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ORIGINAL PAPER

Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.)

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Abstract
Key message **With a novel elite genotype, SA37, and an improved transformation protocol, it is now possible to routinely and efficiently engineer switchgrass using biolistic transformation.**
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 of this study 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 medium sets were compared and a previously published medium set provided cultures with >96 % embryogenic callus, and data on transient and stable gene expression of RFP were used to optimize biolistic parameters, and further validate the switchgrass (*PvUbi1*) promoter. SA37 proved to be the most transformable, whereas eight transgenic calli on average were recovered per bombardment of 20 calli (40 % efficiency) when using a three-day day preculture step, 0.6 M osmotic adjustment medium, 4,482 kPa rupture disks and 0.4 μm gold particles which traveled 9 cm before hitting the target callus tissue. Regenerability was high, especially for ST2, for which it is possible to recover on average over 400 plants per half-gram callus tissue. It is now possible to routinely and efficiently engineer elite switchgrass genotypes using biolistic transformation.

Keywords Particle bombardment · Switchgrass · *Panicum virgatum* L. · Plant transformation · Genetic engineering · pANIC vector set

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Introduction

Switchgrass (*Panicum virgatum* L.) is a North American perennial, warm-season grass from the *Panicoideae* (Lewandowski et al. 2003). It is a C_4 species adapted to a wide range of environments (Lewandowski et al. 2003). Research interest is due to its potential as a dedicated bioenergy crop. Specifically, switchgrass can be used as a source of cellulose to produce cellulosic ethanol; however, there are many limitations that reduce the efficiency of production, including the degree of lignification and the complexity of cell-wall components (Himmel et al. 2007).

Versatile and efficient transformation systems for switchgrass are helpful for the rapid development of switchgrass that are more amenable to biofuel production. For example, silencing of caffeic acid 3-*O*-methyltransferase led to reduced lignin and subsequently 38 % more ethanol from switchgrass fermentation (Fu et al. 2011).

Both type II callus and immature somatic embryos are amenable to transformation (Li and Qu 2010; Somleva 2007; Somleva et al. 2002, 2008; Xi et al. 2009) with the supervirulent *Agrobacterium tumefaciens* strains AGL-1 (Somleva et al. 2002, 2008) and EHA-105 (Burris et al. 2009; Fu et al.

2011; Li and Qu 2010; Xi et al. 2009). Tissue type and AS concentration have been shown to be critical to increase *A. tumefaciens*-mediated transformation, which was 97 % efficient when somatic embryos were engineered (Somleva et al. 2002). In this study, the transformation efficiency was determined as the number of bialaphos-resistant plants recovered from the initial explants. More recently, efforts have focused on the use of embryogenic callus (Li and Qu 2010).

Over 90 % transformation efficiency was obtained using type II callus derived from the seed of 'Performer;' however, only 1.5 % of Performer callus was highly transformable (Li and Qu 2010). Performer was originally selected for low lignin content (Burns et al. 2008); some genotypes provide a unique type II callus that is yellow, friable and highly regenerable and transformable (Li and Qu 2010). Other contributing factors included vacuum infiltration of *Agrobacterium*, desiccation after inoculation, a 5-day resting phase, and the use of L-proline during selection, although the largest factor appears to be the use of Performer over Alamo, accounting for 42 % of the increase.

In contrast, no optimization of microprojectile-mediated transformation has been done for switchgrass, where the efficiency was originally reported as ~4 % (Richards et al. 2001). Nevertheless, microprojectile-mediated transformation has been a highly efficient transformation method for a number of grass species (e.g., rice and corn), many of which were deemed recalcitrant to transformation (Fu et al. 2000; Lowe et al. 2009).

Regardless of the methodology employed, the current challenge is to apply advances in transformation to engineer elite genotypes. The use of clonally propagated material for transformation makes small transgene effects more easily detectable (Xi et al. 2009). Highly embryogenic callus can be obtained from immature inflorescences of elite genotypes, and up to ~60 regenerants per initial explant (half-internode) can be obtained when a 16-week micropropagation step is included (Alexandrova et al. 1996). However, due to low incidence of embryogenic callus obtained from inflorescence explants (Burris et al. 2009), seeds remain as the primary choice of explant (Li and Qu 2010; Somleva et al. 2008; Xi et al. 2009). The goal of this study was to develop a standard engineering protocol for elite genotypes through particle bombardment.

Materials and methods

Part 1: tissue culture and regeneration from inflorescences of elite genotypes

Medium set selection

Several protocols are available for the initiation and regeneration of embryogenic callus (Alexandrova et al. 1996; Burris et al. 2009; Somleva et al. 2002; Xi et al. 2009) (Electronic Supplementary Material Table 1). Additionally, existing procedures for maize (Che et al. 2006) can be used with switchgrass. Therefore, the media from these protocols were compared to identify the best protocol for establishing highly embryogenic and regenerable cultures from three tissue-culture-responsive genotypes.

Plant material

Genotypes used came from cv. Alamo, and were ST1, ST2; and SA37, which was selected from a cross between ST1 and Alamo 2 (Burris et al. 2009). Plants were grown in C2100 pots (Nursery Supplies Inc., Kissimmee, FL) with a 42 % sand and 58 % Fafard 3B soil mix (Conrad Fafard, Inc., Agawam, MA). Growth conditions consisted of a 12 h photoperiod under 400 W high-pressure sodium lighting. The greenhouse ranged in temperature from 20 to 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 380 l of water. All tillers that matured beyond boot stage (Moore et al. 1991) were discarded.

The last culm node produced the desired immature inflorescences at the E2-R0 stages (Moore et al. 1991), which were identified as previously described (Alexandrova et al. 1996). All tissue 5.0 cm above, and 1.5 cm below the culm node was harvested as the initial explant (Electronic Supplementary Material Fig. 2). Thirty-two boot-stage tillers were harvested per genotype to initiate tissue cultures. The 6.5 cm internode explants were surface-sterilized with 70 % ethanol v/v, for 1 min with gentle agitation. Explants were placed in 15 % Clorox® v/v with supplemented with 0.01 % Tween 20 for 3 min with gentle agitation. Tiller segments were rinsed three times, at 2 min intervals and halved longitudinally (Alexandrova et al. 1996). Explants were randomized within genotype and placed cut-side down in 20 × 100 mm Petri dishes to induce and elongate panicles (Electronic Supplementary Material Table 1). Eight replicate plates of two half-internodes per treatment were plated.

For all comparisons of switchgrass medium sets, 2.5 g l^{-1} Gelzan™ (Caisson Laboratories, North Logan, UT) was used as the solidifying agent, the pH was brought to 5.8 prior to autoclaving, and all Petri dishes were sealed with 3 M Micropore™ tape (St. Paul, MN) (Electronic Supplementary Material Table 1). Cultures were incubated in a growth chamber at 25 °C, with cool-white fluorescent lighting (66–95 μE m^{-2} s^{-1}) and 16 h light for 14 days (Electronic Supplementary Material Fig. 1).

Tissue culture

After 14 days of culture, the number of explants that responded was recorded (Fig. 1), and immature

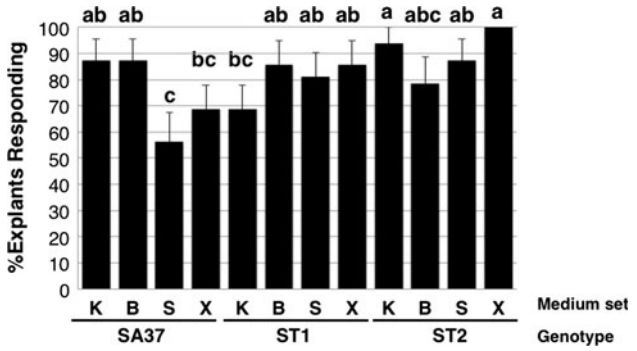

Fig. 1 Initial explants responding by growing inflorescence tissue. Eight replicate plates of two half-internodes per treatment were placed into tissue culture. The percentage of tiller sections that produced inflorescence tissue after 2 weeks of growth on initiation medium is shown. *Error bars* are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Medium sets GA experimental (*K*), Burris et al. (2009) (*B*), Somleva et al. (2002) (*S*), and Xi et al. (2009) (*X*)

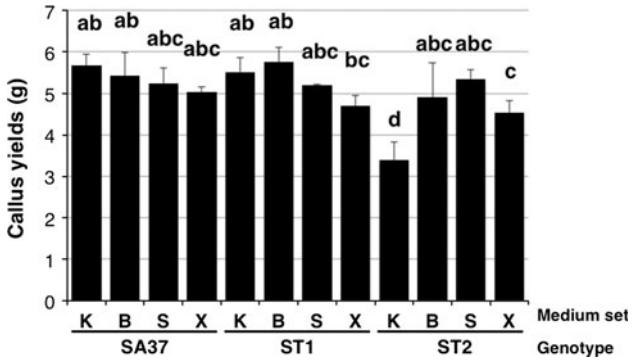

Fig. 2 Average callus yields obtained after 6 weeks. *Error bars* are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Medium sets GA experimental (*K*), Burris et al. (2009) (*B*), Somleva et al. (2002) (*S*), and Xi et al. (2009) (*X*)

inflorescences were dissected out to obtain sections of rachis tissue measuring ~1 cm in length, which were used to initiate embryogenic callus. These were arranged in a 4 × 4-grid pattern in 15 × 100 mm Petri dishes (Electronic Supplementary Material Fig. 1; Table 1). Tissue was incubated at 27 °C in the dark and subcultured at 3 week intervals to induce the growth of embryogenic callus.

After the second subculture, compact, type II calli, some of which already had somatic embryos, were separated from the inflorescences and arranged in a 5 × 5-grid pattern. Embryogenic type II callus tissue was bulked for 6 weeks with one subculture at 3 weeks. At this point, embryogenic callus was separated into six replicate plates per treatment (Electronic Supplementary Material Fig. 3; Table 1). Each replicate plate of callus contained 0.25 g of

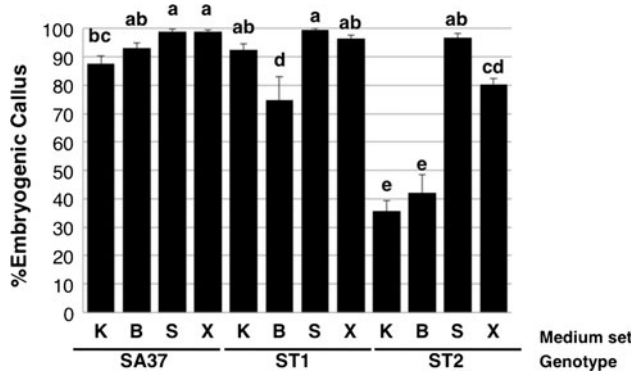

Fig. 3 Average percent embryogenic callus production from various tissue culture medium sets. *Error bars* are standard error. The average was calculated from ten replicate plates of 25 calli per plate. After 3 weeks of callus growth, the percentage of embryogenic callus was determined. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Medium sets GA experimental (*K*), Burris et al. (2009) (*B*), Somleva et al. (2002) (*S*), and Xi et al. (2009) (*X*)

callus arranged in a 5 × 5-grid pattern. All callus material was subcultured after 3 weeks, allowing for 6 weeks of growth (Fig. 2).

To assess callus quality, the remainder of the tissue was used to create ten replicate plates of callus per treatment. For each replicate plate, 25 calli, ~3 mm in diameter were plated in 5 × 5-rows. After 3 weeks in the same conditions as previously described, the percent of embryogenic callus was determined. If a callus had any visible embryogenic sectors using a dissecting scope, it was considered embryogenic (Fig. 3).

Embryogenic calli obtained from the completion of the callus yield trials were selected to determine the regeneration efficiency of plantlets per gram callus over 18 weeks (Electronic Supplementary Material Table 1). Embryogenic calli were arranged in a 5 × 5-grid pattern in six replicate plates containing 0.5 g of callus each per 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 2 weeks. All tissues were transferred to regeneration medium once more and incubated for an additional 2 weeks. All plants were counted from each replicate plate, and the best representative plate from each treatment was photographed (Electronic Supplementary Material Figs. 5, 4). A plantlet was defined as a single green shoot, or shoot-cluster that regenerated from the same crown on a given embryogenic callus. Ten regenerants were selected and rooted per treatment (Electronic Supplementary Material Fig. 1; Table 1). Five plantlets were placed in each Greiner Bio-One vessel, Cat #82051-496 (Baden-Württemberg, Germany) and incubated for 4 weeks.

Part 2: transformation

Particle bombardment optimization

Key factors reported to affect biolistic transformations were evaluated by comparing different treatments 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 transient expression treatment was used in each subsequent experiment (Electronic Supplementary Material Table 2). Each time a particular treatment showed significantly better results than the other treatments tested, the treatment was adopted as part of the protocol.

Vector

The pANIC-6D vector (Mann et al. 2010) was selected for use. It contains a novel DsRed-type, red fluorescent protein (RFP) reporter gene, *pporRFP* (Alieva et al. 2008; Burris et al. 2009) driven by the *P. virgatum* L. ubiquitin 1 promoter (*PvUbi1*) (Mann et al. 2011). The vector was modified by cloning the *hph* gene into the Gateway® cassette as per the supplier's cloning instructions (Invitrogen, Carlsbad, CA), placing *hph* under control of the *Zm*Ubi1 promoter to create the pANIC-6D:*hph*, which was used for all experiments (Electronic Supplementary Material Fig. 6).

Microprojectile bombardment

Bombardment parameters were optimized using SA37 and ST1. Friable, embryogenic type II callus was obtained using the using the GA experimental medium set (K). Calli (2-mm diameter) were subcultured 3 days prior to bombardment. Six hours prior to bombardment, the calli were transferred to N6E osmotic adjustment medium (Electronic Supplementary Material Table 2), which was created by adding equimolar quantities of mannitol and sorbitol as per Chen et al. (1998), and incubated in the dark at 27 °C (Altpeter et al. 1996; Chen et al. 1998).

Each replicate consisted of either 25 or 20 type II calli arranged in a 2.5 cm diameter target depending on the experiment. The PDS-1000/He system was used with 7,584 kPa (1,100 psi) rupture disks, with all reagents produced by Bio-Rad (Hercules, CA). 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 to 150 ng per bombardment. Ten mg of 0.6 μm diameter gold microcarriers were used instead of 12 mg of 1 μm microcarriers. Each bombardment consisted of a 10 μl aliquot, which was completely dried on the macrocarrier.

Eighteen hours post-bombardment, calli were transferred to N6E, maintaining their original orientation, for 10 days prior to being placed on selection medium. RFP expression was observed in vivo 2–5 days post-bombardment using a microscope equipped with an RFP filter cube, using 545/25 nm excitation and visualized at 605/70 nm. All tissues were placed on N6E medium supplemented with 35 mg l^{-1} hygromycin, breaking up calli during selection as per Chen et al. (1998).

Each treatment for every experiment was replicated three times. A factorial experimental design was used to evaluate both flight distance and osmotica (Electronic Supplementary Material Table 2). Osmotic levels were 0.09, 0.4, 0.6 and 0.8 M and flight distances were 6, 9 and 12 cm. The first treatment contained 0.09 M sucrose, while all others also contained equimolar quantities of mannitol and sorbitol.

Microcarrier size and helium pressure were optimized using a $2 \times 2 \times 3$ factorial design with a microcarrier flight distance of 9 cm and calli that were incubated on 0.6 M N6E osmoticum. Both 0.6 and 0.4 μm diameter gold microcarriers (Bio-Rad Laboratories, Hercules, CA and InBio Gold, Eltham VIC, Australia), respectively were tested on genotypes ST1 and SA37. Microcarrier treatments were calibrated to deliver equivalent surface area, which was calculated as $A = 4\pi r^2$. Therefore, 10 mg of 0.6 and 4.5 mg of μm microcarriers were used per treatment. Each gold size was bombarded at one of two different rupture disk pressures, either 4,482 or 7,584 kPa (Electronic Supplementary Material Table 2).

All bombarded tissue was screened at 2-week intervals post-bombardment to monitor for the presence of stable events. An event was defined as an independently obtained callus that expressed both RFP and hygromycin resistance on selection medium. The number of callus events was determined after ~2 months of selection for all experiments (Figs. 5, 6, 7). Transgenic calli were regenerated on selection-free MS medium supplemented with B5 vitamins and 5 μM BAP. Plants were rooted on solid ½MS medium supplemented with B5 vitamins as described above.

Southern blot protocol

DNA for Southern blot analysis was extracted from 0.7 g of leaf tissue following a modified urea (7 M) based plant DNA miniprep (Chen and Dellaporta 1994). Thirty micrograms of genomic DNA and 75 pg of plasmid DNA were digested with *Spe*I, which cleaves once in the gene cassette region. The digested DNA fragments were separated by gel electrophoresis and transferred to a Zeta-Probe GT nylon membrane as per the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). For detection of the

hph gene, an *hph* probe was made from plasmid DNA using forward and reverse primers to amplify a 0.8 kb region of the gene. The *hph* probe was [a-32P]-dCTP labeled (PerkinElmer, Waltham, Massachusetts) using the Amersham Rediprime II DNA Labeling System and purified using an Illustra MicroSpin G-25 Columns (GE Healthcare, Pittsburgh, PA). The membrane was hybridized with a [32P]-labeled *hph* probe overnight using ULTRAhyb Ultrasensitive Hybridization Buffer (Invitrogen, Grand Island, NY) and exposed to Amersham Hyperfilm MP.

Experimental design and statistical analyses

A randomized complete block factorial design was used to conduct each experiment unless otherwise stated. If a significant difference was detected ($p \leq 0.05$) using ANOVA, the least significant difference test (LSD) was employed to analyze for significant differences between treatments within an experiment ($p \leq 0.05$) using SAS® (Version 9.3 SAS Institute Inc., Cary, NC).

Results

Tissue culture

In order to establish a high-throughput transformation system, ample amounts of high-quality callus are required. Each medium set and genotype combination produced high-quality inflorescence-derived tissue that could readily be induced to form callus, except for SA37 on the S medium (Fig. 1).

Significant treatment effects for callus yields were observed ($p < 0.05$); and the 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 (Fig. 2). Additionally, over 96 % of calli from the Somleva et al. (2002) callus induction and maintenance medium had embryogenic sections. Only the maintenance medium from ST2 on the GA experimental medium (K) failed to produce sufficient callus yields (Fig. 2; Electronic Supplementary Material Table 1). Additionally, ST2 produced poor quality callus on the N6-based callus media, with less than 42 % of the callus having embryogenic sections (type II callus) or somatic embryos (Fig. 3; Electronic Supplementary Material Table 1).

Callus from each genotype in all treatments was highly regenerable, with a minimum of 50 plants recovered from each half-gram (Fig. 4). The greatest difference in response was due to genotype. Whereas ST2 could yield over 480 plantlets from a half-gram of callus, SA37 and ST1 never exceeded 150 plants; SA37, performed uniformly on all

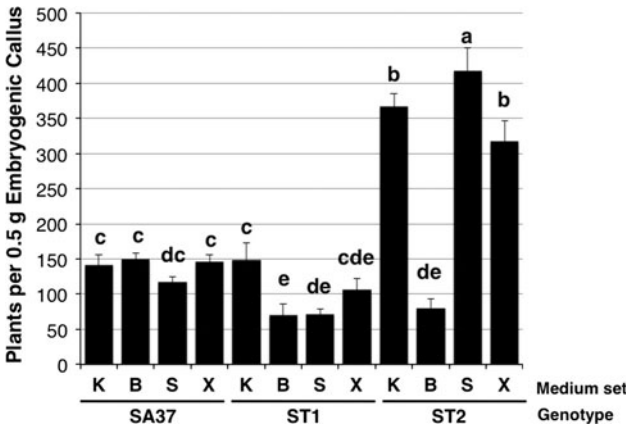

Fig. 4 Average number of plants per 0.5 g of embryogenic callus. *Error bars* are standard error. Only two replicates were available for ST2 on the (*X*) media set. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$). Medium sets GA experimental (*K*), Burris et al. (2009) (*B*), Somleva et al. (2002) (*S*) and Xi et al. (2009) (*X*)

regeneration media, while ST1 performed optimally only on the GA experimental medium (Fig. 4). ST2 significantly outperformed all other genotypes except that of Burris et al. (2009), which highlights the importance of proper genotype and medium selection (Fig. 4). The *K* experimental medium produced the most consistent response for all genotypes, yielding ~150 plants per half-gram callus from SA37 and ST1, and 370 plants from ST2 (Fig. 4). The plantlets on Somleva et al. (2002) medium (S) had elongated and brittle shoots, which may have been caused by the gibberellic acid in the medium (Electronic Supplementary Material, Figs. 5, 4; Electronic Supplementary Material Table 1). All treatments produced phenotypically normal rooted plants after 22 weeks (data not shown).

The use of immature inflorescences makes it possible to obtain quality explants throughout the year. Alternatively, (Somleva et al. 2008) continually screened seed-derived genotypes to find those that regenerate over 175 plantlets per half gram of callus, ~2.5-fold less regeneration efficiency than that of ST2 (Electronic Supplementary Material Figs. 5, 4). In contrast, boot stage switchgrass tillers can be harvested weekly and for many years by reporting at 3–6 months intervals.

The most difficult part of switchgrass tissue culture and transformation is retaining callus quality and yields over time (Burris et al. 2009; Li and Qu 2010). The genotype SA37 was selected for its superior callus response from inflorescence tissue and addresses this bottleneck, and may allow for the development of a high-throughput transformation pipeline. SA37 was favored due to its high callus yields and quality (Electronic Supplementary Material Figs. 4, 2, 3) across all media; over 85 % of calli produced by SA37 on all media were embryogenic. Specifically,

SA37 produced more embryogenic calli, and higher quality callus, as compared to ST1 and ST2, regardless of the medium (Electronic Supplementary Material Figs. 4, 2, 3).

The callus quality, yields and regeneration measurements allowed several conclusions. Firstly, superior genotypes and medium sets can be selected based on the quality of embryogenic callus (Fig. 3), callus yields (Fig. 2) and regeneration response (Fig. 4). These figures can be simply and accurately quantified to generate informative data to evaluate media and genotypes. Although there are many types of callus (Electronic Supplementary Material Fig. 3), the type II callus is the most desirable due to its competency for both transformation and regeneration (Armstrong and Green 1985; Burris et al. 2009; Li and Qu 2010), and therefore must be selected for upon each subculture, leaving the other types behind.

Particle bombardment optimization

This is the first report that tracks transgenics using the same tissue for both transient expression and stable transformation. Transient foci data for osmoticum for the genotype SA37 reflected what was observed for stable events, as 0.6 and 0.8 M osmoticum outperformed 0.09 and 0.4 M osmoticum media in both experiments. ST1 data were more difficult to interpret for osmoticum level due to the low number of stable events recovered (Fig. 5). For complete microcarrier flight distance no significant differences were found for transient foci, or stable transformation when comparing treatments within genotype, however, the 9 and 12 cm distances had on average, over twice the number of stable events when compared to the 6 cm microcarrier flight distance (Fig. 6). Finally, the comparison of rupture disk pressure and microcarrier size showed no difference for transient foci for SA37, however, stable events were recovered at a significantly higher rate for SA37 when 0.4 µm Au and a pressure of 4,482 kPa were used compared to treatments that used 0.6 µm Au. For ST1, 0.6 µm Au particles propelled at 7,584 kPa had significantly more foci than the same particle size propelled at 4,482 kPa. However, there was no significant difference observed when stable transformants of ST1 were observed (Fig. 7). Therefore, SA37 experiments that documented transient foci to optimize osmoticum and microcarrier flight distance were predictive of the best treatment(s) obtained by recovering stable transformants. Alternatively, the experiment, which examined gold size and particle speed, did not have a correlation for transient and stable transformation data. This may be due to the fact that many of the cells transiently expressing RFP were injured beyond the point of recovery when bombarded with the 0.6 µm Au particles as opposed to the 0.4 µm particles.

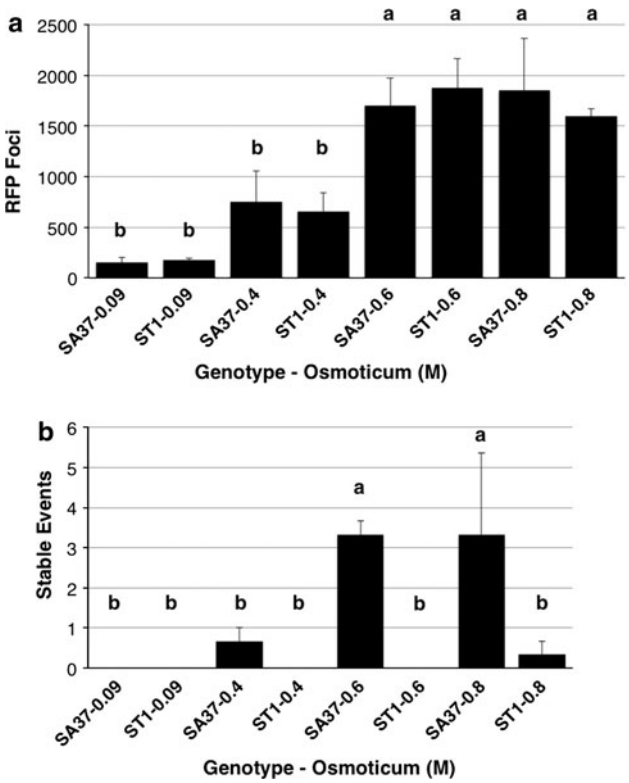

Fig. 5 Osmotic medium optimization. The average number of RFP foci counted per plate (**a**) and the stable number of callus transformants recovered are shown (**b**). *Error bars* are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$)

The osmotic adjustment medium treatments used are within range of those previously tested for rice (Chen et al. 1998). The transient expression results showed a significant difference between treatments due to osmotic adjustment µ medium ($p < 0.05$). Calli incubated on 0.6 or 0.8 M osmotic adjustment medium showed significantly more foci then those incubated on 0.09 or 0.4 M media. There were no significant differences between genotypes at any given osmotic treatment (Fig. 5a). SA37 outperformed ST1 in terms of stable transformation efficiency on every osmoticum ($p < 0.05$); the osmoticum level of 0.6 or 0.8 M provided the best transformation efficiency for SA37 (Fig. 5b).

No significant difference in the number of foci was observed between treatments due to complete microcarrier flight distances, although the 9 cm distance consistently showed the highest number of foci for both ST1 and SA37. SA37 produced significantly more transgenics than ST1 for the 9 cm treatments. A distance of 9 cm was selected as it produced acceptable results for ST1 and the highest mean of events for SA37 (Fig. 6b).

When rupture disk pressure and microcarrier size were evaluated, a significant difference was detected within a

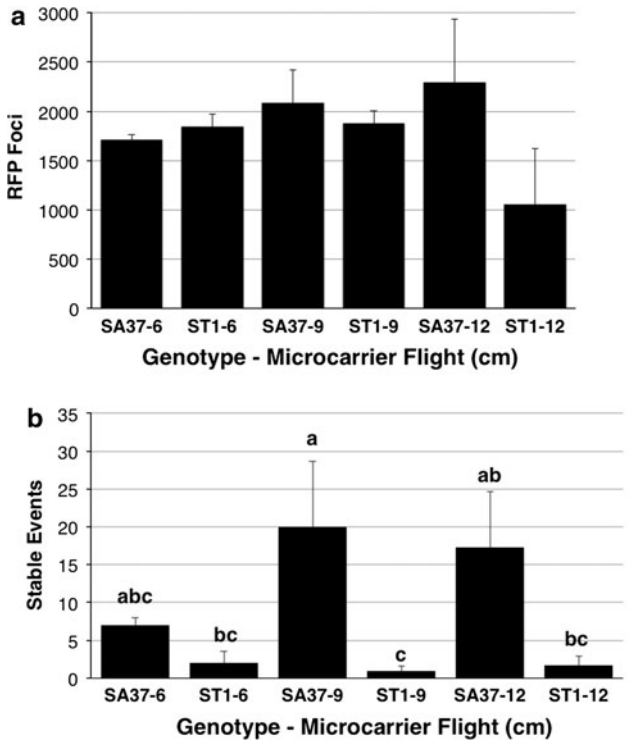

Fig. 6 Microcarrier flight distance optimization. The average number of RFP foci counted per plate (**a**) and the stable number of callus transformants recovered are shown (**b**). *Error bars* represent standard error and treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$)

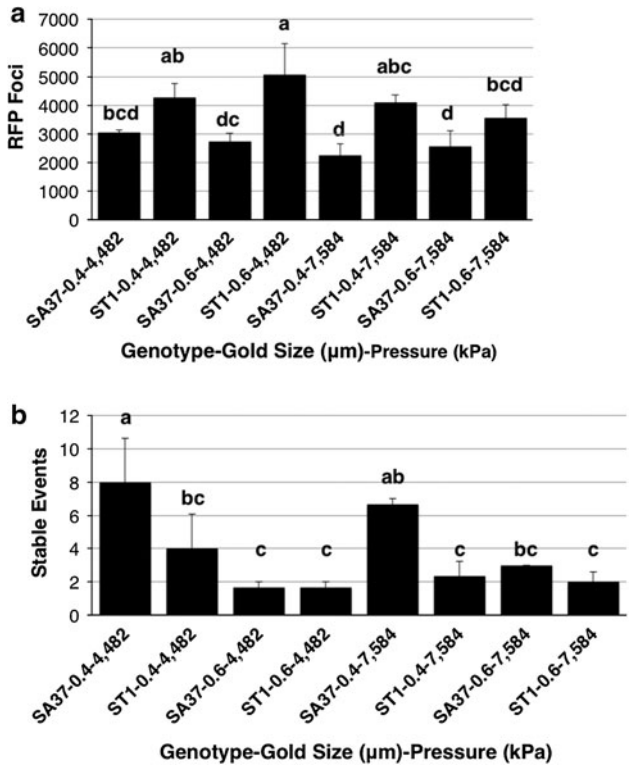

Fig. 7 Microcarrier size and rupture disk pressure optimization. The average number of RFP foci counted per plate (**a**) and the stable number of callus transformants recovered are shown (**b**). *Error bars* are standard error. Treatments that share the same letter are not significantly different as calculated by LSD ($p \leq 0.05$)

genotype for transient foci, whereby ST1 outperformed SA37 ($p < 0.05$) (Fig. 7a). Over 6,000 foci were observed across 20 pieces of tissue from one plate of ST1 (Figs. 7, 8). Bombarding SA37 or ST1 with 0.4 µm microcarriers at a pressure of 4,482 kPa gave the best results in terms of highest mean transformation efficiency (Fig. 7b). The use of 0.4 µm gold significantly outperformed the use of 0.6 µm gold particles ($p = 0.004$).

Southern blot

Southern blot hybridization of randomly selected plants regenerated from hygromycin-resistant calli was used to confirm *hph* insertion, as well as to determine transgene insertion number. The number of insertions averaged three per plant, and ranged from two to six. Based on equivalent copy number and band size, plants labeled 2a and 2b appear to be from the same event (Fig. 9).

Discussion

The tissue culture procedures presented here provide a framework for the selection of a medium set for the optimal production of highly regenerable, embryogenic callus for biolistic transformation. All medium sets worked well for explant initiation, other than SA37 on S initiation medium (Fig. 1). The S medium for both embryogenic callus production (Fig. 3) and callus yields (Fig. 2) provided optimal results for all genotypes and is considered a bottleneck in the process of switchgrass transformation. The S regeneration medium also provided excellent regeneration results for all genotypes. For these reasons, the S medium set is a good choice for these diverse switchgrass genotypes and potentially other switchgrass genotypes as well. Whereas other switchgrass publications used MS-based callus growth and maintenance medium (Richards et al. 2001; Li and Qu 2010; Somleva 2007; Somleva et al. 2002, 2008; Xi et al. 2009), the Georgia experimental medium set (K) and the medium set developed by Burris et al. (2009) (B) used N6 based media. Burris et al. (2009) showed that their N6-based callus medium (LP9) promoted long-term maintenance of type II switchgrass callus that was transformable and highly regenerable. The N6-based callus induction and maintenance medium of the Georgia experimental medium set (K) was based on work done in corn (*Zea mays* L.), which in preliminary experimentation data outperformed MS-based media for callus induction from immature

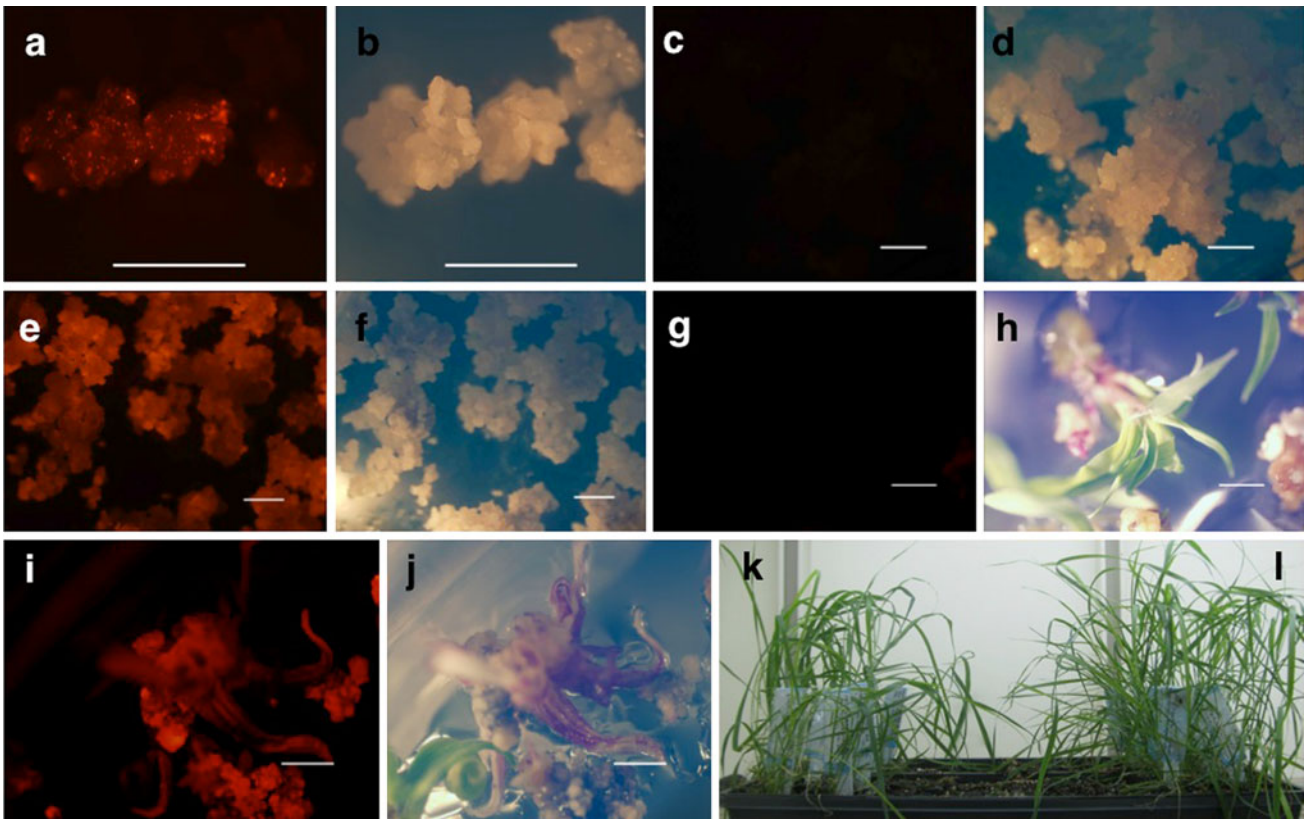

Fig. 8 Red fluorescent protein expression of transgenic SA37 and the wild-type control. Transient RFP expression 2 days post-bombardment on a highly embryogenic callus (RFP filter) (**a**), the same bombarded callus (*white light*) (**b**), wild-type callus tissue without hygromycin selection (RFP filter) (**c**), the same wild-type callus tissue (*white light*) (**d**), stable RFP expression of transgenic callus growing on hygromycin selection medium (RFP filter) (**e**), the same transgenic callus (*white light*) (**f**), non-transgenic plantlet regenerated from tissue culture without selection (RFP filter) (**g**), the same non-transgenic plantlet (*white light*) (**h**), transgenic plantlet with stable RFP expression on regeneration medium (RFP filter) (**i**), the same transgenic plantlet (*white light*) (**j**), transgenic plants regenerated from tissue culture (**k**), and non-transgenic control plants regenerated from tissue culture without hygromycin selection (**l**). *Bar* 2 mm

inflorescences (data not shown). For maintenance, however, the MS-based callus medium of Somleva et al. (2002) within the S medium set produced the highest callus yields and highest proportion of embryogenic calli across genotypes (Figs. 2, 3).

A perceived limitation to 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 can deliver low concentrations of linear DNA cassettes, such that biolistic 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). In this study, 150 ng of plasmid DNA were delivered per bombardment. Transgenic lines regenerated on average showed approximately three insertions per event and the number of insertions ranged from two to six copies (Fig. 9). Although microprojectile-mediated transformation is commonly thought of as producing an unwieldy number of insertions (Altpeter et al. 2005), the range of transgene insertions observed in this biolistic transformation study (2–6) is well within the range obtained with *Agrobacterium*-mediated transformations, which includes Li and Qu (2010) (1–7 insertions); Somleva et al. (2002) (1–8 insertions); Somleva et al. (2008) (1–5 insertions); and Xi et al. (2009) (1–5 insertions). The use of less DNA for transformation reduces the number of insertions (Lowe et al. 2009).

Only limited research exists on the optimal size of the gold microcarriers, which may differ between species (Southgate et al. 1995). The recovery of transgenic maize lines increased 13 % when 0.6 μm microcarriers were used instead of 1.0 μm (Frame et al. 2000). In this study, the use of 0.4 rather than 0.6 μm particles increased the number of

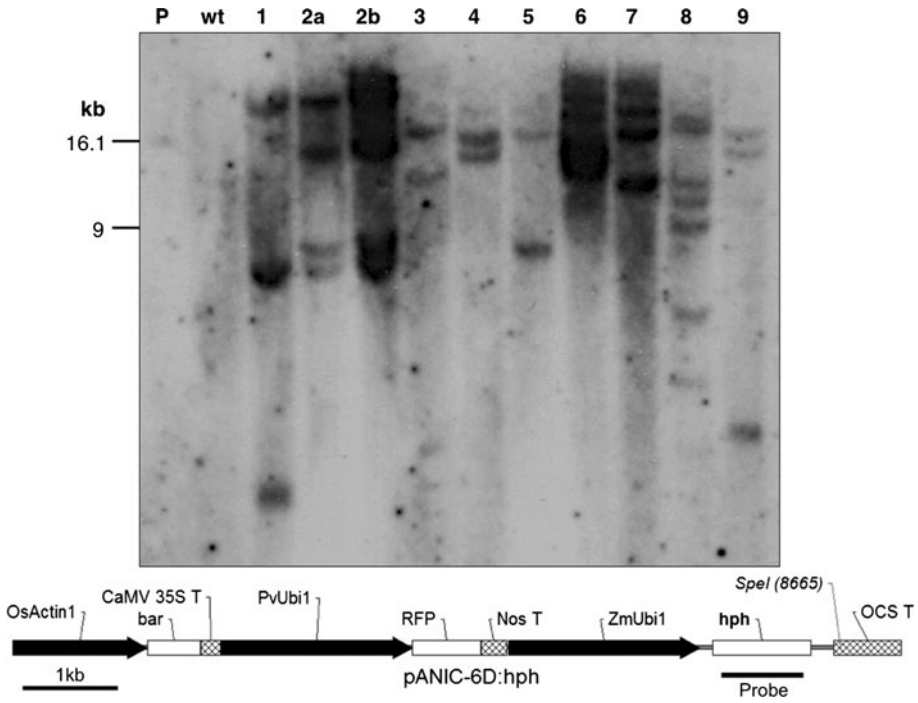

Fig. 9 Southern hybridization of switchgrass DNA extracted from transformed plants and digested with *Spe*I. Digested DNA was hybridized with a p32 labeled probe to the *hph* transgene. The pANIC-6D:*hph* plasmid (*P*) used for transformation is 16.1 kb, which includes three marker cassettes between the left and right borders (9 kb). Shown here is only the 9 kb sequence between the left and right borders. Wild type (*wt*), non-transformed, DNA from switchgrass was probed as a negative control. Nine independent transgenic plants were probed; 2a and b appear to be the same event. Below is a linearized diagram of the pANIC-6D:*hph* vector used to generate the transgenic switchgrass plants

events twofold for SA37 (Fig. 7b), and may be an important factor to test when looking at species recalcitrant to biolistic transformation. In the future transformations will be done with 0.6 M osmoticum, a complete macrocarrier flight distance of 9 cm and a gold size of 0.4 μm propelled at a pressure of 4,482 kPa, which provide the highest numbers of transgenics. These experiments also confirm the importance of genotype choice for transformation, as SA37, a progeny of ST1, was more transformable in every experiment conducted when compared to ST1 (Figs. 5b, 6b, 7b).

In summary, the recovery of transgenic switchgrass was improved by determining the optimal procedures for various steps in the process. The best treatment in this study produced on average eight independent transgenic callus events per 20 SA37 calli bombarded, as determined by RFP expression and hygromycin resistance on selection medium, resulting in an efficiency of 40 %. The procedures presented here resulted in approximately fourfold more independent events per bombardment than in the previous particle bombardment study, whereby the best treatment yielded 1.4 independent events per 30 calli bombarded (Richards et al. 2001). The effect of genotype selection made a significant difference in both tissue culture (e.g., regeneration) and in stable transformation efficiency.

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Conflict of interest The authors declare that they have no conflict of interest.

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