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Agrobacterium-Mediated Transformation of Switchgrass and Inheritance of the Transgenes

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Abstract Switchgrass (*Panicum virgatum* L.) has been developed into an important biofuel crop. Embryogenic calli induced from caryopses or inflorescences of the lowland switchgrass cultivar Alamo were used for *Agrobacterium*-mediated transformation. A chimeric hygromycin phosphotransferase gene (*hph*) was used as the selectable marker and hygromycin as the selection agent. Embryogenic calli were infected with *Agrobacterium tumefaciens* strain EHA105. Calli resistant to hygromycin were obtained after 5 to 8 weeks of selection. Soil-grown transgenic switchgrass plants were obtained 4 to 5 months after *Agrobacterium* infection. The transgenic nature of the regenerated plants was demonstrated by PCR, Southern blot hybridization analysis, and GUS staining. T1 progeny were obtained after reciprocal crosses between transgenic and untransformed control plants. Molecular analyses of the T1 progeny revealed various patterns of segregation. Transgene silencing was observed in the progeny with multiple inserts. Interestingly, reversal of the expression of the silenced transgene was found in segregating progeny with a single insert.

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Keywords *Agrobacterium* - Biofuel crop - *Panicum virgatum* - Switchgrass - Transgene inheritance - Transgenic plant

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid - *BAP* 6-Benzylaminopurine - *PCR* Polymerase chain reaction

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Introduction

Cellulosic biomass is an attractive energy feedstock because it is an abundant, domestic, renewable source that can be converted to liquid transportation fuels [1]. Realization of the potential of cellulosic biofuels can be expedited by genetic improvement of biofuel crops [2]. Transgenic approaches are considered imperative for the development of biofuel crops [3]. Biofuel crops will only be cost-effective in the long run if they are further domesticated transgenically to be more productive and have the right properties as fuels, as well as have residues that have value [3].

As a productive warm-season C4 species, switchgrass has been developed into a model herbaceous biofuel crop [2, 4]. Switchgrass is an outcrossing, polyploid monocot species with a high degree of self-incompatibility. Such biological properties make conventional selection and breeding schemes complex, especially for quantitative traits with low heritability. Transgenic approaches offer the opportunity to generate unique genetic variations; however, compared with major crops, efforts in developing transformation systems in switchgrass have been very limited [5, 6]. To date, there have been only three reports on stable transformation of switchgrass, one based on particle bombardment [7] and the other two based on *Agrobacterium*-mediated transformation of calli derived from mature caryopses [8, 9]. In these reports, the phosphinothricin acetyltransferase (*bar*) gene was used as the selectable marker and bialaphos as the selection agent [7–9]. Considering the importance of switchgrass as a dedicated biofuel crop, more research efforts are urgently needed to optimize the transformation system for this species.

Because of the rapid development of sequencing technology and gene isolation methods, it has become a relatively easy task to isolate large numbers of genes in a short time [10]. However, functional characterization of the genes has become a bottleneck in many recalcitrant species including switchgrass. Regeneration and transformation of recalcitrant species generally require considerable training of the practitioner to develop the skills needed to generate sufficient numbers of transgenic plants [11]. Interlaboratory transfer of grass transformation technology has been very challenging, particularly for species with limited information. The establishment of protocols using alternative selectable markers and different explant sources is an important step toward advancing switchgrass transformation. The objective of the present study was to develop an *Agrobacterium*-mediated switchgrass transformation system using hygromycin for selection and using different explant sources. Detailed characterization of the transgenic progeny revealed interesting findings about gene silencing and reversal of gene expression in the segregants.

Methods

Plant Material, Explant Sterilization, and Callus Induction

A commercial lowland switchgrass cultivar, Alamo, was used for the study. Mature caryopses of Alamo were surface-sterilized and used for callus induction. Surface sterilization of mature caryopses was carried out on two consecutive days. After an initial surface sterilization in 3% calcium hypochlorite (containing 0.1% Tween 80) for 2.5 h, caryopses were rinsed three times with sterile water and left overnight at 4°C. The caryopses were sterilized again as described above for 1 h on the second day and rinsed three times with sterile water. The caryopses were placed on M5 medium consisting of MS basal medium [12] (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) supplemented with 22.6 μM 2,4-D, 3% (*w*/*v*) sucrose and solidified with 0.75% (*w*/*v*) agar (Agar-Agar Sigma-Aldrich, Inc., St. Louis, MO, USA). The cultures were kept in the dark at 24°C. Embryogenic calli were obtained after 8–12 weeks of culture.

Inflorescence and node from the switchgrass genotypes ST1 and ST2 were also used for callus induction. ST1 and ST2 were obtained by selection (based on tissue culture responsiveness) from a large number of plants regenerated from callus cultures of the switchgrass cultivar Alamo. ST1 and ST2 have been maintained by vegetative propagation. For callus induction from inflorescences, top internodes were collected from E4–E5 stage [13] plants in the greenhouse, surface-sterilized with 70% ethanol for 10 min, and precultured on MSB medium (MS medium supplemented with 13.3 μM BAP, 3% sucrose and solidified with 0.75% agar) for 10–15 days under light conditions. The precultured inflorescences were cut into small pieces, transferred onto SM5 medium (M5 medium supplement with 0.67 μM BAP), and cultured for 20–

30 days under dark conditions before subculturing onto fresh medium for another 30 days. For callus induction from nodes, top three nodes at E3 or E4 stage were collected and cut into 3-cm pieces with 1.5 cm above and 1.5 cm below the node. The nodal segments were surface-sterilized with 70% ethanol for 2 min, followed by 20% bleach for 15 min, and then rinsed three times with sterile water. About 0.5 cm above and below the node was again cut, split longitudinally, and placed on M5 or SM5 medium in such a way that the cut surface is touching the medium. After 3 to 4 weeks of culture under dark conditions, the developed calli were subcultured onto the same medium for another 4–5 weeks.

Agrobacterium Preparation and Genetic Transformation

Binary vectors pCAMBIA 1301 and pCAMBIA 1305.2 were introduced into *Agrobacterium tumefaciens* strain EHA105 [14] and used for genetic transformation. Single colonies of *A. tumefaciens* were transferred to liquid Luria–Bertani medium containing 50 mg/L kanamycin and 25 mg/L rifampicin. The cultures were grown at 28°C with shaking (250 rpm) until OD_{600} reached 0.8–1.0. Freshly prepared 100 μM acetosyringone (3′,5′-dimethoxy-4′-hydroxyactophenone, ACROS Organics, Morris Plains, NJ, USA) was added to the cultures, and the shaking was continued for 1 h. Cells were then pelleted by centrifugation at 2,400×*g* for 15 min and resuspended in liquid M1 medium (MS basal medium supplemented with 9.0 μM 2,4-D, 3% (*w*/*v*) sucrose) containing 100 μM acetosyringone. The density (OD_{600}) of the resuspended *Agrobacterium* was adjusted to approximately 0.5. To test the effectiveness of different *Agrobacterium* strains, the binary vector pCAMBIA 1301 was introduced into EHA105, AGL1, and LBA4404. To test the possible effects of the timing of acetosyringone addition for *vir* gene induction, acetosyringone was added to *Agrobacterium* cultures 1 or 12 h before centrifugation.

Embryogenic calli of switchgrass were immersed in *Agrobacterium* suspensions in 6.6-cm culture vessels (Greiner Bio-One, Longwood, FL, USA). The culture vessels were placed in a vacuum chamber and vacuum was drawn for 10 min. After releasing vacuum, the callus pieces and *Agrobacterium* were incubated for 30 min with gentle shaking. Alternatively, the calli were collected into a tea ball and then placed in *Agrobacterium* solution with agitation [15]. Excess bacteria were removed after the incubation, the infected callus pieces were transferred onto filter papers wetted with M1 and placed in empty Petri dishes in the dark at 25°C for co-cultivation. The amount of callus pieces on each filter paper was equivalent to approximately 20 original intact calli.

Selection and Regeneration of Transgenics

Two days after co-cultivation, filter papers supporting the infected callus pieces were transferred onto M1 selection medium—MS basal medium [12] supplemented with 9.0 μM 2,4-D, 3% (*w*/*v*) sucrose, 75 mg/L hygromycin (PhytoTechnology Laboratories), 250 mg/L cefotaxime, and solidified with 0.75% (*w*/*v*) agar. One week later, the callus pieces were transferred to fresh M1 selection medium, and the selection process was continued. Hygromycin-resistant calli obtained after 5 to 8 weeks of selection were transferred onto regeneration medium MSK—MS basal medium supplemented with 0.9 μM kinetin, 3% (*w*/*v*) sucrose, and solidified with 0.75% (*w*/*v*) agar.

The regenerated shoots or plantlets were transferred to plastic vessels containing hormone-free half-strength MS medium. All the regenerating cultures were kept at 25°C under fluorescent light (140 μE m^{-2} s^{-1}) at a photoperiod of 16 h in the growth chamber. After 4 to 5 weeks, plants with well-developed roots were transferred to soil and grown in the greenhouse (16-h light, 390 μE m^{-2} s^{-1}).

Molecular Analyses of Transgenic Plants

For polymerase chain reaction (PCR) analysis, the DNeasy® plant mini kit (Qiagen, Valencia, CA, USA) was used for purification of DNA from greenhouse-grown plants. PCR were carried out by amplifying the coding regions of transgenes using the following sets of oligonucleotide primers: *hph* 5′-CGCATAACAGCGGTCATTGACTGGAGC-3′ (forward) and 5′-GCTGGGGCGTCGGTTTCCACTATCCG-3′ (reverse); *gusA* AACAGTTCCTGATTAACCACAAACC-3′ (forward) and 5′-GCCAGAAGTTCTTTTTCCAGTACC-3′ (reverse); *GUSPlus* 5′-CACGGTGCCGGCCTATCTGA-3′ (forward) and 5′-GCTTGCGACCACTTTGCCTTCCT-3′ (reverse). The transgenes *hph* and *gus*A were amplified together in the same reaction. The expected PCR products were 375 bp for *hph*, 634 bp for *gusA*, and 472 bp for *GUSPlus*. The total volume of reaction mixtures was 50 μL, including 400 ng genomic DNA, 0.5 μL of each primer (50 μM), 1 μL of dNTP mix (10 mM each), 10 μL green GoTaq® reaction buffer (5×), and 0.25 μL GoTaq® DNA polymerase (Promega, Madison, WI, USA). For positive controls, 5 ng plasmid DNA was added to the reaction mix. Cycling parameters began with an initial hot start at 95°C for 2 min, then 30 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s), followed by a final extension of 5 min at 72°C. PCR amplification products were analyzed by electrophoresis in 1% agarose/ethidium bromide gels.

Total RNA was extracted from leaves of the transgenic and the control plants with MRC Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For reverse transcription PCR (RT-PCR) analysis of transgene expression, 2 μg of total RNA was transcribed into cDNA using Omniscript® RT Kit and oligo (dT) primer (Qiagen). Two microliters of cDNA was used in each PCR amplification (25 μL). Primers used for amplifying the *elf1* gene were 5′-TCAGGATGTGTACAAGATTGGTG-3′ (forward) and 5′-GCCTGTCAATCTTGGTAATAAGC-3′ (reverse). PCR conditions were 95°C for 2 min, 23 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

For Southern blot hybridization analysis, genomic DNA was isolated from freeze-dried leaf material of greenhouse-grown plants following the modified 2×CTAB procedure [16]. DNA was digested with the restriction enzyme *Hin*dIII which only cleaves once in the multiple cloning site located between *hph* gene and *gusA* gene in the binary vectors. Twenty micrograms DNA from each sample was digested overnight and loaded in each lane. The hybridization probe (*hph*) was labeled with digoxigenin (DIG) by PCR (Roche Applied Science, IN, USA). Gel electrophoresis, DNA blotting, and hybridization were carried out following the manufacturer's instruction manual (DIG High Prime DNA labeling and detection starter kit II). Hybridization signals were detected using the chemiluminescent substrate CSPD-*Star* (Roche Applied Science).

For histochemical assay of GUS activity, the leaves of greenhouse-grown plants were submerged in a substrate solution containing 100 mM sodium phosphate, pH 7.0, 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (*w*/*v*) X-Gluc, and 0.1% (*v*/*v*) Triton X-100 and incubated at 37°C overnight [17, 18]. GUS expression was visualized after the tissues were destained by soaking and washing several times in 70% ethanol. Hygromycin dip test was used to detect *hph* expression. Tip of a leaf blade was cut off, and the blade was dipped into a 150 mg/L hygromycin solution with 1% Tween 80 for 10 s. The leaf blade was examined 5 days after the dipping treatment.

Results

Regeneration of Transgenic Switchgrass Plants

Embryogenic calli were induced from different explants including mature caryopses, inflorescences, and nodes. The M5 medium was used for callus induction from caryopses. The medium was modified by either adding BAP or replacing sucrose with maltose; however, no significant improvement in callus induction was observed. The SM5 medium was used for callus induction from inflorescences. Adding $CuSO_4$, casamino acid and proline to the SM5 medium did not lead to consistent improvement in the production of embryogenic callus from inflorescences.

Embryogenic calli were infected with *Agrobacterium* strains carrying the binary vectors pCAMBIA1301 and pCAMBIA1305.2 (Fig. 1a). The pCAMBIA1301 vector contains a chimeric hygromycin phosphotransferase gene (*hph*) and a β-glucuronidase gene (*gusA* from *Escherichia coli*), both under the control of the CaMV35S

promoter. The pCAMBIA 1305.2 vector contains an *hph* gene and a *GUSPlus* gene (*GUSPlus* from *Staphylococcus* sp.) under the control of the CaMV35S promoter (www.cambia.org). The *gusA* and *GUSPlus* reporter genes contain a catalase intron inside the coding sequence to ensure eukaryote-specific expression.

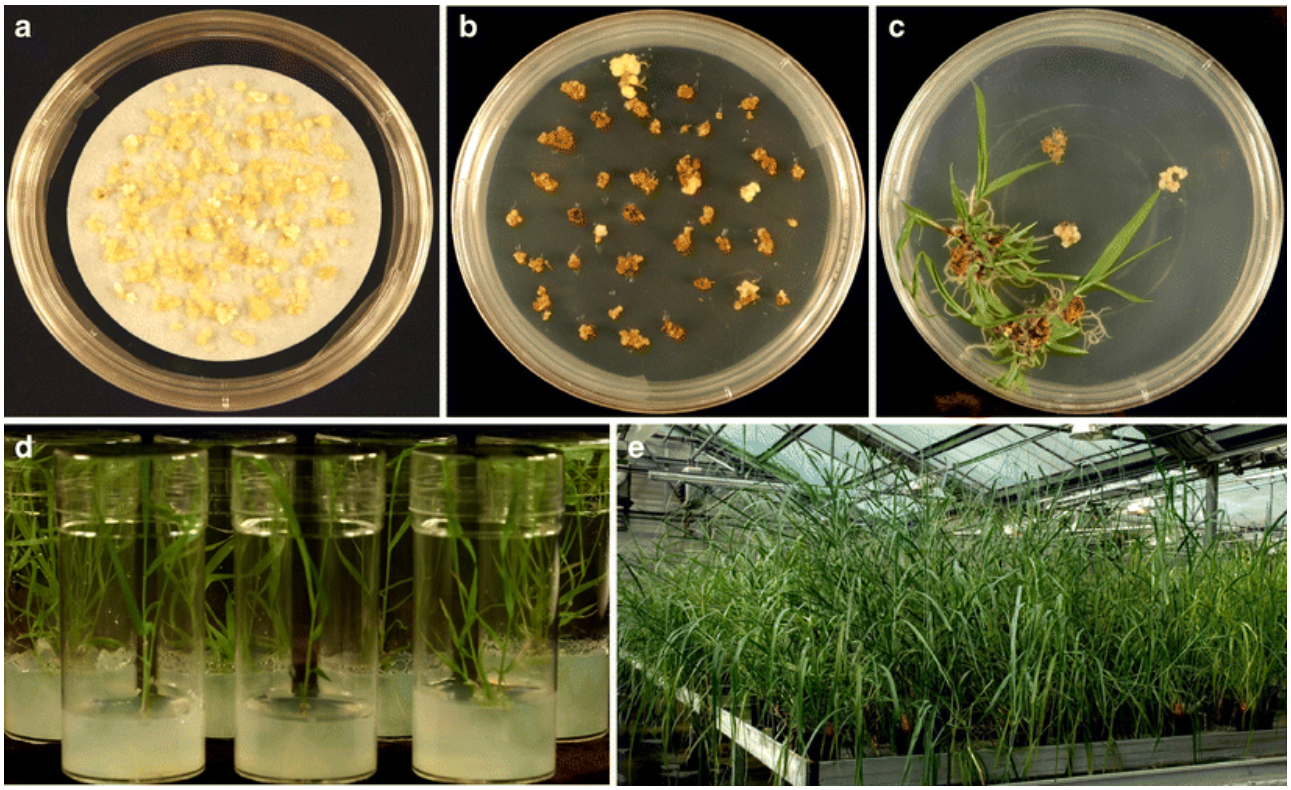

Fig. 1 Transgenic switchgrass (*Panicum virgatum* L.) plants obtained after *A. tumefaciens*-mediated transformation. **a** Embryogenic calli co-cultivated with *A. tumefaciens* on a filter paper disk. **b** Hygromycin-resistant calli obtained 6 weeks after *Agrobacterium*-mediated transformation and selection. **c** Regeneration of shoots/plantlets from hygromycin-resistant calli. **d** Well-rooted transgenic plants obtained 4 weeks after transferring the regenerated shoots to rooting medium. **e** Greenhouse-grown transgenic plants obtained 5 months after *Agrobacterium*-mediated transformation

Preliminary experiments were carried out using transient expression analysis to test the effects of different treatments/parameters (e.g., *Agrobacterium* strains, vacuum or tea ball method during infection, timing of acetosyringone addition to induce *vir* genes). Unlike biolistic transformation in which individual blue spots could be clearly identified after particle bombardment [19], transient GUS expression by *Agrobacterium*-mediated transformation could not be precisely quantified [20]. Nevertheless, differences in GUS expression were observed between different treatments, and the protocol was partially optimized. The *Agrobacterium* strains EHA105 and AGL1 had higher efficiency than LBA4404. During the *Agrobacterium* infection process, placing calli in a tea ball with agitation [15] rather than vacuum treatment [21] led to slightly increased transient expression. No difference in transient expression was found by adding acetosyringone at different time points prior to callus infection. Placement of infected calli directly on medium led to more transient activity than when calli were placed on filter paper; however, more contamination or over growth of *Agrobacterium* was observed accordingly.

For stable transformation, embryogenic calli were infected with EHA105 and then placed on media containing the selection agent hygromycin. Hygromycin-resistant calli were obtained after 5 to 8 weeks of selection (Fig. 1b). The resistant calli were transferred to regeneration medium, and green shoots were regenerated 4 to 6 weeks later (Fig. 1c). The shoots were transferred to half-strength MS medium, and well-rooted plants were obtained after 4 to 5 weeks (Fig. 1d). The plants with well-developed roots were transferred to soil and established in the greenhouse (Fig. 1e). The time from callus infection until the establishment of soil-grown plants took 4 to 5 months.

Different hygromycin (PhytoTechnology Laboratories) concentrations (25, 50, 60, 75, and 100 mg/L) were tested for stable transformation of switchgrass. Several hundred plants were regenerated from resistant calli selected at 25, 50, 60, and 75 mg/L hygromycin. No plants were recovered when the hygromycin level was more than 100 mg/l. The generated putative transgenics were subjected to molecular analysis.

Molecular Characterization of Transgenic Switchgrass Plants

The greenhouse-grown plants were subjected to PCR screening using *hph*, *gusA*, and *GUSPlus* primers. Strong and clear bands of expected sizes (375 bp for *hph*, 634 bp for *gusA*, and 472 bp for *GUSPlus*) were obtained for true transgenic plants after PCR amplification (Fig. 2a). In some cases, faint bands were obtained after PCR amplification. This issue appears to be specific to switchgrass, as we never encountered such a problem in analyzing transgenics in other grasses [19–23]. Much effort was devoted to resolve this issue, and it was found that plants with faint bands were false positives. True transgenics have always showed clear and strong PCR bands.

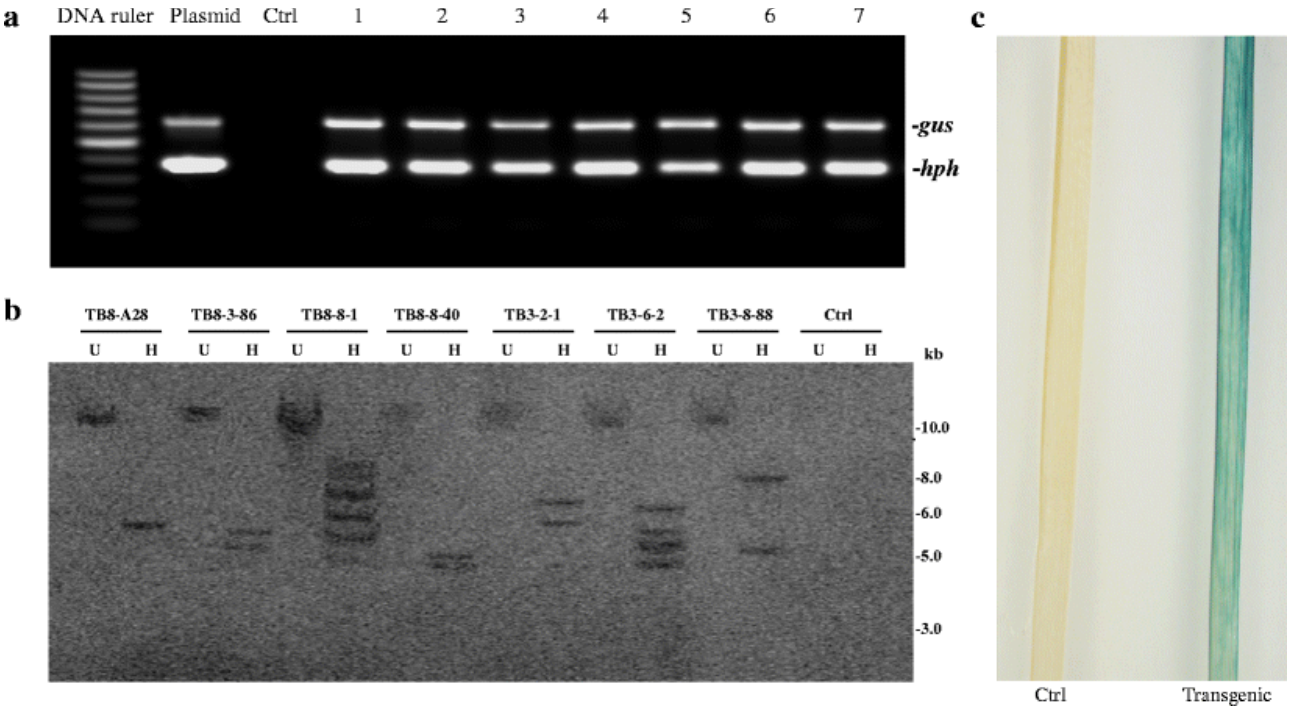

Fig. 2 Molecular characterization of transgenic switchgrass plants. **a** Polymerase chain reaction (*PCR*) analysis of DNA samples from regenerated switchgrass plants after *Agrobacterium*-mediated transformation. *DNA ruler* 100-bp DNA molecular markers (Bio-Rad), *plasmid* pCAMBIA1301 plasmid serving as positive control, *Ctrl* non-transformed plants serving as negative control. **b** Southern blot hybridization of a DNA blot containing undigested and *Hin*dIII digested genomic DNA isolated from regenerated switchgrass plants and hybridized with *hph* probe. *U* undigested genomic DNA, *H* genomic DNA digested with *Hin*dIII, *Ctrl* non-transformed plant serving as control. **c** GUS staining of control (*left*) and transgenic (*right*) leaves

Molecular analysis revealed that plants regenerated from calli obtained under 25 mg/L hygromycin selection were all escapes. Most of the plants (up to 80%) derived from resistant calli under 50 and 60 mg/L hygromycin selection were also escapes. Hygromycin at 75 mg/L concentration provided stringent selection for true transgenic events. The molecular data presented below were from plants obtained under 75 mg/L hygromycin selection regimen.

Southern blot hybridization analysis was used to confirm the transgenic nature of the PCR-positive plants. Undigested genomic DNA and genomic DNA digested with a restriction enzyme (*Hin*dIII) that only cleaves once in the T-DNA region were loaded for each sample and hybridized with the *hph* probe. Hybridization signals corresponding to the high-molecular-weight bands and to the *Hin*dIII fragments of different molecular weights were observed (Fig. 2b). The results confirmed that the transgene was stably integrated into the plant genome and the regenerated switchgrass plants were independent transformants. Both single-copy and multi-copy integrations of the transgene occurred in the genome of the transgenics. The transgenic plant TB8-A28 had single-copy integration, plants TB8-3-86, TB8-8-40, TB3-2-1, TB3-8-88 had two copies, and plants TB8-8-1, TB3-6-2 had more than four copies of the transgene (Fig. 2b).

Transgene Inheritance in Switchgrass

Switchgrass is a self-incompatible wind-pollinated species. To study transgene inheritance and possible gene silencing in switchgrass, the transgenic plants were reciprocally crossed with untransformed control plants. Seeds were harvested from the crosses, and T1 plants were obtained after germination of the seeds. The T1 progeny were analyzed by PCR and Southern blot hybridization. Because of the multi-copy integrations of the transgene, an interesting array of segregations was observed (Table 1). The parental line TB8-8-40 had two copies of the transgene; its progeny showed a 1:1 segregation ratio and had the same banding pattern (Fig. 3a and Table 1). The results indicate that the two transgene copies in TB8-8-40 were integrated into one chromosomal location. The segregation ratio of the parental line TB3-8-88 was very close to 3:1, indicating that the two transgene copies were likely integrated at independent loci. Although the segregation ratio of TB3-2-1 is somewhat close to 3:1, Southern blot hybridization analysis showed that progeny contain either one or two copies of the transgene (Fig. 3b), indicating that the two transgene copies in the parental plant were inserted at different loci but still have some linkage. The TB8-8-1 line had multi-copy integrations, and the majority of the progeny-derived from TB8-8-1 were PCR-positive (Electronic Supplementary Material, Fig. 1). Southern blot hybridization data showed various transgene copies in the progeny (Fig. 3c). Although several T1 plants of TB8-8-1 showed two copies of the transgene, no common band was observed for these plants, indicating single-copy plant could be detected if a larger number of progeny were analyzed. A segregation ratio of 3:1 was observed for TB3-6-2, although the plant contained five transgene copies (Table 1 and Fig. 3d). Southern blot hybridization analysis revealed three banding patterns in the progeny: five copies (parental pattern), single insert, and four transgene copies (Fig. 3d). The four transgene copies segregated together and behaved like one locus. This is consistent with the observed 3:1 segregation ratio.

Table 1 Segregation analysis of T1 progeny derived from reciprocal crosses between transgenic and control switchgrass plants

Cross	Copy number based on Southern blot	T1 plants analyzed by PCR		P/N ratio (assumed)	χ^2
		No. of T1 plants	Positive (P)/Negative (N)		
TB8-8-40× Alamo	2	42	22:20	1.1:1 (1:1)	0.10 ($P > 0.05$)
Alamo×TB8-8-40		48	23:25	0.9:1 (1:1)	0.08 ($P > 0.05$)
TB3-8-88×Alamo	2	36	24:12	2.6:1 (3:1)	1.33 ($P > 0.05$)
Alamo×TB3-8-88		40	26:14	2.3:1 (3:1)	2.13 ($P > 0.05$)
TB3-2-1×Alamo	2	52	34:18	1.9:1 (3:1)	2.56 ($P > 0.05$)

Alamo×TB3-2-1		64	40:24	1.7:1 (3:1)	5.33 ($P > 0.01$)
TB8-8-1×Alamo	5 or more	28	26:2	13:1	–
Alamo×TB8-8-1		16	16:0	16:0	–
TB3-6-2×Alamo	5	24	18:6	3.0:1 (3:1)	0.26 ($P > 0.05$)
Alamo×TB3-6-2		46	32:14	2.3:1 (3:1)	0.72 ($P > 0.05$)

χ^2 (0.05) = 3.84, χ^2 (0.01) = 6.64
P/N ratio ratio of positive and negative T1 plants

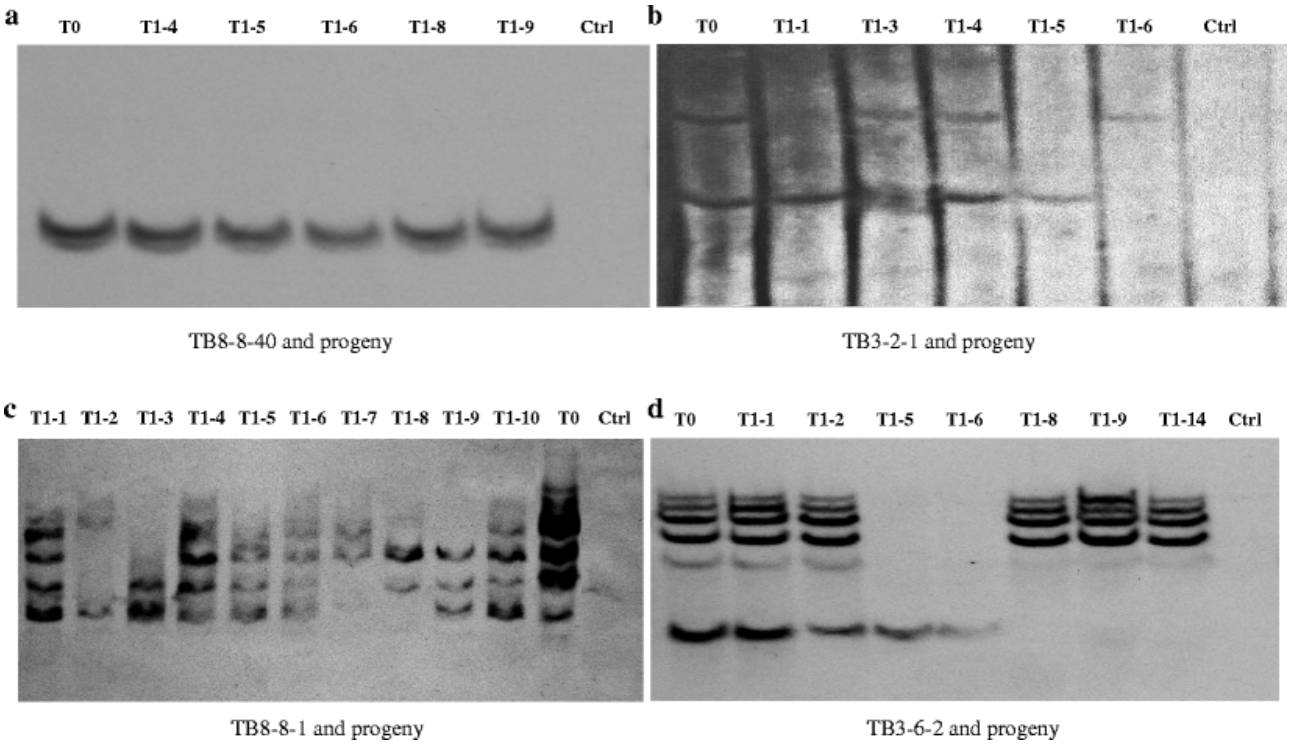

Fig. 3 Southern blot hybridization of a DNA blot containing *Hin*dIII digested genomic DNA isolated from different transgenic plants and their T1 progeny. **a** TB8-8-40 and its progeny (plant numbers 4, 5, 6, 8, and 9). **b** TB3-2-1 and its progeny (plant numbers 1, 3, 4, 5, and 6). **c** TB8-8-1 and its progeny (plant numbers 1–10). **d** TB3-6-2 and its progeny (plant numbers 1, 2, 5, 6, 8, 9, and 14). *Ctrl* segregated negative T1 plant serving as control, *hph probe* hybridization probe

RT-PCR analysis revealed transgene expression in the progeny. An example is illustrated in plant TB-8-840; the parental plant and the progeny showed transcript accumulation and resistance to hygromycin (Electronic Supplementary Material, Fig. 2). An interesting phenomenon was observed for plant TB3-6-2. The multi-copy parental plant and its multi-copy progeny had almost no transcript accumulation and lost resistance to hygromycin (Fig. 4). However, the single-copy segregants showed transcript accumulation and were resistant to hygromycin (Fig. 4). Therefore, expression of the silenced transgene could be reversed in the progeny with a single-copy insert.

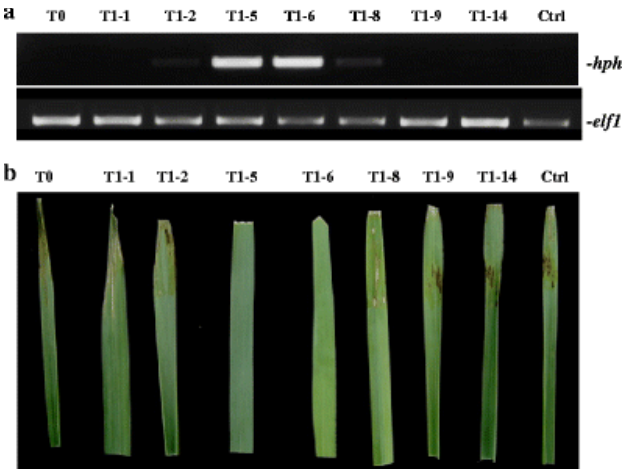

Fig. 4 Expression of the *hph* transgene in the transgenic plant TB3-6-2 and its progeny. **a** RT-PCR analysis of *hph* transcriptional level in the TB3-6-2 plant and its T1 progeny. **b** Hygromycin resistance of leaves from TB3-6-2 and its progeny

Discussion

The establishment of an efficient selection scheme by applying suitable selection pressure for an appropriate length of time is one of the critical aspects of successful grass transformation. Because many grasses have high endogenous tolerance to antibiotics, particularly kanamycin, most of the reports in grass transformation used *hph* or *bar* as selectable marker gene [5]. The stable integration and expression of a chimeric *hph* or *bar* gene results in the ability to phosphorylate hygromycin or the acetylation of phosphinothricin (PPT), thus rendering transformed cells resistant to the antibiotics hygromycin or the herbicide PPT, respectively. In switchgrass, transgenic plants have only been obtained by using the *bar* gene and the herbicide bialaphos for selection [7–9]. In this study, we developed a hygromycin selection scheme and successfully obtained transgenic plants. The development of an alternative selection system is beneficial for transgene stacking and for the ease of use of gene constructs with an antibiotic marker. Herbicide tolerance has been a sensitive issue for native forage and biofuel grasses because these species are highly adaptable and may be considered "weeds" if they grow in fields of major food crops with the same herbicide trait. To avoid potential problems in the future, it is important to consider other production systems when choosing traits and markers for engineering biofuel grasses.

Because of the endogenous tolerance to antibiotics, the selection window for transformed grass cells is somewhat narrow. Low selection pressure tends to result in the regeneration of escapes, while a too high selection level may kill the resistant cells. The use of strong promoters, such as the maize ubiquitin promoter or the rice actin promoter, may help solve this problem. Strong promoters have the potential to increase the antibiotic resistance level of transformed cells and thus allow for more efficient selection.

The choice of explants is also important for switchgrass transformation. Seeds or caryopses are the most readily available source of explants. However, in outcrossing species, individual seeds within a cultivar could represent different genotypes [24]. The use of inflorescence or node explants from defined plants allows for minimizing genotypic effects in transformants. Because it is difficult to identify suitable inflorescences based on plant phenotypic observation, young inflorescences were first cultured in vitro to let them grow into larger sizes and then used for callus induction. To date, caryopsis and inflorescence have been successfully used as sources of explants for producing embryogenic calli and generating transgenic plants. Nodes were used for in vitro propagation of switchgrass [25]. Callus induction from nodes was tested in this study; although embryogenic and highly regenerable calli could be obtained, most of the node-derived calli were not embryogenic. Since nodes are available at different developmental stages and a single tiller contains several nodes, it may be worthwhile to explore the use of node as explant by optimizing tissue culture conditions.

Transmission of foreign genes to progeny is critical for any potential use of transgenic material to develop novel germplasm. Transgenic progeny were readily obtained after crossing transgenics with non-transgenic plants. Molecular characterization and segregation analysis revealed that the multi-copy inserts usually reside at different loci and the segregates had various copy numbers. This is different from biolistic transformation in which multi-copy transgenes tend to be inserted in one chromosomal location [19]. Furthermore, the results showed that *Agrobacterium* transformation in switchgrass could lead to multiple and independent insertions of the transgene into the same transformed cell. It appears that certain types of grass cells were easily infected multiple times by *Agrobacterium*, while infection may not take place in most of the other cells. Further research on identification and characterization of such easily infectable cell types is worthwhile and may lead to breakthroughs in monocot transformation.

The integration of foreign DNA into the genome occurs at random and, frequently, in repeat arrangements [26]. Epigenetic gene silencing is frequently observed in transgenic plants [27]. Studies in *Arabidopsis* showed that arrangements of neither the tandemly repeated transgenes nor the inverted T-DNA structures were sufficient to trigger gene silencing. The silencing effect was attributed to multiple insertions or high transgene doses [26, 28, 29]. It is not surprising to find gene silencing in transgenic progeny of switchgrass. What was unexpected is the reversal of transgene expression in segregating progeny with a single insert. To our knowledge, this is the first report on such natural reversal (without chemical treatment) of gene silencing in monocot species. The results also indicate that analysis of progeny is important for applied projects involving switchgrass transformation.

Transgenic technology can be used in many ways to redesign switchgrass for more efficient biofuel production. For example, efforts have been made to reduce the lignin content in biofuel plants. Lignin negatively affects enzymatic degradation of polysaccharides; reduction of lignin by transgenic approaches is likely one of the most effective ways of reducing costs associated with pretreatment and hydrolysis of lignocellulosic feedstocks. Genetic engineering can also be employed to increase biomass production, manipulate cellulose and hemicellulose biosynthesis, and overexpression of enzymes for cellulose degradation in plants [30, 31].

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Electronic supplementary material

Below is the link to the electronic supplementary material.

ESM Fig. 1. **a** PCR analysis of T1 progeny derived from the transgenic plant TB8-8-1. **b** Southern blot hybridization analysis of DNA samples from the transgenic plant TB8-8-1 and its T1 progeny. Hybridization probe: *hph* probe. Fig. 2. Integration and expression of the *hph* transgene in the transgenic plant TB8-8-40 and its progeny. **a** Southern hybridization of a DNA blot containing *Hin*dIII digested genomic DNA isolated from the TB8-8-40 transgenic plant and its T1 progeny. **b** RT-PCR analysis of *hph* transcriptional level in the TB8-8-40 plant and its T1 progeny. **c** hygromycin resistance of leaves from TB8-8-40 and its progeny (PPTX 3122 kb)

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