a mean of 17.4 µm. Polyethylene glycol (PEG)-mediated transformation of PAH protoplasts revealed an optimum transformation efficiency of 46.7 ± 5.5 % with switchgrass protoplast transformation efficiency of 9.3 ± 1.9 %. The methods in this project provide an essential step toward using *P. hallii* as a C4 panicoid model species.

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Chapter 1 Introduction

Background

With increasing global population and thus greater reliance on fossil fuels, many are looking towards alternative, renewable sources for energy, such as wind, solar, geothermal, and biofuels. *Panicum virgatum* L. (switchgrass) is one of the lignocellulosic candidate feedstocks that is a native C4 perennial polyploid grass with distribution over much of the United States. Switchgrass is a potential choice for cellulosic biofuels because of its high yielding biomass on marginal lands (Parrish and Fike 2005). At present, the energy used in processing switchgrass is far greater than the energy reaped from the biomass (Pimentel and Patzek 2005; Zhong et al. 2016). Cell wall recalcitrance, or the resistance of the plant cell wall to degradation via chemical processes, is the current limiting factor for biomass conversion, making switchgrass feedstocks an expensive alternative to petroleum feedstocks (Himmel et al. 2007). Currently, recalcitrance is not completely understood and clarifying the biochemical and molecular contributions is ongoing (DeMartini et al. 2013; Wuddineh et al. 2015) . Understanding this recalcitrance is necessary for the implementation of switchgrass as a viable biofuel and value-added chemicals feedstock (Keshwani and Cheng 2009; Lu et al. 2015). With polyploid switchgrass having over 80,000 genes (v 1.1, Phytozome 11), it is essential to implement a high-throughput system for screening genes of interest that could contribute to recalcitrance. However, this polyploid, highly heterozygous genome poses problems for genomic analysis (Bouton 2007). Model plant systems are used because they are more experimentally manageable, often in tissue culture as well as genetic manipulation (Mandoli and Olmstead 2000). At present, there is a need for a robust C4 model system.

Potential model plants

Panicum hallii Vasey is a small perennial C4 grass that has two different recognized varieties, var. hallii (PAHAH) and var. filipes (PAHAF) that are related to switchgrass. These grasses are smaller in stature than switchgrass, growing in the southwestern areas of the United States, with PAHAH growing in xeric conditions and PAHAF growing in mesic conditions (Lowry et al. 2015; Waller 1976). Of the two ecotypes, PAHAF has a longer flowering time, smaller seeds, and larger plant size, and is intermediate in size between PAHAH and switchgrass (Lowry et al. 2013; Waller 1976). Because of the highly self-fertilizing tendencies, short seed-to-seed time frame, comparatively low genome size to switchgrass (PAHAF=554Mb, v 2.0, Phytozome 10.3), and its diploid nature, PAH has been slated as a potential model system to study polyploid C4 grasses, such as switchgrass, and their use (Lowry et al. 2015; Meyer et al. 2012; Zhang et al. 2011).

Foxtail millet (*Setaria italica* (L.) P. Beauv.) has been considered for use as a C4 model system because of its relationships to bioenergy grasses, including switchgrass (Doust et al. 2009). Like PAH, foxtail millet is a small statured, selfing, diploid C4 grass (Till-Bottraud et al. 1992) with a small genome (~515Mb, v 2.2, Phytozome 11) and short generation time (Doust et al. 2009). In contrast to PAH, foxtail millet is considered to be one of the oldest cultivated millets, and is grown primarily in eastern Asia (Oelke et al. 1990). Foxtail millet is primarily used for forage, while also being used for human food consumption in Asia and Africa (Marathee et al. 1994; Oelke et al. 1990). However, foxtail millet was introduced to the United States in the middle of the nineteenth century

and is considered to be an invasive plant, and is therefore often a pest in North American agricultural fields (Dekker 2003). Protoplasts from foxtail millet are often not used, even when characterizing proteins from foxtail millet itself, instead opting for a more well-established system such as *Nicotiana tabacum* (tobacco) cv. Bright Yellow-2 (BY-2) (Mishra et al. 2012). Mesophyll tissue from foxtail millet is likely not amenable for multiple mesophyll isolations from regrowth. Foxtail millet is an annual plant and does not grow back from cutting, which is a common weed management technique (Baltensperger 1996). However, root protoplasts of foxtail millet can be obtained and transfected via PEG, but the system is not often used, and no transformation efficiency has been reported (Wang et al. 2014). Considering the above issues, foxtail millet does not appear to be a vigorous C4 model system.

Another proposed model system for the grasses is *Brachypodium distachyon* (L.) P. Beauv (Brachypodium), a small grass native to Europe, Africa, and Asia (Draper et al. 2001). Brachypodium has a very small, diploid genome (~272Mb, v 3.1, Phytozome 11), as well as a short stature, short generation time, and ability to self (Draper et al. 2001), which is a common theme among the projected model systems. *Agrobacterium tumefaciens*-mediated transformation has been reported with Brachypodium callus (Pacurar et al. 2008; Vain et al. 2008; Vogel and Hill 2008). Mesophyll protoplast isolation has been accomplished from Brachypodium, but tissue was grown using a hydroponic system, which makes the system more complicated and takes 25 days for sufficient tissue growth (Jung et al. 2015). Further, Brachypodium protoplast transformation is often only used for studies on localization and standard *Agrobacterium*

callus transformation is conducted for further characterization (Jung et al. 2014; Ryu et al. 2014). Finally the major drawback for using Brachypodium as a model plant system for C4 grasses is that Brachypodium undergoes C3 photosynthesis, therefore it cannot be directly compared to other grasses that use the C4 photosynthetic pathway (Brkljacic et al. 2011). With this major issue, another C4 model plant system must be developed.

Transformation using *Agrobacterium*

Callus-based *Agrobacterium tumefaciens*-mediated transformation is the primary method for generating transgenic switchgrass, however the method has several disadvantages. Currently, *Agrobacterium*-mediated transformation can take 6-12 months from callus to regeneration of whole plants, which makes characterizing high numbers of genes practically impossible (Burris et al. 2009). Further, the reliance on a plant pathogen to complete the DNA integration introduces numerous regulatory hurdles (Garrett 1987; Jaffe 2004). Currently, in the United States the framework for regulation of transgenic plants falls to three government agencies: U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS), Environment Protection Agency (EPA), and Food and Drug Administration (FDA) (Jhansi Rani and Usha 2013). For a transgenic plant to be introduced commercially, it must adhere to standards set by these agencies, and extensive studies must be done (Fernandez-Cornejo et al. 2014). In addition to regulatory concerns, the control of where and how many time the T-DNA is inserted into the host genome is not very tightly controlled, resulting in a wide variation of transgene insertion and expression (Hobbs et al. 1993). In addition, there is a high risk of recovering chimeric plants with *Agrobacterium*-mediated transformation, where the non-

transgenic plant material can outgrow the transgenic plant material (Zhang et al. 2010). For switchgrass, the transformation efficiency is highly variable based on genotype and type of material from which the callus is generated, and the process that involves selecting a specific callus, with yellow, friable embryogenic callus from seeds of switchgrass 'Performer' cultivar being the best for transformation and regeneration (Li and Qu 2011). A high frequency of false positives, up to 30%, have been reported from *Agrobacterium*-mediated transformation of switchgrass callus (Ogawa et al. 2014; Somleva et al. 2002). To address these problems, a different transformation system must be considered that allows for determination of positively transformed cells at an earlier time. A single cell system, such as protoplasts, would allow for homogeneity and selection of only transformed cells early, thus reducing the risk of chimeric recovery.

Protoplast isolation

For both *P. virgatum* and *P.* hallii, a single-cell transformable system would be an important tool for the rapid screening of cell wall genes for the development of a feasible switchgrass biofuel feedstock. In order to effectively develop a reproducible system for screening switchgrass or PAH cells, it is necessary to develop a homogeneous population (Menges and Murray 2002). Protoplasts are cells from which the cell wall has been digested, enzymatically or otherwise, leaving the nucleus and the cytoplasm surrounded by the plasma membrane (Brenner et al. 1958). Until their first cell division, cultures of protoplasts are composed entirely of single cells, thus a homogeneous population is created. The first protoplast isolation was conducted in 1892 by Klercker (cited in Cocking 1960), where leaf tissue of *Stratiotes aloides* was plasmolysed, the cell wall was

mechanically cut, and protoplasts were released. More extensive protoplast research began when protoplasts were isolated 56 years ago from tomato root tips and have proven to be a highly useful tool for transient genetic screening, genetic modification through fusion, as well as understanding processes in single cells such as virus infection (Cocking 1960; Kao and Michayluk 1974; Takebe 1975; Yoo et al. 2007). More recently, there has been a surge in the use of protoplasts for site-directed mutagenesis via clustered regularly interspaced short palindromic repeats (CRISPR) systems, as well as the use of transcription activator-like effector nucleases (TALEN) technology (Jiang et al. 2013; Nicolia et al. 2015; Subburaj et al. 2016). While protoplasts have been previously isolated from switchgrass, the process was expensive and plants were not regenerated (Mazarei et al. 2011; Mazarei et al. 2008). There have been no reported protoplast studies on either ecotype of PAH.

Cost of protoplast isolation

To use protoplasts in a transgene screening system, it must be a cost-effective procedure that is highly reproducible. The common protocol for isolating protoplasts involves the use of expensive enzymes that are combined with isolation buffer immediately prior to extraction, with variable enzymatic activity (Hamlyn et al. 1981). Viable protoplasts from plants and fungi can be extracted using commercial enzymes, which are often cheaper than lab-grade enzymes (Hamlyn et al. 1981; Shenk and Hildebrandt 1969). In particular, food-grade enzymes, Rohament CL, Rohament PL, and Rohapect UF were shown to be effective in replacing traditional cell wall digesting enzymes with a cost reduction of more than 100-fold in *Nicotiana tabacum* “Bright

Yellow-2" (BY-2) protoplast extraction (Buntru et al. 2014). If these food-grade enzymes can be applied to switchgrass and PAH protoplast extraction systems, the current cost could be greatly reduced, and more transgene screening could be conducted.

Protoplast transformation

Protoplast transformation is often used for transient expression screening (Abel and Theologis 1994; Chen et al. 2006), and a transient screening system would be useful for switchgrass and PAH as a model for C4 grasses. It has been routinely demonstrated that plant protoplasts can take up naked DNA (Lurquin and Kado 1977). There are several different ways this can be achieved, including electroporation (Fromm et al. 1985) and polyethylene glycol (PEG) mediation (Negrutiu et al. 1987).

PEG-mediated transformation

PEG- mediated transformation is a common method to transiently express vector DNA in protoplasts (Cao et al. 2016; Junker et al. 1987; Lee et al. 1989). The mechanism of PEG-mediated transformation of protoplasts involves precipitation of the DNA in a PEG/divalent cation solution, which also protects the DNA from degradation, then the precipitated DNA enters into the protoplast (Maas and Werr 1989). Previous work on switchgrass protoplasts focused primarily on PEG-mediated transformation, but the reported method was not highly proficient as 40 µg DNA was used for transformation with a 5.6 kb vector, and transformation efficiency was not optimized (Mazarei et al. 2008). Several factors can influence the transformation efficiency of protoplasts using PEG, such as vector size (Mazarei et al. 2008; Sheen 2001), vector DNA amount (Armstrong et al. 1990; Damm et al. 1989; Maas and Werr 1989), $MgCl_2$ concentration

(Armstrong et al. 1990; Masani et al. 2014; Negrutiu et al. 1987), molecular weight of PEG (Lazzeri et al. 1991; Zhang et al. 2008), and PEG concentration (Masani et al. 2014). All of these variables must be considered when developing a protoplast PEG-mediated transfection system.

Electroporation

Electroporation is another common method of inserting exogenous DNA into plant protoplasts. Electroporation works in the application of an electric pulse to the cells, thus creating reversible permability of the membrane of the cells, allowing for DNA uptake (Xie et al. 1992). Electroporation is sometimes considered superior to PEG-mediated transfection, because of the toxicity of PEG to cells, but damage is also done to cells during the electric shock, so that must be taken into account (Fromm et al. 1985). Similar considerations must be made as with PEG-mediation, such as the size and amount of vector DNA, with the addition of optimization of the electric pulse that is applied to the protoplasts. This method of transfection has been seen to be successful in protoplasts from both monocots and dicots, but there appears to be some decrease in efficiency in monocot protoplasts (Fromm et al. 1985). Additionally, electroporation has been seen to stimulate regeneration from protoplasts in several crops (Chand et al. 1988; Ochatt et al. 1988; Rech et al. 1987). However, electroporation is not conducive to high-throughput transfection, with no adaptability to a robotic platform.

Chapter 2 Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.)

This chapter is based on this published paper:

Burris, K. P., Dlugosz, E. M., Collins, A. G., Stewart, C. N., & Lenaghan, S. C. (2016). Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). *Plant Cell Reports*, *35*, 693–704. http://doi.org/10.1007/s00299-015-1913-7

My primary contributions to this paper were the protoplast isolation experiments and culture and maintenance of cell suspension lines.

Abstract

Key message **A switchgrass protoplast system was developed, achieving a cost reduction of ~1000-fold, a threefold increase in transformation efficiency, and a fourfold reduction in required DNA quantity compared to previous methods.**

Abstract In recent years, there has been a resurgence in the use of protoplast systems for rapid screening of gene silencing and genome-editing targets for siRNA, miRNA, and CRISPR technologies. In the case of switchgrass (*Panicum virgatum* L.), to achieve economic feasibility for biofuel production, it is necessary to develop plants with decreased cell wall recalcitrance to reduce processing costs. To achieve this goal, transgenic plants have been generated with altered cell wall chemistry; however, with limited success owing to the complexity of cell walls. Because of the considerable cost, time, and effort required to screen transgenic plants, a protoplast system that can provide data at an early stage has potential to eliminate low performing candidate genes/targets

prior to the creation of transgenic plants. Despite the advantages of protoplast systems, protoplast isolation in switchgrass has proven costly, requiring expensive lab-grade enzymes and high DNA quantities. In this paper, we describe a low-cost protoplast isolation system using a mesophyll culture approach and a cell suspension culture. Results from this work show a cost reduction of ~ 1000-fold compared to previous methods of protoplast isolation in switchgrass, with a cost of $0.003 (USD) per reaction for mesophyll protoplasts and $0.018 for axenic cell culture-derived protoplasts. Further, the efficiency of protoplast transformation was optimized threefold over previous methods, despite a fourfold reduction in DNA quantity. The methods developed in this work remove the cost barrier previously limiting high-throughput screening of genome-editing and gene silencing targets in switchgrass, paving the way for more efficient development of transgenic plants.

Keywords Switchgrass · Protoplasts · Transformation · Enzymatic digestion

Introduction

Over the last decade, associated with the rapid boom of ‘‘omics’’ technologies, there has been an increasing trend in the development of protoplast systems, for numerous plant species, for rapid gene screens and reverse genetics. Recently, protoplast isolation and transfection systems have been developed/improved for maize (*Zea mays*) (Cao et al. 2014), carrot (*Daucus carota*) (Maćkowska et al. 2014), poplar (*Populus euphratica*) (Guo et al. 2015), grape (*Vitis vinifera*) (Wang et al. 2015), oil palm (*Elaeis guineensis*) (Masani et al. 2014), lettuce (*Lactuca sativa*) (Sasamoto and Ashihara 2014), and mustard (*Brassica juncea*) (Uddin et al. 2015), just to name a few. The reemergence of

protoplast systems is directly related to their utility in the analysis of protein subcellular localization (Chen et al. 2015; Nieves-Cordones et al. 2014) protein–protein interactions (Fujikawa et al. 2014; Li et al. 2015), transcriptional regulatory networks (Nakashima et al. 2014; Pruneda-Paz et al. 2014), signal transduction pathways (Cao et al. 2014) and rapid analysis of gene expression(Yoo et al. 2007). With the advent of genome-editing and gene silencing technologies, protoplast systems have found further utility due to the ease in screening the efficiency of numerous targets, e.g., dsRNA (Cao et al. 2014), siRNA (Bart et al. 2006), miRNA (Martinho et al. 2015), or gRNA (Xing et al. 2014) prior to the development of transgenic plants. With the renewed interest in protoplasts, significant progress has been made into the regeneration of protoplasts into whole plants, which further allows for the establishment of transgenic plants without the need for *Agrobacterium*-mediated transformation. For crop species, this is a crucial advantage, as transgenic plants that have been transformed by non-pathogen-related methods are not as heavily regulated. Despite these advantages, the widespread use of protoplasts is often hampered by the high cost of cell wall degrading enzymes, the large quantity of DNA required for transfection, the need for a constant source of tissue (leaves or roots) for isolation, and regeneration and fertility of regenerated plants, particularly in monocots. As an example of an important lignocellulosic bioenergy feedstock that could significantly benefit from a protoplast screening system, switchgrass (*Panicum virgatum* L.) was chosen for further study.

Previous research has demonstrated the economic viability of switchgrass as both an agricultural and biofuel crop (McLaughlin and Kszos 2005). Unfortunately, a major

economic barrier to the broad use of switchgrass as a lignocellulosic feedstock is the recalcitrance of cell walls to digestion. In order to reduce recalcitrance, numerous studies have focused on the generation of transgenic plants with altered lignin and cell wall bound phenolics(Fu et al. 2011; Ragauskas et al. 2014; Shen et al. 2012; Shen et al. 2013). In addition, since switchgrass is a non-model crop, it has been necessary to identify promoters that can effectively regulate the expression of transgenes in switchgrass (Mann et al. 2011; Mann et al. 2012a). While some success has been attained in the generation of transgenic switchgrass with altered cell wall architecture, the current path from identification of target genes and promoters, through callus transformation, followed by phenotypic characterization in the greenhouse is extremely laborious and slow (Burris et al. 2009; Li and Qu 2011). While previous research has attempted to utilize switchgrass protoplasts for transient screening, the procedure was cost prohibitive, slow, and not very efficient (Mazarei et al. 2008). Considering the importance for rapid screening of promoter efficiency, genome-editing and silencing targets, and gene expression in switchgrass, the development of a rapid, low-cost protoplast isolation and transformation system was the primary objective of this work.

Materials and methods

Plant material

Panicum virgatum cv. Alamo seeds were obtained from Bemert Seed (Muleshoe, Texas, USA). For initial optimization, Alamo seeds were planted at an approximate density of 20 mg/cm^2 in Fafard 3B soil mix (Sun Gro Horticulture, Agawam, Massachusetts, USA), and grown with a 16 h light, 4 h dark cycle at 22 °C to generate lawns of switchgrass

plants in flats. For initial harvests, the plants were grown for 2 weeks, and then the leaves were cut with a scalpel to approximately 1.5 cm above the soil and used for protoplast isolation (see **Fig. 1**). For time-course experiments, each flat was divided into four quadrants in which tissue was harvested from each quadrant at 8, 14, 22, and 29 days after planting (**Fig. 1**). Regrowth was assessed 7, 14, 21, and 28 days following initial harvest.

Panicum virgatum cv. Alamo genotype ST1 cell suspension cultures were established from node culture as described previously (Alexandrova et al. 1996) and maintained in KM8 medium (Kao and Michayluk modified basal medium, Phytotechnology Laboratories, Overland Park, Kansas, USA) with the addition of 20 % sucrose, 10 % glucose, 0.025 % fructose, 0.025 % sorbitol, 0.025 % mannitol, 0.2 mg/L zeatin, 1 mg/L NAA, 0.1 mg/ L 2,4-D (Kao and Michayluk 1975). Suspension cultures were incubated in the dark at 30 °C on a rotary shaker at 105 rpm. Liquid cell suspension cultures were subcultured every 5–7 days and callus cultures were subcultured monthly. Five days after subculture, ST1 cell suspensions were used to produce protoplasts.

Protoplast isolation

Isolation of protoplasts from leaf tissue was adopted from the procedure described for *Arabidopsis thaliana* (Sheen 2001) with several modifications. Leaf protoplasts were isolated from mesophyll tissue in a buffer solution (0.6 M mannitol, 10 mM MES, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 0.1 % BSA, pH 5) containing food-grade enzymes at the manufacturer's suggested concentrations (Rohament CL 1320 ECU, Rohapect 10L 840 ADJU, and Rohapect UF 0.0065 ADJU) (AB Enzymes, Darmstadt, Germany) and

filtered through a 0.22 µm syringe filter (Millipore Express PES Membrane, Merk Millipore Ltd, Tullagreen, Carrigtwohill Co. Cork, Ireland). Leaf tissue was harvested from each quadrant at 8, 14, 22, and 29 days after planting (**Fig. 1**), cut into 2 mm strips in a Petri dish and weighed. Additionally, regrowth was assessed at 7, 14, 21, and 28 days following the initial harvest to determine whether the switchgrass lawn system could be used repeatedly over time without a decrease in the protoplast yield. Cut leaf tissue was added to the enzyme buffer solution (ca. 200 mg tissue/10 mL solution) and incubated with shaking at 80 rpm for 30 min to 24 h, at 28, 37, or 55 °C (maximum optimal temperature of food-grade enzymes was 60 °C) with or without protection from ambient light. Following incubation, tissue and buffer mixture was filtered through a 40 µm filter (Fisherbrand, Fisher Scientific, Hampton, New Hampshire, USA). Five milliliters of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) was then passed through the same filter to dilute the enzyme solution and maximize protoplast collection. Protoplasts were collected and the enzyme solution was removed using centrifugation at 150×*g*, 22 °C for 10 min. Protoplasts were then resuspended in W5 solution, enumerated, and viability was assessed using propidium iodide (PI) staining (working solution: 1 mg/ 50 mL, Sigma–Aldrich, St. Louis, Missouri, USA). Protoplasts were placed on ice following isolation and prior to transfection.

Protoplasts were obtained from cell suspension cultures using similar methods as those for leaf mesophyll protoplasts. Twenty milliliters of a 5 or 8-day-old ST1 suspension culture was removed from a 200 mL culture and cells were allowed to settle for approximately 15 min. Most of the medium was removed from the cell suspension

and approximately 10 % of the initial volume remained as cells. Twenty milliliters of buffer solution containing food-grade enzymes (Rohament CL 7920 ECU, Rohapect 10L 5040 ADJU, and Rohapect UF 0.039 ADJU) (AB Enzymes, Darmstadt, Germany) was added to the remaining cells (ca. 2 mL cells/20 mL solution) and incubated for 2 h at 30 °C. Following incubation, cells and buffer mixture were filtered through a 40 µm filter (Fisherbrand, Fisher Scientific, Hampton, New Hampshire, USA). Twenty milliliters of W5 solution was then added to the tube containing cells, mixed by inverting and passed through the same filter to dilute the enzyme solution and maximize protoplast collection. Protoplasts were collected and the enzyme solution was removed using centrifugation at 150×*g*, 4 °C for 10 min. Protoplasts were then resuspended in W5 solution, enumerated, and viability was assessed using propidium iodide (PI) staining (working solution: 1 mg/50 mL). Protoplasts were placed on ice following isolation and prior to transfection.

Plasmid

The pANIC10A plasmid containing the *pporRFP* orange fluorescent reporter gene (OFP) under the control of the PvUbi1+3 switchgrass constitutive promoter was used in this study (Mann et al. 2011). To create a plasmid that could be readily isolated from standard *Escherichia coli*, the *mGFP5-ER* gene was inserted in reverse orientation using Gateway cloning, to remove the ccdB cassette, to generate the 16 kb pANIC10A GFPuv stuffer plasmid which was used for all transfection experiments. This plasmid was propagated in *E. coli* and purified using a ZymoPURE Giga Prep kit (Zymo Research, Irvine, CA).

PEG-mediated transfection

PEG-mediated DNA transfection was performed as previously described (Sheen 2001) with modifications. Protoplasts were resuspended in MMg (0.4 M mannitol, 25–150 mM $MgCl_2$, 4 mM MES (pH 5.7)) at a concentration of 1×10^6 protoplasts/mL (leaf) or 2×10^5 protoplasts/mL (cell suspension). Plasmid DNA (0–40 µg) was mixed with 200 µL of protoplasts (approximately 2×10^5 protoplasts for mesophyll and 4×10^4 protoplasts for cell suspension). Approximately 0–50 % PEG solution (0.6 M mannitol, 100 mM $CaCl_2$, 0–50 % PEG 4000 (Sigma– Aldrich, St. Louis, Missouri, USA)) was added to the protoplasts to a final PEG concentration of approximately 0–25 %. After a 20 min incubation at room temperature, protoplasts were washed twice with approximately 1–4 mL of W5 and collected by centrifugation at 100×*g* for 5 min. Protoplasts were resuspended in 1 mL WI (0.6 M Mannitol, 4 mM KCl, 4 mM MES, pH 5.7), transferred to 12-well Falcon culture plates (Corning Incorporated, Corning, New York, USA) and incubated at 28 °C in the dark for 15–20 h. Microscopic evaluation of expression of the *pporRFP* reporter was conducted using an Olympus IX71 microscope with the Chroma 49004 CY3/TRITC filter set.

Statistical analysis

A completely random experimental design was used for leaf protoplast optimization experiments, growth and regrowth experiments, and transformation experiments, with all containing at least three independent biological and technical replicates. Results were analyzed using mixed model ANOVAs (SAS 9.4, Cary, North Carolina, USA). Least

significant differences (LSD) were used to determine significant differences among means when the ANOVA results were statistically significant ($p < 0.05$).

To calculate viable protoplasts per mg of starting tissue, the following equation was used:

$$\frac{viable\ protoplasts}{mg\ tissue} + \frac{total\ \#\ of\ protoplasts\ x\ \%\ viability}{mg\ starting\ tissue}$$

The number of protoplasts expressing the OFP and the number of protoplasts not expressing OFP were counted using a hemocytometer. To ensure that a statistically significant distribution of protoplasts was counted on the hemocytometer, samples were collected from individual wells and centrifuged at 100×g prior to resuspension in a minimal volume ~100 µL. Using this strategy, an average of 78.9 protoplasts, across all transformation experiments, were counted on each hemocytometer grid. Transformation efficiency was calculated as: $\left(\frac{\#\ of\ protoplasts\ expressing\ OFP}{total\ \#\ of\ protoplasts}\right) x\ 100 =$ $\%\ transformation\ efficiency.$

Results

Optimization of protoplast isolation using food-grade enzymes

Recent research has demonstrated that the food-grade cell wall degrading enzymes Rohament CL, Rohament PL, and Rohapect UF may provide a low-cost alternative to labgrade enzymes for protoplast isolation (Buntru et al. 2014; Buntru et al. 2015). To test this hypothesis, isolation of protoplasts from switchgrass leaf tissue was tested using Rohament CL, Rohapect 10L, and Rohapect UF. At concentrations of 1320 ECU (Rohment CL), 840 ADJU (Rohapect 10L), and 0.0065 ADJU (Rohapect UF), >1.6 g of 2-week old leaf tissue could be digested without a loss in the protoplast yield per mg of

tissue (**Fig. 2**). Based on this data, a trend line was fit to the dataset (R^2 = 0.94) to obtain the protoplastation efficiency of 8.4×10^5 protoplasts per gram of tissue. In order to optimize the method of protoplast isolation using these enzymes, the temperature of the digestion was analyzed, along with digestion in either light or dark conditions (**Fig. 3**). It was determined that digestion at 37 °C was optimal for both light and dark conditions ($p < 0.05$), with a maximum protoplast yield of 1702 ± 50 viable protoplasts per mg of tissue in the light and 1375 ± 62 viable protoplasts per mg of tissue in the dark. Surprisingly, at 37 °C, there was a significant increase in protoplast yield with incubation in the light, compared to the dark conditions ($p < 0.05$). At both 28 and 55 °C, there was no significant difference between the light and dark treatments; however, incubation at 55 °C resulted in a decrease in viability leading to less than 200 viable protoplasts per mg of tissue, a > 9-fold decrease compared to the 37 °C treatment (**Fig. 3**). To further optimize the procedure, the duration of digestion was tested over 24 h to identify the time required to maximize the yield of viable protoplasts. From these results, it was determined that the maximum number of viable protoplasts per mg of tissue (2424 ± 56) was recovered after digestion for 180 min ($p < 0.05$) (**Fig. 4**). While there was a slight reduction of 7.7 % in the number of viable protoplasts per mg of tissue at 240 min, digestion at >240 min and <180 min resulted in less than half of the maximum yield (**Fig. 4**). It should be noted that since the yield has been converted to the number of viable protoplasts per mg of tissue, at <180 min there are less total protoplasts due to incomplete digestion, whereas at >240 min there is a decrease in viability but not total protoplasts. Based on the results from these experiments, it was determined that the optimum protoplast isolation procedure

with Rohament CL, Rohapect 10L, and Rohapect UF for switchgrass was a 180 min digestion at 37 °C in the light.

Analysis of a renewable source for switchgrass leaf tissue

The need for a renewable supply of tissue with a limited footprint, i.e., without the need for greenhouse space, was a consideration of this work. As such, switchgrass ‘‘lawns’’ were established for the generation of leaf tissue for protoplast isolation (**Fig. 1**). Harvesting of tissue at weekly intervals showed a gradual decrease in the protoplast yield over a 4-week period, with a maximum (2230 ± 204 viable protoplasts per mg of tissue) at 8 days after initial planting (**Fig. 5a**). After 14–22 days, approximately a 33 % reduction in yield was observed, with a reduction of 72 % after 29 days. After identifying the ideal time for first harvest, to test the sustainability of the lawn system, the yield of protoplasts from re-growth after the initial harvest was also examined. After re-growth for 14 days, the yield of protoplasts was similar to the initial harvest at 14–22 days (1560 ± 758 viable protoplasts per mg of tissue) (**Fig. 5b**). While the maximum protoplast yield from the re-growth was achieved at 21 days (2480 ± 363 viable protoplasts per mg of tissue), there was no significant difference in yield from 7 days (**Fig. 5b**). The lack of significance in the yield for the re-growth data is most likely due to difficulty in manually cutting at the same level in the initial harvest. However, even at the minimal yield attained in the re-growth study (1010 ± 87 viable protoplasts per mg of tissue), the level was not significantly different from the initial 22 day harvest (1270 ± 117 viable protoplasts per mg of tissue) ($p = 0.09$). Based on this data, the same lawn can be used for multiple harvests, which reduces the need for continuous planting. Further, continued

experiments have determined that repeated cutting/re-growth did not decrease the yield of protoplasts for up to four cycles, extending the sustainability of a single planting to ~ 3.5 months.

Optimization of switchgrass protoplast transformation

Optimization of a transformation protocol for switchgrass mesophyll protoplasts was conducted to study the effects of plasmid concentration, transfection duration, $MgCl_2$ concentration, and PEG 4000 concentration on the transformation efficiency of switchgrass protoplasts. The first variable that was optimized was the amount of pANIC10A GFPuv stuffer plasmid (0–40 µg) required for transformation. The highest transformation efficiency (21.8 ± 2.3 %) was achieved with a DNA concentration of 10 µg, although there was no significant difference between 10 and 20 µg of DNA ($p = 0.34$) (**Fig. 6a**). Surprisingly, at a concentration of 40 µg, transfection efficiency decreased 2.4 times and was not significantly different from the reactions with 5 µg of DNA ($p = 0.98$) (**Fig. 6a**). The second variable that was optimized was the duration of the transfection procedure. Based on the results from these experiments, there was no significant difference in the transformation efficiency from 10 to 40 min ($p > 0.05$); however, after 60 min, the transformation efficiency was reduced by 1.8 times compared to the shorter duration reactions ($p = 0.006$) (**Fig. 6b**). Similar to the results for the reaction duration, at initial PEG 4000 concentrations of 20–50 %, there was no significant difference in the transformation efficiency (21.8 ± 8.4 %, $p > 0.05$) (**Fig. 6c**). However, below a concentration of 20 % PEG 4000 no transformation was observed, identifying this concentration as the minimal PEG 4000 required to achieve transformation of

switchgrass protoplasts (**Fig. 6c**). While duration of the reaction and PEG 4000 concentration had little effect on increasing the transformation efficiency, a significant increase was observed when the $MgCl_2$ concentration was increased from 25 to 100–125 mM ($p < 0.05$) (**Fig. 6d**). A maximum transformation efficiency (30.4 ± 2.5 %) was observed at 125 mM and was 1.65 times greater than $MgCl_2$ concentrations ranging from 25 to 75 and 150 mM (18.4 ± 4.2 %) (**Fig. 6d**). Based on the data obtained for optimization of transformation in switchgrass protoplasts, the optimal method was found to be incubation of 10 µg of plasmid for 10–40 min with an initial PEG 4000 concentration of 20–50 % and a $MgCl_2$ concentration of 100–125 mM. Using this method, a maximum transformation efficiency of 30.4 % was attained from switchgrass mesophyll protoplasts.

Isolation and transformation of cell culture-derived protoplasts

Since cell suspensions have proven to provide a constant source of sterile, rapidly growing cells, capable of generating protoplasts in other systems (Doelling and Pikaard 1993; Wang et al. 2015), a switchgrass cell culture system for generation of protoplasts was developed. Switchgrass cell suspension cultures were established from callus of the clonal Alamo ST1 cultivar following previously established methods (Gupta and Conger 1999) with several variations. Briefly, after initiation of callus on LP9 media (Burris et al. 2009), callus was transferred to liquid KM8 media and axenic cultures were allowed to establish for a period of 1 month, followed by subculturing every 5–7 days thereafter. ST1 cell suspension cultures were comprised of large aggregated cells (**Fig. 7a, b**), and displayed rapid growth, ideal for protoplast harvesting. After establishing the cultures,

isolation of protoplasts from the cell suspensions were attempted using the optimized method for leaf mesophyll protoplast isolation described above. Unfortunately, the mesophyll protocol failed to release protoplasts from the cell culture, leaving predominately intact cells. Therefore, the enzyme concentrations were increased sixfold, similar to previous work on cell culture protoplasts (Mazarei et al. 2011), to 7920 ECU for Rohament CL, 0.039 ADJU for Rohapect UF, and 5040 ADJU for Rohapect 10L. Results from digestion with the elevated enzyme concentrations found that $3.14 \times 10^5 \pm 3.35 \times 10^4$ viable protoplasts could be harvested from a packed cell volume (PCV) of 3 mL, with no significant difference between isolation at 28 and 37 °C ($p = 0.94$). The protoplastation efficiency of the suspension cultures was 9.6×10^5 protoplasts per gram of cells, as determined by the weight of a 3 mL PCV after filtration through a 3 µm mesh to remove excess water. Unlike the difference in protoplast isolation methods between the mesophyll and cell culture-derived protoplasts, the optimized transfection protocol was significantly more efficient with the cell culture-derived protoplasts isolated at 28 °C, with an efficiency of 46.4 ± 3.3 % ($p < 0.05$) (**Fig. 7c–e**). Surprisingly, there was a significant reduction ($p < 0.05$) in the transformation efficiency of cell culture-derived protoplasts (25.4 ± 3.3 %) isolated at 37 °C.

Discussion

Traditionally, protoplast isolation from plants and fungi use highly purified lab-grade cell wall-digesting enzymes, with many protocols specifying a vendor to ensure success of a procedure (Yoo et al. 2007). Often lab-grade enzymes for protoplast isolation are very costly with the enzyme cost often prohibitive to high-throughput research. For example,

based on the previous methodology for switchgrass protoplast isolation (Mazarei et al. 2008) from approximately 130 mg of leaf tissue, the cost per reaction was $11.59 for the enzymes alone. Considering that each reaction generated ~8×10^5 protoplasts, a maximum of four transfection experiments (typically 2×10^5 protoplasts are used for transformation) could be conducted per reaction, with a cost per transfection of $2.89 for the enzymes alone. Recent research has demonstrated that the use of the low-cost food-grade enzymes, Rohament CL, Rohament PL, and Rohapect UF provides a significant reduction to the cost of protoplast isolation for the Bright Yellow 2 (BY-2) tobacco cell culture line (Buntru et al. 2014; Buntru et al. 2015). In this system, Rohament CL provides the cellulase activity, Rohament PL provides the pectinase activity, and Rohapect UF supplements the other enzymes with specialized pectinases and arabinases (Buntru et al. 2014). Since food-grade enzymes have successfully been used to isolate protoplasts from tobacco, with significantly reduced costs, similar food-grade enzymes (Rohament CL, Rohapect 10L, and Rohapect UF) were tested in this work for their ability to release protoplasts from switchgrass leaves. Using these enzymes, it was possible to reduce the cost of mesophyll protoplast isolation to <$0.01 per reaction (based on current pricing from AB Enzymes), a greater than 1000-fold decrease compared to previous methods. Further, the concentration of enzymes used were able to digest >1.6 g of tissue (**Fig. 2**), releasing ~1.5×10^6 protoplasts per reaction, nearly doubling the yield of mesophyll protoplasts compared to previous methods. The development of a low-cost protoplast isolation system represents an important step in realizing high-throughput screening of transgene expression and promoters in switchgrass; however, to realize this

goal, a reliable transformation system is required. While callus-based *Agrobacterium* tumefaciens-mediated transformation is standard for plant transformation, including switchgrass (Burris et al. 2009; Li and Qu 2011), this method has many disadvantages, including regulatory restrictions (Garrett 1987; Jaffe 2004), limited control of insertion rates resulting in variation in transgene insertion and expression (Hobbs et al. 1993), and potential recovery of chimeric plants (Domínguez et al. 2004). Specifically for switchgrass, *Agrobacterium*-based transformation efficiency is inconsistent and can depend upon genotype, callus type, and callus age (Burris et al. 2009; Li and Qu 2011) Additionally, a high frequency of false positives, up to 30 %, has been reported from callus transformation of switchgrass (Ogawa et al. 2014; Somleva et al. 2002). Since protoplasts are devoid of cell walls, a necessary attachment point for *Agrobacterium*, protoplasts cannot be transformed via *Agrobacterium*. However, the lack of a cell wall opens the door for non-*Agrobacterium*-based transformation protocols, which are routinely used in mammalian systems. Previous studies have used electroporation- (Fromm et al. 1985; Negrutiu et al. 1987), polyethylene glycol (PEG)- (Armstrong et al. 1990; Negrutiu et al. 1987), nanoparticle- (Silva et al. 2010), and lipofection- (Felgner et al. 1987) mediated transformation of plant protoplasts with varying success. Specifically, previous work on switchgrass protoplasts used PEG-mediated transformation with 40 µg of a 5.6 kb plasmid, and achieved very low efficiency transformation (Mazarei et al. 2008). Similar to the high enzyme cost, 40 µg of plasmid DNA per reaction represents a significant hurdle to highthroughput screening of protoplasts, and will discourage many labs from utilizing this protoplast system. Therefore, optimization of a transformation

protocol for switchgrass mesophyll protoplasts was conducted to study the effects of plasmid concentration, $MgCl_2$ concentration, PEG 4000 concentration, and transfection duration on transformation efficiency.

As a ‘‘worst-case’’ scenario the 16 kb pANIC10A GFPuv stuffer plasmid was chosen for evaluation of transformation efficiency. A large plasmid would likely be necessary for CRISPR genome-editing studies, or more complex multi-gene expression studies. Typically, smaller plasmids in the 5 kb range are used for PEG-mediated transformation (Mazarei et al. 2008; Sheen 2001), which may bias the efficiency reported towards these simpler systems. Based on the results obtained from the optimization experiments, a fourfold reduction in the DNA content increased the switchgrass protoplast transfection efficiency by twofold, over the previous methodology (Mazarei et al. 2008). Compared to grape and maize protoplasts, the DNA content required for optimal transformation efficiency in switchgrass was two to tenfold lower, respectively (Cao et al. 2014; Wang et al. 2015). Previous research has noted that decreasing DNA titer often reduces labor and material costs, while potentially increasing efficiency of protoplast transformation (Armstrong et al. 1990; Damm et al. 1989; Maas and Werr 1989). Unlike the increased transformation efficiency observed with a reduction in DNA content, the concentration of PEG 4000 in the reaction mixture had little effect on the efficiency of transformation. Whereas in previous protoplast systems where lower transformation efficiencies have been observed when PEG 4000 surpasses 25 % (Masani et al. 2014), due to toxicity of PEG itself, no PEG toxicity was observed with switchgrass protoplasts even with the highest levels tested. Not surprisingly, the most significant

increase in transformation efficiency was achieved by increasing the $MgCl_2$ concentration from 15 to 100–125 mM. Previous studies have demonstrated that $MgCl_2$ concentration contributes significantly to the efficiency of PEG-mediated transient gene expression in tobacco (Negrutiu et al. 1987), maize (Armstrong et al. 1990) and oil palm protoplasts (Masani et al. 2014). Through optimization of the transfection procedure, it was possible to increase protoplast transformation efficiency from 9.1 to 30.4 %, while also reducing the quantity of DNA by fourfold.

In addition to the differences in the transformation efficiency between the mesophyll and cell culture-derived protoplasts, several other considerations were made when analyzing transformed protoplasts from each source. First, the average fluorescent intensity of the cell culture-derived protoplasts was greater than the mesophyll protoplasts. Since quantitative data was not obtained for fluorescence, this observation was made by using the same exposure setting for analyzing transgenic protoplasts from each source. This increased intensity may be due to higher metabolic activity and more rapid growth in the cell culture protoplasts, or may also be due to the more consistent protoplast size. In general, protoplasts isolated from leaves had a wider size distribution than protoplasts isolated from the cell culture, which is not surprising due to the more consistent environment of a cell culture. Second, the mesophyll protoplasts had numerous chloroplasts present in the cell, while the cell culture protoplasts (grown in the dark) were devoid of chloroplasts. The presence of chloroplasts in isolated protoplasts was a factor in the choice of a fluorescent reporter, and led to the selection of *pporRFP*, which has an excitation maximum at 578 nm and emission maximum at 595 nm (Mann et al. 2012b).

The use of *pporRFP* allowed selection of a filter set (Excitation 545/25x, Longpass 565, and Emission 605/70) that cut-off chlorophyll autofluorescence, while still allowing imaging of the marker. The combination of *pporRFP* with the filter set chosen for this work allowed imaging of transgenic protoplasts from both the cell cultures and leaf tissue, without any observable autofluorescence (**Fig. 7c– e**). It should also be noted that if mesophyll protoplasts were examined using a traditional Texas Red filter set, the chlorophyll autofluorescence dominated and prevented analysis of the *pporRFP* marker. Finally, as anticipated, transformed mesophyll protoplasts could only be screened for ~36 h before bacterial and fungal contamination dominated the cultures and killed the protoplasts. While antibiotics could be added to the protoplast isolation media to reduce contamination, this was not attempted in this work. Similarly, growth of aseptic seeds on agar in a sterile environment could be achieved, but would add additional costs and labor, and thus was not conducted in this study. Unlike the mesophyll protoplasts, the cell suspensionderived protoplasts could be maintained in soft agar cultures for up to 21 days (maximum duration tested) without contamination or a loss in expression of fluorescent marker. Despite the long duration of these cultures, no cell division or regeneration was observed; however, cytoplasmic streaming was evident throughout. Based on these comparisons, either system may function in rapid screening applications; however, for longer-term studies, the use of cell culture-derived protoplasts has a distinct advantage.

High efficiency transformation is essential for rapid screening, as typical reactions contain 2×10^5 protoplasts, and the previous transformation efficiency (9.1 %) would generate 1.8×10^4 OFP expressing protoplasts, below the level of detection of most plate

readers. The increase in transfection efficiency demonstrated in this work (30.4 %) would result in 6.1×10^4 OFP expressing protoplasts, within the range of standard plate readers. In addition, the reduction in DNA content to 10 µg will further reduce the cost of the entire procedure, and considering that a 16 kb plasmid was used for optimization, higher transformation efficiencies would be expected with smaller plasmids. Similarly, to achieve similar transformation efficiencies with an 8 kb plasmid would require half the DNA content as a 16 kb plasmid, as two times the number of individual plasmids would be present per reaction. The broader impact of a high-throughput protoplast screening system for switchgrass would be the ability to collect data at an earlier stage; therefore, screening out ineffective transgenes/promoters decreasing the number of plants to be recovered. For example, in a CRISPR study targeting recalcitrant genes, screening of gRNA targets in a protoplast system prior to the generation of transgenic plants would allow selection of targets with the highest efficiency of silencing. In this way, poor performing gRNA targets could be removed from the pool of candidates, generating a better chance of success in recovering the desired phenotype in greenhouse and field studies.

While the development of a low-cost mesophyll protoplast isolation system for switchgrass represents a significant improvement over current methodologies in both yield and cost, to obtain axenic protoplasts for long-term studies and potential regeneration, a switchgrass cell culture is necessary. Previous attempts at protoplast isolation from cell cultures in switchgrass were only successful with a single genotype, Alamo 2, and required four times the cellulase, two times the macerozyme, and the

addition of driselase and pectolyase (Mazarei et al. 2011). The use of higher enzyme concentrations and the addition of other enzymes to the digestion increased the cost to >$60 per reaction, making the procedure cost prohibitive. Further, the cell cultures derived from Alamo 2 exhibited different morphologies (sandy, fine, and milky) from the same primary culture with only the milky culture yielding viable protoplasts (Mazarei et al. 2011). In order to develop a cell culture that was more feasible for large-scale protoplast isolation, in this work a cell suspension culture was established using the ST1 cultivar. Unlike the previous work, in which MS-maltose media was used to generate switchgrass callus, the callus used for initiation of the cell cultures was grown on LP9 media with sucrose as the sugar source. LP9 media has a decreased level of 2,4 dichlorophenoxyl-acetic acid (2,4 D; 5 mg L^{-1}), increased proline (500 mg L^{-1}), and no benzyladenine (BAP) or myo-inositol, which has been shown to be more effective for culturing switchgrass callus (Burris et al. 2009). The change in callus initiation and cultivation medium led to a more consistent type of culture, similar to the BY-2 tobacco cell culture (**Fig. 7a, b**) (Nagata et al. 1992). The fine, milky, and sandy types of culture observed for the Alamo 2 derived cultures were not observed in the ST1 suspension cultures established in this work, even after passage for over 6 months.

Unfortunately, application of the optimized mesophyll protoplast isolation procedure to the ST1 suspension cultures was not successful in isolation of the protoplasts. Considering that similar results were observed for Alamo 2 suspension cultures, the concentrations of Rohament CL, Rohapect 10L, and Rohapect UF were increased sixfold to match the cellulase concentrations used for digestion of previous

switchgrass cell cultures. As indicated earlier, at this level, without the addition of driselase or pectolyase, it was possible to obtain 3–4 × 10^5 protoplasts from a packed cell volume of 3 mL. The cost associated with the increased concentrations of the low-cost enzymes was minimal, with an overall cost of $0.018 per reaction. Considering the advantages of axenic switchgrass protoplasts, and the marginal increase in the cost of the reaction, the use of the ST1 switchgrass suspension culture provides an ideal method for rapid, bulk harvesting of switchgrass protoplasts for high-throughput studies.

While the protoplast isolation system developed in this work has utility in high-throughput screening applications, future research will be aimed at examining the potential to regenerate protoplasts isolated using this methodology. It is well established that monocot protoplast regeneration is difficult, with limited success in rice, wheat, and grasses (Dalton 1988; Harris et al. 1988; Kyozuka et al.). Often nurse cultures or a complex series of different media is necessary to initiate regeneration, with the majority of regenerated plants being infertile. Specifically for switchgrass, protoplasts have not previously been regenerated, although suspension cultures have successfully been used to regenerate fertile plants (Gupta and Conger 1999). Of further concern would be impurities in the food-grade enzymes, not present in lab-grade enzymes that may interfere with the process of regeneration. However, if methods for regeneration of these axenic protoplasts could be developed, then it will be possible to extend the procedures developed in this work for the generation of transgenic plants without the need for *Agrobacterium*-mediated transformation. This would represent a fundamental shift in the

generation of transgenic switchgrass, and increase the potential to overcome current limitation of recalcitrance in the cell walls of switchgrass.

Chapter 3 Efficient mesophyll-derived protoplast isolation and transformation of *Panicum hallii* Vasey

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Proposed journal: Plant Cell Reports

My primary contributions to this work were gathering and initiating seeds, conducting protoplast isolation and PEG-mediated transformation experiments, along with protoplast size comparisions and experiment cost-analysis.

Abstract

Protoplasts are appropriate targets for genome editing, DNA functional screens and transgenesis. *Panicum hallii* Vasey (PAH) is a close relative of the polyploid cellulosic feedstock *Panicum virgatum* L. (switchgrass). PAH has great potential as a C4 panicoid model species for crop and bioenergy research. Here we develop an inexpensive PAH mesophyll protoplast isolation and transformation system; the first for this species. We show an average fivefold increase in protoplast yield from PAH leaf tissue over the optimum switchgrass tissue protoplast isolation with PAH yielding an average 7340 viable protoplasts per mg mesophyll tissue and switchgrass yielding 1468 viable protoplasts per mg mesophyll tissue with both species having greater than 95% viable protoplasts. PAH mesophyll protoplasts have a diameter from 3.9- 28.1 µm, with a mean of 13.5 µm, which are significantly smaller than switchgrass mesophyll protoplasts which range from 6.5- 39.4 µm with a mean of 17.4 µm. This system shows a further reduced cost compared to previous methods to $0.001 (USD). Polyethylene glycol (PEG)-mediated transformation of PAH protoplasts revealed an optimum transformation efficiency of 46.7% with switchgrass protoplast transformation efficiency of 9.3%. The

methods in this paper provide an essential step toward using *P. hallii* as a C4 panicoid model species.

Introduction

Despite its potential as a cellulosic bioenergy feedstock, *Panicum virgatum* L. (switchgrass), like other perennial grasses is recalcitrant to conversion to biofuel (Himmel et al. 2007). Switchgrass has over 80,000 genes (1,230 Mb, v. 1.1, Phytozome 11), and conducting *Agrobacterium*-mediated transformation experiments for each gene that contributes to recalcitrance is a herculean task given the various non-model features of the species, such as long seed-to-seed time, large tetraploid genome, and large mature plant stature, in addition to inordinate time (>6 mo) for regeneration of transformed plants (Burris et al. 2009). Since switchgrass is an obligate outcrossing polyploid species, there are additional consideration for analyzing T1 plants as well as performing genome analysis (Bouton 2007). A facile C4 grass model would be a beneficial research tool for both bioenergy grasses as well as C4 grain crops.

Panicum hallii Vasey is a small, diploid, perennial C4 grass with two recognized ecotypes, var. ‘hallii’ (PAHAH) and var. ‘filipes’ (PAHAF). Both ecotypes are shorter in stature than switchgrass (**Fig. 8**). Unlike switchgrass, PAH has model plant characteristics including self-fertilization (selfing) (mean F_{IS}=0.895), short flowering time (PAHAF mean days until flowering=81.65, ‘Alamo’ mean days until flowering >200), and a small, diploid genome (Lowry et al. 2015; Lowry et al. 2013; Meyer et al. 2012; Taliaferro 2002). As most model plants, PAH provides more ease in genetic

research compared to switchgrass, as there are only two sets of chromosomes (2n = 2x = 18); the latter exists as a tetraploid or octaploid (Gould 1958). PAHAF has approximately 50,000 protein-coding transcripts and a sequenced genome (554 Mb, v. 2.0, Phytozome 11), whereas PAHAH has not been sequenced. PAHAF grows in mesic conditions, has a longer flowering time and smaller seeds when compared to PAHAH, and is intermediate in size between PAHAH and switchgrass (Lowry et al. 2013; Waller 1976) (**Fig. 8)**. This phenotypic pattern of larger plants growing in areas with more water availability is seen in switchgrass as well between the upland and lowland varieties, with the lowland varieties, such as *P. virgatum* cv. 'Alamo', growing larger in riparian areas than the upland varieties (Porter 1966; Zalapa et al. 2011; Zhang et al. 2011).

Mesophyll protoplasts have been proven to be a highly useful tool for transient gene expression via polyethylene glycol (PEG)-mediated transformation in many different plant species (Cao et al. 2014; Jeon et al. 2007; Sheen 2001; Yoo et al. 2007). Mesophyll protoplasts have been isolated from switchgrass previously, both with expensive lab-grade enzymes and more cost-effective food-grade enzymes (Burris et al. 2015; Mazarei et al. 2008). For high-throughput transient gene screening, protoplasts must be transformed at a high efficiency and with detectable expression of a reporter gene. Mesophyll protoplasts of the lowland switchgrass cultivar 'Alamo' were optimized with PEG-mediated transformation to an efficiency of ~30%, with a large 16 kb vector (Burris et al. 2015). The goal of the present study was to produce a suitable switchgrass model transformation system by way of PAHAF protoplasts.

Materials and methods

Plant material

P. hallii var. filipes (Scribn.) Waller (PAHAF) seeds from an inbred population received by Dr. Thomas E. Juenger of The University of Texas at Austin, hereafter called FIL2, were gathered from greenhouse plants grown under a 16 hour light cycle and 4 hour dark cycle at approximately 26°C (Meyer et al. 2012). *P. virgatum* (switchgrass) cv. Alamo seeds were acquired from Bamert Seed (Muleshoe, Texas, USA). Both 'Alamo' and FIL2 seeds were planted in Farfard 3B mix and grown with a 16 hour light, 4 hour dark cycle at 22°C with irradiance cool white fluorescent lights in a growth room. Above ground biomass was harvested at 7, 14, 21, 28, and 35 days after planting and used for protoplast isolation.

Protoplast isolation

Protoplast isolation from switchgrass tissue has been described previously (Burris et al. 2015). Protoplasts were isolated from mesophyll tissue in buffer solution (0.6 M mannitol, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 0.1% bovine serine albumin (BSA), pH 5) with the food-grade enzymes Rohament CL, Rohapect 10L, and Rohapect UF (AB Enzymes, Darmstadt, Germany). Enzyme concentrations were optimized using 'Alamo' tissue, with Rohament CL increasing over a 5-fold gradient (0-51562.5 endocellulase units (ECU)) and Rohapect 10L and Rohapect UF over a 10-fold gradient (0-70000 and 0-1.91 apple depectinase juice units (ADJU) respectively). Leaf tissue was harvested, cut into 2 mm strips, weighed and added to enzyme-buffer solution with 200 mg tissue per 10 ml

solution. The tissue and buffer mixture was incubated at 37°C on a shaker at 80 rpm in a clear Falcon tube (Fisherbrand, Fisher Scientific, Hampton, New Hampshire, USA) for 3 hours. After incubation, tissue and buffer were then passed through a 40 µm Falcon cell strainer (Fisherbrand, Fisher Scientific), and 5 ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) was passed through the same strainer to increase protoplast recovery. Recovered protoplasts and buffer were centrifuged at 150×*g* at 4°C for 10 minutes, enzyme mixture was removed, and protoplasts were resuspended in W5 solution for viability assessment using propidium iodide (PI) staining (working solution: 1 mg/50 mL). Protoplasts that were to be used for transformation were kept on ice prior to use.

Size distribution

After isolation, protoplasts were filtered through a 100 µm filter to ensure that a wide range of sizes of protoplasts would be obtained, without including large undigested cells. Micrographs of protoplasts were obtained using an Olympus IX71 light microscope, then the diameter of the protoplasts was calculated, using a calibration slide to convert pixels to µm with Fiji ImageJ software (Schindelin et al. 2012). Box plots were then created from 200 random protoplasts using the median, first and third quartiles, and the range of the size of protoplasts.

PEG transformation

PEG-mediated transformation has been described previously for *P. virgatum* and the same plasmid DNA was used for this study, pANIC10A GFPuv stuffer, that contains orange fluorescent protein gene *pporRFP* driven by the *PvUbi1+3* promoter (Burris et al.

2015). First, protoplasts were resuspended in Mmg solution (0.4 M mannitol, 110 mM $MgCl_2$, 4 mM MES, pH 5.7). Next, plasmid DNA (10 µg) was mixed with approximately 2 x 10^5 protoplasts and an equal volume of 40% PEG solution (0.6 M mannitol, 100 mM $CaCl_2$, 40% PEG 4000), for a final PEG concentration of 20%, and incubated for 20 min at 22ºC. Finally, protoplasts were washed once with 1 mL of W5 and then again with 5 mL W5, then resuspended in 1 mL WI (0.6 M mannitol, 4 mM KCl, 4 mM MES, pH 5.7). Protoplasts in WI were moved to 12-well Falcon culture plates and incubated at 28°C for 20 hours prior to screening for orange fluorescent protein (OFP) expression using an Olympus IX71 microscope with the Chroma 49004 CY3/TRITC filter set. (Excitation 545/25x, Longpass 565, and Emission 605/70).

Statistical analysis

A completely random experimental design was used for enzyme optimization, growth and transformation experiments, with each having 3 biological replicates of independent isolations and 2 technical replicates of independent counts of each isolation on the hemocytometer. Results were analyzed using a mixed model ANOVA (SAS 9.4, Cary, North Carolina). Tukey's honest significant difference (HSD) was used to determine significant differences among the means ($p<0.05$). Further t-tests were calculated within the same time point to determine significant differences among the means ($p<0.05$). Viable protoplasts per mg of starting tissue were calculated as follows:

$\frac{viable\ protoplasts}{mg\ tissue} = \frac{total\ number\ of\ protoplasts - number\ of\ dead\ protoplasts}{mg\ starting\ tissue}$. The percent difference in protoplast yield was calculated as:

$\frac{\text{viable protoplasts per mg tissue PAHAF} - \text{viable protoplasts per mg tissue Alamo}}{\text{viable protoplasts per mg tissue PAHAF}} \text{ x } 100.$

Transformation efficiency was calculated using

$$\left(\frac{number\ of\ protoplasts\ expressing\ OFP}{total\ number\ of\ protoplasts}\right) x\ 100 = percent\ transformation\ efficiency.$$

Results

The optimum concentration of the cellulase component, Rohament CL, for protoplast extraction was found to be 2062.5 ECU (**Fig. 9a**). For both Rohapect 10L and Rohapect UF, when using this optimum Rohament CL concentration, there was no significant difference ($p<0.05$) between omitting each respective enzyme and any of the tested concentrations (**Fig. 9b, c**). Using only Rohament CL at 2062.5 ECU, PAHAF had significantly higher protoplast yield than *P. virgatum* at 7, 14, and 21 days after planting (**Fig. 9d**). After 21 days, there was no significant difference in protoplast yield between PAHAF and *P. virgatum* (**Fig. 9d**). The highest yield of PAHAF protoplasts was between 7 and 14 days after planting, in which PAHAF produced 4.32 times more viable protoplasts per mg tissue (10,557 ± 1,381 viable protoplasts/mg tissue) compared to *P. virgatum* (2,445 ± 258 viable protoplasts/mg tissue) (**Fig. 9d**). Average viability of PAHAF protoplasts was 97.73 ± 1.06% and average viability of 'Alamo' protoplasts was 96.44 ± 0.74% under optimized isolation conditions. Qualitative data based on visual observation of the growth over time of lawns of 'Alamo' and PAHAF of three flats showed that 'Alamo' lawns tended to grow taller than PAHAF, and PAHAF grows more densely (**Fig. 10**). Further, the morphology of mesophyll cells qualitatively appear to differ between 7 day old leaves of PAHAF and 'Alamo' (**Fig. 11**). Protoplasts isolated from PAHAF ranged from 3.90- 28.11 µm, with a mean of 13.47 µm (**Fig. 12 a, b**). Protoplasts isolated from 'Alamo' tissue ranged from 6.53- 39.45 µm with a mean of

17.34 µm (**Fig. 12 c, d**). The mean size of PAHAF protoplasts was significantly smaller than the mean size of ‘Alamo’ protoplasts ($p<0.0001$).

After determining the optimal conditions for protoplast extraction from PAHAF, the PEG-mediated transformation efficiency of PAHAF was compared to P. virgatum. PAHAF had higher transformation efficiency when compared to ‘Alamo’ (**Fig. 13**). At 7 days of growth, PAHAF had a transformation efficiency of 39.90 ± 2.37% that was not significantly different than transformation efficiency at 14 or 21 days of growth (39.94 ± 4.08 and 46.68 ± 5.51%, respectively) (**Fig. 13**). For 7, 14, 21 and 28 days of growth, PAHAF had higher transformation efficiency than ‘Alamo’ at the same time point ($p<0.05$) (**Fig. 13**).

Discussion

Previous research on the use of food-grade enzymes, Rohament CL, Rohapect 10L, and Rohapect UF, for cell wall degradation focused mainly on the procedure of protoplast isolation, not the enzyme concentration (Burris et al. 2015). Optimizing enzyme concentration could further reduce the cost of isolation when compared to when lab-grade enzymes are used (Buntru et al. 2014; Buntru et al. 2015; Burris et al. 2015). Results from enzyme optimization indicate that Rohapect 10L and Rohapect UF can be left out of the leaf tissue digestion entirely, reducing the cost of the previous method to $0.00151 (current AB Enzymes pricing) (Burris et al. 2015). Rohament CL, under the old name Econase (AB Enzymes, Darmstradt, Germany), has been shown to have peptidase activity as well as xylanase activity (Treimo et al. 2009). Plants such as grasses have been seen to contain more D-xylose in their cell wall than other angiosperms (Popper

2008), the xylanase activity that exists in the Rohament CL must help to completely digest the cell wall of both of these species (Treimo et al. 2009). Further, the cell walls of grasses have been seen to have significantly less pectins than dicots, so the pectinase activity provided by Rohapect 10L and UF is secondary to the cellulase and xylanase activity provided from just Rohament CL (Smith and Harris 1999; Vogel 2008).

Model systems are often chosen for studies because they outperform a species of interest in certain aspects of *in-vitro* experiments (Barker et al. 1990; Meinke et al. 1998). In order to begin to validate PAHAF as a model system for mesophyll protoplast isolation, protoplasts were isolated from both PAHAF and switchgrass tissue at 7, 14, 21, 28, and 35 days using the optimum concentration of 2062.5 ECU (Rohament CL) and PAHAF was shown to yield more viable protoplasts/mg starting tissue for all time points. Frequently, plants that grow in dry habitats display accelerated growth when compared to habitats with more readily available water to avoid devastating drought at important points in a plants life history (Franks 2011; Ludlow 1989). Since PAHAF is adapted to the mesic conditions of the southwestern US, it displays over twofold more rapid progression to flowering than switchgrass (PAHAF mean days =81.65, ‘Alamo’ mean days >200) (Lowry et al. 2013; Taliaferro 2002). Preliminary data gathered for one replication indicated that PAHAF had more tissue mass per cm^2 of soil over 28 days of growth with 254.68 mg/cm^2, while switchgrass accumulated only 164.91 mg/cm^2. In wheat, it has been seen that ploidy level affects the size and, inversely, the number of mesophyll cells in leaves (Pyke et al. 1990). From this information it can be assumed that in diploid PAHAF, there are more mesophyll cells per leaf than in polyploid switchgrass.

More mesophyll cells would allow for more protoplasts to be extracted from the tissue. Also, PAHAF tissue was observed to be easier to slice than switchgrass tissue during all stages of growth. This indicates that the cell wall structure of PAHAF is likely to be slightly different than switchgrass, namely less lignified, leading to better digestion of the cell wall by Rohament CL (Buxton 1990). Additionally, differences in leaf cell structure between species can affect digestibility (Wilson and Hattersley 1989). We can visibly see differences in the cell structures between the two species, so this may a further reason for the increased protoplast yield from PAHAF (**Fig. 11 a, b**).

PEG-mediated transformation of switchgrass cv. Alamo has been previously described (Burris et al. 2015). As mentioned before, high transformation efficiency with a fluorescent protein reporter gene is required for high-throughput transient gene screening. To determine if PAHAF would be an efficient transient model system via PEG-mediated transformation, transformation efficiencies were compared between PAHAF and ‘Alamo’ using a large vector for a “worst-case scenario”. This large vector likely has a detrimental effect on transformation efficiency because of the inherent difficulty of transporting large molecules across the cell membrane (Ahmed et al. 1997; Miao and Jiang 2007). Thus, the transformation efficiency could be improved even further using smaller vectors. Also, the size difference between protoplasts of PAHAF and ‘Alamo’ likely has to do with transformation efficiency as well as seen with the yield. It has been previously noted that larger protoplasts were seen to burst during PEG incubation in barley, and this could contribute to the higher transformation efficiency of the significantly smaller PAHAF protoplasts (Lazzeri et al. 1991).

With all of this new information on *Panicum hallii* var. filipes low-cost mesophyll protoplast isolation and transformation via PEG, there can be further experimentation done using new genome editing techniques. If protoplasts of *Panicum hallii* var. filipes can be edited with these new technologies, it can be implemented as a C4 model species and be used for the improvement of switchgrass as a viable lignocellulosic biofuel crop.

Chapter 4 Conclusions and Recommendations

After the dip in crude oil prices in late 2015-early 2016, some are forgetting that there remains a potential oil shortage crisis with supply and demand from an ever-growing world population. Research must continue with other alternative forms of energy, especially renewable energy. The US Environmental Protection Agency has listed lignocellulosic biomass as one of the ways to obtain biofuels that would reduce greenhouse gas emissions and *Panicum virgatum* L., switchgrass, remains on the list of potential crops that can be used for such a purpose (EPA 2014; Zhong et al. 2016). However, the high recalcitrance of switchgrass makes it still economically unviable, as the processing cost before ethanol extraction remains high. Recently, there have been advances in the bioconversion process that allow for higher sugar liberation from the pretreatment, but this technology could still benefit from reduced recalcitrance in the starting biomass (Frederix et al. 2016).

Research into the genomic design of switchgrass with reduced recalcitrance for use for biofuels continues to be slow. A low-cost, high throughput protoplast system provides a crucial step towards speeding up this research. Protoplasts are often chosen for research to look at protein localization and stability or to understand cellular processes at a single cell level (Cui et al. 2016; Jayaraman et al. 2016; Planchais et al. 2016). However, protoplasts also allow for easy genome editing using various technologies, and crops resulting from these technologies are not regulated as GMO crops like crops arising from *Agrobacterium*–mediated transformation are, as they are not considered to be "genetically modified" in the US, where herbicide resistant canola currently on the market from genome editing of protoplasts (Bortesi and Fischer 2015; Cao et al. 2014;

Gocal 2014; Martinho et al. 2015; Sauer et al. 2016). Thus, the establishment of a protoplast system in switchgrass provides a way to apply genome editing technologies which will allow an improved switchgrass line to be utilized quickly for biofuel use.

Along with the aforementioned advantage of using protoplasts for genome editing, the protoplast system outlined in the previous chapter holds other benefits for researchers. The use of food-grade enzymes instead of the enzymes that are habitually used for protoplast isolation in other plant species reduces the cost over 1000-fold, thus allowing for many more protoplast isolations experiments. This also allows for the budget that would have gone towards purchasing expensive enzymes to be used for other experiments. In addition to reducing cost, the particular food-grade enzymes used in this research (Rohament CL, Rohapect 10L, and Rohapect UF) are delivered in liquid form that is stable at room temperature. This is important as high-throughput robotic systems are becoming desirable in many laboratories, and these food-grade enzymes can be directly hooked to a liquid handling system and protoplast isolations and PEG-mediated transfections can be done with only the initial user input (Dlugosz et al. 2016). Further, the use of mesophyll tissue for protoplast isolation and PEG-mediated transfection bypasses the time and effort required to establish embryogenic cell suspensions, which can often take months of optimization (Mórocz et al. 1990; Taylor et al. 1992). Mesophyll protoplasts have been seen to regenerate whole plants in several species, therefore it may not be necessary to isolate protoplasts from an embryogenic suspension (Bokelmann and Roest 1983; Kartha et al. 1974; Maćkowska et al. 2014; Shepard and Totten 1975).

Finally, this research provides the first protoplast system for *Panicum hallii* Vasey var. filipes (PAHAF), the potential model system for C4 grasses. Of course, the earlier mentioned benefits of this low-cost high-throughput protoplast system apply here, but in addition the PAHAF protoplast system is more efficient than the switchgrass system, which is ideal for a model species.

With switchgrass being a candidate for a lignocellulosic biofuel feedstock, these types of technologies would be useful for greater understanding of the genetic control of recalcitrance and creating an improved transgenic line of switchgrass for biofuel use.

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Appendix

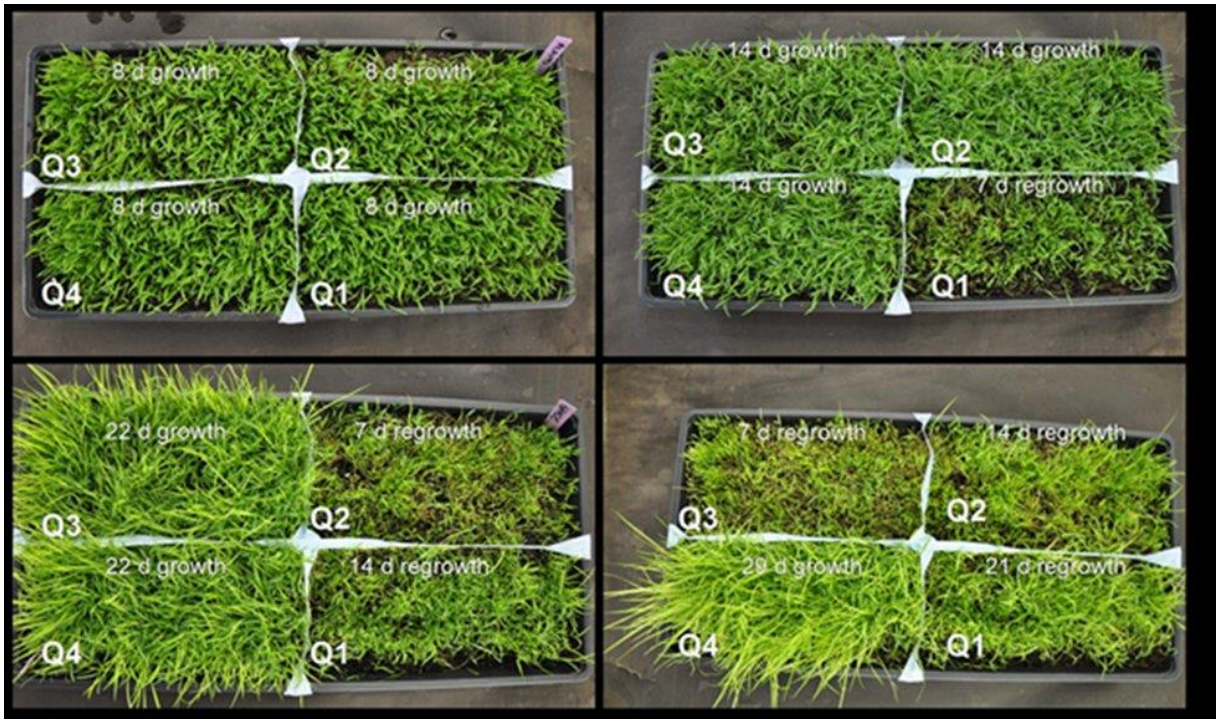

Figure 1. Schematic of switchgrass “lawns” demonstrating stage of growth of leaf tissue when harvested from each quadrant (Q1, Q2, Q3, Q4) at 8, 14, 22, and 29 days after planting and regrowth at 7, 14, 21, and 28 days following complete cutting of tissue.

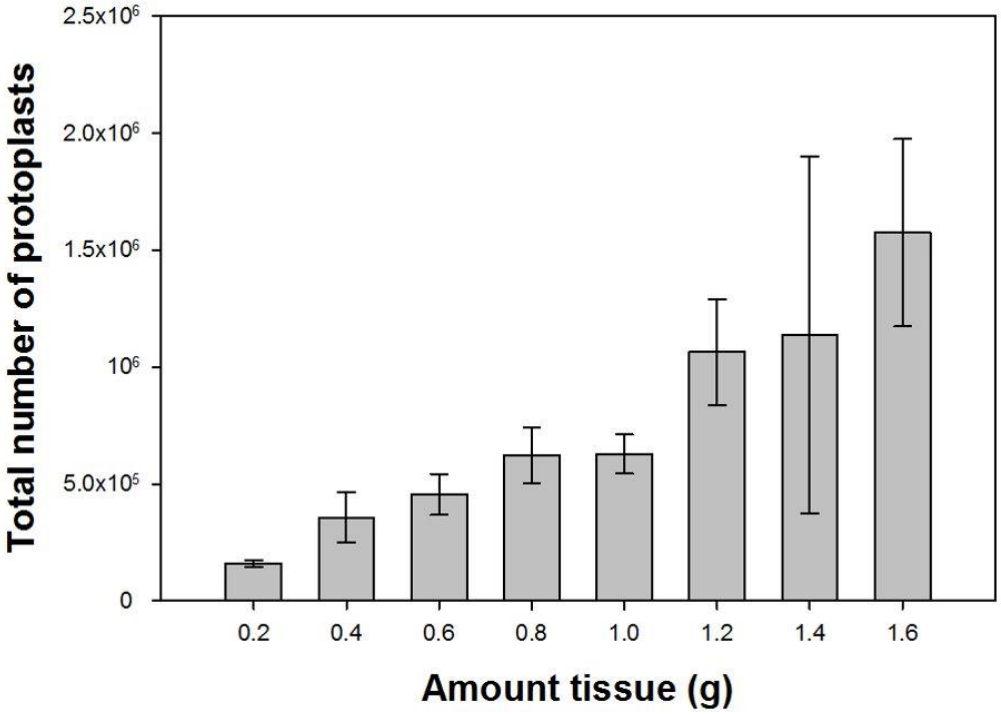

Figure 2. Total protoplast yield for varying amounts of leaf tissue. A concentration of 1320 ECU Rohament CL, 840 ADJU Rohapect 10L, and 0.0065 ADJU Rohapect UF, was able to digest > 1.6 g of leaf tissue, without a change to the yield per milligram of tissue (n =3). At the upper limit tested, ~1.6×10^6 protoplasts could be generated from a single reaction. Incubation conditions: CL=1320 ECU, 10L=840 ADJU, UF=0.0065 ADJU, 3 hour digestion, temperature 37°C, in dark.

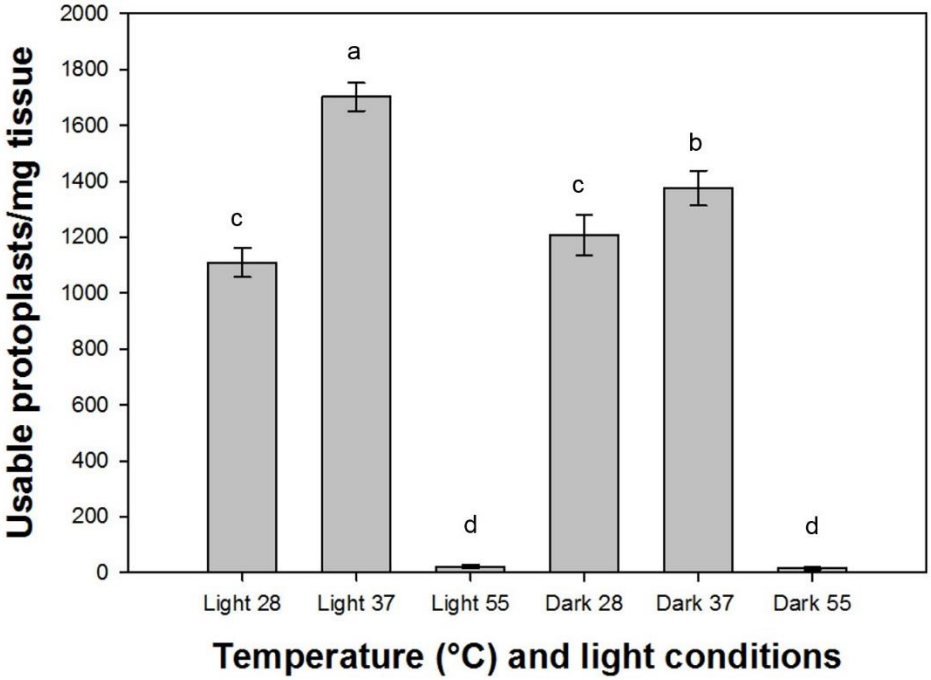

Figure 3. Temperature (28, 37 or 55°C) and light conditions (light or dark) and the effect on viable protoplast recovery per mg starting tissue. Incubation conditions: CL=1320 ECU, 10L=840 ADJU, UF=0.0065 ADJU, and 3 hour digestion. Error bars represent standard error (n=6). Same letters above bars indicate no significant difference according to the LSD test ($p<0.05$).

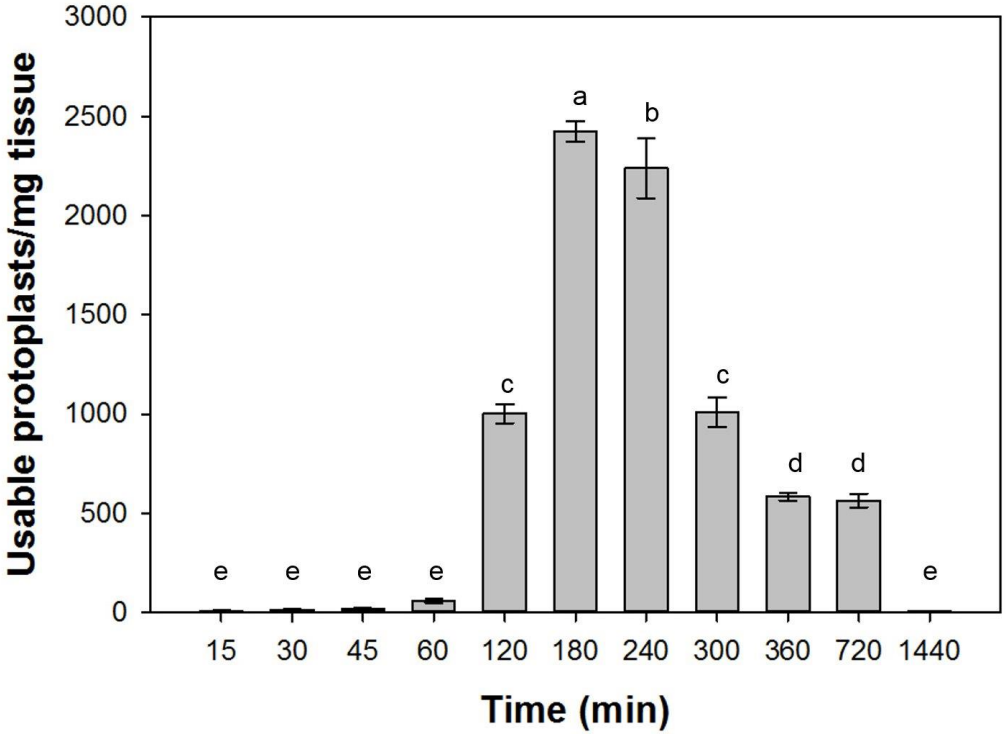

Figure 4. Time of incubation in enzyme mixture (minutes) and its effect on the number of viable protoplasts recovered per mg tissue. Incubation conditions: CL=1320 ECU, 10L=840 ADJU, UF=0.0065 ADJU, temperature 37°C, in light. Bars represent standard error (n=6). Same letter above bar indicates no significant difference ($p<0.05$) according to the LSD test.

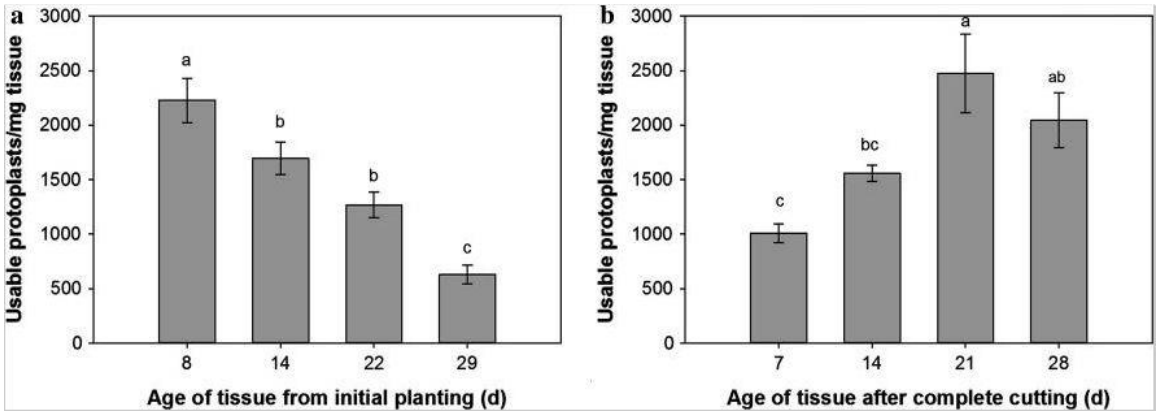

Figure 5. Effect of tissue age on viable protoplast recovery per mg of starting tissue. a) Age (in days) at switchgrass tissue harvest and its effect on the viable protoplast recovery per mg of starting tissue. b) Age (in days) at switchgrass tissue harvest after complete cutting (regrowth) and its effect on the viable protoplast recovery per mg starting tissue. Protoplastation conditions: CL = 1320 ECU, 10L = 840 ADJU, UF = 0.0065 ADJU, 3 h digestion, temperature 37 °C, in light. *Error bars* represent standard error (n = 9). *Same letters above bars* indicate no significant difference ($p < 0.05$) according to the LSD test

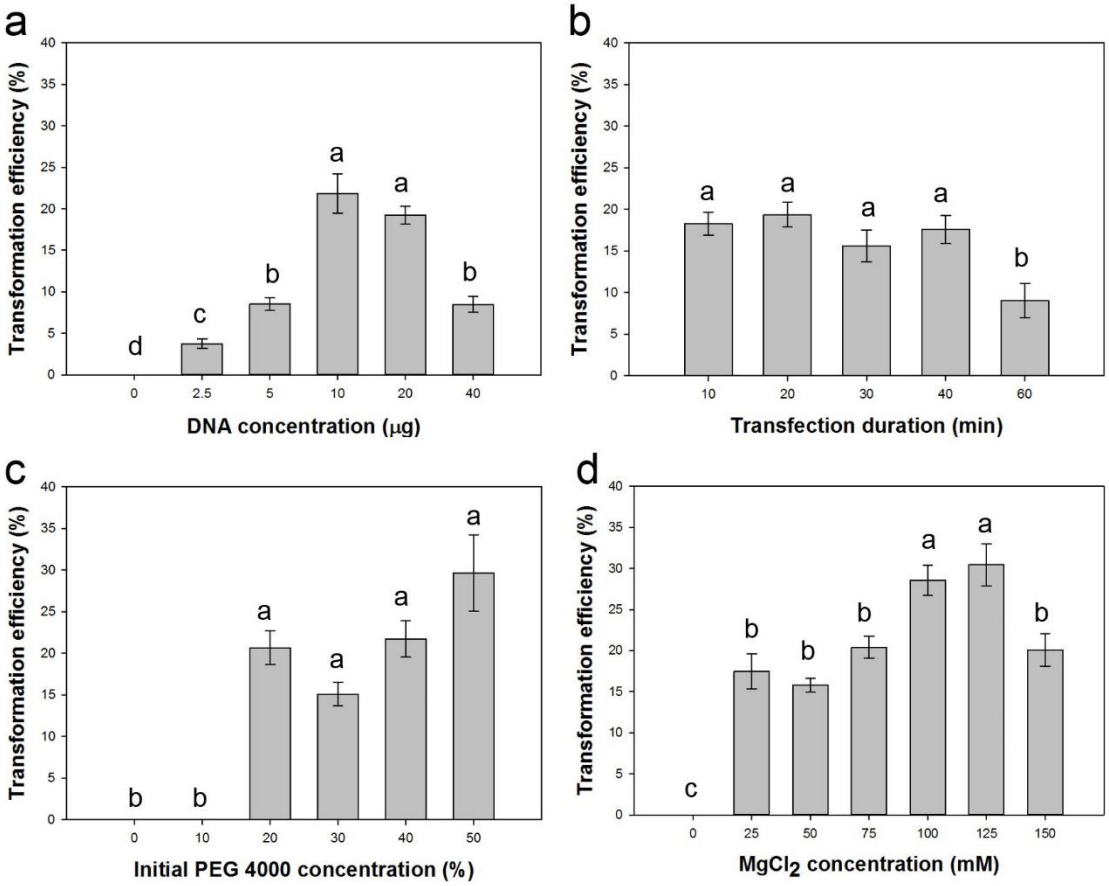

Figure 6. Optimization of transformation for switchgrass protoplasts. a) Effect of DNA concentration on transformation efficiency. b) Effect of duration of transfection (minutes) on efficiency. c) Effect of PEG 4000 concentration on transformation efficiency. d) Effect of $MgCl_2$ concentration on transformation efficiency. Error bars represent standard error (n=6). Same letters above bars indicate no significant difference ($p<0.05$) according to the LSD test.

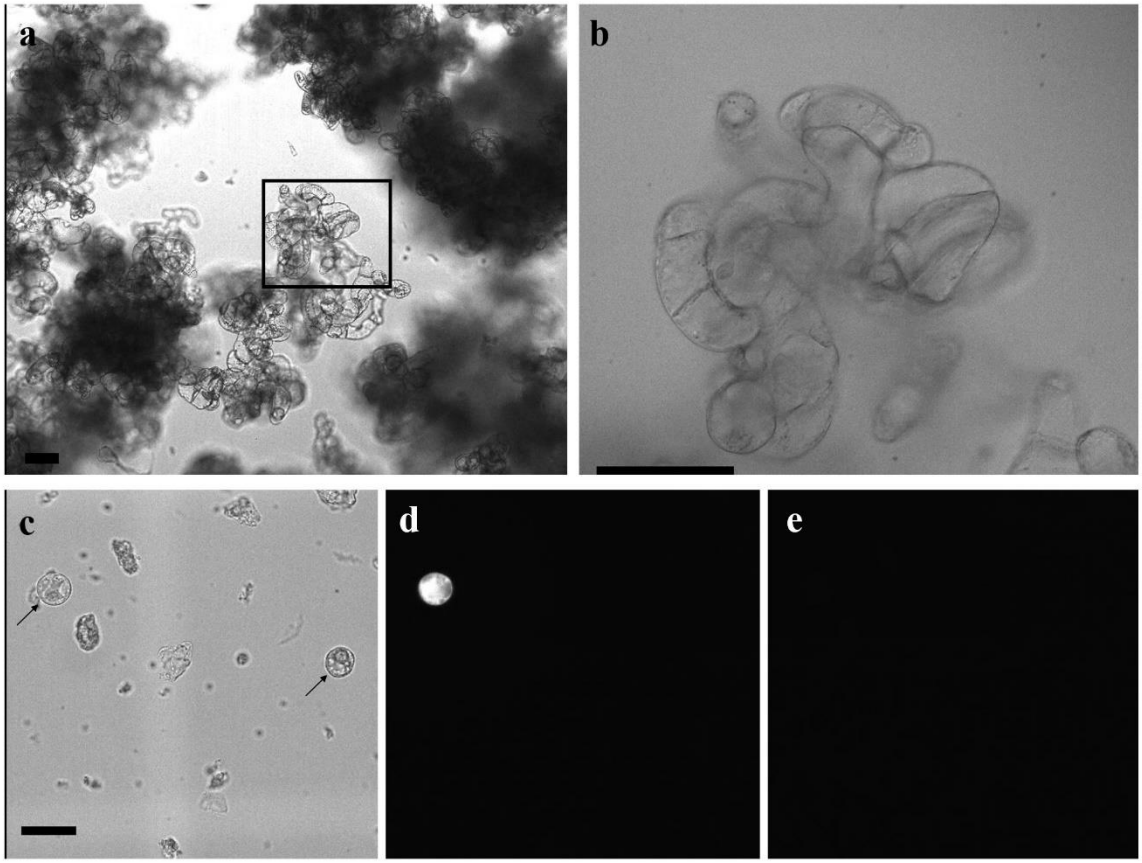

Figure 7. ST1 cell culture and protoplasts isolated from culture. a) Low-magnification (10X) image of population of 8 day old ST1 cell culture grown in KM8. Scale bar is 10 µm. b) High-magnification (40X) image of boxed portion of cell culture in A. Scale bar is 100 µm. c) Expression of OFP reporter in protoplasts isolated from ST1 cell suspension culture 18 hours following transfection with 10 µg pANIC10A GFPuv stuffer plasmid DNA was visualized using a tdTomato filter set: 535/30 nm excitation and 600/50 nm band pass emission and GFP filter set: 535/30 nm excitation and 600/50 nm band pass emission. The exposure time was 20 ms under white light (c), tdTomato filter (d) and GFP filter (e). Protoplasts shown with arrows in (c). Scale bar is 10 µm.

Figure 8. Greenhouse-grown *Panicum hallii* and *Panicum virgatum* L. From left to right: *Panicum hallii* var. '*hallii'* (PAHAH), *Panicum halli* Vasey var. '*filipes'* (Scribn.) Waller (PAHAF), *Panicum virgatum* L. cv.'Alamo'

Figure 9. Enzyme concentration and age effect on mesophyll protoplast isolation. a) Effect of Rohament CL enzyme concentration on viable protoplast recovery per mg *Panicum virgatum* cv. 'Alamo' tissue. Conditions of incubation: 37°C unprotected from ambient room light for 3 hours. Error bars represent standard error (n=6). Same letter above bar indicates no significant difference ($p<0.05$) according to Tukey's HSD test b) Effect of Rohapect 10L enzyme concentration on viable protoplast recovery per mg *Panicum virgatum* cv. 'Alamo' tissue. Conditions of incubation: CL=2062.5 ECU, 37°C unprotected from ambient room light for 3 hours. Error bars represent standard error (n=6). c) Effect of Rohapect UF enzyme concentration on viable protoplast recovery per mg *Panicum virgatum* cv. 'Alamo' tissue. Conditions of incubation: CL=2062.5 ECU, 37°C unprotected from ambient room light for 3 hours. Error bars represent standard error (n=6). d) Effect of tissue age on viable protoplasts per mg recovered. Conditions of incubation: CL=2062.5 ECU, 37°C unprotected from ambient room light for 3 hours. Error bars represent standard error (n=6). Same letter above bar indicates no significant difference ($p<0.05$) according to Tukey's HSD test

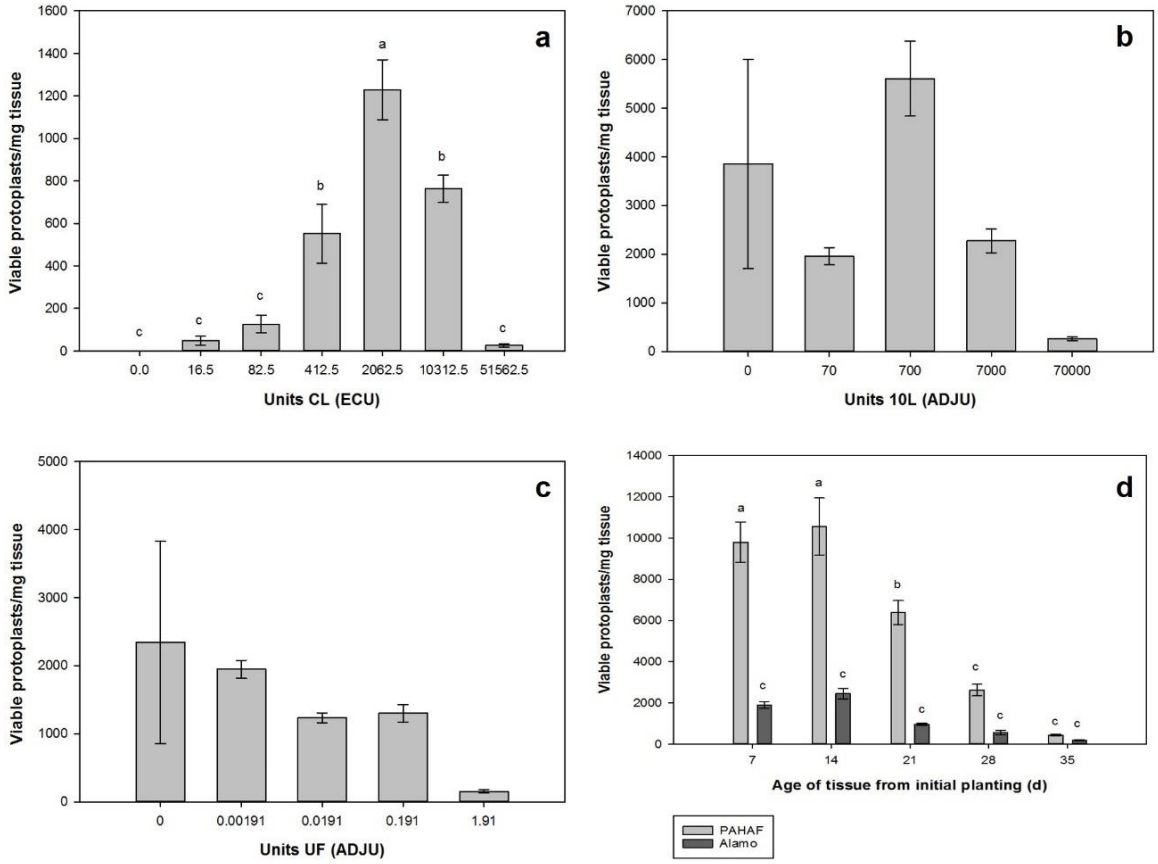

Figure 9 continued

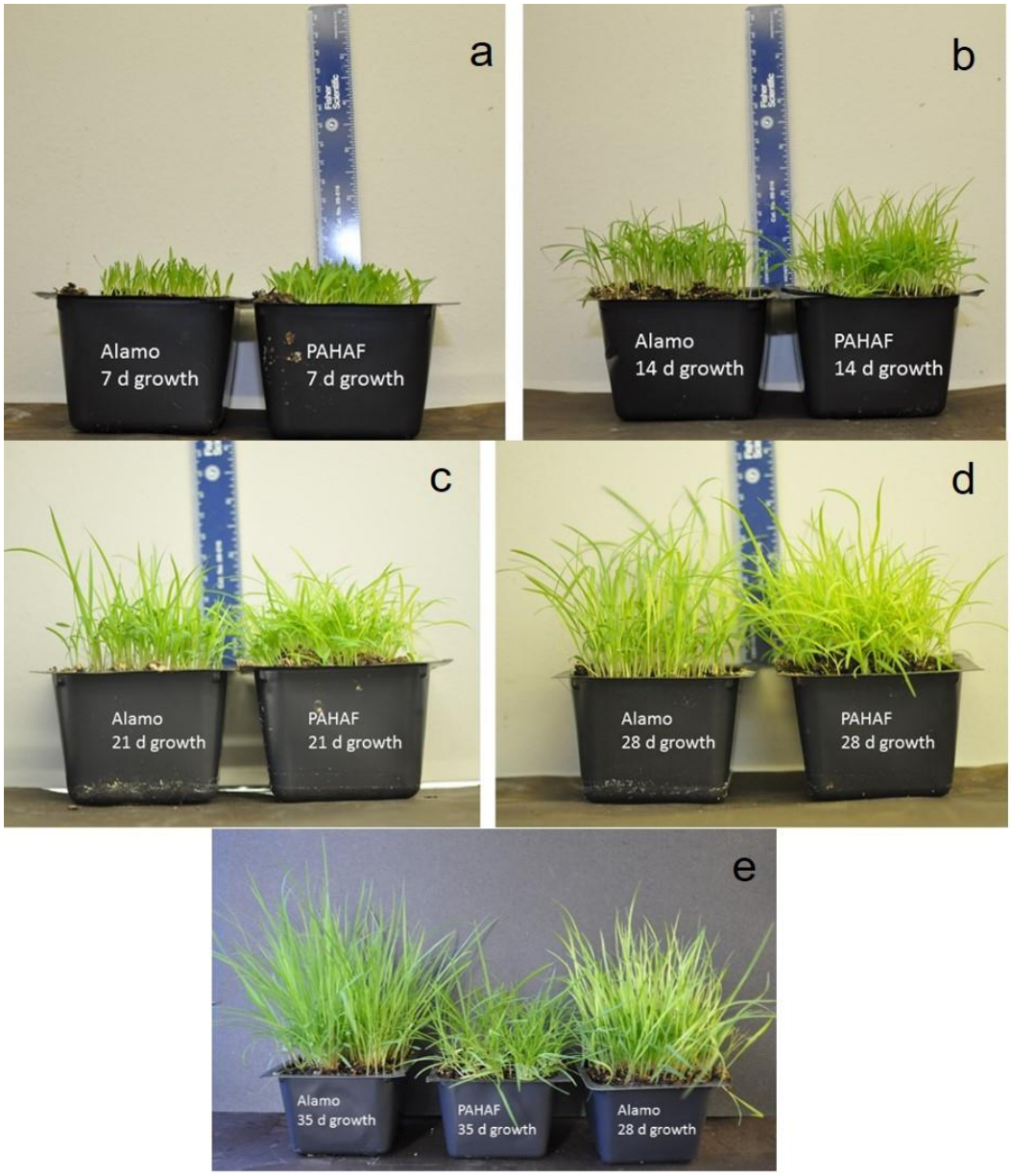

Figure 10. *Panicum virgatum* L. cv. ‘Alamo’ and *Panicum hallii* var. filipes (PAHAF) growth. Above ground biomass was harvested after each time point to obtain protoplasts. a) Tissue growth after 7 d. b) Tissue growth after 14 d. c) Tissue growth after 21 d. d) Tissue growth after 28 d. e) Tissue growth after 35 d for *Panicum virgatum* L. cv. ‘Alamo’ and *Panicum hallii* var. filipes (PAHAF) and after 28 d for *Panicum virgatum* L. cv. ‘Alamo’

Figure 11. Mesophyll cells and protoplast micrographs. a) *Panicum hallii* var. filipes mesophyll cells. Age of tissue was 7 days. b) *Panicum virgatum* cv. ‘Alamo’ mesophyll cells. Age of tissue was 7 days. c) Size (µm) distribution of *Panicum hallii* var. filipes (PAHAF) protoplasts. d) *Panicum hallii* var. filipes (PAHAF) protoplasts. e) Size (µm) distribution of *Panicum virgatum* cv. ‘Alamo’ protoplasts. f) *Panicum virgatum* cv. Alamo protoplasts. Scale bars represent 50 µm

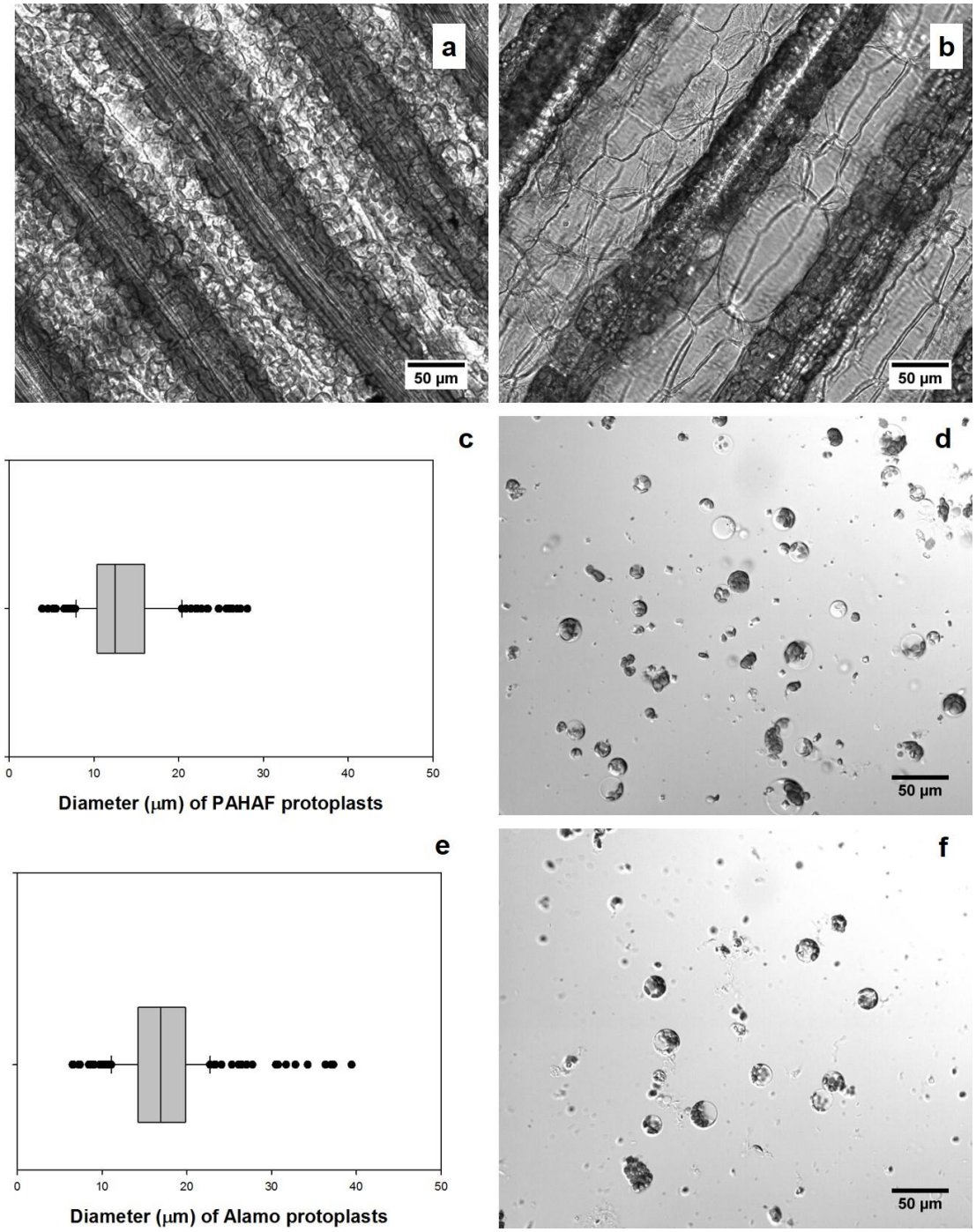

Figure 11 continued

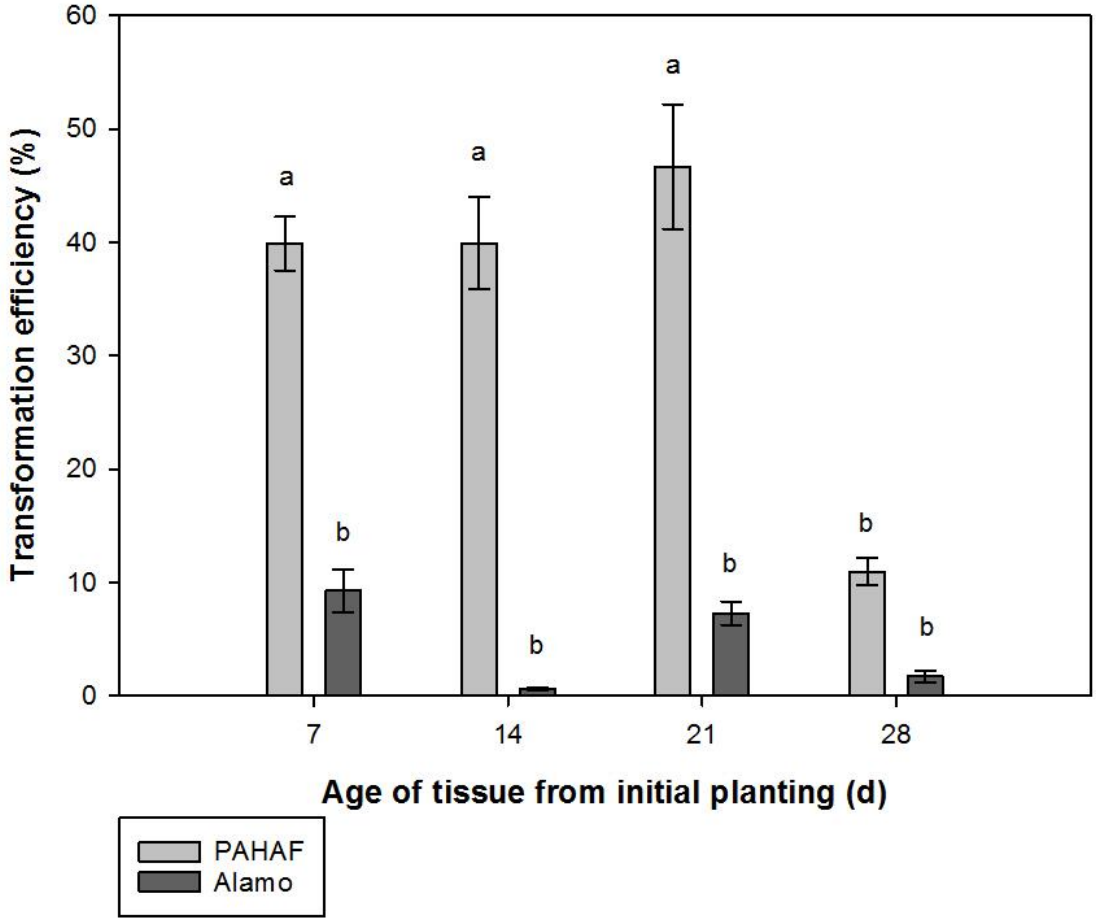

Figure 12. Mesophyll protoplast transformation efficiency. PEG- mediated transformation efficiency of *Panicum hallii* var. filipes (PAHAF) and *Panicum virgatum* cv. 'Alamo'. Error bars represent standard error (n=5). Same letter above bar indicates no significant difference ($p<0.05$) according to Tukey's HSD test

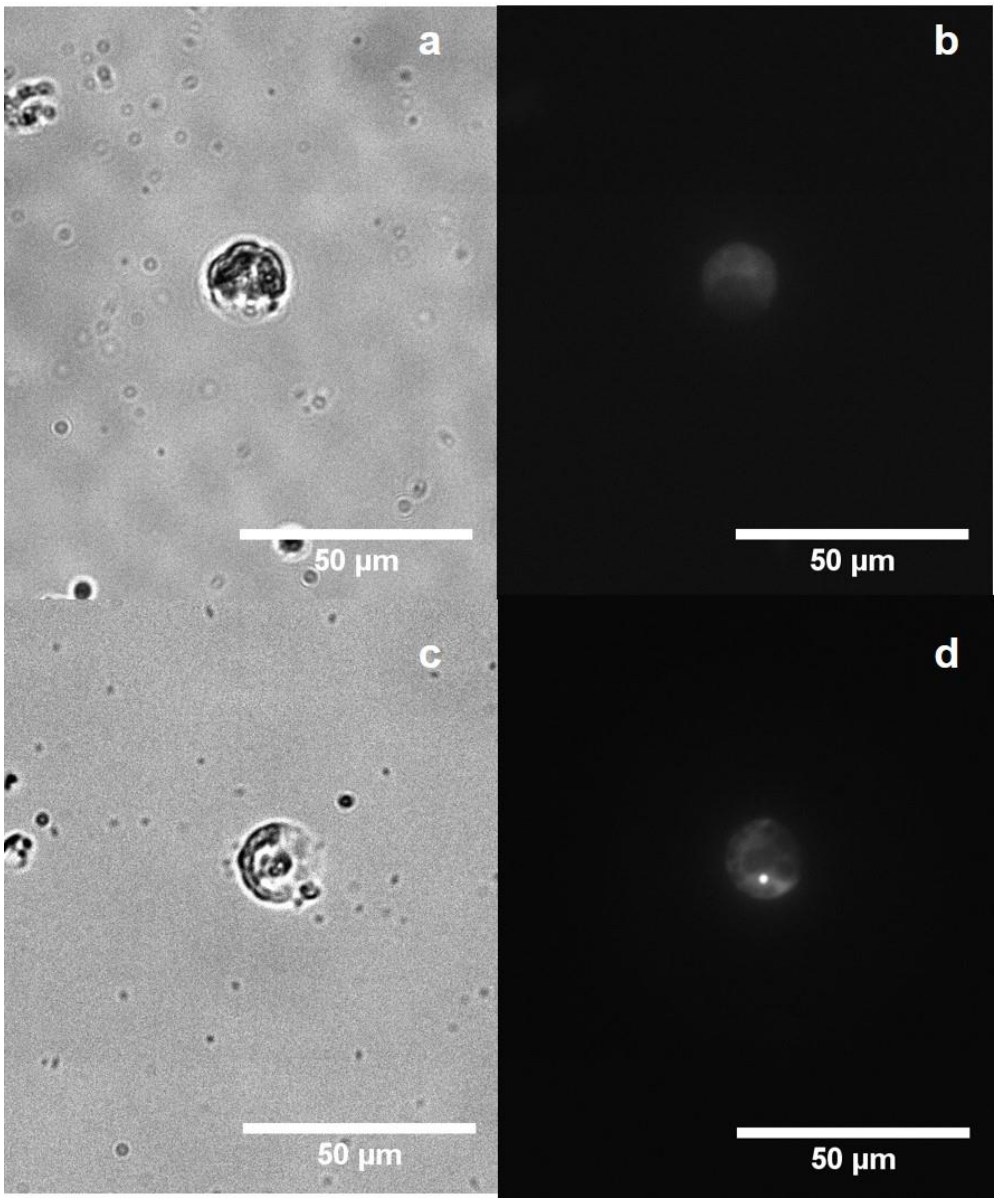

Figure 13. Expression of OFP reporter in protoplasts isolated from *Panicum virgatum* cv. Alamo mesophyll tissue (a-b) and *Panicum hallii* var. filipes (c-d) 18 hours following transfection with 10 µg pANIC10A GFPuv stuffer plasmid DNA was visualized using a tdTomato filter set: 535/30 nm excitation and 600/50 nm band pass emission and GFP filter set: 535/30 nm excitation and 600/50 nm band pass emission. The exposure time was 10 ms under white light (a and c) and 100 ms with tdTomato filter (b and d). Scale bar represents 50 µm.

Table 1. Age of mesophyll tissue of *Panicum hallii* var. filipes (PAHAF) and *Panicum virgatum* L. cv. 'Alamo' and the effect on viable protoplasts per mg starting tissue recovered after isolation.

Age of tissue (d)	**Viable protoplasts/mg tissue PAHAF**	**Viable protoplasts/mg tissue 'Alamo'**	**p-value (two-tailed t-test)**
7	**9799 ± 989**	**1904 ± 162**	**0.00053**
14	**10558 ± 1382**	**2446 ± 258**	**0.002195**
21	**6384 ± 583**	**963 ± 62**	**0.000249**
28	**2622 ± 286**	**559 ± 111**	**0.000525**
35	**436 ± 45**	**189 ± 22**	**0.001685**

Table 2. Age of mesophyll tissue of *Panicum hallii* var. filipes (PAHAF) and *Panicum virgatum* L. cv. 'Alamo' and the above ground biomass of tissue harvested per cm^2 soil.

Age of tissue (d)	**Mass (mg) of PAHAF tissue per cm^2 soil**	**Mass (mg) of 'Alamo' tissue per cm^2 soil**
7	**37.6**	**17.9**
14	**85.4**	**91.8**
21	**194.3**	**163.2**
28	**254.7**	**164.9**

Electroporation

In addition to PEG-mediated transformation of protoplasts from *P. virgatum* L. var. ‘Alamo’ and *P. hallii* Vasey var. filipes, electroporation of mesophyll protoplasts was attempted. Various electroporation parameters were tested on both ‘Alamo’ and PAHAF mesophyll protoplasts using the Biorad Gene Pulser Xcell™ total electroporation system. As a control, *Nicotiana tabacum* BY-2 suspension-derived protoplasts were electroporated according to a previously optimized protocol (Miao and Jiang 2007). Mesophyll protoplasts of either species of *Panicum* were not transiently transfected with the same pANIC10A plasmid containing the *pporRFP* orange fluorescent reporter gene (OFP) under the control of the PvUbi1+3 switchgrass constitutive promoter that was used for PEG-mediated transfection using this protocol. This was to be expected as a smaller plasmid is recommended by previous work (Miao and Jiang 2007).

With the consideration that BY-2 is not a monocot like the *Panicum* species, various electroporation protocols of monocot protoplasts were attempted. The first protocol attempted was one that is currently used for maize mesophyll protoplasts (Sheen 1991)with ~1×10^5 ‘Alamo’ and PAHAF protoplasts in 300 µL of electroporation buffer (0.6 M mannitol, 4 mM MES, 20 mM KCl, pH 5.7) with 10 µg of plasmid. Protoplasts were incubated with DNA in buffer at room temperature as well as in ice for 10 minutes before electroporating protoplasts with 400 V and 200 µF. Protoplasts were then incubated both on ice and at room temperature before resuspending in incubation buffer (0.6 M mannitol, 4 mM MES, 4 mM KCl, pH 5.7) in a 6-well dish. Protoplasts were

screened for transient OFP expression after overnight incubation. With each of these various conditions, protoplast survival was very low and no OFP expression was seen.

Other monocot electroporation protocols were attempted with both *Panicum* protoplasts, with a wide variety of parameters. Buffers were maintained as previous maize protocol (Sheen 1991) with variations in the voltage and capacitance. One variation was a 450 V pulse with a 200 µF capacitance, both with and without an incubation period on ice prior to the electric shock that has been seen to be successful in barley mesophyll protoplasts (Teeri et al. 1989). The next combination tested was seen to be successful with *Panicum maximum* protoplasts, where a 400 V pulse with a 510 µF capacitance was applied to the protoplasts after an incubation on ice for 10 minutes before electric shock (Hauptmann et al. 1987). The third protocol tested is one that has been used for maize protoplasts with a 250 V pulse for 70 ms (Huang and Dennis 1989). The last electroporation condition tested with switchgrass and PAHAF protoplasts was a 2000 V pulse and a 10 nF capacitance which has been seen to be sufficient for electroporation of rice protoplasts (Zhang et al. 1988). These protocols were tried twice, with no OFP expression being seen in any of the treatments.

Finally, an attempt was made to purify intact, viable protoplasts from cell debri and non-viable protoplasts. Protoplasts were isolated and washed and a sucrose-sorbitol gradient was attempted (SERVA-Electrophoresis). First the protoplast pellet was resuspended in 20 mL of a sucrose solution (500 mM sucrose, 1 mM $CaCl_2$, and 5 mM MES-KOH pH 6). On top of this, 5 mL of a sucrose-sorbitol solution (400 mM sucrose, 100 mM D-sorbitol, 1 mM $CaCl_2$, and 5 mM MES-KOH pH 6) was gently layered. For

the final layer, 5 mL of a sorbitol solution (500 mM D-sorbitol, 1 mM $CaCl_2$, 5 mM MES-KOH pH 6) was gently added. Then the tubes were centrifuged at 300 g for 5 minutes. The protoplasts were supposed to be seen in a band at the interface of two layers, but after several attempts, there was no protoplast band seen with this gradient.

At this time, it was determined that electroporation of mesophyll protoplasts of PAHAF and ‘Alamo’ was to be postponed, as PEG-mediated transfection appeared more efficient.

Vita

Andrea Grace Collins was born in Voorhees, NJ to parents, Nick and Melinda Collins. She is the middle of five children, her siblings being Laura, Paul, Mary, and Sarah. She attended Sullivan South High School in Kingsport, TN, and decided to attend the University of Tennessee, Knoxville, her parent's alma mater, and where her grandfather worked in Soil Science Extension. She got a job in the Stewart Plant Molecular Genetics laboratory as an undergraduate, where her interest in plants was sparked. She received her Bachelors of Science degree in Biological Sciences with a focus on Plant Biology in August 2013. She accepted a graduate research assistantship in the Plant Molecular Genetics program at the University of Tennessee, Knoxville, which she graduated with a Masters in Science in December 2016.