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High throughput *Agrobacterium*-mediated switchgrass transformation

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ABSTRACT

Switchgrass is one of the most important biomass/bioenergy crops. For its improvement as a feedstock through biotechnological approach, we have developed a high throughput *Agrobacterium*-mediated transformation system for cv. Alamo and two new elite cultivars, Performer and Colony. Highly regenerable and transformation-competent embryogenic calli were identified and used for genetic transformation. *GFP* reporter gene was employed to identify transformation events at early stages and to guide modifications at various stages for improvement of transformation efficiency. The modifications included infection under vacuum, co-cultivation at desiccation conditions, resting between co-cultivation and selection, and supplement of L-proline in the callus culture and selection media. Transformation efficiency over 90% was routinely achieved for Performer, and around 50% for Alamo and Colony. The new system substantially improved switchgrass transformation efficiency and will significantly contribute to the genetic improvement of this important biofuel feedstock via biotechnological approach.

1. Introduction

Switchgrass (*Panicum virgatum* L.) is a C4 perennial grass native to North America [1]. It is recognized as a major biofuel crop because of its high yield of lignocellulosic biomass, which was estimated to yield 5.6 m^3 ha^{-1} of ethanol [2,3]. In the past, switchgrass breeding was targeted at its improvement as a forage grass [4,5]. Along with recognition of switchgrass as a major biomass/bioenergy crop, recent switchgrass breeding has been focused on its improvement for bioethanol production using conventional [6–8] and molecular approaches [9,10]. So far, a few reports regarding genetic transformation of switchgrass using particle bombardment or *Agrobacterium*-mediated approach have been published [11–14]. *Agrobacterium*-mediated transformation has been considered as a preferred technique for plant genetic engineering mainly due to its low transgene copy number and thus less chance to induce transgene silencing [15,16]. Great progress has been made for *Agrobacterium*-mediated transformation of grass species, once considered recalcitrant, such as rice [17], maize [18,19], wheat [20], sorghum [21], and creeping bentgrass [22]. Relatively high transformation frequencies were reported in tall fescue [23,24], *Brachypodium distachyon* [25], and maize [19]. However, most transformation efforts of monocots still suffer from inefficiency, which is also a major obstacle for switchgrass transformation. *Agrobacterium tumefaciens* strain AGL1 carrying binary vector pDM805 was utilized to transform somatic embryo or embryogenic callus of various selected genotypes of cv. Alamo using the *GUS* gene as a reporter gene and the *bar* gene for selection [12]. The transformation efficiency varied a great deal depending on the explants and genotypes used in the experiments. Certain genotypes from Alamo were reportedly more competent for *Agrobacterium*-mediated transformation. However, these materials seem to

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go to the private sector [13] and are no longer available to the public (J. Zale, personal communication). Antibiotic hygromycin B (hyg B) selection in *Agrobacterium*-mediated transformation of switchgrass was reported but it is not clear what the transformation efficiency was [14].

In this correspondence, we report a high-throughput transformation system of Alamo, together with two newly-released, elite cultivars, Performer [26] and Colony [8]. Performer has less lignin content and Colony has more cellulose content than Alamo. They could serve as excellent starting materials for further improvement by genetic engineering for their use as biofuel feedstock. The system we developed uses random seeds of a cultivar and does not require specific genotypes of the plants. It includes identification of embryogenic callus highly competent for transformation and regeneration, improvement of callus growth and selection by supplement of L-proline in the media, vacuum treatment at infection stage, desiccation treatment during co-cultivation stage, resting between co-cultivation and selection stages, and selection of transformants with hyg B. In addition, utilization of *GFP* as a reporter gene greatly facilitates early identification of transformation events and guides the development of the system. As the result, transformation efficiency reached 50% for Alamo and Colony, and was as high as 90% for Performer. Using this system, the time period from *Agrobacterium* infection to transplantation of transgenic plants to the soil could be as short as 3 months. The new system substantially improved switchgrass transformation efficiency and will significantly contribute to the genetic improvement of this important biofuel feedstock via biotechnological approach.

2. Materials and methods

2.1. Callus induction and culture

Seeds of cultivars Alamo, Performer, and Colony were kindly provided by Dr. J. Burns of NCSU and USDA/ARS Forage Program, surface-sterilized with full strength Clorox® (6% sodium hypochlorite, Clorox, Oakland, CA, USA) for two and an half hours with gentle stirring. After rinsing with distilled water three times, seeds were kept overnight in the dark at 26 °C and then sterilized again for another 80 min. With another round of rinsing with distilled water three times, seeds were placed on callus induction medium (MB) (MB and other media used in the experiments are listed in Table 1). Six to eight weeks later, the embryogenic calli were picked and subcultured on MP medium.

2.2. Regeneration test of subcultured callus

Embryogenic calli, which were subcultured once every 3 or 4 weeks, were placed in REG medium for regeneration. The cultures were kept under cool white fluorescent light (140 μmol m^{-2} s^{-1}) with a photoperiod of 16/8 h (light/dark) at 25 °C in a growth chamber. A month later, number of calli having green or albino shoots were recorded. In each experiment, 20 to 30 pieces of calli (0.5–1 cm in diameter) were used per replicate and the experiment was replicated three times.

Table 1 – Media composition used in the experiments.

Medium	Composition
MB	MS basic medium with 30 g l^{-1} maltose, 5 mg l^{-1} 2, 4-D, 1 mg l^{-1} BAP, 3 g l^{-1} phytagel, pH 5.8
MP	MB with 2 g l^{-1} L-proline
CTMP	MP with 200 mg l^{-1} carbencillin and 150 mg l^{-1} timentin
REG	MS with 0.2 mg l^{-1} NAA, 1 mg l^{-1} BAP, 0.5 mg l^{-1} GA, 30 g l^{-1} maltose, pH 5.8
½ TM	½ MS with 30 g l^{-1} maltose, 150 mg l^{-1} timentin

Sources of the chemicals: MS basal medium (Caisson Laboratories, North Logan, UT, USA), timentin (GlaxoSmithKline, Research Triangle Park, NC, USA), carbenicillin (Apollo Scientific, Stockport, UK), maltose (Caisson Laboratories North Logan, UT, USA), L-proline (Fisher, Fair Lawn, New Jersey, USA). All other chemicals used in the experiments were purchased from Sigma (St. Louis, MO, USA).

2.3. L-proline effects on callus growth and transformation selection

The effects of L-proline supplement with concentrations from 10 mg l^{-1} up to 32 g l^{-1} were tested on Alamo callus growth in MB medium in a pilot experiment. Based on the results, supplement of 2 g l^{-1} L-proline was chosen and formally evaluated on callus growth of both Alamo and Performer. Ten pieces of callus per plate, five plates per treatment, were weighed before the treatment and cultured in the dark at 25 °C. The calli were weighed again 4 weeks later. The growth index was calculated as the final fresh weight minus the initial fresh weight, and divided by the initial fresh weight. The experiment was replicated three times. The effect of proline supplement in callus selection medium during transformation was also evaluated.

2.4. Agrobacterium strain and vectors

Binary vector pJLU13 [27] was used in the transformation experiments. It is a derivative of pCAMBIA1300 containing the hyg B selectable marker gene, *hpt*, and a green fluorescent protein reporter gene *sGFP* (S65T) [28], driven by the rice *rubi3* promoter [29]. The freeze-thaw method [30] was used to mobilize pJLU13 and another plasmid, pTOK47, into *Agrobacterium* strain EHA105 [31]. Plasmid pTOK47 carries a 20 kb *Kpn*I fragment of Ti plasmid from pTiBo542, which contains *virB*, *virC*, and *virG* virulence genes [24]. The resulted *Agrobacterium* strain, EHA105 (pTOK47, pJLU13), was grown in YEP medium in the presence of 20 mg l^{-1} rifampicin, 5 mg l^{-1} tetracycline, and 50 mg l^{-1} kanamycin. The culture was grown at 28 °C with shaking (250 rpm) until the OD_{595} reading reached 0.8–1.0, and then centrifuged at 4000 g for 10 min. The pelleted cells were resuspended in liquid MP medium (Table 1) to adjust OD_{595} to 0.5–0.6. Acetosyringone (3′, 5′–dimethoxy-4′-hydroxyactophenone, Aldrich, Milwaukee, WI, USA) was then added to the suspension to 100 μM.

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2.5. Transformation procedure

In pilot experiments, application of vacuum at *Agrobacterium* infection, desiccation treatment during co-cultivation, resting before selection stage and supplement of proline in the culture medium showed positive impacts in transient and stable transformation with Alamo. Based on these observations, a formal experiment was designed to evaluate the effects of these factors on transformation efficiency. Eighty pieces of embryogenic calli, 0.5–1 cm in diameter, with vigorous growth were picked and pre-cultured for 2 d on a fresh MP medium. The calli were then immersed in 10 ml *Agrobacterium* suspension in a 50 ml sterile plastic tube. And they were either directly incubated with gentle agitating for 30 min at 1.25 Hz at 28 °C, or under vacuum for 10 min followed by agitating for 20 min. Vacuum treatment was conducted by placing the open tubes in the center of a Nalgene 5311 desiccator with vacuum (approximately 80 kPa) applied from a pump. After incubation, excessive *A. tumefaciens* cells were removed by blotting with sterile Whatman No. 3 filter papers. The calli were either transferred onto MP medium or desiccation-treated in the dark at 26 °C for 2 d for co-cultivation. For desiccation treatment, a piece of sterile Whatman No. 1 filter paper was placed in a Petri dish. One hundred mm^3 of sterile water was loaded in the middle of the filter paper. After 1 min, forty pieces of calli were placed on the paper. The plates were immediately sealed with Parafilm® (Pechiney plastic packaging, Chicago, IL. USA).

2.6. Selection and regeneration of putative transgenic plants

After co-cultivation, the calli were either directly transferred to the selection medium, which is CTMP medium containing 100 mg l^{-1} hyg B (CAS 31282-04-9, A. G. Scientific, San Diego, CA, USA), or to the CTMP medium for resting for 3 d before going to selection medium. Two weeks after on selection medium (100 mg l^{-1} hyg B), the calli were broken into small pieces and all the calli were transferred to CTMP medium containing 200 mg l^{-1} hyg B for 2 more rounds of selection (2 weeks/round). Care was taken so the callus originated from the same piece of callus was always placed together as a callus line. Number of *GFP*-expressing callus lines and hyg B resistant callus lines were counted at the end of the selection. The vigorously growing, hyg B resistant calli were transferred to the regeneration medium (REG) containing 200 mg l^{-1} carbenicillin and 150 mg l^{-1} timentin. The regenerated shoots were transferred onto ½ TM medium with 50 mg l^{-1} hyg B for rooting and further selection. The cultures for regeneration and rooting were maintained under cool white fluorescent (140 μmol m^{-2} s^{-1}) at a photoperiod of 16/8 h (light/dark) at 25 °C in a growth chamber. Three to four weeks later, plants with well developed roots were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH, USA) and grown in a greenhouse at 25 °C under natural light. Overall transformation efficiency is counted as the number of regenerated hyg B resistant callus divided by the total number of callus infected.

2.7. Visualization of GFP gene expression

The fluorescence of *GFP* was visualized using a Leica MZ FLIII dissecting microscope coupled with a Hamamatsu (Bridgewater, NJ, USA) cooled color CCD Camera (C5810) connected to a computer system at NCSU Cellular and Molecular Imaging Facilities. The *GFP*2 filter set (excitation filter: 480 ± 40 nm, barrier filter: 510 LP) was used for imaging. The light source was an HBO 100 W mercury lamp.

2.8. Molecular analysis of transgenic plants

For Southern blot analysis, DNA was extracted from plant leaves based on the CTAB method [32] with addition of DNase-free RNase A treatment (Sigma, 10 mg ml^{-1}, 30 min). Twenty μg of genomic DNA from each sample was digested with *Eco*RV (Promega, Madison, WI, USA) overnight and was subjected to electrophoresis in a 1% agarose gel. *Eco*RV has one restriction site on *hpt* gene construct outside the *hpt* coding sequence. The fractionated DNA was transferred to a positively charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. A 591 bp fragment of *hpt* coding sequence was amplified by PCR using a set of primers (CGTCTGTCGAGAAGTTTC; CAGGACATTGTTGGAG) from plasmid pJLU13. The probe was labeled with [α-^{32}P]-dCTP (PerkinElmer, Boston, MA, USA) using the Prime-It® II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX, USA) and hybridized with the blotted membrane in MiracleHyb™ hybridization solution (Stratagene) according to the manufacturer's instructions. The hybridized membranes were exposed to CL-X Posure™ X-ray film (Thermo, Rockford, IL, USA) for autoradiography.

2.9. PCR analysis of offspring plants

Seeds were collected from transgenic plants in the greenhouse, sterilized, and placed on a piece of filter paper in a sealed Petri dish moisturized with 20 μM H_2O_2 for about a week [33] before germinating on MS medium. After plants were well rooted, they were transplanted to the soil. Genomic DNA was extracted from leaves of the seedlings using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). *GFP* gene was detected by PCR with a pair of primers (GTCTAGACCATGGGATCGATGCATCATC; ACGAGCTCTTACTGTACAGC). The expected size of the amplified fragment is 715 bp. *AccuPower* ™ PCR Premix kit (Bioneer, Alameda, CA, USA) was used in a standard 35-cycle reaction with annealing temperature of 58 °C. The positive control for PCR used 50 ng plasmid pJLU13 as the template while the negative control had genomic DNA extracted from a non-transgenic plant, or just water.

2.10. Experimental design and statistical analysis

Randomized block design was used for all the applicable experiments. ANOVA was performed using SAS software (ver.9.1, 2003, SAS Institute, Cary, NC, USA) for significance analysis. When significant difference ($p < 0.05$) was observed in a treatment, the least significant difference test (LSD) [34] was performed to evaluate the effect of the treatment.

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3. Results

3.1. *Identification of highly competent embryogenic callus for transformation*

Callus was induced on MB medium from mature caryopsis. After 6–8 weeks of culture, approximately 15% of them were white and compact embryogenic calli with numerous embroids on the surface. This type of callus was transferred to MP medium for subculture, where approximately 50% of the callus became white and friable in the culture of Alamo and Colony (Fig. 1A). Although both types are embryogenic and highly regenerable, pilot experiments suggested that the white friable embryogenic calli were much more competent for genetic transformation and were subsequently used for future experiments. In Performer callus culture on MP medium, a third type of callus, yellow, friable embryogenic callus (Fig. 1B), was observed. Although it consisted of only about 10% of the subcultured calli, it was highly regenerable and highly competent for transformation. Therefore, only this type of callus was employed in Performer transformation and the highest transformation efficiency was achieved (see below). In addition, the yellow, friable embryogenic calli from Performer maintained the ability to regenerate into green shoots in long-term cultures. Dozens of the callus lines tested in a replicated experiment were able to regenerate green shoots after 14 months in culture without producing albinos (Fig. 1C; Table 2). In comparison, 16% of the white,

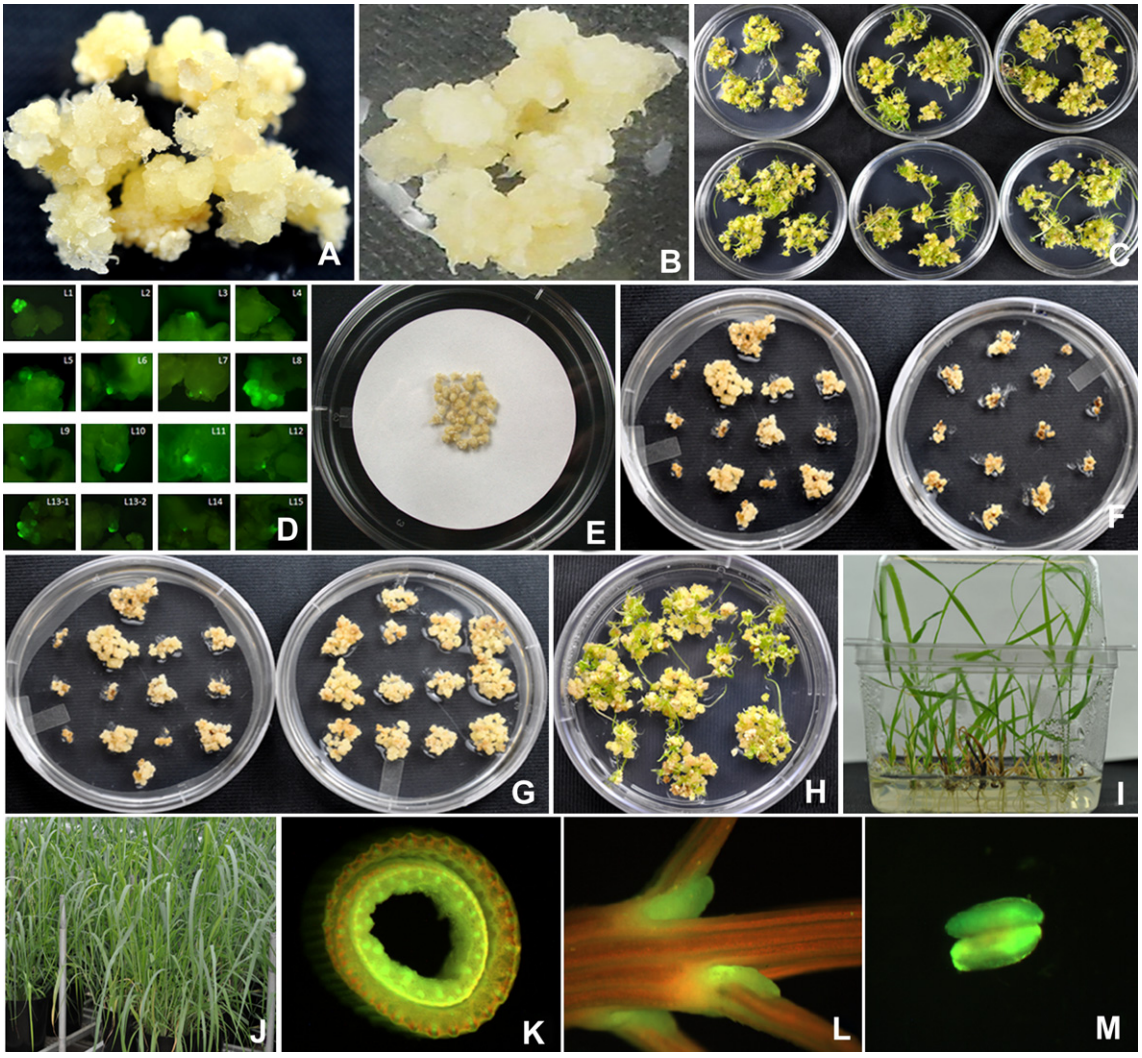

Fig. 1 – *Agrobacterium tumefaciens*- mediated transformation of switchgrass with *GFP* biomarker and hyg B selection. A. Yellow, friable embryogenic callus from Performer; B. White, friable embryogenic callus from Alamo; C. Regeneration of 14-month-old embryogenic calli of Performer; D. Detection of *GFP* expression in cell clusters during selection; E. Co-cultivation under desiccation condition; F. Resistant calli of Alamo growing with (left side) and without proline (right side) after 4 weeks of hyg B selection; G. Resistant calli of Alamo (left side) and Performer (right side) after 4 weeks of hyg B selection; H. Regeneration of hyg B resistant calli of Performer; I. Root development of regenerated plants under hyg B selection; J. Transgenic plants growing in greenhouse; K. stem cross-section of an Alamo transgenic plant showing *GFP* expression; L. *GFP* expression in axillary buds of a transgenic plant of Alamo; M. An anther from a transgenic plant of Alamo expressing *GFP*.

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Table 2 – Regeneration ability of long-term subcultured callus of Alamo and Performer.

Cultivar	Age of callus (month)	Regenerated callus (%)	Regenerated callus with albino shoots (%)
Alamo	4	$100 \pm 0^{a*}$	16 ± 11.5^{b}
	14	98.3 ± 2.9^{a}	100 ± 0^{a}
Performer	14	100 ± 0^{a}	0^{c}

*Each value represents means of the three replicates of 20–30 pieces callus per replicate. Values followed by the same letter within each column were not significantly different from each other according to the LSD analysis ($p = 0.05$).

Table 3 – L-proline effect on callus growth of cv. Alamo and Performer.

Cultivar	Alamo	Performer
2 g l^{-1} L-proline	$20.2 \pm 1.9^{a*}$	18.74 ± 1.0^{a}
No proline	15.9 ± 1.6^{b}	18.24 ± 1.2^{a}

*Each value represents means of growth index from three replicates, 50 pieces of callus per replicate. Values followed by the same letter within each column were not significantly different from each other according to the LSD analysis ($p = 0.05$).

friable embryogenic callus lines from Alamo regenerated albino only plants after four-month culture and all the regenerated plants were albino after calli being 14 months in culture (Table 2). The results indicated that the yellow, friable embryogenic callus is superior over the white ones in maintaining ability to regenerate green shoots. Moreover, the stably-transformed yellow, friable embryogenic callus from Performer grew fast under selection and usually only needed a total of 4 weeks of selection (2 weeks on 100 mg l^{-1} hyg B and 2 weeks on 200 mg l^{-1}) before being transferred to the regeneration medium.

3.2. *L-proline effects on callus growth and transformation selection*

Supplement of 2 g l^{-1} L-proline to the callus culture medium not only significantly promoted the callus growth rate of Alamo by 30% (Table 3), but also promoted formation of friable embryogenic callus. Supplement of L-proline also enhanced transformation selection (see below). The effect of L-proline supplement on callus growth and selection of Performer was not obvious though, most likely because the selected Performer callus already had good growth rate.

3.3. *Employment of GFP reporter gene to guide transformation experiments*

To closely monitor the transformation progress and assess the effects of variable treatments on transformation efficiency, *GFP* was used as a reporter gene in the transformation efforts. High fluorescence signals were clearly observed, particularly in callus and root. The non-destructive nature of the reporter gene is very valuable in early detection of the transformation events and in evaluating treatments for transformation improvement. Transient *GFP* expression could be observed as early as 2 d after infection (right after co-cultivation) using fluorescence microscopy, which would help to evaluate the effects of the treatment during infection stage. Frequently monitoring *GFP* expression (Fig. 1D) also facilitated assessment of treatment at selection stage (Table 4). For example, in an early experiment, although approximately 36% calli showed stable transformation based on *GFP* expression in cell clusters after 6-week selection, only about 1/3 of them showed hyg B resistance, indicating the low transformation efficiency was mainly due to poor growth of the transformed cells during selection stage. The results eventually guided us to supplement L-proline in the selection medium to promote growth of the transformed cells to have a more efficient selection. Since we were able to monitor *GFP* expression and hyg B resistance independently during the whole process of transformation and selection, it was observed that the growth of some *GFP*-expressing calli was inhibited under hyg B selection (Tables 4 and 5). The discrepancy was often higher when the protocol was not optimized (Table 4). It was also true vice versa. But this discrepancy was minimized when the protocol was optimized (Table 5).

3.4. *Development of a high-throughput switchgrass transformation system*

In our practice, it was observed that 200 mg l^{-1} of hyg B was able to completely inhibit callus growth while 100 mg l^{-1} of

Table 4 – Effects of different treatments on transformation of Alamo and Performer after 6 wks of selection.

Treatment	Vacuum	Desiccation	Resting	Proline	Alamo		Performer	
					GFP callus (%)[e]	Hyg B^R callus (%)	*GFP* callus (%)	Hyg B^R callus (%)
With all	+	+	+	+	$58.7 \pm 6.5^{a**}$	48.5 ± 4.9^{a}	94.7 ± 4.2^{a}	90.3 ± 5.6^{ab}
No vacuum	–	+	+	+	46.3 ± 4.8^{ab}	34.0 ± 3.4^{b}	98.7 ± 1.3^{a}	95.5 ± 1.7^{a}
No desiccation	+	–	+	+	34.3 ± 8.1^{bc}	22.8 ± 4.7^{c}	93.3 ± 4.0^{a}	80.0 ± 6.5^{c}
No resting	+	+	–	+	31.6 ± 4.7^{c}	23.1 ± 3.3^{c}	96.0 ± 1.2^{a}	92.5 ± 0.4^{a}
No proline	+	+	+	–	35.8 ± 6.3^{bc}	13.4 ± 2.6^{d}	92.9 ± 3.6^{a}	83.0 ± 5.0^{bc}

**Each value represents means of three replicates, 80 pieces of callus per replicate. Values followed by the same letter within each column were not significantly different from each other according to the LSD analysis ($p = 0.05$).
e Callus with *GFP* sectors after 6-wks selection.

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Table 5 – Transformation of switchgrass cv. Alamo, Performer and Colony under optimized conditions.

Cultivar	Experiment	Callus infected	*GFP* + callus line[a]	Hyg B^R callus line	Hyg B^R plant[b]	*GFP* + plant	Overall transformation efficiency (%)[c]
Alamo	1	79	58	52	46	46	58.2
	2	76	56	56	51	51	67.1
	3	80	59	52	36	36	45
	Total	235	173	160	133	133	56.6
Performer	1	75	75	75	74	74	98.7
	2	80	79	77	73	68	91.3
	3	80	78	78	68	68	85.0
	Total	235	232	230	215	210	91.6
Colony	1	75	36	34	26	22	34.7
	2	80	46	45	38	36	47.5
	3	78	61	46	40	40	51.3
	Total	233	143	125	104	98	44.6

a Callus line with *GFP* sectors after 6 wks of selection.
b Each callus line which regenerated plants that showed resistance to hyg B in rooting medium is counted as one.
c Overall transformation efficiency = No. of regenerated hyg B resistant callus line/total No. of callus infected × 100%.

Table 6 – Summary of transformation protocol with optimized conditions.

Stage	Treatment	Media	Period
Infection	10 min vacuum	Liquid MP with 100 μM acetosyringone	30 min
Co–cultivation	desiccation	H_2O	2 days
Resting	with resting	CTMP	3 days
Selection	addition of 2 g l^{-1} proline	CTMP + 100/200 mg l^{-1} hyg B	4–6 wks, subculture every 2 wks
Regeneration	–	REG with 200 mg l^{-1} carbenicillin and 150 mg l^{-1} timentin	3–4 wks
Rooting	–	1/2TM with 50 mg l^{-1} hyg B	3–4 wks

hyg B in culture medium can only partially do so. Based on the performance of the transformed cells as monitored by the *GFP* expression, a two-step selection scheme was carried out: 100 mg l^{-1} hyg B in the first selection (2 weeks) and 200 mg l^{-1} in the second and third rounds of selection (2 weeks each).

To improve transformation efficiency, effects of vacuum treatment at infection stage, desiccation during co-cultivation (Fig. 1E), resting between co-cultivation and selection, and L-proline supplement in the selection medium were investigated with both cv. Alamo and Performer. As shown in Table 4, each single treatment significantly improved transformation efficiency of Alamo, among which supplement of L-proline had the highest positive impact. When all other treatments were included, only 13.4% hyg B resistant calli were recovered from the selection medium without proline whereas 48.5% of such calli were obtained from selection medium supplemented with proline (Table 4, Fig. 1F). As for

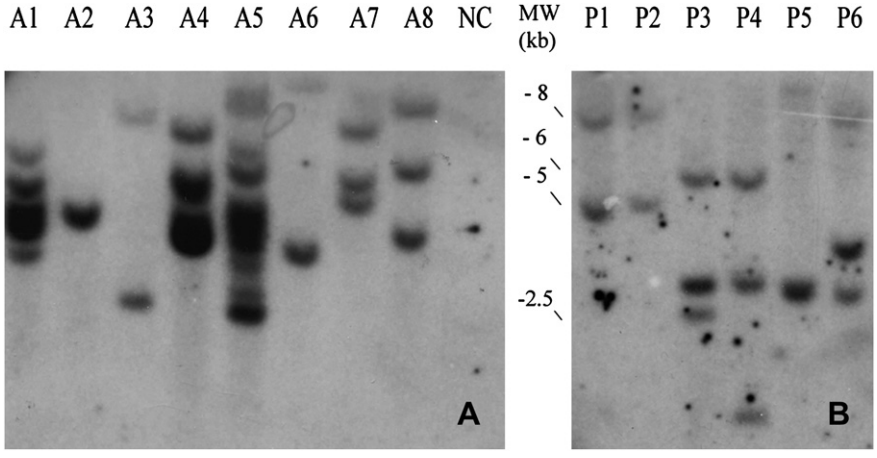

Fig. 2 – Southern blot analysis of transgenic plants of Alamo and Performer with *hpt* probes from its coding sequence. Twenty μg of genomic DNA was digested with *Eco*RV, which cuts outside the *hpt* coding sequence. The probes are PCR products of a fragment of the *hpt* coding sequence labeled with ^{32}P. A. A1 to A8: DNA from 8 putative transgenic plants of switchgrass cv. Alamo. NC: negative control. DNA from a non-transformed switchgrass plant. B. P1 to P6: DNA from 6 putative transgenic plants of switchgrass cv. Performer. MW: molecular markers.

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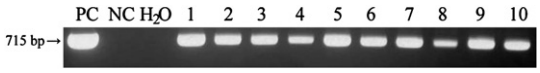

Fig. 3 – Polymerase chain reaction (PCR) analysis of T_1 switchgrass progenies showing the presence of *GFP* gene. PC: positive control, diluted pJLU13 plasmid DNA. NC: negative control, DNA from a non-transformed switchgrass plant. H_2O: another negative control without addition of DNA template. 1–10: DNA from 10 seedlings of T_1 generation.

Performer, probably because the yellow friable callus was already highly competent for *Agrobacterium*-mediated transformation and fast-growing in selection, only desiccation treatment had a significant effect on its transformation efficiency. When the protocol was optimized by including all the four treatments, 48.5% callus of Alamo and 90.3% callus of Performer were hyg B resistant (Table 4, Fig. 1G) after *Agrobacterium*-mediated transformation.

When using the optimized protocol as summarized in Table 6, 50% of transformation efficiency was routinely achieved for Alamo and 90% for Performer. Transformation efficiency of another new, elite cultivar, Colony, also reached about 45% (Table 5). The resulted hyg B-resistant calli were highly regenerable. Ninety-three percent of Performer callus, 80% of Alamo, and 83% of Colony, were able to regenerate into green shoots and the plantlets grew well after being transplanted to soil (Table 5, Fig. 1H–J). *GFP* expression was routinely detected in transgenic plants (Table 5, Fig. 1K–M).

3.5. *Confirmation of transgenic nature of the hyg B resistant plants and transgene inheritance*

Southern blot analysis was performed on putative transgenic plants of Alamo and Performer. All of the plants tested were confirmed for its transgenic nature (Fig. 2). The various hybridization patterns suggest the plants were from independent transformation events and no escape was observed, indicating the tightness of the selection scheme. PCR analysis (Fig. 3) of GFP-positive (data not shown) seedlings germinated from T1 seeds collected from the transgenic plants indicated that the transgene was inherited to the next generation.

4. Discussion

We have developed a high-throughput switchgrass transformation protocol using *Agrobacterium*-mediated approach and hyg B selection, which performed well for all the three cultivars tested. An advantage of our system is that it does not depend on certain genotypes within a cultivar. Instead, it induces calli from random seeds of a cultivar and selects certain types of callus based on morphology for transformation. With addition of L-proline, friable embryogenic callus emerged. The yellow, friable embryogenic callus from Performer was superior in green shoot regeneration and particularly competent for transformation. Tracking *GFP* expression provided early feedback to the treatments and greatly facilitated the efforts for transformation improvement. Optimized treatments at various transformation stages, especially the supplement of L-proline in selection medium, substantially contributed to the improvement. As a result, over 90% transformation efficiency was achieved for Performer and around 50% for Alamo and Colony.

Although *GFP* was used as a reporter gene in early reports of switchgrass transformation, its role was limited to demonstration of transgenic nature of the recovered plants [11]. In this research, we took full advantage of the non-destructive nature of *GFP* detection and were able to observe its expression at very early stages of the transformation, which facilitated us to assess the effects of various treatments on transformation efficiency. Because of the non-destructive nature of the *GFP* reporter gene, it was observed that quite some calli with stable *GFP* expression did not grow further under hyg B selection. Although some could be due to weak or no *hpt* gene expression in the transformed cells, we speculated that growth inhibition of the transformed cells by the surrounding dying non-transformed cells maybe a main cause for the phenomenon. By adding L-proline in the selection medium to promote growth of the transformed cells, substantial improvement was achieved. The s*GFP* is much brighter than the conventional *GFP* in plants [28] and the *rubi3* promoter is a strong, constitutive promoter in monocots [27,29]. Both made the fluorescence signals more observable. In addition, the model of the fluorescence microscope and the corresponding filter set used in the experiments seemed to be a right combination for sensitive detection of the *GFP* fluorescence.

Choosing the right callus type(s) is a key for high frequency transformation in switchgrass. We identified two types of highly competent callus for transformation in switchgrass: white friable embryogenic callus and yellow friable embryogenic callus. The yellow friable embryogenic calli found in Performer is especially suitable for *Agrobacterium* transformation, which also maintains the ability to regenerate green plantlets for a long period. Subculturing such callus lines for transformation would save time, efforts, and resource than starting callus induction from seeds every time. The transformation frequency using this type of callus is among the best in plant transformation. This type of callus was occasionally also seen from Alamo culture, which could further facilitate transformation of Alamo, the most widely used low-land cultivar of switchgrass. The high throughput transformation of the yellow, friable callus could also serve as a platform for functional genomics studies in this important bioenergy crop.

Various supplements were tested in the callus culture and selection media, and only L-proline had an obvious, positive effect on callus growth and transgenic selection in Alamo. With proline supplement, the callus growth was accelerated. The enhanced growth of transgenic callus made the selection more efficient. Without proline in the selection medium, only about 25% of the *GFP*-positive calli (having a cluster of *GFP*-expressing cells, cv. Alamo) survived hyg B selection after 6 weeks whereas about 83% of such calli were hyg B resistant with proline as a supplement to the selection medium.

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However, proline supplement did not have an obvious effect on Performer (Table 4), reflecting the difference in nutrition requirements between the two cultivars or between the two types of callus. Another important role of proline we observed is that it promoted the formation of more friable embryogenic callus, which is more competent for transformation in our experiments. The situation was similar to the formation of maize type II callus in the presence of L-proline [35]. But in switchgrass, we observed two types of friable, embryogenic calli, white and yellow. The yellow one showed even greater transformation competence. How proline affects plant tissue culture still remains to be elucidated. It could serve as an osmotica in plant cells to buffer high ion concentration in vacuoles, or simply as a nitrogen source in plant metabolism [35]. Friable, type II embryogenic callus was observed in switchgrass cultured on N6-based medium supplemented with 100 mg l^{-1} L-proline [36]. Proline at this low level had little effect in our culture and we found 2 g l^{-1} is optimal. The discrepancy may reflect the effects of interactions between medium salts (MS vs. N6) and L-proline on callus growth as proposed previously [37,38].

Importantly, vacuum application during infection, desiccation at co-cultivation stage, and resting after infection also facilitated switchgrass transformation and selection, especially for cv. Alamo. There has been some reports on using vaccum [23], desiccation [20] and resting [19,24] in transformation of other plant species. Vacuum infiltration is often used to enhance pathogen infection in plant pathology studies, and was successfully applied for *Agrobacterium*-mediated transformation of adult *Arabidopsis* plants [39]. Desiccation at co-cultivation was first demonstrated effective in wheat transformation [20] and later applied to transformation of tall fescue [23], Darnel ryegrass [40], and *Brachypodium* [41]. In switchgrass, we found that the transformation efficiency of Alamo could increase from 22% to 48% by desiccation (Table 4). The mechanism might be that desiccation significantly suppressed *A. tumefaciens* growth and subsequently facilitated better recovery of the infected plant cells. Alternatively, the effect may be related to osmotic stress or elevated ABA level induced by the desiccation treatment [20].

Hyg B selection in switchgrass transformation was recently reported [14], in which up to 75 mg l^{-1} hyg B was used for selection of transformed callus, and no transgenic plants were recovered from 100 mg l^{-1} selection. However, in our practice, even 100 mg l^{-1} was not sufficient to inhibit growth of non-transformed cells, and selection at this level was inefficient. We adopted a two-step selection scheme (100 and 200 mg l^{-1}), supplemented L-proline in the selection medium, and added a short period of resting treatment before the selection, and believed that the fine-tuned selection scheme maximized the growth of the transformed cells and minimized the negative effects of the dying, non-transformed cells surrounding the transformed cells.

Overall, we established a high throughput *Agrobacterium*-mediated transformation system for switchgrass by selecting callus type, altering medium composition, and optimizing conditions during infection, co-cultivation and selection. The approaches may have applications to various switchgrass cultivars and other plant species.

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