BIOLISTIC TRANSFORMATION OF ELITE GENOTYPES OF SWITCHGRASS (*PANICUM VIRGATUM)*

by

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(Under the Direction of Wayne Parrott)

ABSTRACT

Transformation of elite switchgrass (*Panicum virgatum* L.) genotypes would facilitate the characterization of genes related to cell wall recalcitrance to saccharification. However, transformation of explants from switchgrass plants has remained difficult. Therefore, the objective was to develop a biolistic transformation protocol for elite genotypes. Three switchgrass genotypes (ST1, ST2, and AL2) were previously selected for tissue culture responsiveness. One genotype, SA37, was selected for further use due to its improved formation of callus amenable to transformation. Various media sets were compared and the Somleva et al. (2002) media provided cultures with >96% embryogenic callus and high regenerablility. Data on transient gene expression and stable transformation were used to optimize biolisitic parameters, and test novel switchgrass (*Pv*Ubi) promoters in switchgrass and rice. SA37 and ST2 produced transgenic plants at a frequency of >1%. It is now possible to engineer elite switchgrass genotypes at a frequency commonly reported for maize.

INDEX WORDS: Particle bombardment, quantitative gene expression, switchgrass, *Panicum virgatum* L., plant transformation, pANIC vector set, ubiquitous promoter

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DEDICATION

I dedicate this work to my late Grandfather Homer Keith, for inspiring me to pursue a career in agriculture, and teaching me a man can build his own future from the ground up. In addition, I thank my loving girlfriend Emelie and wonderful family for all their support throughout the last few years.

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CHAPTER 1

INTRODUCTION, RESEARCH OBJECTIVES AND LITERATURE REVIEW

In 2006, the USA began a program to achieve a measure of energy independence (DOE, 2006). A rapid allocation of research resources ensued, and efforts are currently directed towards identifying efficient and renewable resources for the production of alternative fuels. The USA consumes 25% of the world's oil reserves, while harboring 3% of its supply (Greene et al., 2004). One key to achieve energy independence will lie in the production of domestically renewable fuels in the form of cellulosic ethanol from biofuel feedstocks, to partially supplement the fuel necessities of the USA. The US Congress, Biomass Research and Development Act of 2000 established a technical advisory committee, which aspired to replace 30% of US petroleum consumption by 2030 (Perlack et al., 2005). Currently, renewable energy resources including geothermal, wind, solar, hydroelectric, and biomass generate 6% of America's energy needs (Perlack et al., 2005). These figures include 172.37 metric tons of dried biomass produced from forest residues and managed agricultural stands; however, only 3% of the USA's total energy utilization is currently allocated from biomass.

Food and Fuel Competition

Currently, ethanol production from maize (*Zea mays* L.) has the potential to create an optimal net yield of 26% renewable energy due to advancements of agricultural techniques and high-yielding hybrids (Farrell, 2006). Although these data are promising, ethanol from maize faces several uncertainties. The use of starch derived from maize for the production of energy raises bioethical issues to many concerning the competition of resources between food and feed

with fuel sources.

The increased use of biofuels derived from food crops is not directly associated with the recent spike in the food prices. Recent spikes in food prices are directly related to a number of independent events, including a 0.9% decline in growth of agricultural production since 1990, and unfavorable weather conditions from 2006-2007 in Australia and Europe that produced substantial losses to grain and oil crops. Additionally, adverse climatic conditions contributed to a decline in world-wide grain stocks, which was complemented with an increased demand from emerging economies, and higher energy prices for commodities such as agricultural fertilizers and fuel (Mittal, 2009).

Therefore, current strategies have been expanded towards the production of ethanol from renewable, native, perennial, lignocellulosic biomass crops such as switchgrass (*Panicum. virgatum* var. *virgatum* L.) and poplar (*Populus trichocarpa*) which generate desirable feedstocks along with variety of other plant resources (Yuan et al., 2008). Unlike first-generation biofuel crops such as soybean (*Glycine max* (L.) Merr.) and maize, switchgrass is a perennial crop that reduces soil erosion, supports wildlife, and complements ethanol fuel from food- and feed-based agriculture (Yuan et al., 2008).

Ethanol-based fuels have a significant impact on the USA economy. Without additional energy from ethanol fuel, the US Department of Energy (DOE) estimates gasoline prices would raise $0.20-0.35 per 3.8 liters (gallon), costing each resident on average $150-300 more in annual fuel costs. These increases are equivalent to $28-49 billion for the USA as a whole, which consumes approximately 530 billion liters (140 billion gallons) of gasoline annually (DOE, 2008c). These estimates rely on data generated using the current difference in price between ethanol and gasoline, as well as the elasticity associated with petroleum resources.

In 2007, the US imported 65% of its necessary crude oil supply at a cost of over $333 billion; this in turn represented almost one-half of the US trade deficit. The impact of the 24.6 billion liters (6.5 billion gallons) of ethanol produced in the US that same year aided in cutting foreign petroleum imports by 16 billion liters (4.3 billion gallons), and in turn reduced the trade deficit by $9 billion (DOE, 2008b). This economic impact supports the need for additional research in cellulosic feedstocks. Cellulosic ethanol crops have the power to expand agricultural productivity beyond the grain-belt to areas across the country and can generate up to 20,000 new jobs for every additional billion gallons of ethanol fuel (Lewandowski et al., 2003; Perlack et al., 2005).

Cellulosic ethanol production has many benefits besides economic gains. Holistically, cellulosic ethanol has the potential to reduce greenhouse gas emissions 86% relative to gasoline emissions when examining CO_2-equivalent grams per million British Thermal Units of fuel generated and utilized (Wang et al., 2007). Cellulosic ethanol produced from various sources can displace gasoline consumption in the US by over 27.25 billion liters (7.2 billion gallons) per year (DOE, 2008a; DOE, 2008b). It is estimated that switchgrass can produce 540% more renewable energy than nonrenewable energy consumed. Furthermore, the average net energy yield from established switchgrass fields is 60 GJ·ha^{-1}·y^{-1} (Schmer et al., 2008).

Switchgrass requires approximately 25% of the irrigation and fertilization of row crops with a net energy gain calculated by Schmer et al. (2008) to be approximately 343% (DOE, 2008a). Although this will inevitably change once switchgrass plantings become widespread, virtually no pesticides are currently needed for switchgrass, whereas row crops such as maize require substantially more irrigation, herbicide applications and pesticide inputs (Wiebe and Gollehon, 2006). Additionally switchgrass requires herbicide application only during

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establishment from seed, which is followed by potentially ten years of productivity. Compared to row crops, switchgrass requires 10-fold less herbicide, and can reduce soil erosion 20-100%, and nitrogen losses associated with surface and ground-water 20-35 fold (Lynd et al., 2009).

For these reasons and others, switchgrass is being currently being pursued as a renewable cellulosic biomass crop (Dixon, 2009; Miller and Keller, 2009). If implemented into the current agronomic infrastructure, switchgrass could reduce the US dependence on imported petroleum by diversifying and expanding renewable energy sources, which is anticipated to jump 50% by the year 2030 (DOE, 2008a).

The Selection of Switchgrass for Renewable United States Energy

In 1984 the Oak Ridge National Laboratory established the Herbaceous Energy Crops Research Program (HECP). HEPC focused on characterizing plants that could be utilized as biomass feedstocks for the production of renewable fuels and economically high yields in diverse landscapes with relatively limited agricultural inputs (Lewandowski et al., 2003). Another focus was to have readily available agronomic practices, as well as adequate infrastructure in place for the crop of choice. Throughout the first five-years, the HECP program evaluated 35 promising herbaceous feedstock species. Of those species analyzed, 18 were perennial grasses, with equal numbers of C_4 and C_3 species. Switchgrass was selected as the best herbaceous candidate due to superior biomass yields (Pfeifer et al., 1990). Additionally, switchgrass comes with a suite of biological traits that boost its agronomic value including broad adaptation and a high genetic diversity for biomass yields and other traits (Lewandowski et al., 2003). Breeding efforts for lowland switchgrass varieties have produced genetic gains for yield ranging from 3-7.4% yr^{-1} through recurrent phenotypic selection depending on the ecotype and environment (McLaughlin and Kszos, 2005).

Based on switchgrass yield studies conducted in Texas (6 locations over 2 years), Alabama (2-year average), and the upper south, 'Alamo' had the highest average biomass yields. Upper south yields represent an average from seven locations in four states (Virginia: three sites; West Virginia: one site; North Carolina: one site; and Tennessee: two sites). In both Texas and Alabama, Alamo produced significantly higher yields than all other cultivars (p = 0.05). In the upper south region Alamo out-yielded all other switchgrass cultivars (p = 0.05) except 'Kanlow' where no significant difference was observed. Because of the ability of Alamo to produce biomass quickly, switchgrass has become a leading crop for the production of cellulosic ethanol, and is the model selected by US DOE Joint Genome Institute and the DOE BioEnergy Science Center (BESC) (Shen et al., 2009). The production of ethanol fuel from switchgrass biomass is viewed as carbon-neutral when considering agronomic inputs, processing the biomass, and transportation costs of the ethanol (Schmer et al., 2008; Tilman et al., 2006). However, it is naïve to think that optimal biomass yields of switchgrass can be attained without supplemental additions of nitrogen to the soils, or adequate amounts of water, and land to sustain productivity over time (Sinclair, 2009).

The first real introduction of switchgrass to the US public may have been the 2006 State of the Union address by then President George W. Bush Jr. (Dixon, 2009). In 2007 BESC was awarded a 5-year, $135-million grant when the Secretary of Energy announced it as one of three bioenergy research centers (Dixon, 2009). BESC is focused on conducting research to increase the production of bioethanol from cellulosic biomass feedstocks, focusing primarily on poplar and switchgrass feedstocks (Dixon, 2009). Thus, current goals are focused on the genetic transformation of Alamo using vectors for the overexpression (OE) or RNA interference (RNAi)-gene silencing of putative genes. These experiments focus on the manipulation of

biosynthetic pathways involved in polysaccharide and lignin production, which are key cell-wall components (Miller and Keller, 2009; Shen et al., 2009). Either pathway if manipulated properly, could improve switchgrass biomass by lowering its recalcitrance to enzymatic saccharification, i.e., allowing it to be degraded into fermentable sugars with greater ease, thus, creating higher yields of bioethanol production per unit biomass (Miller and Keller, 2009). Recalcitrance to saccharification still remains the limiting factor in the biofuel industry (Dixon, 2009).

Primary Research Objectives

1) The overall goal was to improve tissue culture protocols for the regeneration of elite genotypes of switchgrass from embryogenic callus cultures derived from vegetative tissues of three tissue-culture-responsive genotypes of cv. Alamo.

2) To identify parameters which may significantly affect stable transformation efficiency using microprojectile-mediated transient expression assays and stable transformation.

3) To determine the relative strength of transgene expression in switchgrass and rice driven by novel *P. virgatum* L. polyubiquitin promoters (*Pv*Ubi) and their variations. These promoters will be compared to well-characterized monocot promoters in transient expression assays using switchgrass and rice callus cultures. Additionally, rice callus will be stably transformed to analyze the promoter expression of *Pv*Ubi promoters.

Literature Review

Switchgrass is a native North American perennial, rhizomatous, warm-season pasture grass, from the *Poaceae*; subfamily *Panicoideae* (Lewandowski et al., 2003). Switchgrass has a distinct appearance with coarse tillers, a caespitose growth habit, and large, broad leaves with parallel venation and panicle-type inflorescences. This C_4 forage grass has the ability to grow in

a wide range of environments including marginal agricultural land. In fact, switchgrass is native to 47 of the 50 states of the USA, excluding Washington, California, and Oregon. Additionally, switchgrass is found in regions of eastern Canada, and is endemic from Quebec to Mexico (Lewandowski et al., 2003; USDA, 2006).

Like most grasses, switchgrass forms arbuscular mycorrhizal associations, which allow for robust adaptation to edaphic conditions due to efficient use of the rhizosphere, primarily due to better phosphorus uptake from supporting hyphal networks (Finlay, 2004; Parrish and Fike, 2005).

Switchgrass follows the normal stages of growth and development of perennial forage grasses that are clearly separated by five distinct developmental stages [germination, vegetative growth, elongation, reproductive maturity, ripening and maturation of caryopses] (Moore et al., 1991). Once switchgrass plants reach the elongation-stage, morphological distinctions can be defined by the number of observable culm nodes, commonly referred to as joints. Specifically, these stages range from E0, defined as initiation of tiller elongation, to E1, E2, E3, E4 etc., where E1 represents the first culm node (Moore et al., 1991). Elongation is followed by reproductive development, whereby the first reproductive stage, R0, known as the boot stage, is defined by the development of panicles prior to their visible emergence from the top of the tiller, thus exposing the spikelets. The boot stage occurs after the E2-E4 developmental stages (Alexandrova et al., 1996b).

At maturity, switchgrass plants can be 1 to 1.5-m high, with extensive and vigorous root systems extending 3 m down (Heath et al., 1978; Jensen et al., 2007; Parrish and Fike, 2005). The rhizomes produced by switchgrass are common to the *Poaceae*, and add to the aggressive growth habits of the plant. Each node from a rhizome can potentially generate a new flowering

tiller (Raven et al., 1999); both vegetative and sexual tissues produce biomass. Among other factors, high yields of caryopses, high seedling vigor, and the extensive ability to produce biomass have made switchgrass the preferred species for extensive study and biomass production.

Switchgrass is anemophilous and almost exclusively cross-pollinated. Strong gametophytic self-incompatibility (SI) barriers do not allow self-fertilization (Martinez-Reyna and Vogel, 2002). Switchgrass SI is believed to be controlled by two independently segregating, polyallelic S and Z genes that are commonly responsible for SI systems in the *Poaceae* (Lundqvist, 1956).

Advantages and Limitations of Switchgrass as a Biofuel Crop

Switchgrass is established from seed, thus allowing less input for establishment over other biomass crops, such as miscanthus (*Miscanthus × giganteus*), which is vegetatively propagated (Lewandowski et al., 2003). Miscanthus is a sterile triploid (2n = 57), thus breeding efforts for plant improvement of this species are limited (Greef et al., 1997). In addition miscanthus is not adapted to many distinct regions of the US (Parrish and Fike, 2005).

Farmers already have the necessary infrastructure to plant switchgrass seed, maintain the plants, and harvest the biomass (Lewandowski et al., 2003). As a C_4 plant, switchgrass will likely be less productive in cold climates and may not be able to grow at temperatures below 10°C. Below this threshold, C_3 biomass crops such as poplar and *Eucalyptus* spp. Are the best candidates for biomass production (Rubin, 2008).

History of Switchgrass Usage and Commercial Importance

Switchgrass has a high prevalence in the Great Plains naturally (Heath et al., 1978). Later, Europeans established switchgrass in other North American areas (Hitchcock, 1971).

Switchgrass has been used as high-quality livestock forage (Burns et al., 1984), silage (Luginbuhl et al., 2000), and as excellent hay and summer pasture. However, switchgrass has low nutritional value when used as standing winter-feed due to decreased nitrogen levels (Heath et al., 1978; Parrish and Fike, 2005).

Paradoxically, higher levels of nitrogen make the biomass lower in value for combustion to produce energy. Therefore, harvest time for biomass quality depends on its end use. If harvesting biomass for fuel purposes, late-season harvests are ideal and more sustainable.

Switchgrass grows best on fertile, moist soil, and will form sod and an extensive root system which may extend over 3.3 m deep (Ma et al., 2000). To control soil erosion, switchgrass has been planted near permanent waterways, and is capable of growing in drought conditions (Heath et al., 1978). Alone, or with other grasses, (e.g. sand lovegrass (*Eragrostis pilifera* Scheele)), switchgrass is planted on high-risk erosion areas such as strip-mine spoils, steep embankments, sand dunes, and dikes (Parrish and Fike, 2005; USDA, 2006).

Fundamental Genetics and Polyploidy

Switchgrass has a relatively large genome of ~5,600 megabases which is currently being sequenced by the Joint Genome Institute using foxtail millet (*Seratia italica*) as a reference. Foxtail millet has a genome of ~515 megabases, is closely related to switchgrass and holds agronomical importance relative (Kellogg, 2001; Rubin, 2008; Shen et al., 2009). Switchgrass is a polymorphic species, and consists of two ecotypes. The lowland ecotype is almost exclusively dominated by tetraploid varieties ($2n = 4x = 36$) such as Alamo and Kanlow, while the upland ecotype is predominantly composed octoploid varieties ($2n = 8x = 72$) such as 'Cave-In-Rock' and 'Pathfinder,' with DNA (2C) values of 2.7-3.2 pg and 4.8-5.8 pg, respectively (Hopkins et al., 1996; Parrish and Fike, 2005). Interploidy crosses between tetraploid and octoploid plants is

inhibited by postfertilization incompatibility systems, and all seeds produced in controlled crosses are abnormal (Martinez-Reyna and Vogel, 2002). Data are currently inconclusive as to whether upland switchgrass 'Alamo' and 'Summer' are autotetraploid or allotetraploid (Missaoui et al., 2005).

Selection of Reporter Transgenes

Frequently used reporter transgenes encoding enzymes for either colorometric (Broothaerts et al., 2005; Jefferson, 1989; Jefferson et al., 1987), fluorescent (Chiera et al., 2008; Richards et al., 2003; Stewart, 2006; Wenck et al., 2003), or luminescent (Leckie et al., 1994; Ow et al., 1986; Schledzewski and Mendel, 1994; Sivamani and Qu, 2006) activity allow sensitive assays to identify intact transformed cells, or quantify transgene expression. It is possible to use colorimetric and fluorescent reporter genes either separately or in combination to monitor transgenic plant cells and tissues (Wenck et al., 2003).

Fluorescent Protein Visualization of Transgenic Events *in planta*

The cloning of the green fluorescent protein (GFP) gene (*gfp*) from the jellyfish *Aequorea victoria* (Prasher et al., 1992) profoundly impacted plant biotechnology (Alieva et al., 2008; Chiera et al., 2008; Elliott et al., 1999; Finer and Finer, 2007; Finer, 2006; Hernandez-Garcia et al., 2009; Lindbo, 2007; Stewart, 2001; Stewart, 2006; Wenck et al., 2003). In fact, a representative organism from every kingdom has been transformed with *gfp* (Stewart, 2006), and plant biotechnologists now benefit from a plethora of non-toxic fluorescent protein (FP) genes (Wenck et al., 2003). In fact, fertile transgenic oat plants can be regenerated following selection with GFP fluorescence microscopy alone (Kaeppler et al., 2001; Kaeppler et al., 2000).

Fluorescent protein is confined within single cells post-bombardment, whereas histochemical GUS stains can create either discrete single-cell foci or leakage via diffusion to

multiple cells. Additionally, a GUS assay will destroy or damage tissue, and requires an expensive substrate, and incubation times which range from ~1-24 hrs to visualize gene expression (Elliott et al., 1999; Hunold et al., 1994). The use GFP foci to optimize bombardment conditions offers a rapid, non-destructive means for tracking, and potentially recovering, transgenic cell cultures (Tee and Maziah, 2005).

Additionally, GFP cassettes allow real-time, *in planta* gene expression quantification using imagery data. Such techniques can hone in on details, such as gene expression by tissue-specific promoter activity, or even developmental stages (Chiera et al., 2008; Chiera et al., 2007; Finer and Finer, 2007; Stewart, 2001). Indeed, FPs could be used monitor transgenic plants in the environment using simple ultra violet (UV) flashlights or monitor gene-flow (Stewart, 2005).

Quantitative GFP monitoring could also be used in plant breeding by comparative *in vivo* fluorescence imaging to determine transgene expression levels of a FP construct coupled to an agronomically important transgene, such as those currently being pursued for biofuels (Stewart, 2006). GFP expression levels can also distinguish homozygous and hemizygous transgenic lines (Richards et al., 2003).

The *gusA* gene can be directly quantified using a fluorogenic substrate known as 4-methyl umbelliferyl glucuronide (MUG), and direct detection of fluorescence (Jefferson, 1989; Jefferson et al., 1987). For example, MUG assays can be used in quantitative promoter analysis to measure levels of gene expression with high precision (Leckie et al., 1994; Schledzewski and Mendel, 1994; Sivamani et al., 2009).

The colorimetric reporter gene recently selected for routine switchgrass transformation is *GUSPlus™* (Broothaerts et al., 2005; Mann et al., 2010). *GUSPlus™* was isolated from a *Staphylococcus* spp., and plant codon-optimized. The gene product is 603-aa with a 47% amino

acid identity to the traditional 602-aa sequence of *E. coli*-derived *GUS* (Broothaerts et al., 2005).

GUSPlus™ produces β-glucuronidase (GUS), which can be visualized upon the addition of the

5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide (X-Gluc) substrate (Jefferson, 1989; Jefferson

et al., 1987). *GUSPlus™* features many advantages, including increased stability, useful

restriction sites, and high activities in various temperatures (20-60°C) and pH (6-8) ranges, with

near optimal activity at ambient conditions (Cambia, Brisbane, Queensland, Australia).

CHAPTER 2

BIOLISTIC TRANSFORMATION OF ELITE GENOTYPES OF SWITCHGRASS[1]

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[1]King, Z., Lang, A., LaFayette, P.R., Ping, Wu, Bray, A., Parrott, W.A. Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.) To be submitted to *Plant Cell Reports.*

INTRODUCTION AND LITERATURE REVIEW

There are many current limitations which reduce the efficiency of cellulosic ethanol production in biorefineries from second generation biofuels such as switchgrass (Himmel et al., 2007). Himmel et al. (2007) highlight several key biomass components that contribute to the recalcitrant properties of biomass, including epidermally produced waxes, the degree of lignified cell and tissue types, and the heterogeneneity and complexity of cell-wall components. All these components could be targeted separately or in combination to potentially improve the conversion process of raw biomass to ethanol in biorefineries. Suggestions for overall improvements of biorefinery systems partially lie in allowing liquid contact to cellulose microfibrils and enzymatic contact to target sugars for efficient fermentation and lowered conversion costs for the production of cellulosic ethanol (Himmel et al., 2007).

Genetic transformation can have profound effects on cell wall composition, and cellulosic ethanol production downstream. Biomass feedstocks have been genetically engineered for lowered recalcitrance to better facilitate saccharification in consolidated bioprocessing. For example, a well conserved gene in the lignin biosynthesis pathway, hydroxycinnamoyl CoA: shikimate hydroxycin-namoyl transferase (HCT) was silenced in transgenic alfalfa (*Medicago sativa* L.), resulting in over 40% more enzymatic hydrolysis compared to the wild-type plant (Chen and Dixon, 2007). Thus, the biological mechanisms of switchgrass cell-wall biosynthesis can be modified through the production of transgenic plants.

By targeting key genes using RNAi silencing or overexpression vectors, it is possible to validate and explore gene function in relation to the production of cell-wall components and generate elite transgenic switchgrass plants with lowered recalcitrance to enzymes such as cellulases (Stewart et al., 2009). Reverse genetics offers tools to achieve these goals, such as the

analysis of putative genes involved in switchgrass cell-wall biosynthesis (Miller and Keller, 2009).

Genes of high priority are those directly related to enzymes controlling what are believed to be the most critical components of cell-wall biosynthesis, including polysaccharide related components which primarily consist of cellulose, hemicellulose, and pectin; and in a separate class, lignin biosynthesis (Chen and Dixon, 2007; Miller and Keller, 2009). Additionally, reverse genetics goals may focus on traits such as herbicide resistance or male sterility to avoid transgene escape (Gressel, 1999; Gressel, 2008a; Gressel, 2008b).

Properly developed *in vitro* regeneration systems will be key for the genetic transformation of switchgrass using explants from well characterized, and elite genotypes responsive to transformation compared to seed-derived explants for several reasons. Firstly, each seed is a unique genotype, and the different genetic backgrounds makes phenotypic analysis of transgenic lines more difficult. By using clones of the same switchgrass genotype, small gene effects are more easily detectable (Xi et al., 2009), as there are no differences in genetic background to confound the data.

History of Switchgrass Biotechnology and Transformation

The vast majority of work to date has been from seed-derived callus. Currently, *A. tumefaciens*-mediated transformation of switchgrass somatic embryos has been reported to be upwards of 97%, as determined by the number of bialaphos resistant plants recovered from the initial explants (Somleva et al., 2002). It was not indicated by Somleva et al. (2002) whether each regenerant was from an independent event. Microprojectile-mediated transformation efficiency still stands at ~4% (Richards et al., 2001). However, Richards et al. (2001) made little

effort to optimize the transformation process, and several of their transgenic plants appear to have been recovered from the same event.

Switchgrass transformation using the super-virulent *A. tumefaciens* strains AGL-1 (Somleva et al., 2002; Somleva et al., 2008), and EHA-105 (Burris et al., 2009; Fu et al., 2011; Li and Qu, 2010; Xi et al., 2009) has been accomplished. More recently, Somleva et al. (2008) engineered the metabolic pathway of switchgrass plants to produce yields of polyhydroxybutyrate (PHB), a biodegradable plastic precursor. The PHB dry weight produced was 3.72%, and 1.2% of leaves, and tillers, respectively. Somleva provided detailed descriptions of the seed-derived transformation process (Somleva, 2006a), including optimizing acetosyringone (AS) concentrations. Evidence indicates that callus type and AS concentrations are critical to achieve relatively high switchgrass transformation efficiency (Li and Qu, 2010; Somleva, 2006; Somleva et al., 2002; Somleva et al., 2008; Xi et al., 2009). The data show that somatic embryos inoculated and cocultivated in medium supplemented with 200 µM AS show increased transformation frequency (Somleva et al., 2002).

The latest advance in switchgrass engineering achieved over 90% transformation efficiency routinely when using callus derived from the seed of 'Performer,' however, only 1.5% of 'Performer' callus was highly transformable (Li and Qu, 2010). Performer was selected for low lignin content (Burns et al., 2008) and provides a unique callus type that is yellow, friable and highly regenerable and transformable (Li and Qu, 2010). Additionally, the authors improved the transformation system by using a strong constitutive promoter, *rubi3,* to drive the reporter cassette, *sGFP* (S65T), whereas the 35S promoter drove the selection cassette, *hph.* A number of other improvements in the transformation process such as vacuum infiltration of *Agrobacterium* into callus tissue, a desiccation step after inoculation, a 5-day-resting phase, and

the use of L-proline in selection medium added to the significant success of the transformation process; although the largest contributing factor to increased transformation efficiency appears to be the use of cv. Performer over Alamo, accounting for 42% more transformation efficiency under optimized conditions.

Highly embryogenic callus cultures can be obtained from immature inflorescences of switchgrass and regenerated into plants with an efficiency as high as ~60 regenerants per initial explant (half-internode) when a 16-week micropropagation step is included in the regeneration process (Alexandrova et al., 1996b). Using a similar process, switchgrass transformation has been accomplished using embryogenic callus cultures established from mature plants (Burris et al., 2009); however, there are difficulties associated with the transformation of callus cultures derived from vegetative origins versus seed (Xi et al., 2009).

Species-specific parameters affecting optimal transformation efficiency are helium pressure, microcarrier size, and DNA concentration. All parameters need to be optimized for each cell or tissue type (Sanford et al., 1993). Additionally the cell type bombarded must be highly transformable, and in the proper physiological state to receive a gold microcarrier and transgene with high-efficiency. Finally, the cells that receive the transgenes must be highly regenerable to create an efficacious transformation system (Birch, 1997; Kikkert et al., 2004).

The goal of this study was to develop a standard engineering protocol for elite switchgrass genotypes (e.g., ST1, and SA37) through particle bombardment. Optimized transformation parameters need to be developed for efficient reverse genetics studies to investigate recalcitrance related genes. Birch (1997) clearly defines a logistical progression for developing a transformation system, and this approach was used to design these experiments.

MATERIALS AND METHODS PART I – TISSUE CULTURE

Media Set Selection

Several efficient media sets are available for the initiation of embryogenic switchgrass callus cultures that are highly regenerable (Alexandrova et al., 1996; Burris et al., 2009; Somleva et al., 2002; Xi et al., 2009) (Table 2.1). Additionally, existing procedures for maize (Che et al., 2006) tissue culture could be adapted to create highly embryogenic and regenerable cultures of switchgrass from elite plant material. In order to identify the most efficient media set, procedures were compared side-by-side to elucidate the best protocol for establishing highly embryogenic and regenerable cultures of switchgrass from elite plant materials from three tissue-culture-responsive genotypes described below.

Plant Material

Three genotypes from Alamo were maintained in the greenhouse in a 42% sand, and 58% Fafard 3B soil mix (Conrad Fafard, Inc., Agawam, MA), tillers that matured beyond boot stage (Moore et al., 1991) were discarded. The three elite genotypes selected were ST1, and ST2 supplied by the Noble Foundation (Xi et al., 2009), and SA37, a University of Georgia introduction selected for superior tissue culture performance from 215 F_1 progeny from a cross between two tissue-culture responsive genotypes (ST1xAlamo 2). Alamo 2, a highly tissue culture responsive genotype, was supplied by the University of Tennessee (Burris et al., 2009). Growth conditions consisted of a 12-h photoperiod under 400-watt high-pressure sodium lighting. The greenhouse ranged in temperature from 20-27°C and plants were watered daily, and fertilized weekly with 0.453 kg of Peters® Professional All Purpose Plant Food (St. Louis, MO) per 378.54 liters of water (1 lb 100^{-1} gallons).

Harvesting Explants

The last culm node of switchgrass produced the desired immature inflorescences at the

E2-R0 stages (Moore et al., 1991) and were identified as previously described by Alexandrova et

al., (1996b).   All tissue 5.0 cm above, and 1.5 cm below the culm node were harvested as an

initial explant. Thirty-two tillers at boot stage were harvested per genotype to initiate tissue

cultures.

Surface Sterilization of Initial Explants

The 6.5-cm internode explants were surface-sterilized with 70% ethanol, for 1 minute

with gentle agitation. Subsequently explants were placed in 15% Clorox[®] v/v with 2 drops of

Tween 20 per 100 ml, for 3 minutes with gentle agitation. All tissues were then rinsed 3 times,

at 2-minute intervals.

For all comparisons of switchgrass media sets, 2.5 g l^{-1} Gelzan™ (Caisson Laboratories,

North Logan, UT, USA) was used as the solidifying agent, the pH was brought to 5.8 prior to

autoclaving, and all Petri dishes were sealed with 3M Micropore™ tape (St. Paul, MN, USA)

(Table 2.1). Cultures were established to elongate panicles in 20x100 mm Petri dishes. Sterile

tiller segments were halved longitudinally (Alexandrova et al., 1996) to produce initial explants.

Explants were randomized within genotype and placed cut-side down on solid medium to induce

and elongate panicles (Table 2.1).   In this manner eight replicate plates of two half-internodes

per treatment were plated. Cultures were incubated in a growth chamber at 25° C, with cool-

white fluorescent lighting (66-95 μE m^{-2} s^{-1}) 16-hour light and 8 hour dark cycle for 14 days

(Figure 2.1, Table 2.1).

Embryogenic Switchgrass Callus Culture Initiation and Tissue Culture Efficiency Tests

After 14 days of culture, immature inflorescences were used to initiate embryogenic callus cultures. The inflorescences were dissected out and cut to obtain sections of rachis tissue measuring approximately 1 cm in length. Inflorescence pieces were placed on medium to initiate embryogenic callus cultures arranged in a 4x4-grid pattern in 15x100 mm Petri dishes (Figure 2.1, Table 2.1). Tissue was incubated at 27°C in the dark and subcultured at three-week intervals to induce the growth of embryogenic callus.

After the second subculture, the number of inflorescences pieces generated which gave rise to callus was documented. This figure was then used to calculate the total percentage of explants that gave rise to callus (Figure 2.2). Compact, embryogenic callus was separated from the inflorescences and arranged in a 5x5-grid pattern. Embryogenic callus tissue was bulked for six weeks with subcultures at three-week intervals. At this point, embryogenic callus was selected and weighed in order to set up six biological replicates per treatment to determine callus yields per unit time as determined by fresh-weight in grams (Table 2.1). Each replicate plate of callus contained 0.25 g of callus arranged in a 5x5-grid pattern. All callus material was transferred once more after three weeks, allowing six weeks of growth total. At this point all callus tissue per replicate plate was recorded, and a representative group of calli was photographed from each treatment (Figure 2.4, Figure 2.6).

The remainder of the embryogenic callus tissue from the experiment above was used to create ten replicate plates of callus per treatment. For each replicate, 25 calli, approximately 3 mm in diameter were plated in 5x5-rows. The calli were allowed to grow for three weeks in the same conditions as previously described, and were then observed using a dissecting microscope to determine the level of embryogenic callus tissue retained per plate. If a callus had any

embryogenic sections, it was considered embryogenic; this assay was devised as a means to assess the quality of the callus generated (Figure 2.3).

Regeneration of *in vitro* Derived Switchgrass Shoots

Embryogenic calli obtained from the completion of the callus yield trials were selected to determine the regeneration efficiency of plantlets per gram callus, per treatment over 18-weeks (Table 2.1). Embryogenic calli were arranged in a 5x5-grid pattern to establish six replicate plates containing 0.5 g of callus each, on each respective regeneration medium. Cultures were incubated at 26° C, with cool-white fluorescent lighting (66-95 μE m^{-2} s^{-1}) with a 23-h photoperiod for two weeks. All tissue was transferred to regeneration medium once more and incubated for an additional two weeks. All plants were counted from each replicate plate, and the best representative plate from each treatment was photographed (Figure 2.5; Figure 2.7). A plantlet was defined as a single shoot, or shoot-cluster that regenerated from the same crown on a given embryogenic callus, thus suggesting that all shoots arose from the germination of a single somatic embryo. If present, albino shoots were excluded from the total number of plants regenerated.

A regeneration index was used to calculate the efficiency of the media sets. The index is the product of the average amount of callus (g) generated multiplied by the average number of plantlets recovered per 0.5 g of callus for each genotype over the 18-week period (Figure 2.8). The mean and standard error of each treatment was calculated from all possible index value combinations given by the multiplication of callus and plant yields.

Rooting of Switchgrass Plants

Ten regenerants were selected and rooted per treatment (Figure 2.1, Table 2.1). Five plantlets were evenly distributed within each Magenta®GA-7 vessel (Magenta Corporation,

Chicago, USA) and incubated for four weeks, using the same conditions as described for plant regeneration.

Experimental Design and Statistical Analyses

Randomized complete block design was used to conduct each experiment. Data were subjected to Levene's test to check for homogeneity of variance using the software package JMP® (Version 8.0.2 SAS Institute Inc., Cary, NC) (Levene, 1960). If data were not normally distributed ($p \leq 0.05$), they were subjected to a square root transformation and subsequently ANOVA. If a significant difference was detected ($p \leq 0.05$) using ANOVA, the least significant difference test (LSD) was employed to analyze the data for significant differences between treatments within an experiment ($p \leq 0.05$).

MATERIALS AND METHODS PART II – STABLE TRANSFORMATION

Particle Bombardment Parameters for Transformation Efficiency Optimization

Key factors reported to affect biolistic transformations were evaluated by comparing different treatments using a randomized complete block design. The experiments were completed using a strict one-at-a-time approach, whereas a single factor was varied in the transformation process while all other variables remained fixed (Daniel, 1973). This approach assumes factors do not interact; therefore the best treatment was adapted from each experiment and used in each subsequent experiment (Table 2). Each time a particular treatment showed significantly better results than the other treatments tested, the treatment was adopted to become part of the protocol. The adoption of a superior treatment was indicated in the materials and methods section by writing the treatment in a bold, italicized font (e.g. ***treatment***).

The pANIC-6D vector (Mann et al., 2010) was selected for biolistic transformation. This vector contains a novel DsRed-type, red fluorescent protein (RFP) reporter gene, *pporRFP,* isolated from the coral *Porites porites* (Alieva et al., 2008), and the *hph* gene in the Gateway® cassette under control of the *Zm*Ubi1 promoter (Figure 2.9). The *hph* gene was cloned into pANIC-6D as per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The expression of pporRFP can be monitored via *in vivo* fluorescence microcopy using an RFP filter (Alieva et al., 2008). The use of *pporRFP* bypasses the auto-fluorescence associated with traditional GFP (Appendix Figure 1).

Microprojectile Bombardment

Particle bombardment parameters were optimized using the elite genotypes SA37 and ST1. Tissue preparation to obtain type II callus from these genotypes was performed as described above in the tissue culture section using the King et al., unpublished media set.

Embryogenic calli (2-mm-diameter pieces) were subcultured three days prior to bombardment onto fresh N6E medium in 5x5-rows (Figure 2.10). Callus cultures were incubated on 0.6 M N6E osmotic adjustment medium, which was created by supplementing the already present 0.09 M sucrose with equimolar quantities of mannitol and sorbitol as per Chen et al. (1998). Callus cultures were incubated in the dark at 27°C for 6-h prior to bombardment (Altpeter et al., 1996; Chen et al., 1998).

Biolistic-transformation parameters were optimized by bombarding three-replicate plates per treatment; whereas each replicate consisted of a 2.5-cm target of either 25 or 20 type II calli. The PDS-1000 was used with 7,584 kPa (1,100 psi) rupture disks, with all hardware and reagents produced by Bio-rad. Baseline conditions were a microcarrier flight distance of 6 cm, and a vacuum of 97 kPa (27 in) Hg (Kikkert, 1993; Sanford et al., 1993). Microprojectile preparation essentially followed Trick et al. (1997) with the DNA amount decreased from 625 ng to 150 ng per bombardment, and 10 mg of 0.6-μm diameter gold microcarriers used instead of 12 mg of 1-μm microcarriers. Each bombardment consisted of a 10-μl aliquot, which was completely dried on the macrocarrier. When a given parameter was tested (Table 2.2), the remaining ones remained fixed.

Eighteen hours post-bombardment, switchgrass calli were transferred to N6E medium in the same orientation for a ten-day resting period prior to being subjected to selection medium. RFP expression was observed 2-5 days post-bombardment in complete blocks using an Olympus MVX10 microscope equipped with an RFP filter cube, model U-XL49004, all hardware from Olympus. Callus tissue was excited with 545/25 nm light and visualized using a 605/70 nm emissions filter. All RFP foci from each plate were scored manually to generate an average foci

number per treatment. All tissue was placed on N6E medium supplemented with hygromycin B selection (30 mg l^{-1}), and calli were broken up during selection as per Chen et al. (1998).

Four concentrations of osmotic adjustment media were compared consisting of 0.9 M, 0.4 M, 0.6 M and 0.8 M treatment. The first treatment contained 0.09 M sucrose alone, while all other treatments contained 0.09 M sucrose and equimolar quantities of mannitol and sorbitol. Complete microcarrier flight distance was optimized using three distances of 6 cm, 9 cm and 12 cm. All tissue was incubated on *0.6 M* N6E osmotic medium. The size of the microcarriers and helium pressure have a direct correlation with respect to force under Newton's 2nd law whereas *force = mass x acceleration*; assuming there is not a complete vacuum, therefore these factors were tested using a 2x2 factorial design (Table 2.2).

Microcarrier size and helium pressure were optimized using complete microcarrier flight distance of *9 cm*, and calli were incubated on *0.6 M* N6E osmoticum. The gold microcarrier sizes tested were 0.6 μm and 0.4 μm supplied by Bio-Rad (Hercules, Ca) and InBio Gold (Eltham VIC, Australia), respectively. Microcarrier treatments (0.6 μm and 0.4 μm) were made equivalent by surface area using the formula for spherical area given by the formula $A=4\pi r^2$. Therefore, using the standard protocol above, 0.6 μm and 0.4 μm microcarriers were compared by weighing out 10 mg and 4.5 mg of microcarriers, respectively, per treatment. Each microcarrier size was bombarded at one of two different rupture disk pressures, either 4,482 kPa (650 psi), or 7,584 kPa (1,100 psi) (Table 2).

All bombarded tissue was screened at 2-week intervals post-bombardment to monitor for the presence of stable transformants using the RFP reporter. If obtained, transgenic calli were regenerated on selection-free MS medium supplemented with B5 vitamins and 5µM BAP. Plants were rooted on ½ MS0 supplemented with B5 vitamins in GA7 boxes as previously described.

Statistical Analyses

Each experiment was conducted as a randomized complete block with three replicates per plate. Foci data were subjected to Levene's test to check for homogeneity of variance using the software package JMP® (Version 8.0.2 SAS Institute Inc., Cary, NC) (Levene, 1960). If data were not normally distributed ($p \leq 0.05$), they were subjected to a square root transformation and subsequently ANOVA. If a significant difference was detected ($p \leq 0.05$) using ANOVA, the least significant difference test (LSD) was employed to analyze the data for significant differences between treatments within an experiment ($p \leq 0.05$).

TABLES AND FIGURES PART I – TISSUE CULTURE

Table 2.1: Switchgrass tissue culture media sets.

Media Set	Sugars	Panicle Elongation	Induction and Maintenance of Embryogenic Callus	Plant Regeneration	Rooting Medium
Somleva et al., 2000	3% maltose	MS 1 mg/l BAP	MS 1 mg/l BAP 5 mg/l 2,4-D	MS 0.5 mg/l GA3	MS
Burris et al., 2009	3% sucrose	MS 1 mg/l BAP	N6/B5 vitamins 5 mg/l 2,4-D 0.5 g glutamine Proline 0.1 g Casein hydrolysate 0.5 g	MS 1 mg/l BAP	MS
Xi et al., 2009	3% sucrose	MS 3 mg/l BAP	MS 0.15 mg/l BAP 5 mg/l 2,4-D	MS Kinetin 0.2 mg/l	1/2 MS
King et al., unpublished[2]	3% sucrose	*MS/B5 vitamins 1 mg/l BAP	**N6 2 mg/l 2,4-D Proline 2.76 g Casein hydrolysate 0.1 g 25 μM AgNO$_3$	*MS 1 mg/l BAP	1/2 MS/B5 vitamins
Time wks		2 wks	12 wks	4 wks	4 wks

[2]The medium set developed by King et al., unpublished was developed using a series of informal tissue culture experiments and the elite genotypes ST1 and Alamo 2 (data notshown). As the results showed promise, a series of experiments were devised in this manuscript to efficiently compare the King et al., unpublished media set to existing media in the literature. *The MS + 5 μM BAP medium was developed from Alexandrova et al. (1996b) with two exceptions, sucrose substituted maltose, and the pH was raised from 5.5 to 5.8. **N6E medium (Che et al., 2006) was used for callus induction and maintenance.
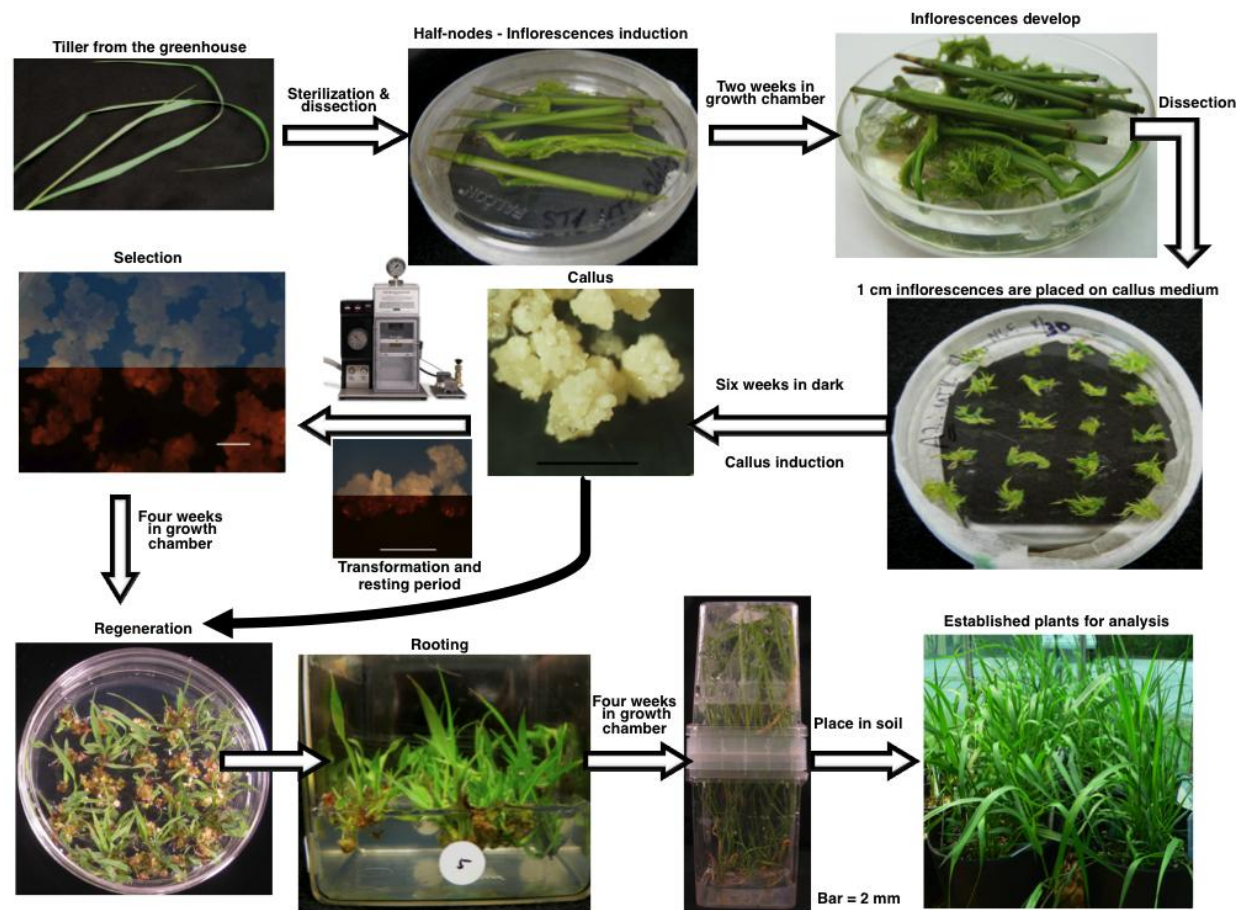
Figure 2.1: Switchgrass somatic embryogenesis, transformation, and regeneration. This figure represents the tissue culture system, which uses N6E (Che et al., 2006) callus induction medium (Table 2.1). Bar = 2 mm.
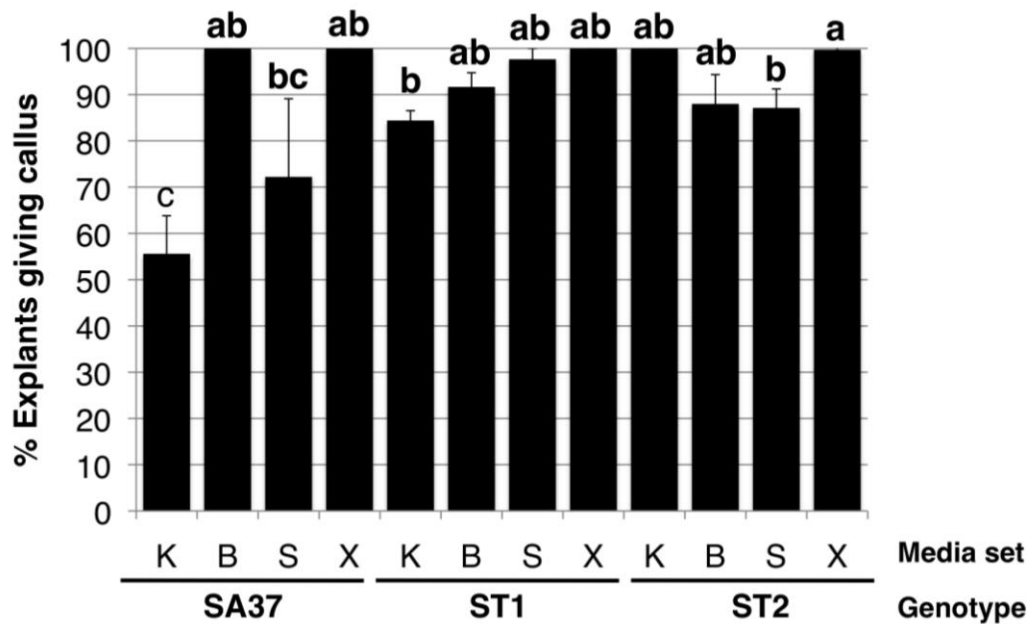
Figure 2.2: Inflorescence explants responding with callus. The number of 1 cm inflorescences pieces which gave rise to embryogenic callus after 6 weeks of growth on callus induction medium was documented, and the percentage of these explants which gave rise to callus were calculated. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Media sets King et al., unpublished (K), Burris et al., 2009 (B), Somleva et al., 2000 (S), and Xi et al., 2009 (X).
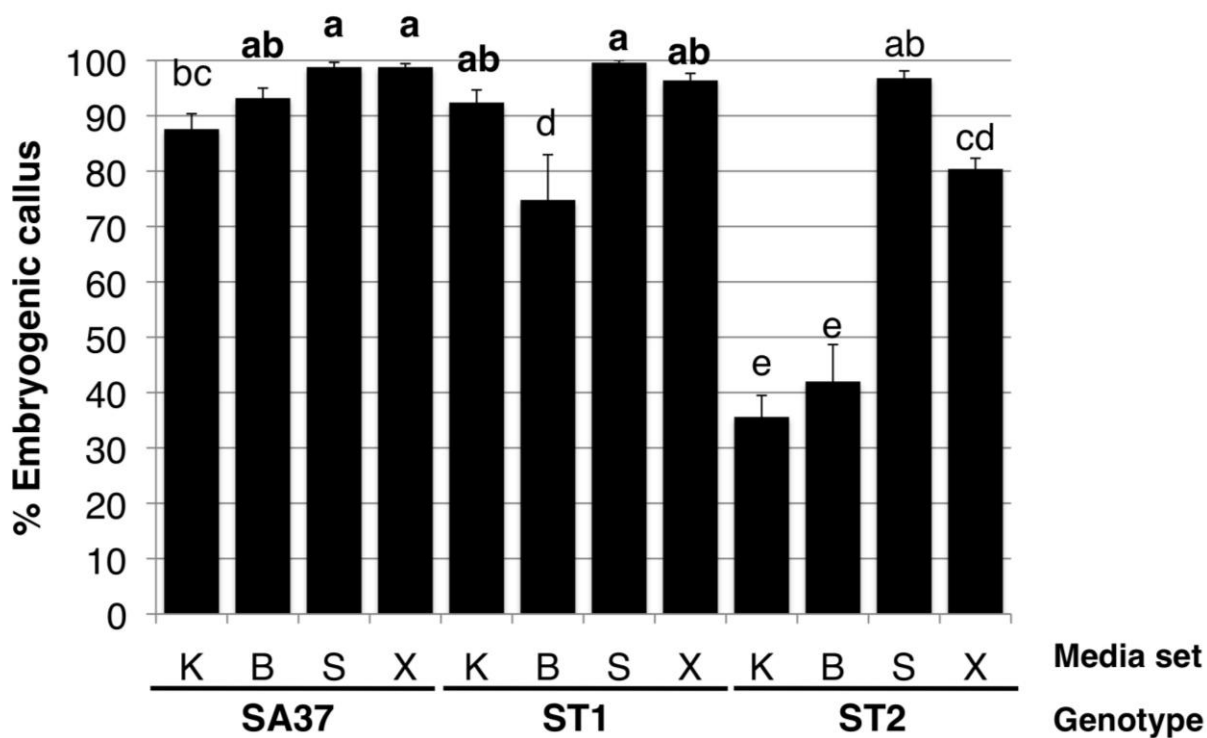
Figure 2.3: Embryogenic callus production from various tissue culture media sets. Bars represent the mean of embryogenic calli counted per treatment with ten biological replicates, each consisting of 25 calli. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Media sets King et al., unpublished (K), Burris et al., 2009 (B), Somleva et al., 2000 (S), and Xi et al., 2009 (X).
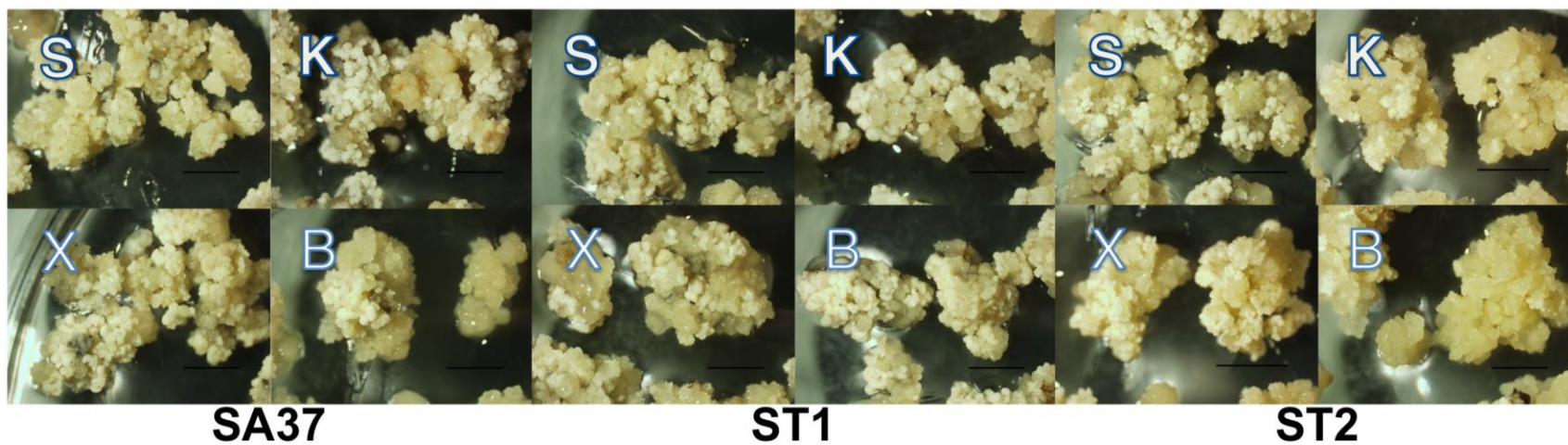
Figure 2.4: Embryogenic callus development. A representative group of calli were photographed from all media set treatments from Somleva et al., 2000 (S), King et al., unpublished (K), Xi et al., 2009 (X) and Burris et al., 2009 (B). Bar = 5 mm.
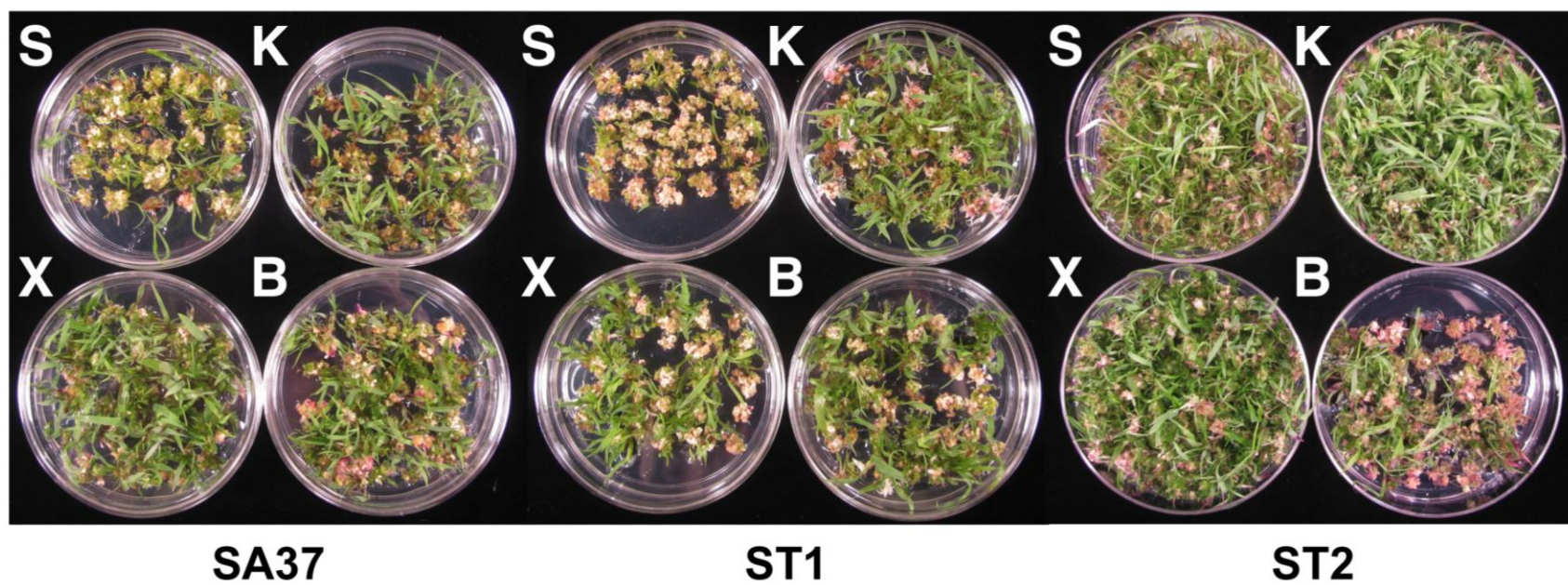
Figure 2.5: Regenerated plantlets from 0.5 g of embryogenic callus per replicate plate. A representative plate of regenerated plants was photographed from all media set treatments from Somleva et al., 2000 (S), King et al., unpublished (K), Xi et al., 2009 (X) and Burris et al., 2009 (B).
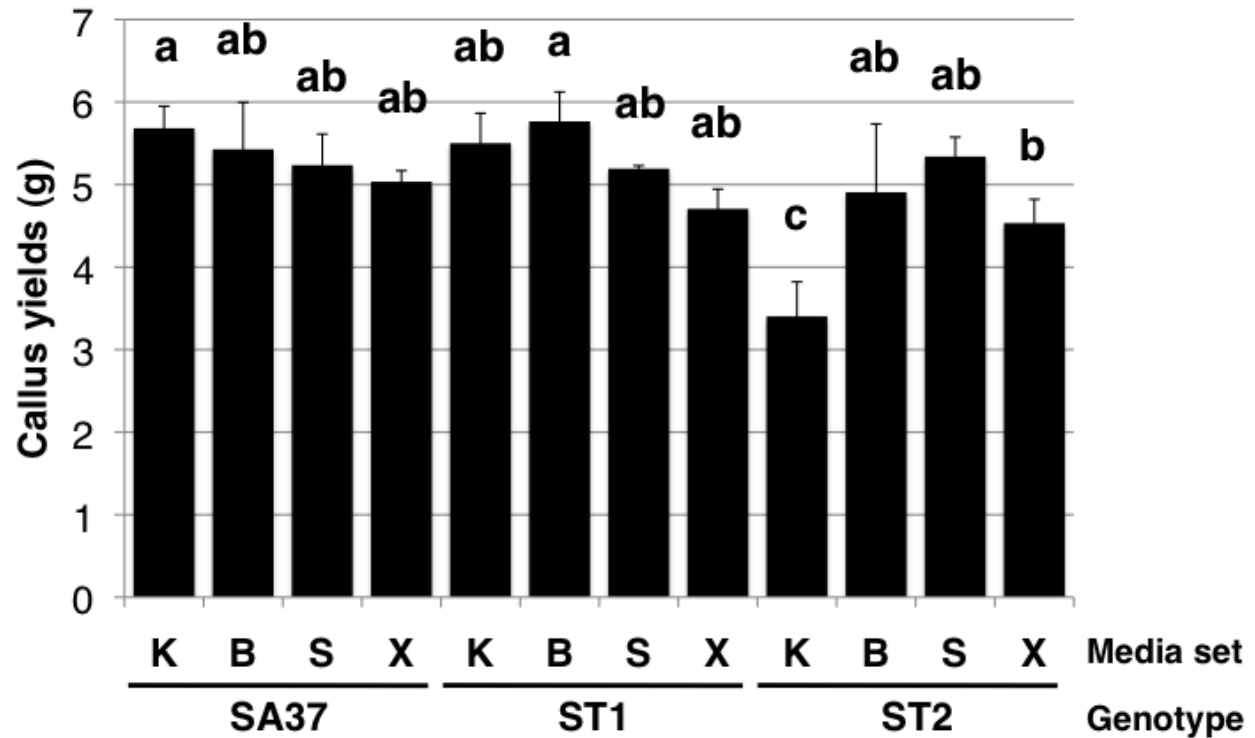
Figure 2.6: Callus yields from six biological replicates obtained after six weeks of growth from 0.25 g of embryogenic callus per replicate. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Media sets King et al., unpublished (K), Burris et al., 2009 (B), Somleva et al., 2000 (S), and Xi et al., 2009 (X).
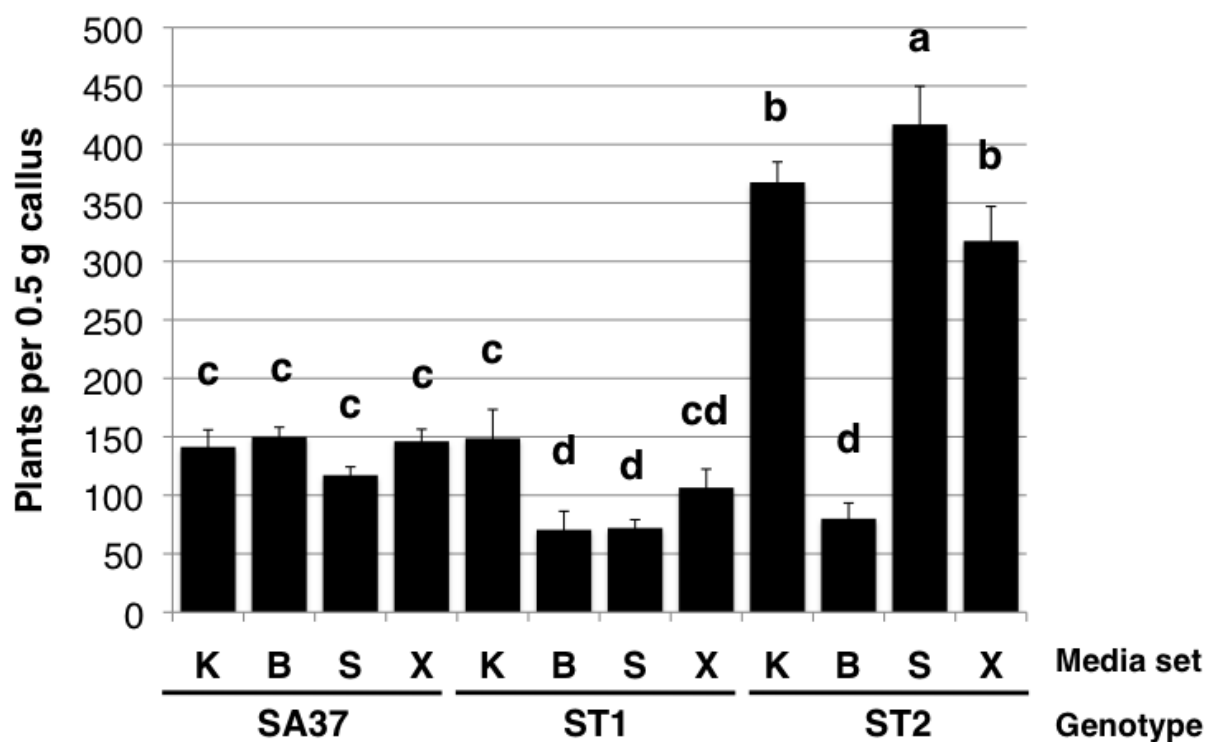
Figure 2.7: Plant regeneration yields from 0.5 g of embryogenic callus. Bars represent the mean yields of plants, which germinated from a single embryo from six biological replicates obtained after four weeks of growth from 0.5 g of embryogenic callus per replicate. Error bars are standard error. Only two replicates were available for ST2 on the (X) media set. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Media sets King et al., unpublished (K), Burris et al., 2009 (B), Somleva et al., 2000 (S) and Xi et al., 2009 (X).
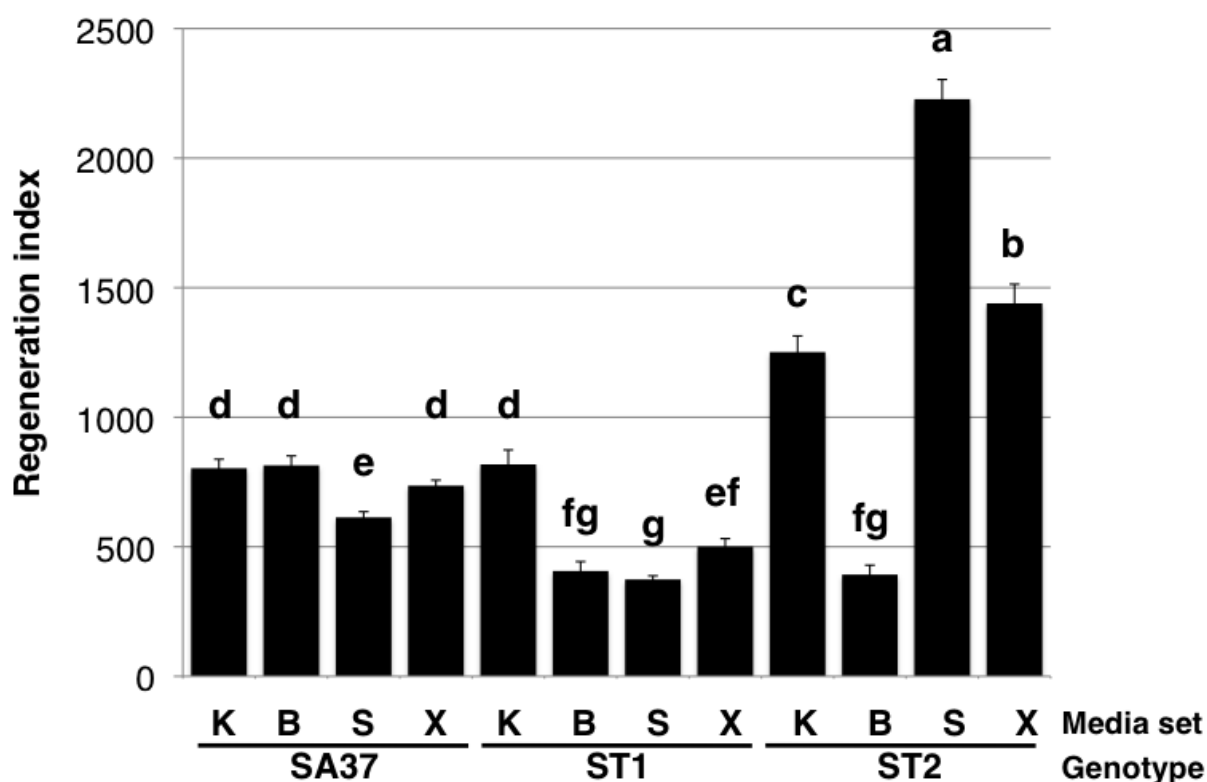
Figure 2.8: The tissue culture regeneration index showing the product of the average amount of callus (g) generated multiplied by the average number of plantlets recovered per 0.5 g of callus for each genotype over the 18-week period. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Media sets King et al., unpublished (K), Burris et al., 2009 (B), Somleva et al., 2000 (S), and Xi et al., 2009 (X).
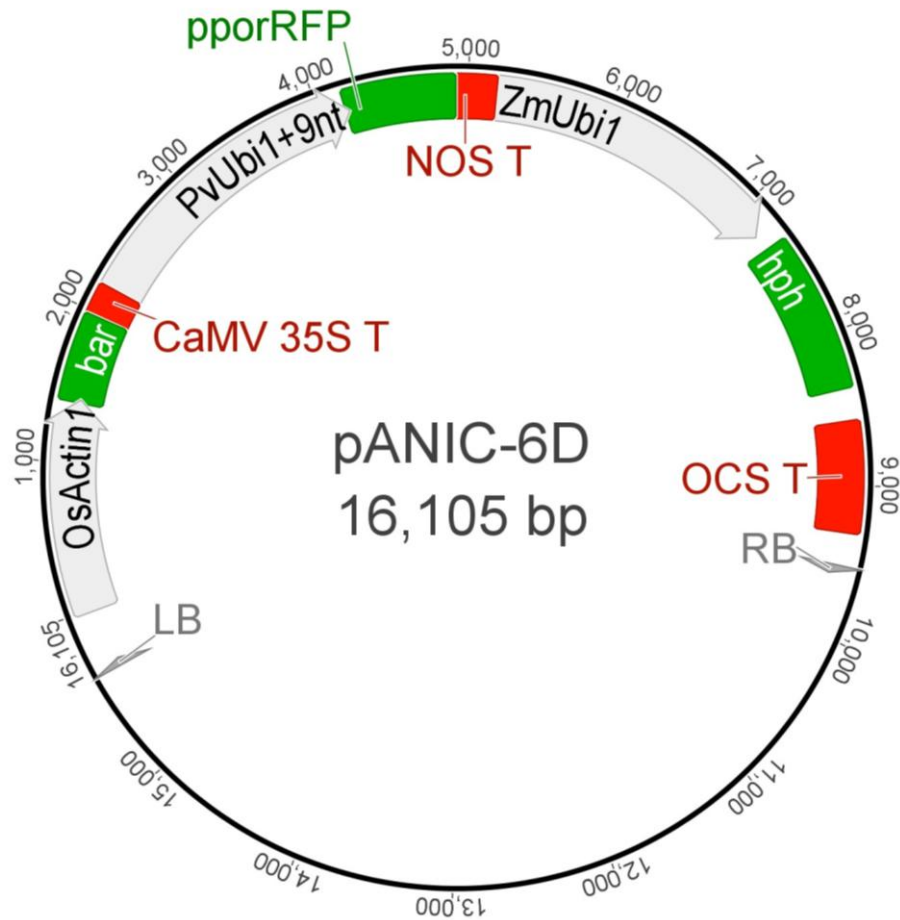
Figure 2.9: The modified pANIC-6D binary vector was used for the biolistic transformation of switchgrass. pANIC-6D has the *Pv*Ubi1+9nt promoter driving *pporRFP,* and is terminated by the *A. tumefaciens* nopaline synthase terminator (*NOS T*). The plant selection cassette, *hph* is driven by *Zm*Ubi1 and is terminated by the octopine synthase terminator (*OCS T*).

Table 2.2: Biolistic parameters tested for switchgrass transformation.

Experiment	Treatment 1	Treatment 2	Treatment 3	Treatment 4
a) Total concentration of sucrose plus mannitol and sorbitol (M)	0.9	0.4	0.6	0.8
b) Microcarrier flight distance (cm)	6	9	12	-
c) Microcarrier size (μm) and helium pressure (psi/kPa)	0.4 and 650/4,482	0.4 and 1,100/7,584	0.6 and 650/4,482	0.6 and 1,100/7,584
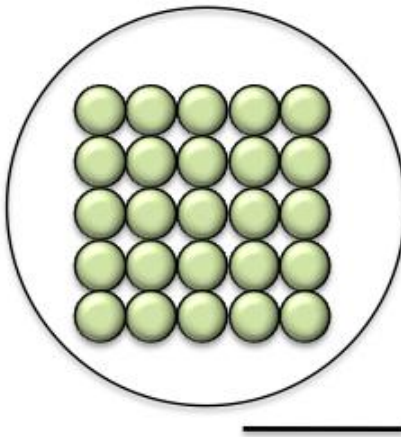
Figure 2.10: The distribution of switchgrass calli prior to bombardment. All calli per replicate were transferred in the same orientation and RFP foci were manually scored. Bar = 1 cm.
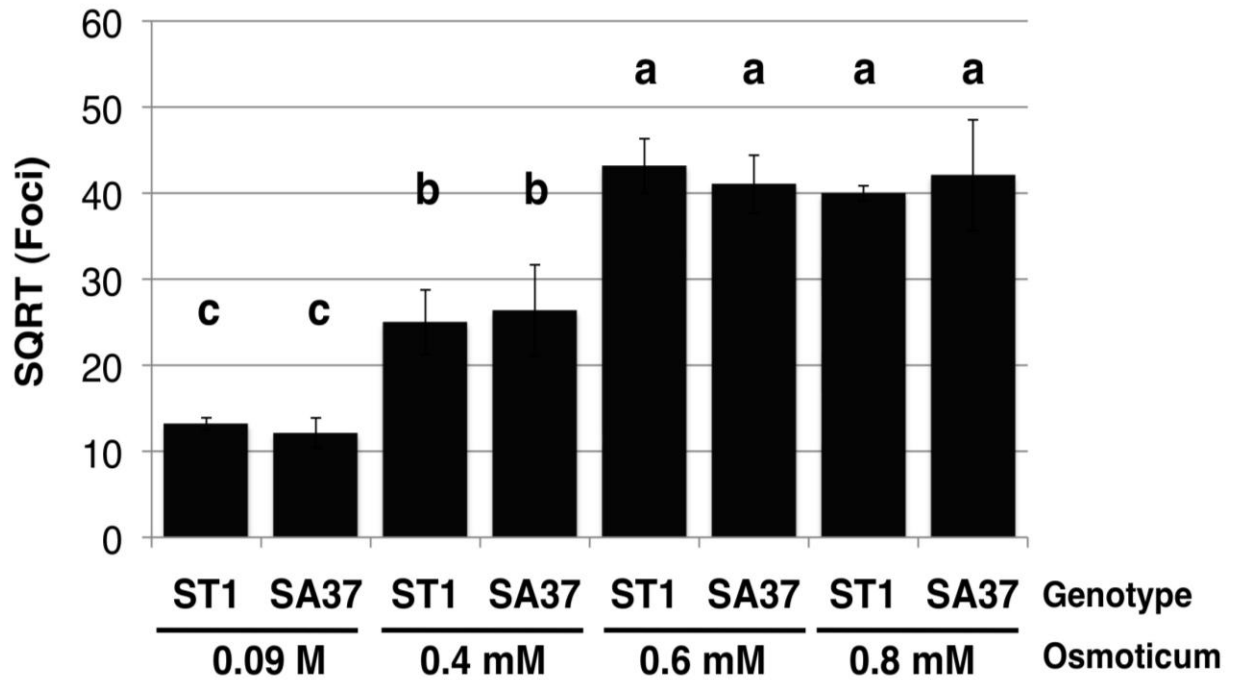
Figure 2.11: Osmotic medium optimization for biolistic transformation. The average number of RFP foci counted per plate of switchgrass callus bombarded. Bars represent the mean number of foci from three biological replicates after a square root transformation was performed. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($P \leq 0.05$).
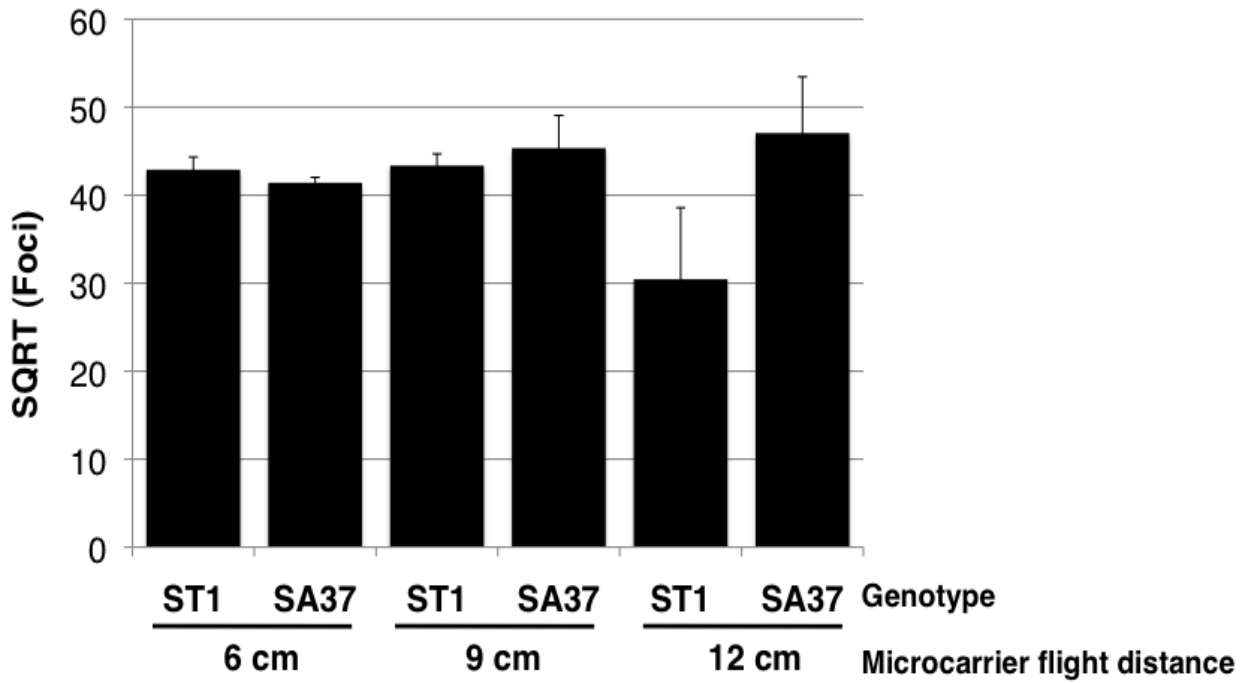
Figure 2.12: Microcarrier flight distance optimization for biolistic transformation. The average number of RFP foci counted per plate of switchgrass callus bombarded. Bars represent the mean number of foci from three biological replicates after a square root transformation was performed. Error bars are standard error.
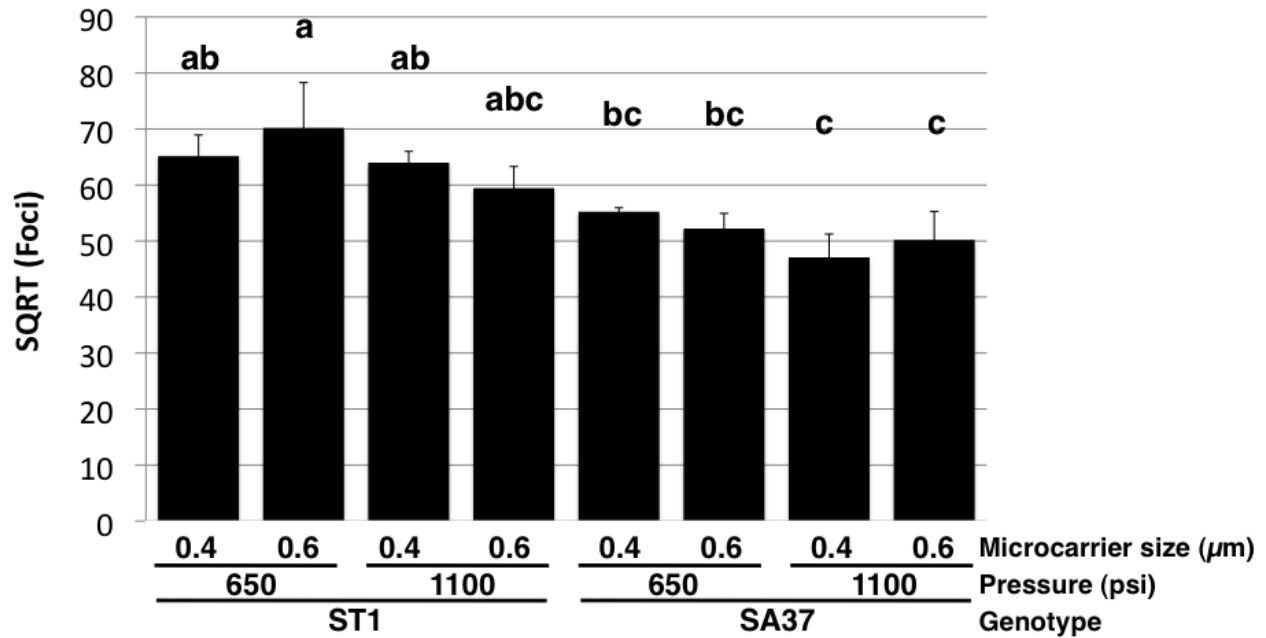
Figure 2.13: Microcarrier size and rupture disk pressure optimization for biolistic transformation. The average number of RFP foci counted per plate of switchgrass callus bombarded. Bars represent the mean number of foci from three biological replicates after a square root transformation was performed. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($P \leq 0.05$).
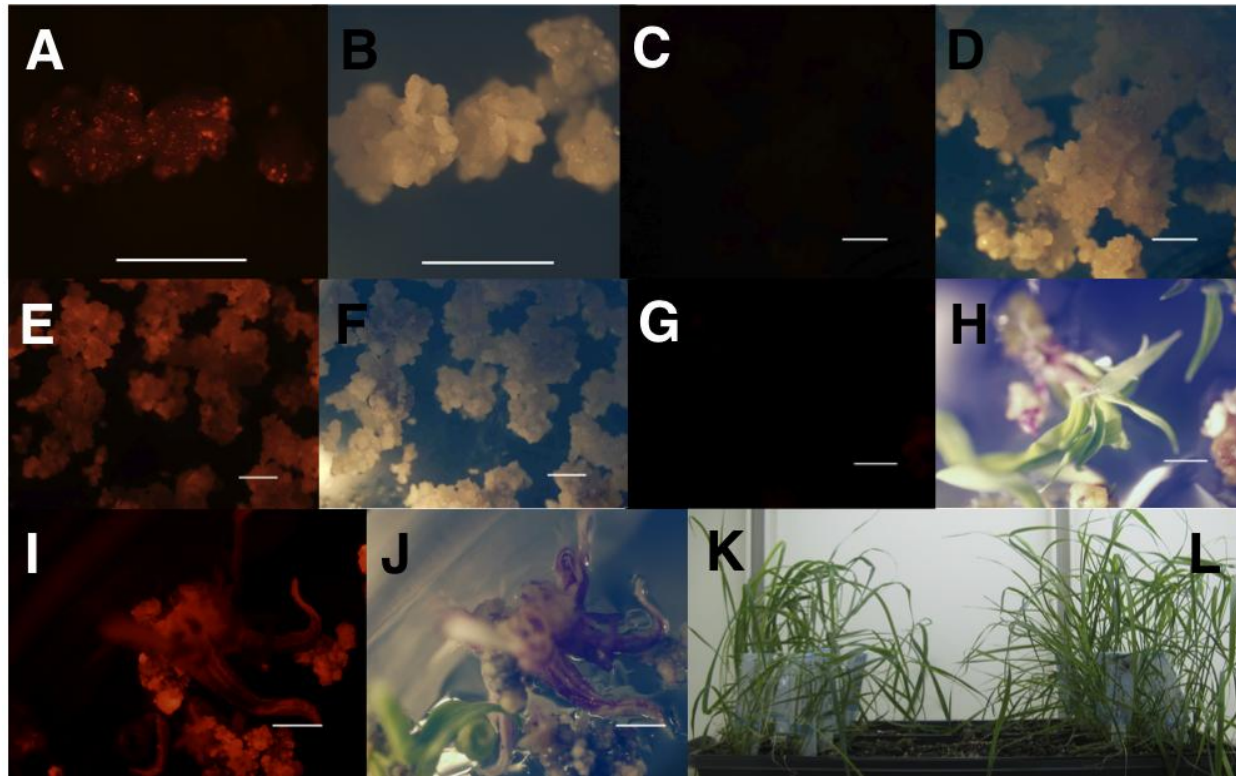
Figure 2.14: Red fluorescent protein expression of transgenic SA37 switchgrass and the wild type SA37 control. Transient expression two days post-bombardment on a highly embryogenic callus (A), non-transgenic callus (RFP filter) (B), non-transgenic callus (white light) (C), transgenic callus (RFP filter) (E), transgenic callus (white light) (F), non-transgenic plant (RFP filter) (G), non-transgenic plant (white light) (H), transgenic plant (RFP filter) (I), transgenic plant (white light) (J), transgenic plant (K), and non-transgenic plant (L).

Establish embryogenic callus

10 weeks

Microprojectile bombardment

1 day

Callus culture without selection

10 days

Callus culture with selection

4-8 weeks

Plant regeneration and rooting

6 weeks

Transgenic plants in soil
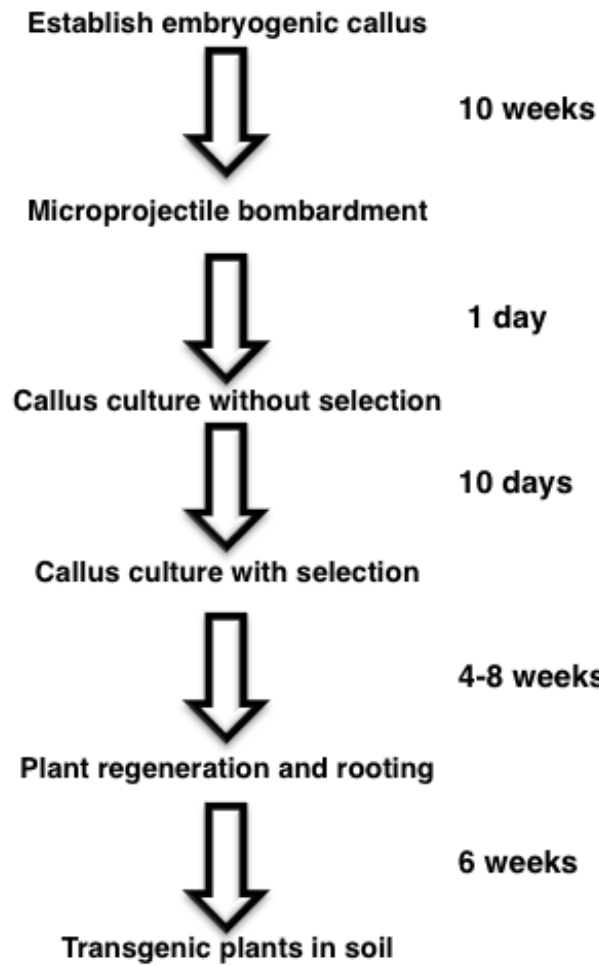
Total time in culture ca. 22-26 weeks

Figure 2.15: Switchgrass biolistic transformation timeline. Figure adapted from Birch (1997).

RESULTS AND DISCUSSION PART I – TISSUE CULTURE

Generally, monocot species such as maize and switchgrass produce two morphotypes of embryogenic callus (Armstrong and Green, 1985). Type I callus is made of compact cells, which can generate somatic embryos and can develop organized structures, but may not have long-term regenerability, thus limiting their use in high-throughput systems for the production of transgenic plants. Type II callus is preferred for its ability to produce somatic embryos from embryogenic callus cultures (Emons, 1994) with highly retained germination for over six months in species such as maize, and switchgrass (Armstrong and Green, 1985; Burris et al., 2009; Li and Qu, 2010). Additionally, type II callus permits the use of multiple tissue culture systems for transformation via cell suspension and protoplast cultures (Carvalho et al., 1997) which can be used for successful transformations e.g. Chen et al. (1998).

Along with optimizing transformation methods, there is substantial benefit to improve the regeneration protocols of switchgrass, which depend heavily on the media employed. Specifically, some compounds such as silver nitrate ($AgNO_3$) supplemented into *in vitro* cultures allow for highly productive, embryogenic and regenerable type II callus cultures to form in maize (Songstad et al., 1991). However, Zhao et al. (2002) found that $AgNO_3$ in the inoculation medium reduces the efficiency of *A. tumefaciens*-mediated transformation of maize by two to four-fold. Moreover, the addition of $AgNO_3$ was found to increase stable transformation efficiency up to 1.7 times when added post-inoculation (Frame et al., 2002; Zhao et al., 2002).

Many studies suggest hygromycin B kills sensitive plant cells more efficiently in some species then the antibiotic, kanamycin, and may prevent non-transgenic escapes. Therefore hygromycin B is commonly used for selection as opposed to kanamycin (Rosellini et al., 2004; Vasil, 1994; Wilmink and Dons, 1993) and has been successful in obtaining fertile transgenic

switchgrass plants with high efficiency (Burris et al., 2009; Fu et al., 2011; Li and Qu, 2010; Xi et al., 2009).

The callus quality and regeneration index metrics presented here allowed several conclusions. Firstly, superior genotypes and media sets can be selected based on the quality of embryogenic callus (Figure 2.3), callus yields (Figure 2.6) and regeneration response (Figure 2.7). These figures can be simply and accurately quantified to generate informative data to evaluate media and genotypes, and the regeneration index can be used to demonstrate both callus yields and regeneration capacity (Figure 2.8).

Based on these data, ST1 is not an effective genotype due to lower performance in comparison to other genotypes such as SA37 and ST2 (Figures 2.7; 2.8). In regards to the regeneration index, ST2 significantly outperformed all other genotypes on all media sets except for Burris et al. (2009), which highlights the importance of genotype and medium selection in switchgrass transformation (Figure 2.8).

The results shown here demonstrate each media set and genotype combination produced high quality inflorescence tissue that could readily be induced to form callus (Figure 2.2). The callus generated from each elite genotype in all treatments was highly regenerable, with a minimum of 50 plants recovered from each half gram of callus. ST2 has the potential to yield 450 regenerated plants from a half gram of callus which exceeds the standard set by the commercial switchgrass industry (Metabolix Inc., Baltimore, MD) led by Somleva et al. (2008). Somleva et al. (2008) must continually screen many seed-derived genotypes to find those that regenerate 175 plantlets per gram of callus, approximately 2.5-fold less regeneration efficiency than ST2 (Figures 2.5; 2.7). Additionally, bootstage switchgrass tillers can be harvested weekly

for high-quality explants, for many years by replanting potted genotypes at three-month intervals. This is little effort compared to routinely generating, and harvesting variable seed lots.

Conclusion

In order to establish a high-throughput transformation system, ample amounts of high-quality callus are required to be generated routinely and maintained with relative ease. By using immature inflorescences it is easy to maintain high quantities of quality tissue year round in greenhouse conditions (Figure 2.2). Using known genotypes and quality media that produce predictable responses (Figures 2.2; 2.3; 2.7; 2.8) will make the transformation process and downstream analysis of transgenic plants routine. Explants derived from elite genotypes will not confound data, and allow greater sensitivity to detect subtle gene effects not detected with seed-derived explants (Xi et al., 2009).

When initiating cultures, the Xi et al. (2009) media combination to induce immature inflorescences to grow, followed by callus induction for six weeks cause over 99% of all explants from all genotypes to induce callus (Figures 2.2; Table 2.1). Highly significant treatment effects for callus yields were observed ($p < 0.01$); and Somleva et al. (2002) callus induction and maintenance medium yielded over 5.15 g of tissue from 0.25 g of callus over a period of 6 weeks for all genotypes (Figure 2.6). Additionally, the Somleva et al. (2002) callus induction and maintenance medium produced over 96% of calli with embryogenic sections. All callus induction and maintenance medium treatments other than ST2 on the King et al., (unpublished) media produced sufficient callus yields (Figure 2.6; Table 2.1).

The King et al., (unpublished) regeneration medium produced the most consistent regeneration response for all genotypes; the regenerated plantlets produced by the Somleva et al. (2002) regeneration medium produced elongated and brittle shoots compared to the other

regeneration media tested, which may have been caused by the gibberellic acid in the medium (Figures 2.5; 2.7; Table 2.1). All treatments produced phenotypically normal rooted plants after 22 weeks (Table 2.1).

No single media set performed equally well at each stage for all genotypes tested. These data support tailoring the medium at each stage of the tissue culture process, whereas Xi et al. (2009) would be used to induce callus from inflorescences as mentioned above, Somleva et al. (2002) would be used during the callus maintenance process, and the King et al. (unpublished) regeneration medium would be used to regenerate and root plants (Figures 2.2; 2.3; 2.6; 2.7). The largest differences between genotype response *in vitro* was observed at the plant regeneration stage whereas ST2 regenerated the best on all media except the Burris et al. (2009) regeneration medium (Figure 2.7).

The most difficult part of switchgrass tissue culture and transformation is retaining callus quality and yields overtime (Burris et al., 2009; Li and Qu, 2010). This bottleneck needs to be surpassed in order to have a high-throughput transformation pipeline. The genotype SA37 was selected from 215 F_1 progeny for its superior callus response from inflorescence tissue. SA37 is favored for plant tissue culture for its high callus yields and quality (Figures 2.2; 2.3), specifically; SA37 produced more embryogenic sections on a given callus than ST1 or ST2.

RESULTS AND DISCUSSION PART II – STABLE TRANSFORMATION

Particle Bombardment Optimization

Biolistic transformation offers several advantages over *Agrobacterium*-mediated transformations. Firstly, some cell cultures have a hypersensitive response to *Agrobacterium* (Kikkert et al., 2004). Additionally, when using *Agrobacterium* it is necessary to eliminate the bacterium *in vitro* after transformation, and the elimination of false positives caused by *A. tumefaciens* harboring the binary vector in PCR screening. *A. tumefaciens*-mediated transformation does not lend itself to convenient transient expression assays, as gene expression cannot be differentiated between plant cells and bacteria, or discretely quantified (Wang and Ge, 2005).

However, a perceived concern with biolistic transformation is the ability to produce transgenics with single copy insertions, which comes from the fact that the initial protocols used microgram quantities of plasmid DNA. Today, efficient protocols range considerably in the amount of DNA bombarded, from only a few nanograms (e.g. 2.5 ng μl^{-1}) to hundreds of nanograms per bombardment, and must be optimized for the species and project goals (Chen et al., 1998; Goldman et al., 2003; Lowe et al., 2009; Trick et al., 1997). Furthermore, current protocols deliver low concentrations of linear DNA cassettes, such that biolisitic transformation of maize can produce up to 46% of single-copy transformants with "clean inserts," harboring no vector backbone (Fu et al., 2000; Lowe et al., 2009).

Bombardment parameters, such as the time following subculture prior to bombardment, are important for many plants such as rice, soybean, maize and giant reed (*Arundo donax* L.) (Chen et al., 1998; Dhir et al., 2010; Hazel et al., 1998; Songstad et al., 1996). For example, rice callus was found to be highly transformable with a subculture nine-days prior to bombardment

(Chen et al., 1998). Alternatively, for soybean and maize, a subculture 4 days prior to bombardment was found optimal for the transformation competency of globular-stage somatic embryos and callus, respectively (Hazel et al., 1998; Songstad et al., 1996). Increased mitotic activity resulted after soybean somatic embryos were transferred to fresh culture medium, which peaked on the fourth day post-subculture (Hazel et al., 1998). In this study, embryogenic switchgrass callus was incubated on N6E medium three-days prior to bombardment.

The strict one-at-a-time approach used in these experiments allowed a number of factors known to affect transient and stable transformation efficiencies to be tested using embryogenic switchgrass callus in separate experiments. In this manner the effect of variables on transient expression were observed (Table 2).

The strength of osmotic adjustment medium in which cells are incubated prior to bombardment has been shown to make a significant difference in the number of foci observed, and transgenic plants obtained downstream (Chen et al., 1998; Southgate et al., 1995). The effect of osmotic adjustment medium in the biolistic transformation of switchgrass was never investigated. The only transgenic switchgrass recovered via particle bombardment used MS medium containing 22.5 μM 2,4-D, 5 μM BA and was supplemented with 0.4 M sucrose, mannitol and sorbitol as described above (Richards et al., 2001).

The osmotic adjustment media treatments selected to optimize switchgrass transformation presented here are within range of those tested for rice (Chen et al., 1998). The results showed a highly significant difference between treatments due to osmotic adjustment medium ($p < 0.0001$). Switchgrass calli incubated on 0.6 M or 0.8 M osmotic adjustment media showed significantly more foci then those incubated on 0.09 M or 0.4 M media, and there were no significant differences between genotypes at any given osmotic treatment (Figure 2.11). This

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reflects the result obtained by Chen et al. (1998), as rice transformation was also optimal using a 0.6 M osmotic adjustment medium.

Sanford et al. (1993) found the complete microcarrier flight must be wide enough to not cause excessive cell damage (Kikkert, 1993; Sanford et al., 1993).). In this study, no significant difference in the number of foci was observed between treatments due to complete microcarrier flight distances ($p > 0.2186$) (Figure 2.12). There were no significant differences between genotypes at any given complete microcarrier flight distances.

In biolistics, helium pressure directly affects the velocity of the microcarriers, which results in varying levels of cell penetration and tissue damage, and must be optimized on a species, and target tissue level. However, 7,585 kPa (1,100 psi) is near optimal to transform most cell types and species (Dhir et al., 2010; Kikkert, 1993; Kikkert et al., 2004; Sanford et al., 1993), although other species such as pearl millet (*Pennisetum glaucum* (L.) R. Br.) require higher helium pressure (e.g. 10,342 kPa (1,500 psi)) for optimal transformation (Goldman et al., 2003).

Additionally, only limited research exists on the optimal size of the gold microcarriers, which may have variable optima between species (Southgate et al., 1995). The recovery of transgenic maize lines increased 13% when 0.6 µm microcarriers were used as opposed to 1.0 µm (Frame et al., 2000).

The 2x2 factorial experiment of microcarrier size and helium pressure showed a significant treatment effect ($p = 0.0179$) (Figure 2.13). No significant differences were detected due to microcarrier size or rupture disk pressure within a genotype. The 0.4 µm microcarriers are two-thirds the diameter of the 0.6 µm microcarriers; therefore it is reasonable to expect cell damage may be less severe when using the 0.4 µm microcarriers.

When rupture disk pressure and microcarrier size where evaluated in switchgrass transformation, the treatment which used 4482 kPa (650 psi) rupture disks with 0.6 μm microcarriers produced on average approximately 30 and 16% more foci per bombardment for the genotypes ST1 and SA37, respectively compared to the standard treatment of 7584 kPa (1,100 psi) with 0.6 μm microcarriers, and will therefore be used in future transformation experiments (Figure 2.13). This experiment resulted in over 6,000 foci observed across 20 pieces of tissue bombarded from one plate (Figure 2.13).

Conclusions

Baseline conditions are now optimized, and a genotype independent protocol has been established based on transient expression data for switchgrass. Optimized conditions are a 6-h incubation prior to transformation on 0.6 M N6E osmotic adjustment medium, followed by an 18-h incubation post-bombardment. For the actual bombardment, a 97 kPa (27 in) Hg vacuum, a complete microcarrier flight distance of 9 cm, and 0.6 μm or 0.4 μm microcarriers propelled at 4482 kPa (650 psi) proved to be optimal.

The 0.6 M osmotic adjustment medium showed a significant improvement over the 0.4 M osmotic adjustment medium previously described (Richards et al., 2001). Although not statistically significant, the flight distance of 9 cm consistently showed the highest number of foci for both ST1 and SA37 (Figure 2.12).

Using the 0.6 M osmoticum and the standard procedure prior to optimization, a single SA37 and ST2 event were recovered prior to the optimization process, yielding a transformation efficiency of over 1% for both genotypes. It is too early to know the effect of the optimized treatments in terms of stable transformation efficiency, as the process to obtain switchgrass events is still difficult and can require over two months of selection, however, transient

expression data has been a reliable tool to optimize bombardment parameters for stable transformations in other species (e.g. Chen et al., 1998).

The SA37 and ST2 events showed RFP expression throughout the callus selection and plant regeneration process, and an example showing RFP expression at each stage using SA37 was recorded (Figure 2.14, Figure 2.15). These results are promising as fluorescent protein markers appear to be the key to optimizing transformation conditions for switchgrass or other species recalcitrant to transformation (Harry Richards, University of Tennessee, personal communication) (Li and Qu, 2010). The *pporRFP* reporter gene is now routinely used for rice transformation (Appendix A), and can be useful for event selection or transformation optimization. The optimized transformation protocol described above will be used in complement with the optimized media set described above which adopts the best medium for each stage of the tissue culture process.

The switchgrass transformation community is aware that low transformation efficiencies are caused primarily by the use of genotypes which are recalcitrant to transformation, a barrier that has had substantial improvement as of late (Li and Qu, 2010). However, even with media sets such as those described in Burris et al. (2009), only one-third of the callus generated is desirable, causing excessive labor to maintain cultures of transformable tissue. This hurtle may be overcome by a breeding and selection effort to obtain genotypes more transformable than those present here (Zeng-Yu Wang, Samuel Roberts Noble Foundation, personal communication).

Future studies should address whether or not elite cultivars such as 'Performer' routinely create high-quality callus cultures from inflorescences cultures, as seed-derived callus is highly variable and costly to manage seeing as only 10% of all callus is usable in the transformation process (Li and Qu, 2010). The use of elite genotypes bred for transformation, such as SA37, coupled with well optimized transformation protocols will advance this field.

CHAPTER 3

CHARACTERIZATION OF THE SWITCHGRASS (*PANICUM VIRGATUM L.*) PROMOTERS *PVUBI1* AND *PVUBI2* IN SWITCHGRASS AND RICE TRANSFORMATION[3]

INTRODUCTION AND LITERATURE REVIEW

Advances need to be made in monocotyledonous plant vectors to ensure their usefulness for switchgrass (Mann et al., 2010). Currently, it is not known which promoters will work best to express genes in switchgrass cells and tissues.

Although MUG assays can be used to quantify enzymatic activity, there is a drawback. Due to the heterogeneity associated with particle bombardment (Kikkert, 1993; Russell et al., 1992a; Russell et al., 1992b; Sanford et al., 1993), gene-expression data require normalization. For this reason MUG results are normalized by the expression of the luciferase gene (*luc*) isolated from the North American firefly, *Photinus pyralis* (Ow et al., 1986) in co-transformation. The luciferase luminescence reaction produces light when the substrate luciferin is catalyzed in an adenosine triophosphate-dependent oxidation (Ow et al., 1986).

Schledzewski and Mendel (1994) used biolistic transformation to express the *GUS* gene (*gusA*) in cell-cultures of maize, barley and tobacco. They tested various promoters fused to *gusA,* namely the maize polyubiquitin 1 with intron (*Zm*Ubi1) (Christensen and Quail, 1996); rice actin1 with intron (*Os*Act1) (McElroy et al., 1990); maize *Emu*, a recombinant truncated Anarobic Responsive Element from the maize *Adh1* gene and OCS-elements of the octopine synthase gene of *A. tumefaciens* (Last et al., 1991); and the cauliflower mosaic virus *CaMV 35S* (Topfer et al., 1988). The *Zm*Ubi1 and *Os*Act1 promoters provided upwards of 15-fold more gene expression in barley and maize cultures than in tobacco cultures, supporting the premise that promoter selection is critical to optimize transgene expression in monocots. These data were normalized using *luc* expression driven by the *CaMV 35S* promoter in separate vector in co-bombardment. Luciferase quantification provided lower standard error when compared to normalization using a Bradford assay to quantify total protein (Schledzewski and Mendel, 1994).

Thus, co-bombardment of the reporter genes normalizes for the variability associated with microcarrier-DNA binding efficiency, differences in cell competence, cell fitness, and bombardment variability associated with biolistic transformation (Lanahan et al., 1992). The variability associated with MUG and luciferase assays has been further reduced by using a single lysis buffer (LB) to measure gene expression from both cassettes. Additionally, the authors found a 62% higher level of GUS activity using the LB buffer in comparison to results obtained when GUS extraction buffer was used (Leckie et al., 1994).

Rice has become the model crop for *A. tumefaciens-* and particle bombardment-mediated transformation studies, as rice is relatively easy to transform and regenerate in a short time frame (Chen et al., 1998; Nishimura et al., 2006). Additionally, rice has become a very powerful model species for the routine analysis of large numbers of events, and the effects of transgenes and promoters in reverse genetics (Bajaj and Mohanty, 2005; Biswas et al., 1998; Chen et al., 1998; Christensen and Quail, 1996; LaFayette et al., 2005; Li et al., 1993; Nishimura et al., 2006; Sivamani and Qu, 2006; Sivamani et al., 2009; Wang and Oard, 2003).

To date, high-throughput systems for switchgrass transformation are still difficult, and time consuming. Therefore, agricultural companies such as Ceres Inc. (Thousand Oaks, CA, USA) have developed high-throughput pipelines by performing initial promoter-gene combination screens using the model plant, *Arabidopsis thaliana* (4,000 events/quarter) followed by the model crop, rice (150 events/quarter). Only when desirable phenotypes are obtained using a specific construct with both models is a cellulosic crop transformed (e.g., switchgrass, sugar cane, and miscanthus), as transformation efficiency is too low, and the cost and space needed per event is too great. For these reasons rice was selected in this study to test the *Pv*Ubi- promoters and classically used promoters as opposed to switchgrass.

MATERIALS AND METHODS

Two novel *P. virgatum* polyubiquitin (*Pv*Ubi) promoters were isolated from cv. Alamo*,* and designated *Pv*Ubi1 and *Pv*Ubi2 (Mann et al., 2010). Inasmuch as promoter fusions have been shown to increase the transgene expression and accumulation of the protein product (Hondred et al., 1999; Sivamani and Qu, 2006), variations of the *Pv*Ubi promoters were created. These contain the original promoter sequence, which includes a 9, 18, or 27-nt of the coding sequence fused to the open reading frame downstream of the respective *Pv*Ubi intron.

In order to evaluate candidate *Pv*Ubi- and classic monocot-promoters, the pHLucGWgus vector was constructed containing an internal *Zm*Ubi1:*luciferase* cassette to normalize gene expression data and a Gateway® site to evaluate promoter strength (Invitrogen, Carlsbad, CA) (Figure 3.1).

The reporter gene cassettes were assembled from various sources, sequenced, and annotated using Geneious v5.0.3 software (Drummond et al., 2010). Each unique promoter was cloned into the Gateway® site of the pHLucGWgus vector upstream and in frame of *gusA* as per the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Each promoter was compared to the gene expression of the *CaMV 35S* promoter, which acted as a control to allow promoter strength to be visualized as a dimensionless value of relative strength (Schledzewski and Mendel, 1994; Sivamani and Qu, 2006).

Switchgrass Plant Material and Tissue Culture

A tissue culture responsive Alamo genotype referred to as ST1was supplied by the Noble Foundation (Xi et al., 2009). Plants were maintained in the greenhouse by cutting back tillers which matured beyond boot stage (Moore et al., 1991) in a 42% sand, and 58% Fafard 3B soil mix. Growth conditions consisted of a 12-h photoperiod under 400-watt high-pressure sodium

lighting. The greenhouse was kept at approximately 27°C, and ranged in temperature from 20-27°C. Plants were watered daily, and fertilized weekly with 0.453 kg of Peters® Professional All Purpose Plant Food (St. Louis, MO) per 378.54 liters of water (1 lb 100^{-1} gallons).

Harvesting Explants

The last culm node of switchgrass produced immature inflorescences at the E2-R0 stages (Moore et al., 1991). Culm nodes were identified by the presence of immature inflorescences as previously described by Alexandrova et al. (1996). Culm nodes were identified and all tissue 5.0 cm above, and 1.5 cm below the culm node were harvested as an initial explant.

Surface Sterilization of Initial Explants

The 6.5-cm internode explants were surface-sterilized with 70% ethanol, for 1 minute with gentle agitation. Subsequently explants were placed in 15% Clorox® v/v with 2 drops of Tween 20 per 100 ml, for 3 minutes with gentle agitation. All tissues were then rinsed 3 times, at 2-minute intervals.

For all media, 2.5 g l^{-1} Gelzan™ was used as the solidifying agent, and the pH was brought to 5.8 prior to autoclaving. Cultures were initiated in 20x100 mm Petri dishes. All vessels were sealed with 3M Micropore™ tape. Sterilized internodes were halved longitudinally (Alexandrova et al., 1996) and two explants were placed cut-side down on solid MS medium supplemented with B5 vitamins and 5µM BAP to induce and elongate panicles. Explants were incubated in a growth chamber at 25° C, with cool-white fluorescent lighting (66-95 µE m^{-2} s^{-1}) 16-hour light cycle for 14 days.

Initiation of Embryogenic Switchgrass Callus Cultures

After 14 days of culture, immature inflorescences were used to initiate embryogenic callus

cultures. The inflorescences were dissected out and cut to obtain sections of rachis tissue

measuring 1 cm in length.

Inflorescence pieces were placed on solid N6E medium (Che et al., 2006) arranged in a

4x4-grid pattern to induce and maintain callus in 15x100 mm Petri dishes. Tissue was incubated

at 27°C to in the dark and subcultured at three-week intervals.

After the second subculture, callus was separated from the inflorescences and arranged in

a 5x5-grid pattern. Compact, embryogenic callus tissue was bulked for 8 months with

subcultures at three-week intervals and use in particle bombardment experiments.

Rice Plant Material and Tissue Culture

The United States Department of Agriculture-National Plant Germplasm System

provided seeds of *Oryza sativa* L. japonica cv. Taipei 309 (TP309). Kernels from dehusked

seeds were surface-sterilized in 70% ethanol for 2 minutes rotated at 100 RPM. Kernels were

then transferred to a 60% Clorox® solution (v/v) supplemented with Tween-20 (Fisher Scientific,

Pittsburgh, PA, USA) 0.01% and stirred for 30 minutes. The kernels were subsequently passed

through three 2-minute water rinses. Sterilized kernels were dry. All rice media were brought to

a pH 5.8 prior to autoclaving, solidified with 2.5 g l^{-1} Gelzan™, and all vessels were sealed with

3M Micropore™ tape in 100x15 mm Petri dishes.

Kernels were arranged in a 5x5-grid on modified NB medium (MNB) as per Chen et al.

(1998) and incubated in the dark at 27°C. Prior to particle bombardment rice callus was induced,

selected, and maintained as previously described for 5 months with transfers at 3-week intervals

(Chen et al., 1998).

Genetic Transformation

The PDS-1000 was used for plasmid delivery with 7,584 kPa (1,100 psi) rupture disks, with all hardware and reagents produced by Bio-rad, with a microcarrier flight distance of 6 cm and a vacuum of 97 kPa (27 in) Hg (Kikkert, 1993; Sanford et al., 1993).

Microprojectile preparation essentially followed Trick et al. (1997) with the DNA amount decreased from 625-ng to 300 ng per bombardment, and 10 mg of 0.6 μm diameter gold microcarriers used instead of 12 mg of 1 μm microcarriers . Each bombardment consisted of a 10-μl aliquot prior to being completely dried on the macrocarrier.

Switchgrass and rice callus cultures were incubated for 6 h prior to bombardment on N6 osmotic medium with the total molarity adjusted to 0.6 M (http://www.agron.ias-tate.edu/ptf/web/system.htm), or 0.6 M NB osmotic medium (Chen et al., 1998), respectively. Each vector was used to bombard six replicate plates.

Following bombardment, gene expression was analyzed with luciferase and MUG assays. Thirty-six hours post-bombardment, 25 calli per replicate were ground in 50 μl of 1x lysis buffer (1x LB) (Leckie et al., 1994). For the first two replications, five calli were GUS-stained as per Jefferson (1987); the best five calli from the 10 calli selected were photographed for rice (Figure 3.2). Upon lysing the cells, 350 μl of additional 1x LB were added to each sample. The cell lysates were centrifuged at 13,000 g for 5 minutes at ambient conditions; the tubes were then rotated 180° and spun again. The soluble protein extracts produced from each sample were used for 4-Methylumbelliferyl Beta-D-Glucuronide (MUG) and luciferase assays (Leckie et al., 1994; Sivamani et al., 2009).

For MUG assays, 50-μl of protein extract was added to 50 μl of assay buffer (1 mM MUG in 1x LB). Reactions were incubated at 37°C for 24 hours, and subsequently terminated

with 100 µl of stop buffer (0.2 M Na_2CO_3 in type I water). Samples were read in duplicate with

the BioTek® Synergy 2 fluorometer (BioTek, Winooski, VT, USA) at an excitation of 360/40

nm as per Jefferson (1987) and an emission of 460/40 nm. The fluorometer was calibrated with

MU standards in stop buffer. MUG results were expressed as micromole MU released hour[-1].

Luciferase activity was quantified in duplicate for each replicate by the BioTek® Synergy

2 using 25 µl of protein extract. For each sample reading, 25 µl of sample extract in 1x LB

buffer were diluted in 75 µl of Glo-lysis buffer and mixed with 100 µl of ONE-Glo[TM] Luciferase

Assay buffer (Promega Corporation, Madison, WI, USA) and allowed to incubate at room

temperature for 5 minutes.

Non-specific GUS and luciferase activity was corrected as per Schledzewski and Mendel

(1994). Normalization of the MUG data was accomplished using the luciferase cassette as per

Schledzewski and Mendel (1994). Each unique GUS-expression cassette allowed the

measurement of gene expression to be quantified. The strength of each promoter was reported

relative to that of the *CaMV 35S* control*,* reported as 1, to create a dimensionless value of

promoter strength (Schledzewski and Mendel, 1994; Sivamani and Qu, 2006).

Statistical Analyses

Data for relative gene expression were subjected to Levene's test to check for

homogeneity of variance using the software package JMP® (Version 8.0.2 SAS Institute Inc.,

Cary, NC). The data had significantly unequal variances ($p < 0.05$) and were therefore subjected

to a square root transformation and subsequently ANOVA. Since there were highly significant

($p < 0.001$) differences due to promoter, LSD was employed to determine the presence of

significant difference between the relative gene expression of all promoters within a species ($p \leq$

0.05) (Figure 3.3).

Stable Rice Transformation

Stable transformations were performed as previously described for transient expression assays with three exceptions, three month old cultures were used as apposed to five month old cultures, all vessels were sealed with 3M Micropore™ tape as opposed to Nescofilm (Karlan, Cottonwood, Arizona, USA), and 150 ng of the pHLucGWgus vector variants (*PvUbi*1, *PvUbi*1+*3, PvUbi*1+*3, PvUbi*2, *PvUbi*2+*3, PvUbi*2 +*27nt, ZmUbi*1, and *35S*) were used per bombardment as apposed to 300 ng per bombardment.

Rice callus cultures were incubated for 6 h prior- and 18 h post-bombardment on 0.6 MNB osmotic medium (Chen et al., 1998). Each vector was used to complete three replicate bombardments, whereas each replicate consisted of 50-bombarded calli.

Rice callus cultures were selected on MNBH50 as previously described by Chen et al. (1998) to ensure independent events were recovered. Briefly, 3-6 weeks post-bombardment, a portion of each transgenic callus was confirmed for the presence of *gusA* using a histochemical GUS assay (Jefferson et al., 1987) (Figure 3.4). Transgenic calli expressing GUS were regenerated as per Broothaerts et al. (2005) on RGH6 medium solidified with Phytagel (6 g l^{-1}) without hygromycin selection in 100 x 20 mm Petri dishes. Calli, approximately 3 mm in diameter were arranged in a 5x5-grid on RGH6 medium. Plantlets were rooted on ½ MS medium supplemented with B5 vitamins and hygromycin B (50 mg l^{-1}) solidified with 3 g l^{-1} gelzan in Magenta® GA-7 Plant Culture vessels for four weeks. Transgenic plants were regenerated and rooted under a 23-h photoperiod provided by cool-white fluorescent light (66-95 µE m^{-2}s^{-1}) and incubated at 26°C. Prior to being moved to the greenhouse a root sample was harvested and GUS stained for all transgenics, and an untransformed control regenerated without selection.

Plants were allowed to grow in the greenhouse for approximately 2 months prior to

harvesting tissue for leaf and stem GUS assays. GUS assays were completed using the modified

Kosugi et al. (1990) GUS buffer brought to pH 7 (Hodal et al., 1992), and chlorophyll was

removed from the tissues as per Cervera (2005) (Figure 3.5).

FIGURES
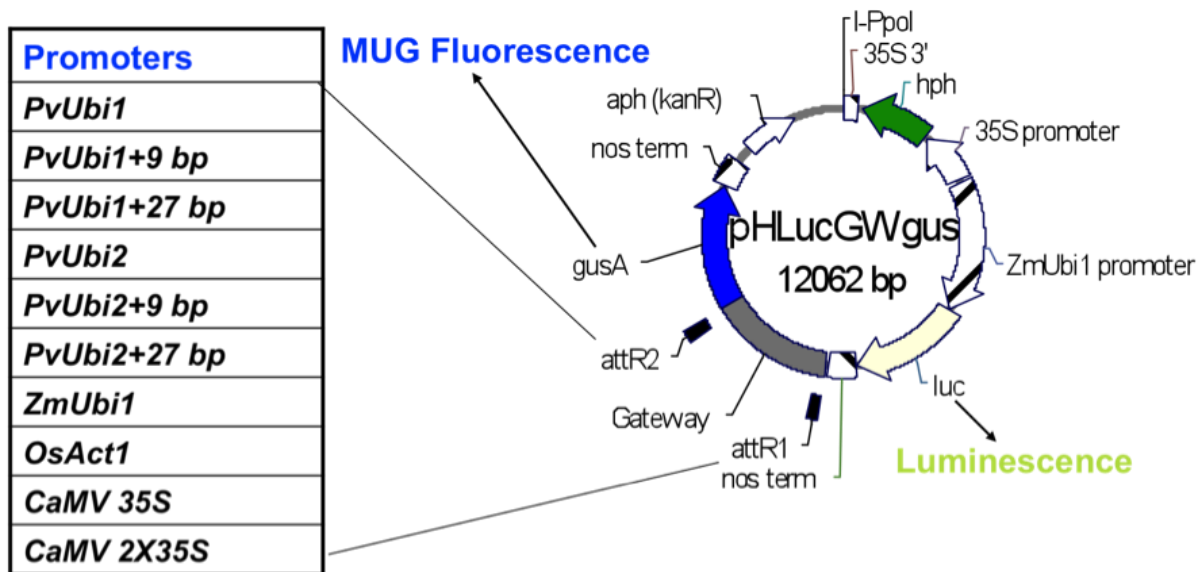
Figure 3.1: The pHLucGWgus vector for promoter testing. Each promoter was separately

cloned into the Gateway® site to drive the expression of *gusA*.
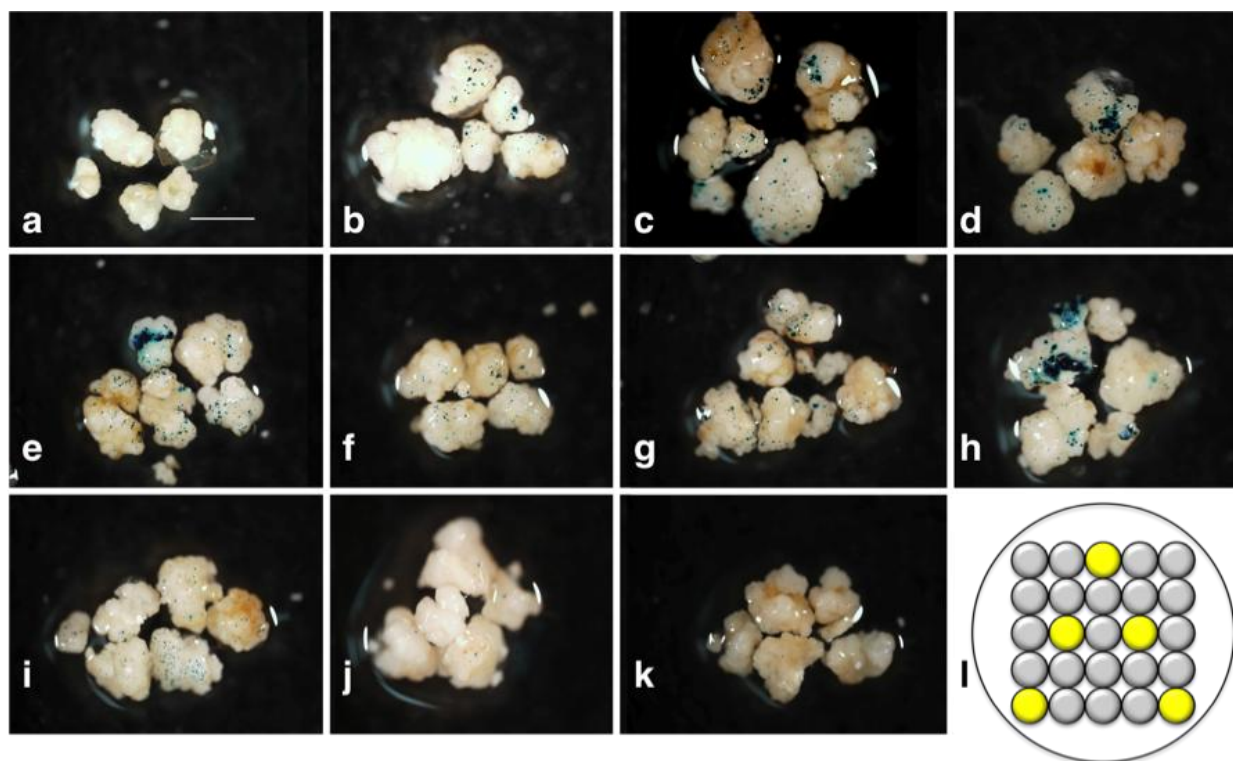
Figure 3.2: Transient GUS expression in rice callus from pHLucGWgus promoter variants. Five rice calli showing the best GUS expression were photographed per treatment. **a)** No DNA bombarded control, **b)** *PvUbi*1, **c)** *PvUbi*1+*9nt,* **d)** *PvUbi*1+*27nt,* **e)** *PvUbi*2, **f)** *PvUbi*2+*9nt,* **g)** *PvUbi*2 +*27nt,* **h)** *ZmUbi*1, **i)** *OsAct1,* **j)** *2X35S, and* **k)** *CaMV 35S*, **l)** sampling distribution of calli selected for the visualization of GUS expression. Yellow circles represents the five calli selected per replicate plate bombarded. Bar = 2 mm.
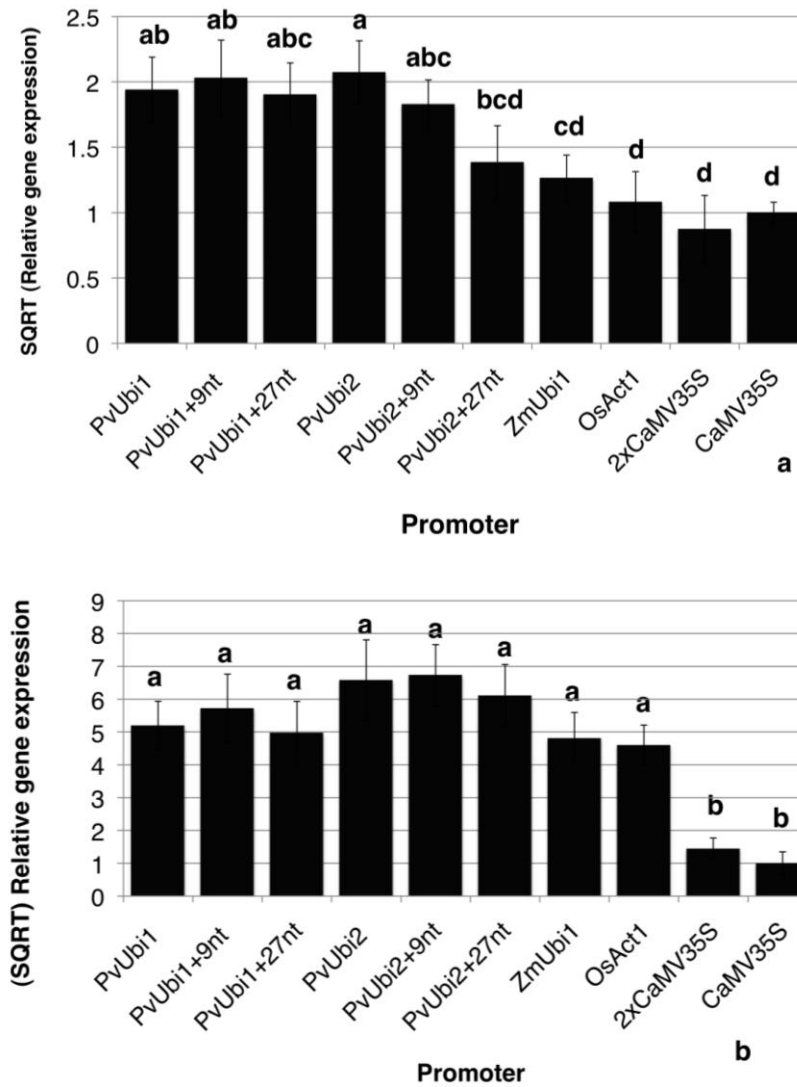
Figure 3.3: Relative rice and switchgrass gene expression from pHLucGWgus promoter variants compared to the *35S* promoter (a and b, respectively). Each mean was generated using a construct with the same pHLucGWgus DNA backbone and a unique promoter. The relative expression levels of *35S* were set to 1, a dimensionless value, for both switchgrass and rice. Bars represent the mean value of relative gene expression from six biological replicates. Error bars are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($P \leq 0.05$).
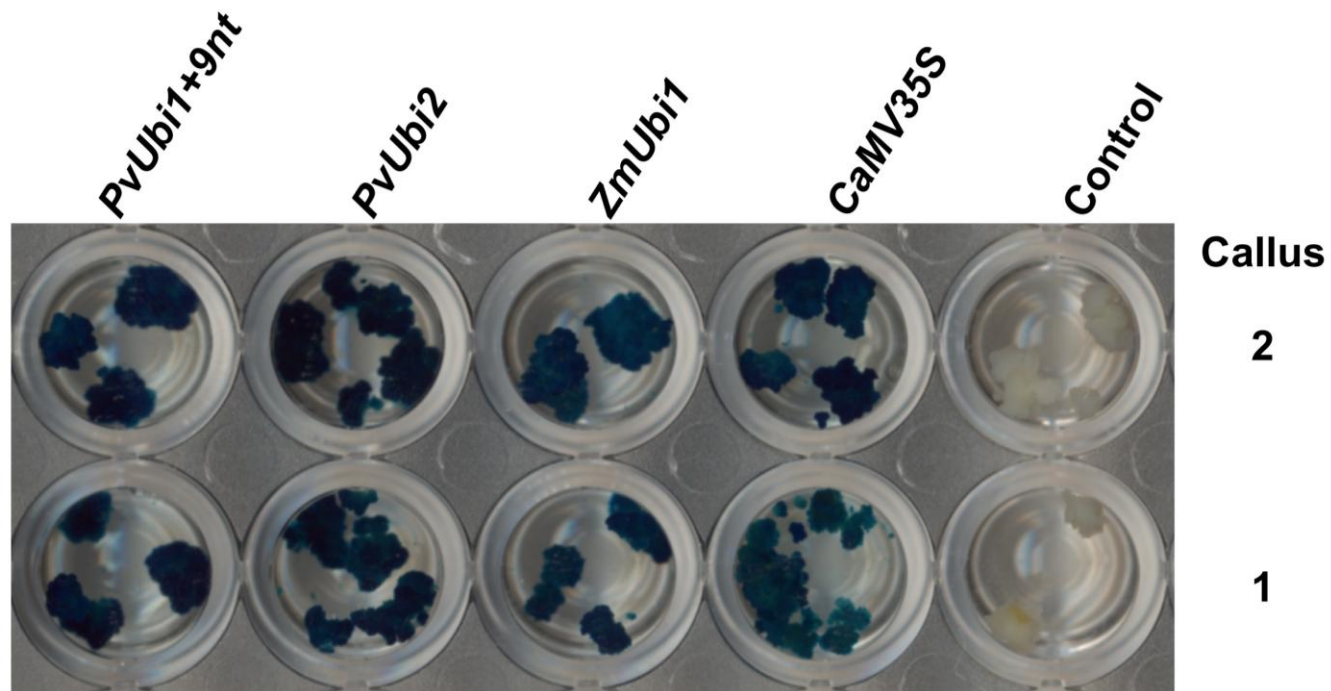
Figure 3.4: GUS expression in stable rice callus events from pHLucGWgus promoter variants driving the expression of *gusA*, or the wild-type control calli.
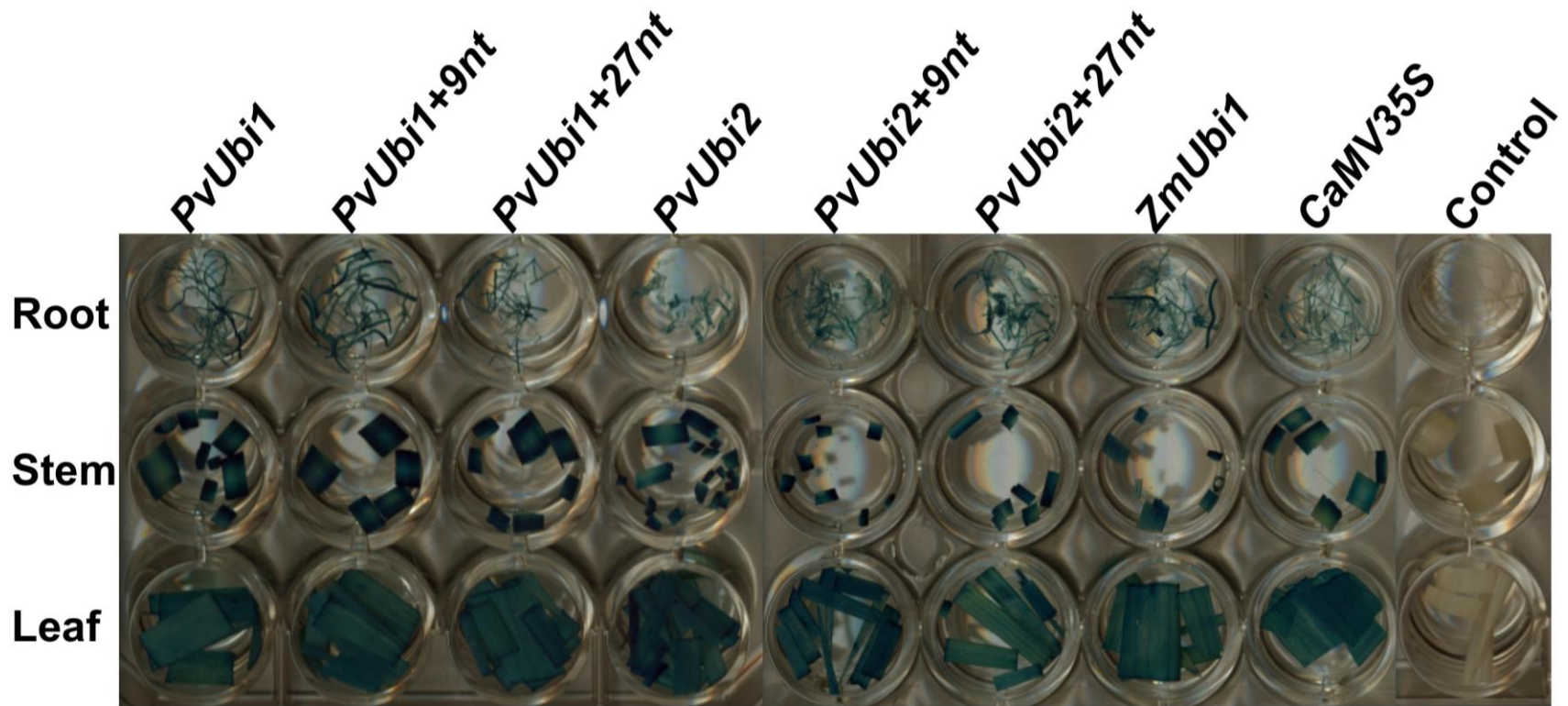
Figure 3.5: GUS expression in rice roots, stems and leaves from pHLucGWgus promoter variants of driving the expression of *gusA,* or a wild-type control plant.

RESULTS AND DISCUSSION

The novel promoter variants *PvUbi1+9nt* and *PvUbi2* showed the highest mean gene expression in both rice and switchgrass, suggesting that these promoters hold potential to outperform classically used rice promoters (*ZmUbi*1, *OsAct1* and *CaMV 35S*) (Figure 3.3). For switchgrass, *PvUbi1+9nt* and *PvUbi2* allowed for significantly more gene expression than non-switchgrass promoters (Figure 3.3). The relative difference in absolute gene expression between rice and switchgrass was in some cases 15-fold higher for rice (Figure 3.3).

This methodology is the first plant transformation study of promoter expression using an internal *LUC* cassette control within the same vector backbone as the experimental promoter:*gusA* cassette, as opposed to the use of co-transformation (Leckie et al., 1994; Sivamani and Qu, 2006; Sivamani et al., 2009). All constitutive promoters expressed GUS in leaves, stems and roots of mature rice plants, and no GUS expression was detected in the untransformed control (Figure 3.5). The promoters *Pv*Ubi2, *Pv*Ubi1+9nt, and *Zm*Ubi1 should be used for studying transgene expression in switchgrass or rice, as all promoters are constitutively expressed and strongly express transgenes in callus tissue (Figures 3.4; 3.5).

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INTRODUCTION TO RESEARCH AND DEVELOPMENT

Transformation of TP309 and ST1 embryogenic callus cultures was accomplished using particle-bombardment with the vectors pANIC-7A (*pporRFP*, Hyg[r], RNAi) and pANIC-7B (*GUSPlus™*, Hyg[r], RNAi) (Mann et al., 2010). Particle-bombardment was completed as described above with 150 ng of DNA delevered per bombardment.

In regards to pANIC-7B transformations, embryogenic calli were given a resting period on osmotic medium for 24-h post-bombardment, and then GUS-stained (Jefferson et al., 1987) and fixed in 95% ethanol until photographed.

General Procedures for the Polymerase Chain Reaction

Tissue harvested for DNA extraction was placed in a 2-ml microcentrifuge tube (MCT), with two glass borosilicate glass beads (4-mm diameter each). A hole was poked in the top of the MCT and snap frozen in liquid nitrogen, and subsequently freeze-dried overnight. The tissue was then ground to a fine powder with a tissue disruptor.

DNA isolation for PCR was performed on the ground tissue as per Lassner et al. (1989), and the DNA was quantified using an Eppendorf® BioPhotometer (Hamburg, Germany), and standardized to a concentration of 30 ng μl^{-1} by dilution with Type I water. Standard PCR was performed using the GoTaq™ kit by Promega, using 1-μl of DNA template at a concentration of 30 ng μl^{-1} or 1 pg μl^{-1} of plasmid DNA. PCR conditions were: (1) 94°C 4', (2) 94°C 30'' (3) Tm X^{o}C 30'', (4) 72°C 1' per kb, steps (2-4) 37 times, (5) 72°C 7' (Table 3). Non-transgenic template DNA, water, and the transformation plasmid were used as PCR controls. PCR products

were visualized on a 0.5xTBE 1% agarose gel supplemented with 0.5 μg ml^{-1} ethidium bromide (Appendix Figure 7).

Screening Putative Events

All putatively transgenic callus tissues were screened in the same manner using hygromycin B selection (30 mg l^{-1}). Calli were screened for pporRFP expression as previously described, or a 2-mm diameter piece of callus was tested for GUS expression. For photographs, the same lighting settings, and exposure times were used for all white light photographs and all RFP filter photographs of a given experiment, respectively.

If the reporter gene was expressed, half of the tissue remained on selection medium, while the remaining portion was subcultured onto regeneration medium with selection as previously described. To verify that the genomic DNA was of the proper quality, a switchgrass-specific primer pair was used to amplify the acetyl-CoA carboxylase (*PvAcc*) gene (Appendix Table 1). Rice genomic DNA extractions were verified using a rice-specific Os_rbcS F primer pair to amplify a region of the ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (EC 4.1.1.39) (Appendix Table 1).

<div align="center">

RESULTS – APPENDIX

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Thirty-three independent TP309 lines were resistant to hygromycin B (Appendix Figure 1) and expressed RFP (Appendix Figure 2). These events were recovered from 100 bombarded calli and subcultured to a size of 0.5 mm 3-weeks post-bombardment on MNB medium supplemented with 50 mg l^{-1} hygromycin B (MNB-H50) (Chen et al., 1998). One piece of callus from each line displaying relatively intense RFP expression was selected for regeneration. TP309 lines were regenerated as per Chen et al. (1998) to characterize the *Pv*Ubi1:*ppor*RFP cassette expression plant tissues. The TP309 RFP-expression pattern may indicate a similar

expression pattern in switchgrass or other plants.

RFP fluorescence in cultures of ST1 embryogenic callus was verified and cell divisions were visible. Circled regions indicate a portions callus expressing RFP, and each side-by-side picture is from the same piece of tissue (Appendix Figure 3).

Switchgrass selection medium consists of N6E medium supplemented with 50 mg l^{-1} hyromycin B (N6EH50). Switchgrass cell divisions ceased after approximately 3 weeks of growth on N6EH50 medium.

In order to investigate the slower growth of ST1 cell clusters expressing RFP, the stability of RFP was investigated. Stably transformed TP309 culutures displaying RFP were freeze-killed at -20°C 1-h, -80°C 2-h and -20°C 1-h to freeze and then thaw the cells. Fifteen-days later, the cells still displayed fluorescence. To further investigate the stability of the protein, the cultures were bleached with 578-nm light for 35-minutes to excite the RFP. The primary emission of 595-nm was then monitored using an RFP filter. It is evident that RFP is highly stable (Appendix Figure 4).

Switchgrass and rice calli showed transient expression of GUSPlus after bombardemnt with pANIC-7B, and no endogenous GUS ativity was observed in the bombarded controls (Appendix Figure 5).

DNA isolation for PCR as per Lassner et al. (1989), provided a high-quality DNA sample. The acetyl-CoA carboxylase (*PvAcc*) gene was successfully amplified using standard PCR conditions and the GoTaq™ kit by Promega using 10 ng of genomic template (Appendix Figure 7).
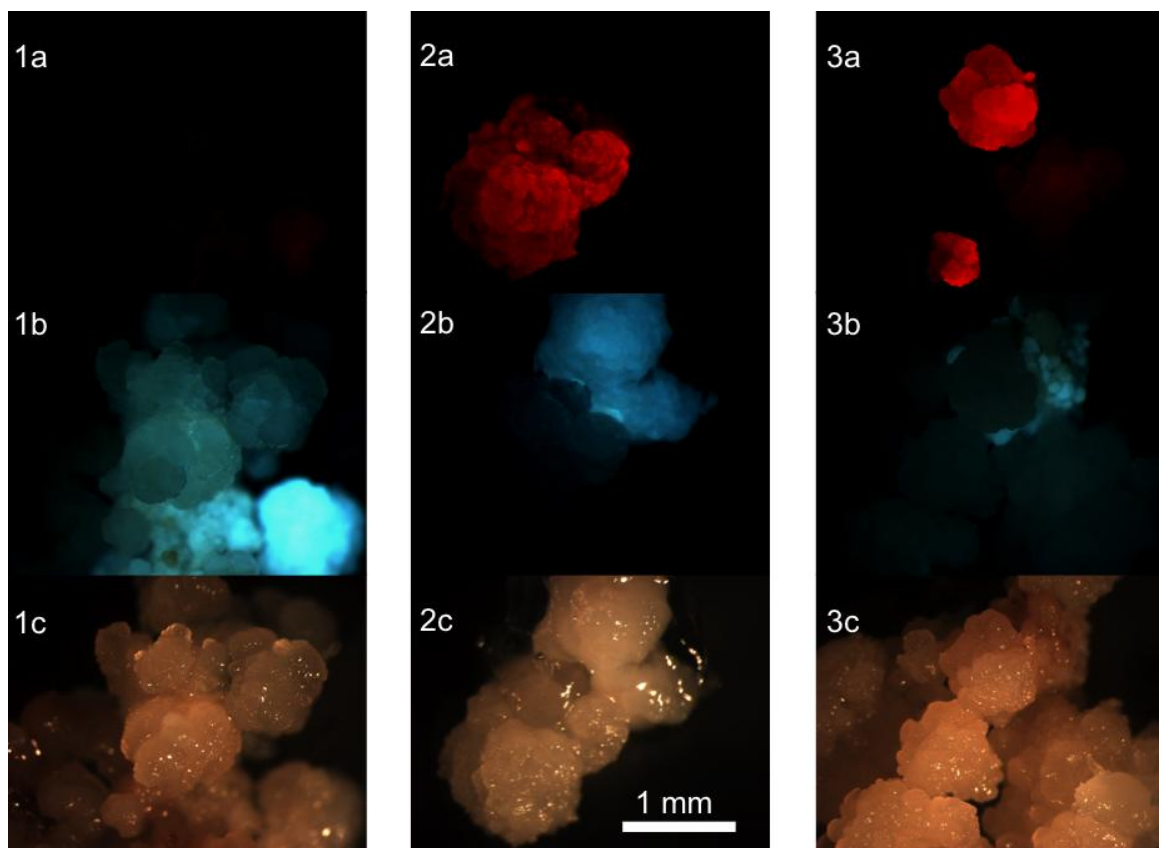
Appendix Figure 1: Rice calli after three weeks of selection on MNBH50 medium. One untransformed callus (1) and two independent transgenic lines (2 and 3) transformed with pANIC-7A, are shown in white light (a), using a GFP filter (b) and an using an RFP filter (c).
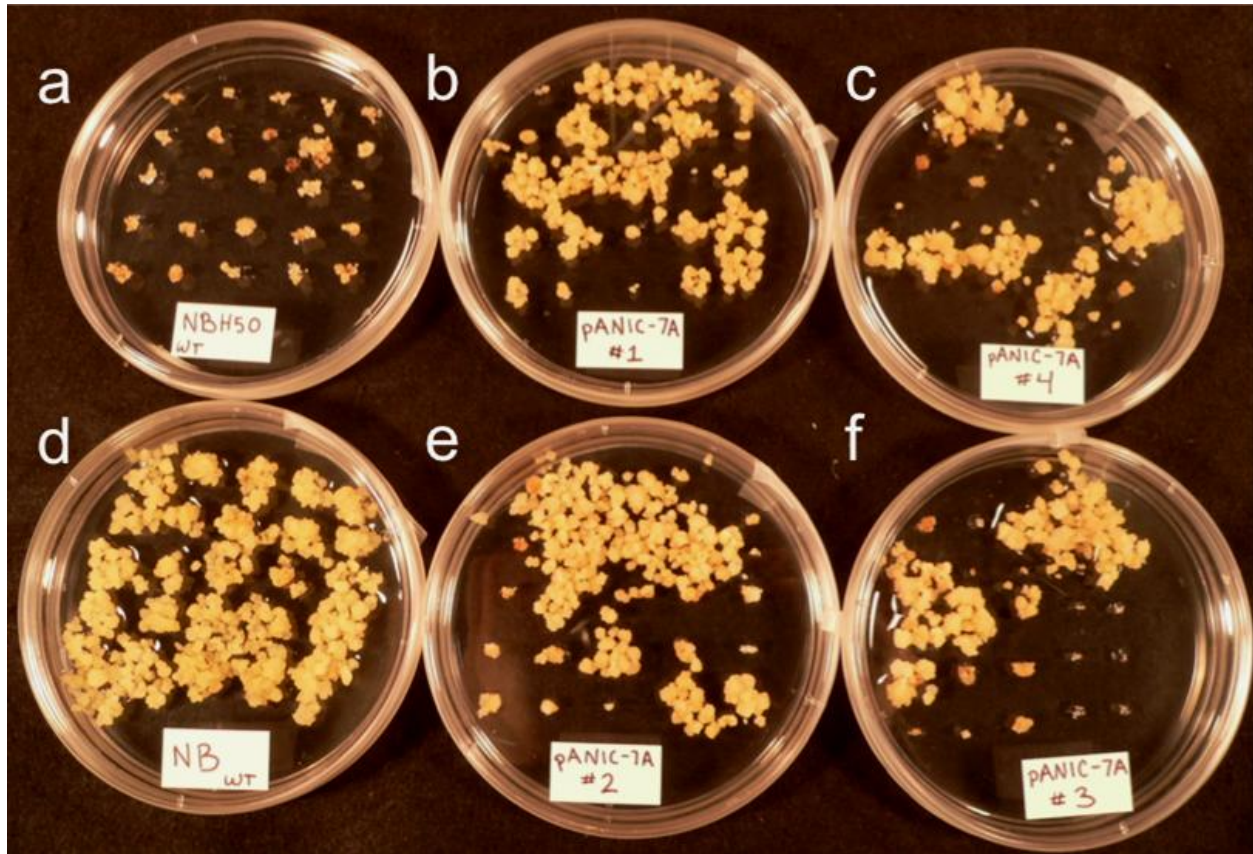
Appendix Figure 2: Independent rice events recovered from selection with pANIC-7A. Thirty-three independent lines were recovered from four bombardments using hygromycin B and RFP selection. Untransformed calli on MNB-H50 medium (a), untransformed calli on MNB medium (d), and four plates of calli transformed with pANIC-7A (b, c, e, and f), which show independent events emerging on MNB-H50 medium.
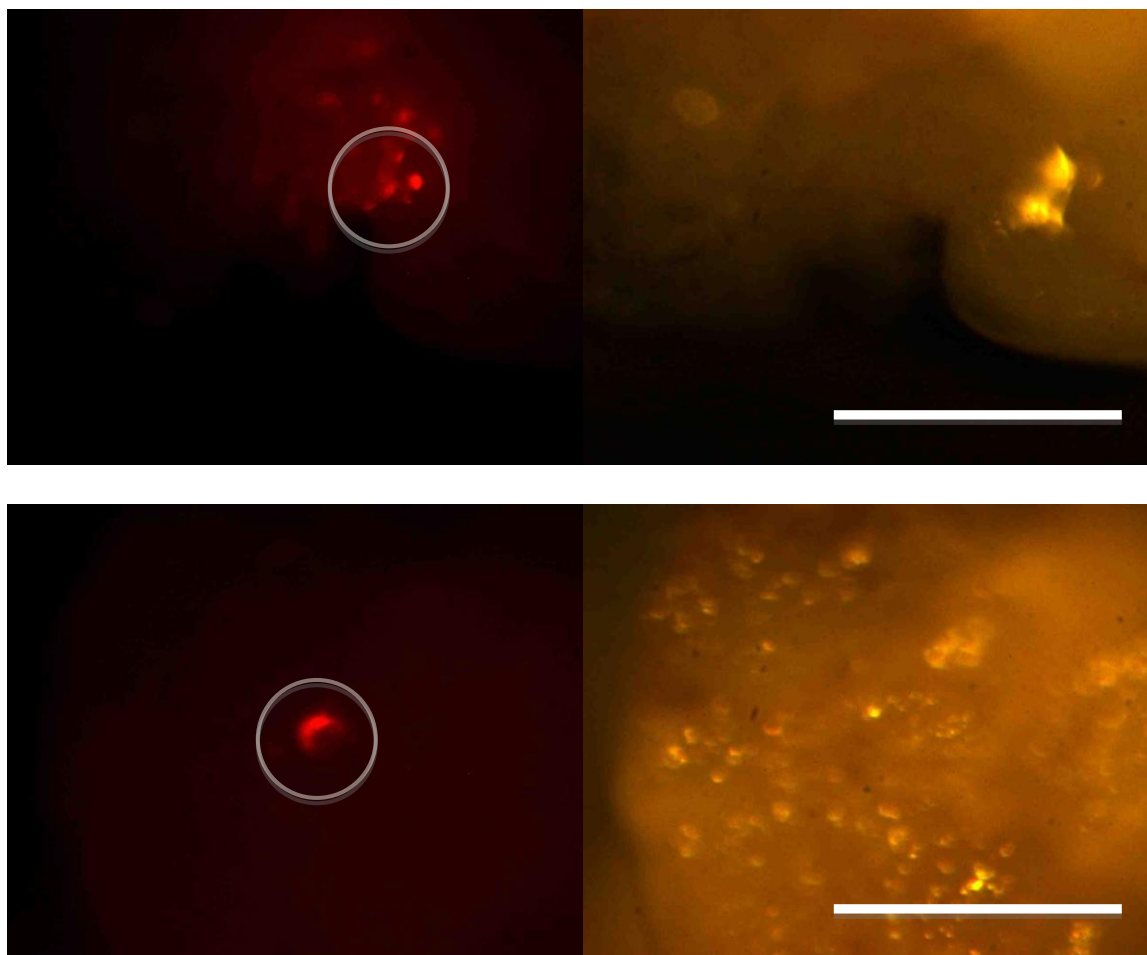
RFP Photo   White Light Photo

Appendix Figure 3: RFP expression of ST1 callus bombarded with pANIC-7A from two separate calli on N6-H50 medium for two months of selection, Bar = 1 mm
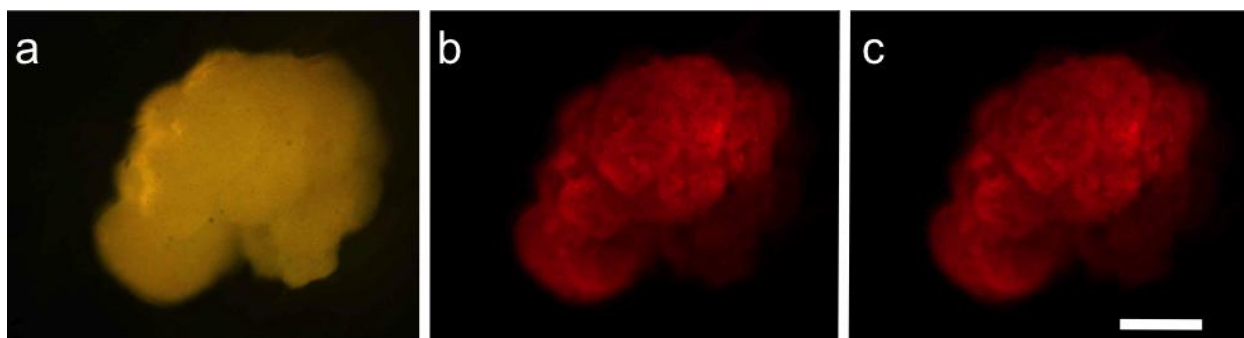
Appendix Figure 4: RFP expression of rice callus transformed with pANIC-7A. Callus is on MNB-H50 selection medium. Photos were taken intially using white light (a), then using 578-nm of ultraviolet light continually at 0 minutes (a), and 35 minutes (b). Bar = 1 mm.
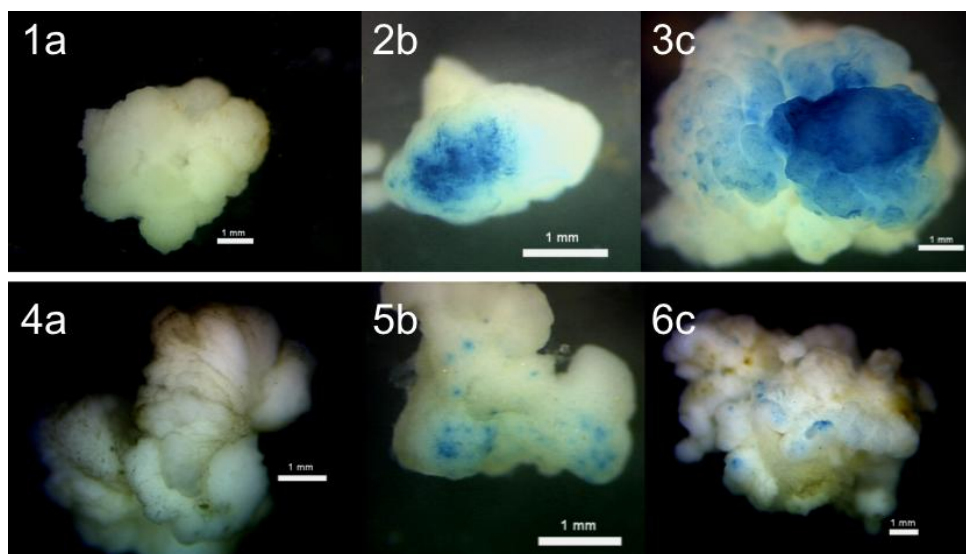

Appendix Figure 5: Validation of pANIC-7B using biolistic transformation and transient expression assays of rice and switchgrass callus (switchgrass genotype ST1). The *Pv*Ubi1:*GUSPlus™* cassette has been validated in transient and stable transformations in rice. Pictured are untransformed controls of rice (1a), and switchgrass (4a) bombarded without the presence of DNA, and two calli of rice (2b and 3b) and switchgrass (5b and 6b), which were bombarded with pANIC-7B. Bar = 1 mm.

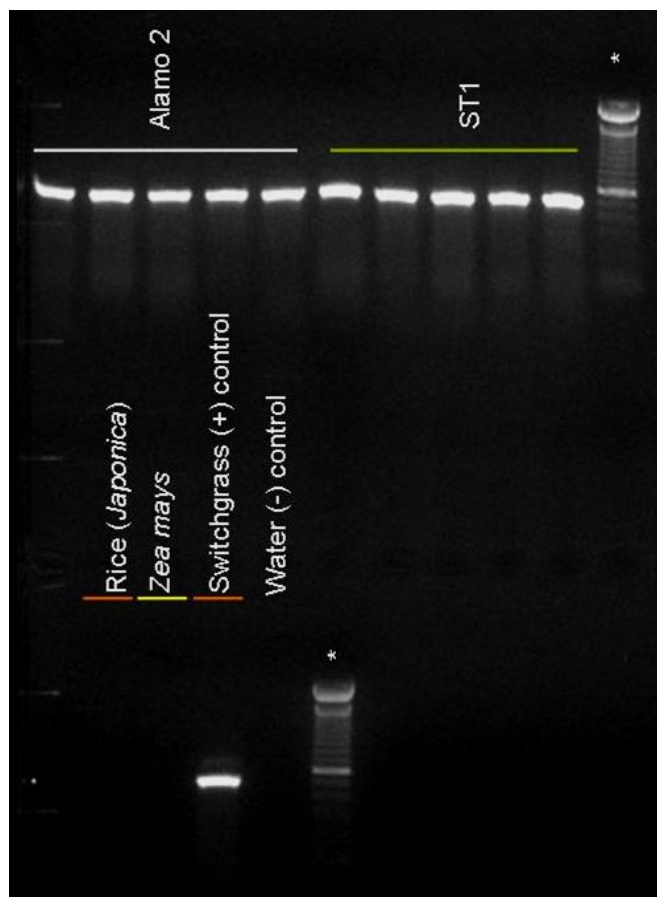**pGWB533-PvUbi1**   **pGWB533-PvUbi1+9nt**   **pGWB533-PvUbi1+27nt**
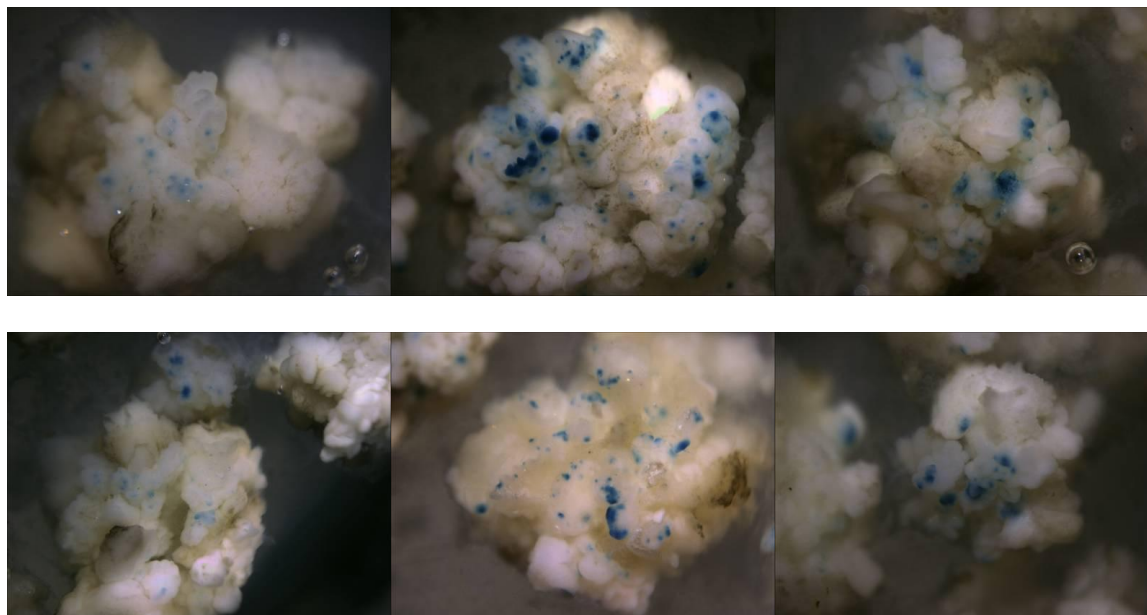
Appendix Figure 6: Foci on bombarded ST1 callus produced from by *Pv*Ubi1 fusions:

*Pv*Ubi1+9nt was selected based on qualitative GUSPlus™ expression. Photos courtesy of Dr.

David J.G. Mann (University of Tennessee).

Appendix Figure 7: PCR amplicons of *PvAcc* from switchgrass. DNA was extracted from callus tissue, samples were standardized to 10 ng μl^{-1}. One microliter of template DNA was loaded for PCR reaction. Seven microliters of PCR product from template DNA and 2 μl of ladder were loaded. PCR bands are amplicons of *PvAcc* from switchgrass. * = 100 basepair ladder.

Appendix Table 1: Primer sets used to generate DNA amplicons of interest in PCR.

Primer Name	Sequence 5'-3'	Tm °C	Amplicon (bp)	Cycles
PvAcc291F	AAGCTGGAGTTGGGATCATGG	56	488	37
PvAcc779R	CAACAGTAACTGGGCCTTCCTC	56	-	-
Hyg117F	CGATGTAGGAGGCGCTGGATA	57	821	37
Hyg938R	CTTCTGCGGGCGATTTGTG	57	-	-
PvUbi1_1462F	GATGATAACATATGCTGCTGGTGG	57	1171	37
RFP 632R	GTAGCACCATCGGTGTTGTG	57	-	-
Os_rbcS F	CTTGGTGAGCTGCAGAGATGG	57	288	37
Os_rbcS R	AGGGTCTCGAACTTCTTGATGC	57	-	-
Zmubi-1900F	TTTAGCCCTGCCTTCATACG	52	774	37
guslink R	CCAAAGCCAGTAAAGTAGAACG	52	-	-
guslink F	GTCGGTGAACAGGTATGGAAT	50	817	37
nosT-Reverse	TGATAATCATCGCAAGACC	50	-	-

DISCUSSION – APPENDIX

The switchgrass promoter *Pv*Ubi1+9nt shows stable expression of pporRFP in embryogenic callus cultures of ST1 and TP309, well over 2-months after bombardment with pANIC-7A (Appendix Figures 1; 3). The *Os*Act1:*hph* cassette in the pANIC vectors is functional in TP309 (Appendix Figure 2) and transgenic tissue can be isolated to regenerate whole plants (Appendix Figure 1). Using pANIC-7A procedures 33 independent TP309 events were recovered (Appendix Figure 2).

Transgenic cell divisions of ST1 expressing RFP were documented, however, cell divisions were no longer visible after 2-months (Appendix Figure 3). On one plate bombarded, 18 of 25 independent ST1 calli showed RFP foci (72**%).**

RFP is highly stable well over 15-days after transgenic lines of TP309 have been freeze-killed. There is no visible change in RFP expression after 35 minutes of continuous photo bleaching. Therefore, it is difficult to discern if the RFP-fluorescing tissue is viable (Appendix Figure 3).

White, opaque, textured, and friable-callus is more embryogenic and thus selected for at each subculture using a dissecting microscope and very fine forceps. Observations, and scanning electron microscopy documented by Denchev and Conger (1994) have previously illustrated the potential of this callus morphotype. Therefore it is best to avoid and discard all brownish or translucent-sticky callus tissue generated on N6E, as it performs poorly in plant regeneration and in transient expression experiments that utilize particle bombardment.

Amplification of *PvAcc* works routinely and provides a genotype independent control when screening putative transgenic switchgrass tissues. Primer pairs listed in Appendix Table 1 can be used to amplify transgenes currently in the pANIC vector set (Mann et al., 2010).