ORIGINAL PAPER

A simple and reliable multi-gene transformation method for switchgrass

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Abstract

Key Message **A simple and reliable *Agrobacterium*-mediated transformation method was developed for switchgrass. Using this method, many transgenic plants carrying multiple genes-of-interest could be produced without untransformed escape.**

Abstract Switchgrass (*Panicum virgatum* L.) is a promising biomass crop for bioenergy. To obtain transgenic switchgrass plants carrying a multi-gene trait in a simple manner, an *Agrobacterium*-mediated transformation method was established by constructing a Gateway-based binary vector, optimizing transformation conditions and developing a novel selection method. A MultiRound Gateway-compatible destination binary vector carrying the *bar* selectable marker gene, pHKGB110, was constructed to introduce multiple genes of interest in a single transformation. Two reporter gene expression cassettes, *GUSPlus* and *gfp*, were constructed independently on two entry vectors and then introduced into a single T-DNA region of pHKGB110 via sequential LR reactions. *Agrobacterium tumefaciens* EHA101 carrying the resultant binary vector pHKGB112 and caryopsis-derived compact embryogenic calli were used for transformation experiments. Prolonged cocultivation for 7 days followed by cultivation on media containing meropenem improved transformation efficiency without overgrowth of *Agrobacterium*, which was, however, not inhibited by cefotaxime or Timentin. In addition, untransformed escape shoots were completely eliminated during the rooting stage by direct dipping the putatively transformed shoots into the herbicide Basta solution for a few seconds, designated as the 'herbicide dipping method'. It was also demonstrated that more than 90 % of the *bar*-positive transformants carried both reporters delivered from pHKGB112. This simple and reliable transformation method, which incorporates a new selection technique and the use of a MultiRound Gateway-based binary vector, would be suitable for producing a large number of transgenic lines carrying multiple genes.

Abbreviations

MS	Murashige and Skoog
OE-PCR	Overlap extension PCR
PCR	Polymerase chain reaction
RT-PCR	Reverse-transcription PCR
SD	Standard deviation

Communicated by H. Ebinuma.

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Introduction

Switchgrass (*Panicum virgatum* L.) is a warm season C_4 perennial grass belonging to the subfamily Panicoideae within the family Poaceae (Gramineae). This species is one of the greatest potential bioenergy crops, along with miscanthus (*Miscanthus* spp.), reed canarygrass (*Phalaris arundiacea* L.) and the giant reed (*Arundo donax* L.) (Lewandowski et al. 2003; McLaughlin and Kszos 2005), and has the nature to combine high net energy efficiency with economic feasibility (Schmer et al. 2008). Because switchgrass is a highly self-incompatible out-crossing species, the use of conventional crossing-based breeding is limited. Therefore, molecular breeding techniques are desirable for this species (Bouton 2007, 2008).

Genetic transformation of switchgrass was first reported using a microprojectile bombardment method (Richards et al. 2001), and subsequently using an *Agrobacterium*-mediated method (Somleva et al. 2002). Recently, various modified *Agrobacterium*-mediated methods have also been reported (Burris et al. 2009; Li and Qu 2011; Ramamoorthy and Kumar 2012; Song et al. 2012; Xi et al. 2009). Although transgenic switchgrass plants overexpressing/suppressing a unique gene were produced by these transformation methods (Fu et al. 2011; Saathoff et al. 2011; Xu et al. 2011), these methods still had limitations due to transformation efficiency, availability of explants, albino formation or complexity of procedures.

In addition, because of recent accumulation of genetic and genomic information in this crop (Casler et al. 2011), introducing multi-gene traits, such as those in a metabolic pathway, into transgenic plants would be of more interest than introduction of single gene traits. Although several strategies (sexual crossing, re-transformation and cotransformation) have been developed to introduce multiple genes into plant genomes (Dafny-Yelin and Tzfira 2007; Halpin 2005), these methods have several drawbacks, such as complex transgene integration patterns, low coexpression and segregative separation of transgenes in the progeny. Because of the limited efficiency of biolistic transformation, delivery of multiple genes on a single T-DNA by a single *Agrobacterium*-mediated transformation is preferable. Several strategies have been employed for assembly of multiple gene expression cassettes on a binary vector, such as traditional type II restriction enzyme-mediated cloning, the *Cre/loxP*-mediated recombination system, rare-cutting enzymes and Gateway-based systems (Dafny-Yelin and Tzfira 2007). The MultiRound Gateway system is based on multiple rounds of two-component Gateway LR recombination (Chen et al. 2006). With this system, multiple transgenes can be delivered sequentially and indefinitely into a Gateway-compatible
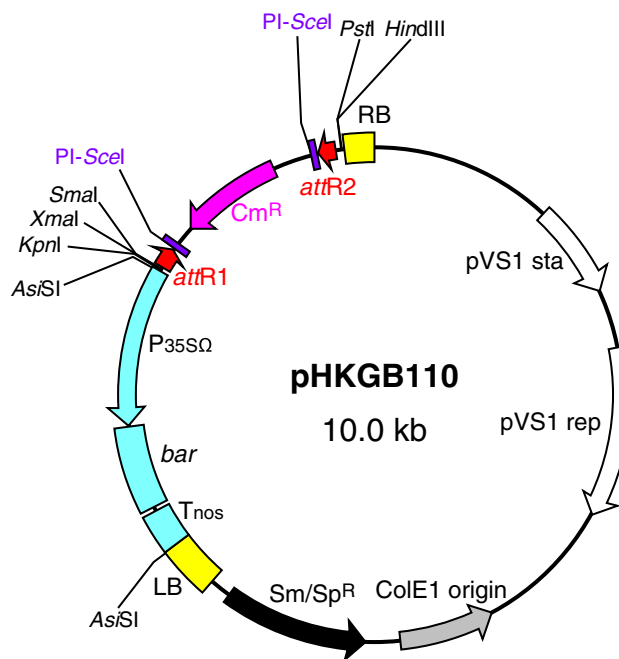

Fig. 1 Physical map of the MultiRound Gateway-compatible destination binary vector, pHKGB110

destination binary vector through alternate use of two series of entry vectors, pL12R34 and pL34R12.

Since caryopsis is the most readily available explant in switchgrass, and Type I callus is the most typical and prevalent type of callus induced from caryopsis (Denchev and Conger 1994; Vasil and Vasil 1994), we tried to establish a simple and reliable *Agrobacterium*-mediated transformation method for caryopsis-derived Type I calli of Alamo switchgrass. A MultiRound Gateway-compatible destination binary vector, pHKGB110, and a derivative expression binary vector carrying reporter genes, pHKGB112, were constructed and used for transformation experiments. To eliminate the production of untransformed escapes, a novel selection method at the rooting step was also developed.

Materials and methods

Vector construction

Two fragments derived from pB2GW7 (Karimi et al. 2002), a 35SΩ promoter ($P_{35SΩ}$) (De Loose et al. 1995) and the selectable marker gene *bar* with a nopaline synthase terminator (*bar*-T_{nos}), were amplified and fused by the overlap extension polymerase chain reaction (OE-PCR) (Horton et al. 1989) to produce the expression cassette, $P_{35SΩ}$-*bar*-T_{nos}. A primer set amplifying the whole fragment was designed for the In-Fusion PCR Cloning System

(Clontech Laboratories, Mountain View, CA, USA) with an additional *Asi*SI recognition sequence. The *bar* expression cassette was cloned into the *Eco*RI site in the T-DNA region of the binary vector pPZP200 (Hajdukiewicz et al. 1994) using an In-Fusion Advantage PCR Cloning Kit (Clontech), and this binary vector was designated pHKB1. An *Asc*I-*Pac*I fragment of the entry vector pL34R12H-Cm, which contained *att*R1-Cm^r-*att*R2 (Chen et al. 2006), was blunted and ligated into the *Bam*HI site of pHKB1 using a Blunting-Convenience Kit (Nippon Gene, Tokyo, Japan), and the resulting destination binary vector was designated pHKGB110 (Fig. 1).

Two reporter genes, *GUSPlus* (Broothaerts et al. 2005) and *gfp* (*sGFP-TYG*) (Chiu et al. 1996), were introduced into pHKGB110 using the MultiRound Gateway system (Chen et al. 2006). A $P_{35S\Omega}$-*GUSPlus*-T_{nos} expression cassette was constructed by OE-PCR using pB2GW7 and pCAMBIA1105.1 (CAMBIA, Canberra, Australia) as templates, and was cloned into the *Hin*dIII/*Asc*I sites of the entry vector pL12R34H-Ap (Chen et al. 2006) by In-Fusion PCR Cloning. A maize *ubi1* gene promoter (P_{ubi1}) (Christensen et al. 1992) -*gfp*-T_{nos} expression cassette was similarly constructed using maize genomic DNA (BioChain Institute, Hayward, CA, USA), pBI221/SP-GFP-HDEL (Mitsuhashi et al. 2000) and pB2GW7, and was cloned into the *Pac*I site of the entry vector pL34R12H-Cm. The *GUSPlus* and *gfp* expression cassettes were sequentially introduced into pHKGB110 by two rounds of LR reactions (Chen et al. 2006), and the resulting binary vector was designated as pHKGB112 (Fig. 2). This binary vector was transformed into *Agrobacterium tumefaciens* EHA101 (Hood et al. 1986) using the freeze–thaw method (An et al. 1988) and was used for transformation experiments.

Callus induction and maintenance

Mature seeds of switchgrass (*Panicum virgatum* L.), cultivar Alamo, were purchased from a local supplier. Embryogenic callus was induced from mature caryopses according to the methods of Denchev and Conger (1994).

Mature caryopses were dehusked with 60 % (v/v) H_2SO_4 for 30 min, rinsed with distilled water and sterilized with 3.5 % (w/v) calcium hypochlorite for 30 min. After rinsing three times with sterilized distilled water, caryopses were placed on PVC medium [Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962), 30 g l^{-1} maltose, 22.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.4 μM 6-benzylaminopurine (BAP) and solidified with 8 g l^{-1} agar] and cultured at 28 °C in the dark for 4 weeks. Because switchgrass is a self-incompatible open-pollinated species, calli formed on individual caryopses were maintained as independent genotypic lines. The regeneration ability of Type I (i.e., compact and embryogenic) calli was confirmed by growing the material on PVS medium [MS salts and vitamins, 30 g l^{-1} maltose, 1.4 μM gibberellic acid (GA$_3$) and 8 g l^{-1} agar], and cell lines with a high regeneration ability were maintained by subculturing every 4 weeks. Seven independent lines of 6- to 18-month-old calli were used for the transformation experiments.

Agrobacterium-mediated transformation

Transformation experiments were performed according to Somleva et al. (2002) and Somleva (2007) with modifications. A glycerol stock of *A. tumefaciens* EHA101 (pHKGB112) was streaked onto a YEP agar plate supplemented with 50 mg l^{-1} kanamycin, 100 mg l^{-1} spectinomycin and 35 mg l^{-1} chloramphenicol and cultured at 28 °C. After 2–3 days, bacterial cells were inoculated into liquid YEP medium containing the proper selective antibiotics and cultured overnight with shaking. Bacterial cells were collected by centrifugation and resuspended in liquid PVC-CO medium (PVC medium supplemented with 100 μM acetosyringone) at a density of $A_{600} = 0.5$.

Type I calli were divided into 3–4 mm clumps and inoculated with the *Agrobacterium* suspension for 3 min with occasional agitation. After blotting on sterilized paper towels to remove excess *Agrobacterium* suspension, calli were transferred to PVC-CO agar plates. After 3–7 days of cocultivation at 28 °C in the dark, calli were transferred to PVC-S medium (PVC medium supplemented with
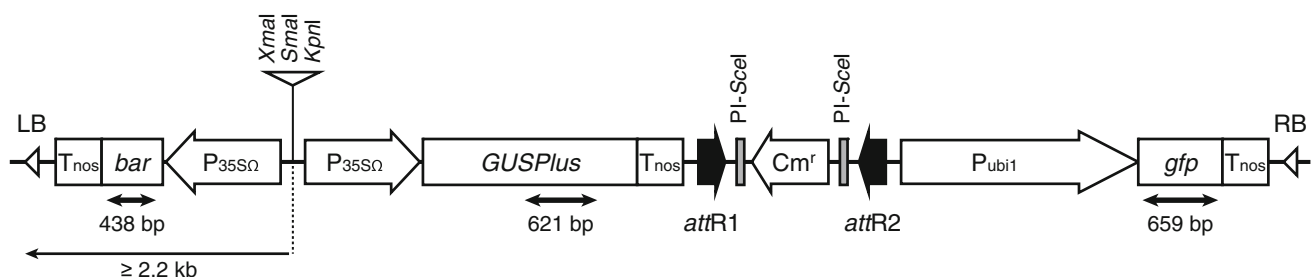

Fig. 2 Schematic representation of the T-DNA region of pHKGB112. *Double-headed arrows* below the map indicate fragments amplified by PCR. The *thin arrow* below the map indicates the fragment detected by southern blot analysis using a *bar*-specific probe

10 mg l^{-1} bialaphos and *Agrobacterium*-suppressing agents) without washing and cultured for 8 weeks at 28 °C in the dark, with bi-weekly subculturing. Bialaphos-resistant (Bar^R) and/or GFP-expressing (GFP$^+$) calli were then transferred to PVS-S medium (PVS medium supplemented with 10 mg l^{-1} bialaphos and *Agrobacterium*-suppressing agents) and cultured for 6 weeks, with bi-weekly subculturing, at 28 °C under a 16/8-h light/dark cycle. The *Agrobacterium*-suppressing agents used in this study were 150 mg l^{-1} Timentin (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), 250 mg l^{-1} cefotaxime sodium salt (Duchefa Biochemie, Haarlem, Netherlands) and 25 or 50 mg l^{-1} meropenem trihydrate (Wako Pure Chemical Industries, Osaka, Japan).

Herbicide selection of putatively transformed shoots

For rooting and selection, shoots regenerated on PVS-S medium were transferred onto PVR-S medium, comprising PVR medium (half-strength MS salts and vitamins, 15 g l^{-1} sucrose and 7 g l^{-1} agar) supplemented with the *Agrobacterium*-suppressing agents used in the PVS-S medium. Two herbicide selection methods were examined as follows: (1) the conventional method—shoots were placed onto PVR-S medium incorporated with 10 mg l^{-1} bialaphos; (2) the herbicide dipping method—shoots were dipped into a filter-sterilized 0.1 or 1 % (v/v) Basta (Bayer CropScience, Monheim am Rhein, Germany) solution for a few seconds, briefly blotted on sterilized paper towels, and then placed onto herbicide-free PVR-S medium. For both selection methods, shoots were cultured at 28 °C under a 16/8-h light/dark cycle, and herbicide resistance was evaluated after 2 weeks. Non-transformed control plantlets and putative transgenic plantlets were acclimatized and grown in a hydroponic solution (Mae and Ohira 1981) supplemented with 0.1 % (v/v) Plant Preservative Mixture (Plant Cell Technology, Washington DC, USA) to inhibit microbial growth. Thereafter plants were potted in soil and grown in the greenhouse.

Observation of GFP expression

GFP expression of inoculated calli at the macroscopic level was monitored and recorded using a ProDoc-LED470GFP macro imaging system (OptoCode, Tokyo, Japan). GFP expression of calli and shoots at the microscopic level was observed using a Leica MZ FLIII fluorescence stereomicroscope with filter sets of GFP Plus (excitation 480 ± 40 nm, emission ≥510 nm) and GFP Plant (excitation 470 ± 40 nm, emission 525 ± 50 nm) and recorded using a DFC310 FX digital color camera (Leica Microsystems, Wetzlar, Germany).

PCR analysis of putatively transformed plants

Small (ca. 3 mm^2) segments of leaf blades sampled from 2- to 4-week-old putatively transformed plantlets were subjected to PCR using a Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR was initially performed using a primer set that amplified the *virC* gene (Sawada et al. 1995) to detect persistent *Agrobacterium* according to Cubero et al. (1999). Then, *virC*-negative plant samples were analyzed for transgene integration by PCR. The primer sequences, their annealing temperatures and the expected fragment lengths for each transgene were as follows: *virC* (VCF: 5′-ATCATTTGTAGCGACT-3′ and VCR: 5′-AGCTCAAACCTGCTTC-3′, 55 °C, 730 bp), *bar* (5′-CTGAAGTCCAGCTGCCAGAA-3′ and 5′-CAT CGTCAACCACTACATCG-3′, 62 °C, 438 bp), *GUSPlus* (5′-CCACTGAACACGTATCTCTACCAG-3′ and 5′-GGT AGCCATCACAAACAGCA-3′, 62 °C, 621 bp) and *gfp* (5′-GTAAACGGCCACAAGTTCAG-3′ and 5′-AGATCT CCCTTGTACAGCTCG-3′, 62 °C, 659 bp). The amplification reaction was performed using a MultiGene Gradient thermal cycler (Labnet International, Edison, NJ, USA) using the following thermal cycling conditions: initial denaturation at 98 °C for 5 min followed by 35 cycles of denaturation at 98 °C for 5 s, annealing for 5 s at the temperatures described above, and extension at 72 °C for 20 s kb^{-1}, with a final extension at 72 °C for 1 min. Amplified products were electrophoresed in a 1.5 % agarose gel and visualized by ethidium bromide staining. Because faint false-positive bands were occasionally detected in nontransformed control samples as reported by Xi et al. (2009), only clear, strong bands were determined to be positive.

Reverse-transcription (RT) PCR

Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized from total RNA using a PrimeScript II 1st Strand cDNA Synthesis Kit with an oligo-dT primer (Takara). RT-PCR analyses were performed using Premix Ex Taq Hot Start Version (Takara) and four primer sets for the *elf1* gene as an internal control (Xi et al. 2009) and the three transgenes used for genomic PCR. All experiments were performed according to the manufacturer's instructions.

Histochemical GUS (β-glucuronidase) assay

GUS assay was performed by 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) staining according to Kosugi et al. (1990). Tissue samples excised from transformed plants were stained with X-gluc solution at 37 °C overnight, and were fixed and cleared with ethanol before observation.

Southern blot hybridization of transgenic plants

Genomic DNA was extracted from leaf blades of PCR-positive plants using a Phytopure DNA Extraction Kit (GE Healthcare, Buckinghamshire, UK) and digested with *Xma* I. 15 μg of digested DNAs were electrophoresed in 0.8 % agarose gel, transferred onto a nylon membrane and hybridized with a *bar* gene-specific probe prepared using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany) and the primer set described above. Hybridization and detection were performed with the DIG system according to the instruction manual (Roche Applied Science).

Experimental design and statistical analysis

All plant tissue culture media were buffered with 1 % (v/v) 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.9) to adjust the pH to ca. 5.7 before and after autoclaving. Plates containing 40 ml of agar medium and cultures were sealed with Micropore surgical tape (3 M, St. Paul, MN, USA). Fifty calli and five shoots were used for transformation and herbicide selection experiments, respectively. The experiments were independently repeated three times and the data were represented as the mean ± standard deviation (SD) of the replicates. Analysis of variance was performed using single-factor ANOVA and followed by the Tukey–Kramer method using Excel 2003 software (Microsoft, Redmond, WA, USA) with the add-in software, Statcel3 (Yanai 2011).

Results

Construction of destination binary vector and expression binary vector

MultiRound Gateway (Chen et al. 2006) is one of the most useful systems for constructing a binary vector carrying multiple expression cassettes. To utilize this system for switchgrass transformation, we first constructed a MultiRound Gateway-compatible binary vector, pHKGB110 (Fig. 1), by introducing an *att*R1-Cm^r^-*att*R2 fragment for the LR recombination reaction as well as a selectable marker cassette, P$_{35S\Omega}$-*bar*-T$_{nos}$, into an empty binary vector, pPZP200. Subsequently, an expression binary vector, pHKGB112 (Fig. 2), was constructed for transformation experiments using a MultiRound Gateway. Two expression cassettes of reporter genes, *GUSPlus* and *bar*, could be easily constructed on two independent entry vectors, pL12R34H-Ap and pL34R12H-Cm, by general molecular techniques, and simply introduced into pHKGB110 by two sequential LR reactions. The resultant

binary vector pHKGB112 was transformed into *A. tumefaciens* EHA101, and this strain was used for all switchgrass transformation experiments.

Effect of *Agrobacterium*-suppressing antibiotics on the transformation of Type I callus of switchgrass

For efficient recovery of transgenic plants, it is essential to select a suitable antibiotic for suppressing the overgrowth of *Agrobacterium* after cocultivation. In the first experiment, calli inoculated with EHA101 (pHKGB112) were cocultivated for 5 days as previously recommended by Somleva (2007) and were then cultured on medium supplemented with various antibiotics to suppress *Agrobacterium* overgrowth. Cefotaxime of 250 mg l^{-1}, Timentin of 150 mg l^{-1} and meropenem of 25 and 50 mg l^{-1} were used in this study, and *Agrobacterium* overgrowth and transformation efficiency were evaluated (Table 1).

Cefotaxime of 250 mg l^{-1} failed to suppress overgrowth of *Agrobacterium* on almost all inoculated calli and also failed to produce any transgenic plants (Fig. 3a). Timentin of 150 mg l^{-1} allowed *Agrobacterium* overgrowth during both the callus selection and shoot regeneration stages at moderate frequency (18.7 %) (Fig. 3b). Meropenem of 25 mg l^{-1} also allowed *Agrobacterium* overgrowth at a similar frequency to Timentin. However, overgrowth of *Agrobacterium* was observed at the callus selection stage but not at the regeneration and rooting stages. Among the antibiotics tested, only 50 mg l^{-1} meropenem completely suppressed the overgrowth of *Agrobacterium* on inoculated calli (Fig. 3c).

Most calli survived and grew slightly on medium containing 10 mg l^{-1} bialaphos during the first 2–4 weeks of the callus selection stage. After the subsequent 6- to 8-week period, vigorously growing bialaphos-resistant (Bar^R) calli and severely browning bialaphos-sensitive (Bar^S) calli could be distinguished by visual inspection (Fig. 3c). Green fluorescent areas expressing the reporter GFP (GFP$^+$) were observed on Bar^R calli (Fig. 3d), which eventually regenerated Bar^R shoots (Fig. 3e). GFP$^+$ cells were also observed in bialaphos-sensitive/partially resistant calli as small foci (<1 mm diameter), but these calli generally failed to regenerate shoots. This result suggests that the expression level is different between the *bar* gene driven by a CaMV 35S-based promoter and the *GUSPlus* gene driven by a maize *ubi1* promoter in switchgrass. The formation rates of Bar^R callus, GFP$^+$ callus and Bar^R shoots did not vary among the antibiotics used except for cefotaxime (Table 1). Although treatment with 150 mg l^{-1} Timentin and 50 mg l^{-1} meropenem occasionally caused regeneration of albino shoots, these shoots formed with a frequency of less than 1.3 % (Table 1).

Table 1 Effect of antibiotics for suppression of *Agrobacterium* during switchgrass transformation using a binary vector pHKGB112

Antibiotic	Overgrowth of *Agrobacterium*[a,b]	Callus		Shoot		Plantlet		Transformation efficiency (A × B)[b]
		Bar[Ra]	GFP[+a]	Green[a,c]	Albino[a,d]	Bar[R] (A)[a]	PCR[+] (B)[e]	
Cefotaxime 250 mg l^{-1}	98.7 ± 2.3[y]	0.7 ± 1.2	0.7 ± 1.2	0 ± 0	0 ± 0	0 ± 0	NA	0 ± 0[y]
Timentin 150 mg l^{-1}	18.7 ± 28.9[z]	32.0 ± 10.4	40.0 ± 12.2	18.7 ± 13.3	0.7 ± 1.2	16.7 ± 11.7	76.1 ± 23.4	11.3 ± 5.0[z]
Meropenem 25 mg l^{-1}	11.3 ± 4.6[z]	27.3 ± 2.3	39.3 ± 1.2	16.7 ± 3.1	0 ± 0	14.7 ± 3.1	94.4 ± 9.6	14.0 ± 4.0[z]
Meropenem 50 mg l^{-1}	0 ± 0[z]	30.7 ± 7.0	48.7 ± 7.0	18.7 ± 4.2	1.3 ± 2.3	17.3 ± 5.0	100 ± 0	17.3 ± 5.0[z]

In total, 150 calli of line 06–05 were inoculated in three independent experiments with 50 calli in each replication. The data are presented in percentage as the mean ± standard deviation of the replicates

NA not available

[a] The data were calculated as the percentage of positive calli among all inoculated calli

[b] Data with same letter are not significantly different according to the Tukey–Kramer method at the 5 % level

[c] Calli that formed both green and albino shoots were included

[d] Calli that formed both green and albino shoots were excluded

[e] PCR was performed using a *bar*-specific primer set. The data were calculated as the percentage of positive plantlets among all rooted plantlets
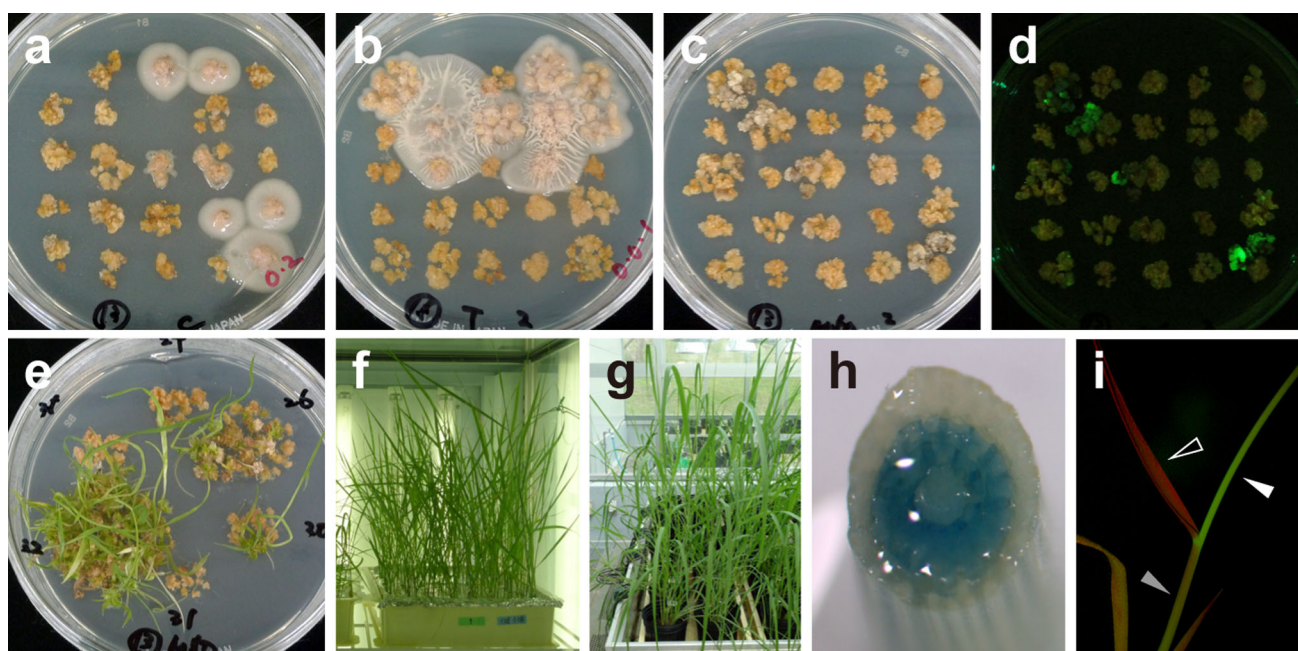

Fig. 3 Transformation of Type I calli of Alamo switchgrass. Calli inoculated with *A. tumefaciens* EHA101 (pHKGB112) and cultured on PVC-S medium supplemented with: **a** 250 mg l^{-1} cefotaxime; **b** 150 mg l^{-1} Timentin; **c** 50 mg l^{-1} meropenem. Overgrowth of *Agrobacterium* appears as *white halos* in (**a**) and (**b**). **d** Fluorescent image of **c** showing GFP expression. **e** Shoot regeneration of inoculated calli cultured on PVS-S medium supplemented with 50 mg l^{-1} meropenem. Transgenic plants cultivated in: **f** hydroponic solution in a growth chamber; **g** potted soil in a greenhouse. **h** X-gluc-stained internode section showing GUSPlus expression with weak blue staining in the stem (*inner section*), strong staining in an immature leaf (*middle section*) and no staining in the leaf sheath (*outer section*). **i** Shoot showing strong GFP expression fluorescence in the emerging leaf blade (*white arrowhead*), no fluorescence in the mature leaf blade (*black arrowhead*), and weak fluorescence in the leaf sheath (*gray arrowhead*)

Bar[R] shoots that were rooted on PVR-S medium containing 10 mg l^{-1} bialaphos and grown in hydroponic solution were subjected to PCR analysis to confirm *Agrobacterium* persistence and transgene integration (Fig. 4).

Although the *virC* gene was detected in small plantlets at both the rooting and acclimatization stages, most of the plantlets grown in hydroponic culture became *virC*-negative 2–4 weeks after acclimatization. In subsequent PCR
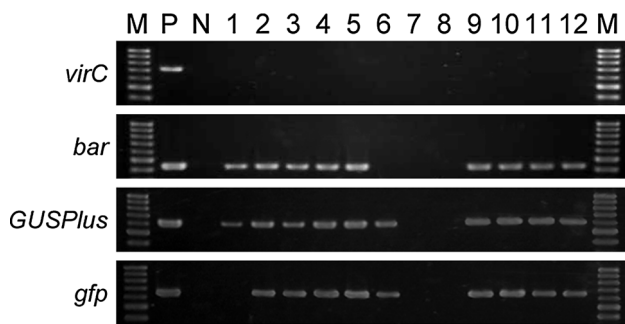
Fig. 4 PCR analyses of putatively transformed Bar^R plantlets for *virC*, *bar*, *GUSPlus* and *gfp* genes. *M* marker, *P A. tumefaciens* EHA101 (pHKGB112), *N* non-transformed control plantlet, *1–6* putatively transformed plantlets treated with 50 mg l^{-1} meropenem, *7–12* putatively transformed plantlets treated with 150 mg l^{-1} Timentin

analyses using the *bar*-specific primer set, positive fragments could not be amplified from some of the Bar^R plantlets, which also did not show GFP fluorescence. Interestingly, these untransformed escapes were most frequently obtained with the 150 mg l^{-1} Timentin treatment (24 % of Bar^R events) (Table 1).

When 50 mg l^{-1} meropenem was used as an *Agrobacterium*-suppressing agent throughout the transformation, Bar^R plantlets were recovered at the highest efficiency (17.3 %) without overgrowth of *Agrobacterium* and the appearance of escape plantlets (Table 1). In addition, 25 mg l^{-1} meropenem successfully suppressed the overgrowth of *Agrobacterium* at the regeneration and rooting stages and produced Bar^R plantlets at a high frequency comparable to 50 mg l^{-1} meropenem. Therefore, 50 mg l^{-1} meropenem was used in the PVC-S medium for callus selection and 25 mg l^{-1} meropenem was used in the PVS-S and PVR-S media for regeneration and rooting, respectively, in subsequent experiments.

Cocultivation period for efficient recovery of transgenic switchgrass

Since we found that meropenem was effective for suppressing *Agrobacterium* overgrowth and producing transgenic switchgrass plants, we subsequently compared different cocultivation periods (3, 5, and 7 days) for further optimization of the transformation protocol. Transformation efficiencies (all Bar^R calli, shoots and plantlets) were positively correlated with the length of the cocultivation period (Table 2). The efficiency of Bar^R-plant recovery obtained with cocultivation for 7 days (17.3 %) was the highest among the tested conditions, and was 3.3- and 1.5-fold higher than with cocultivation for 3 and 5 days, respectively. The effectiveness of prolonged cocultivation was also confirmed in five other cell lines induced from independent caryopses that had different genotypes. All and four out of five lines failed to recover Bar^R plants with 3 and 5 days of cocultivation, respectively. In contrast, all lines produced Bar^R plants with 7 days of cocultivation at 2–12 % efficiency.

Novel selection of putatively transformed shoots

Selection of putatively transformed shoots was conventionally performed by placing shoots onto rooting medium supplemented with a selective agent. In this study, non-transformed switchgrass shoots partially survived and occasionally rooted on PVR-S medium supplemented with 10 mg l^{-1} bialaphos after 2 weeks of culture (Fig. 5). To develop a simpler and more reliable selection method, we tried a new selection technique as follows: shoots were dipped in herbicide solution (0.1 or 1 % Basta) for a few seconds, and then placed onto PVR-S medium without a selective agent. This was designated as the "herbicide dipping method". Using this method, non-transformed shoots showed severe chlorosis within a week and were completely dead after 2 weeks. Conversely, transformed shoots rooted within 1 week after herbicide dipping even with 1 % Basta. After 2 weeks, they developed into healthy plantlets, which were similar to shoots grown without selection and were able to acclimatize. Integration of the *bar* gene into all of the resistant plantlets was confirmed by PCR.

Multi-gene transformation with the MultiRound Gateway binary vector

Additional PCR analyses were performed on *bar*-positive plants (assessed by PCR) using specific primers for the

Table 2 Effect of cocultivation period on switchgrass transformation using a binary vector pHKGB112

Cocultivation period (days)	Overgrowth of *Agrobacterium*	Bar^R callus[a]	Bar^R shoot[a]	Bar^R plantlet[a]
3	0 ± 0	7.3 ± 4.2[x]	5.3 ± 1.2[y]	5.3 ± 1.2[y]
5	0 ± 0	35.3 ± 4.6[y]	11.3 ± 4.2[yz]	11.3 ± 4.2[yz]
7	0 ± 0	50.7 ± 8.1[z]	20.0 ± 7.2[z]	17.3 ± 6.1[z]

In total, 150 calli of line 06–12 were inoculated in three independent experiments with 50 calli in each replication. The data are presented in % as the mean ± standard deviation of replicates

[a] The data with same letter are not significantly different according to the Tukey–Kramer method at the 5 % level
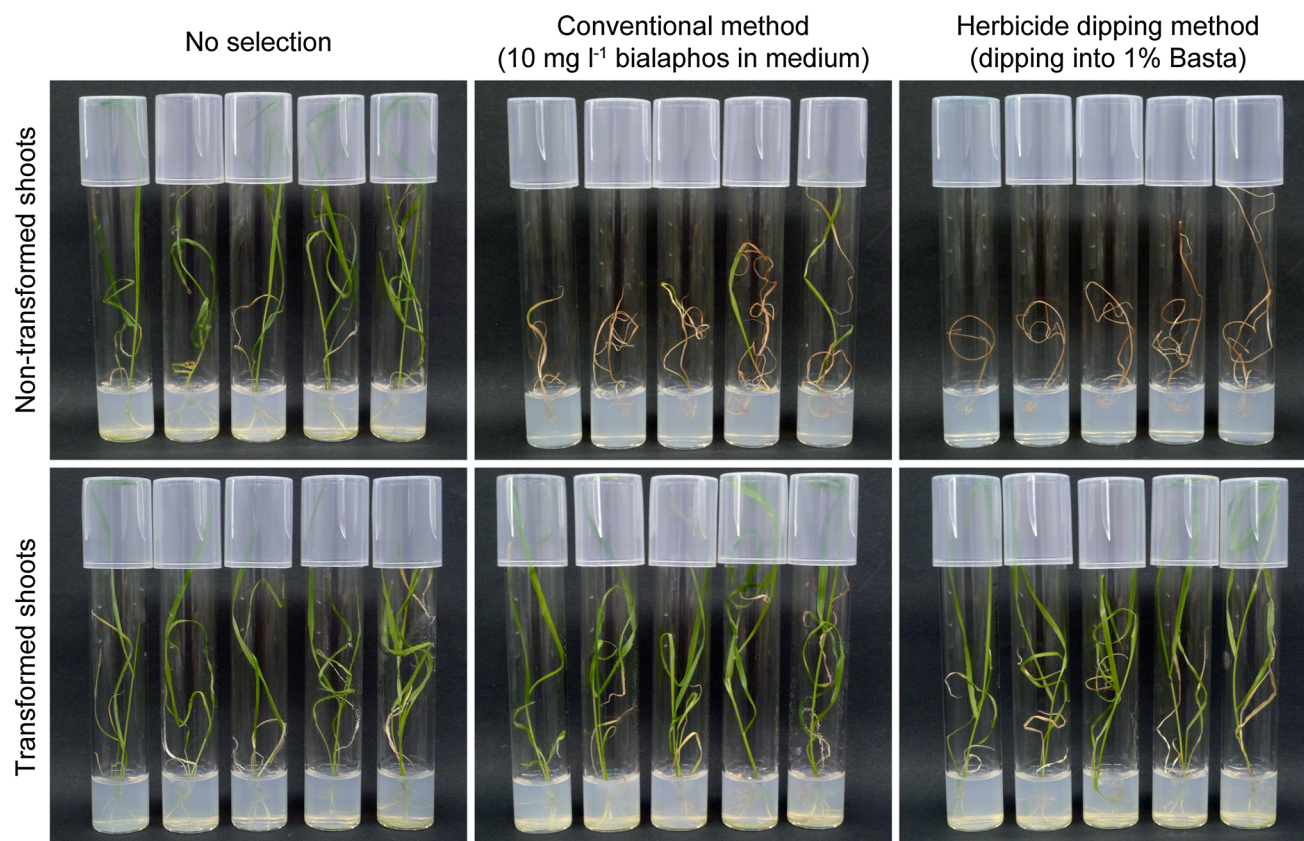
Fig. 5 Selection of regenerated shoots by the conventional and herbicide dipping methods

Table 3 Multi-gene transformation of switchgrass using a binary vector pHKGB112

Transformation condition	Total no. of events	$GUSPlus^+$ gfp^+	$GUSPlus^-$ gfp^+	$GUSPlus^+$ gfp^-	$GUSPlus^-$ gfp^-
Overall	128	119 (93.0 %)	1 (0.8 %)	5 (3.9 %)	3 (2.3 %)
Optimized[a]	40	38 (95.0 %)	0 (0 %)	1 (2.5 %)	1 (2.5 %)

Plantlets formed on an inoculated callus are referred to as a single event. Up to five bar^+ plantlets in each event were subjected to PCR analyses using GUSPlus and gfp primers

[a] Seven days of cocultivation, 50 mg l^{-1} meropenem for callus selection, 25 mg l^{-1} meropenem for regeneration and rooting, and herbicide dipping for selection

GUSPlus and gfp genes (Table 3). Among the 128 bar-positive transformants examined, 119 (93.0 %) carried both reporter genes, and only 6 (4.7 %) and 3 (2.3 %) lacked either one or both reporters, respectively. These frequencies were similar to those of optimized transformation conditions (7 days of cocultivation, the use of meropenem for suppression of Agrobacterium, and selection by the herbicide dipping method).

These transgenic plants grew well in both hydroponic solution and soil (Fig. 3f, g). Expression of transgenes was examined by RT-PCR in transgenic plants carrying the three transgenes (bar, GUSPlus and gfp). Transcription products of all transgenes were detected from all plants tested (Fig. 6). GUSPlus expression in transgenic plants was confirmed by X-gluc staining, with blue staining clearly observed in immature tissues but absent or only slight in mature tissues (Fig. 3h). GFP expression fluorescence was also tissue dependent (Fig. 3i), but strong fluorescence was observed in all parts of the plant in some plants. These results indicated different promoter activities between the CaMV 35S-based promoter for GUSPlus and the ubi1 promoter for gfp in switchgrass, as was similarly observed in GFP-expressing calli showing bialaphos sensitivity/partial resistance. Southern blot hybridization confirmed integration of a T-DNA fragment containing the bar gene in all transgenic lines tested (Fig. 7). Six out of eight transgenic lines carried the bar gene in a single copy or low (2–3) copies. Signals smaller than the expected size
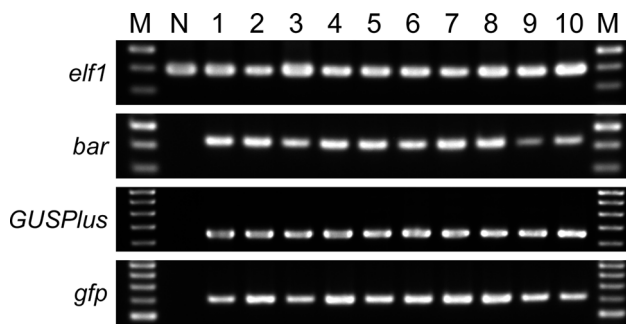
Fig. 6 RT-PCR analyses of transgenic switchgrass plants for *elf1, bar, GUSPlus* and *gfp*genes. *M* marker, *N* non-transformed control plant, *1–10* transgenic plants
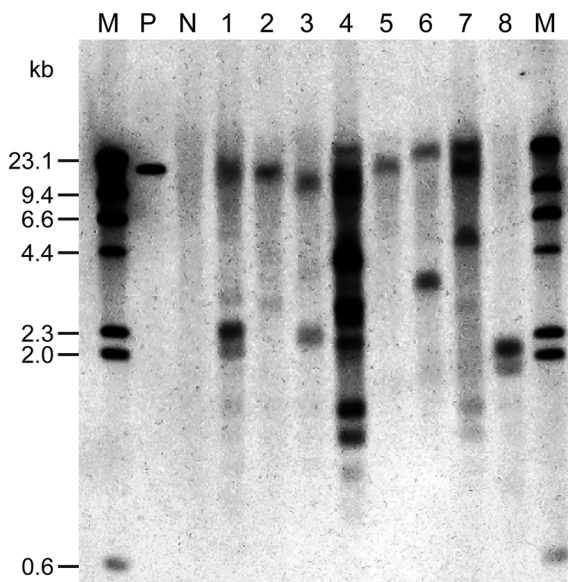

Fig. 7 Southern blot hybridization analysis of transgenic switchgrass plants for integration of *bar* gene. *M* marker, *P* pHKGB112, *N* non-transformed control plant, *1–8* transgenic plants

(≥2.2 kb) were occasionally observed in lines carrying multiple copies of the transgenes, suggesting that the T-DNA was truncated at the left border sequence in these transgenic lines.

Discussion

Here, we established a simple and reliable method for multi-gene transformation of switchgrass that incorporates the construction and use of a MultiRound Gateway-compatible binary vector, the improvement of a transformation method for caryopsis-derived embryogenic Type I calli, and the establishment of a novel selection technique. The Gateway system is widely used for construction of plant transformation vectors (Karimi et al. 2002), and Gateway-compatible vectors for switchgrass and other monocots

have been reported (Mann et al. 2012). However, most of these vectors were designed for construction of a single gene vector. The MultiRound Gateway is a simple, efficient and more flexible system for multi-gene assembly (Chen et al. 2006). Once the DNA fragments of interest are cloned into the two entry vectors, they can be introduced into a destination vector in a defined order and orientation through multiple rounds of Gateway reactions. In principle, the procedure may be repeated an infinite number of times. In this work, the binary vector pHKGB112 (Fig. 2) was constructed by introducing two reporter genes into the destination vector pHKGB110 (Fig. 1) using the Multi-Round Gateway technique. By transformation with pHKGB112, transformants carrying both reporters as well as a selectable marker could be recovered at a high frequency (more than 90 % of Bar-resistant transformants). Although only three genes were stacked in a single T-DNA in the present proof-of principle experiments, it has been reported that eight genes were successfully introduced into a T-DNA by four rounds of LR recombination reactions (Chen et al. 2006). In addition, a series of binary vectors carrying various sets of genes-of-interest could be easily constructed by combining entry vectors harboring different genes (Ren et al. 2010). We have already been constructed various binary vectors carrying multiple genes-of-interest using this system and the work for switchgrass transformed with these binary vectors is in progress. In coupled with these findings, we demonstrated that the MultiRound Gateway would be a useful tool for introducing a series of genes, e.g., genes related to a unique metabolic pathway, in a single transformation for switchgrass.

We chose caryopsis-derived Type I calli for the explants of transformation experiments in this study. Embryogenic Type I callus is the most typical and prevalent type of callus induced from the most readily available explant type in switchgrass, the caryopsis (Denchev and Conger 1994; Vasil and Vasil 1994). Inflorescence-derived callus and friable Type II callus were used in other recent reports, but the former is limited in the time available for callus induction and the latter potentially induces albino shoots, particularly under prolonged cultivation (Li and Qu 2011; Ramamoorthy and Kumar 2012). In the present study, Type I callus lines were successfully used for transformation after at least 18 months of subculture without increasing albino shoot formation.

Cocultivation is an essential period for introducing transgenes from *Agrobacterium* cells to plant cells, and suppression of *Agrobacterium* overgrowth after cocultivation is important to recover transgenic plants. Somleva (2007) suggested that the optimal period for cocultivation is 3–5 days depending on the target explants, and the optimal cocultivation period is shorter for somatic embryos than for callus. Carbenicillin, cefotaxime and Timentin

were employed alone or in combination for the transformation of switchgrass in previous reports. Xi et al. (2009) reported that the placement of inoculated calli directly onto agar-solidified medium during cocultivation led to overgrowth of *Agrobacterium* in subsequent cultivation with 250 mg l^{-1} cefotaxime. Thus, they cocultivated calli on filter papers wetted with medium for only 2 days. Other researchers also performed short cocultivation (2–4 days) and additional treatments after cocultivation such as washing with water/liquid medium/antibiotic solution (Burris et al. 2009; Ramamoorthy and Kumar 2012; Song et al. 2012) and resting on selective agent-free medium (Li and Qu 2011). Meropenem is a derivative of carbapenem and possesses high bactericidal activity against *Agrobacterium* and low phytotoxicity (Ogawa and Mii 2004, 2005, 2007). We found that 50 mg l^{-1} meropenem successfully suppressed the overgrowth of *Agrobacterium* throughout transformation, and that 25 mg l^{-1} meropenem was sufficient to suppress overgrowth at the regeneration and rooting stages, even after 5 days of cocultivation on agar medium (Table 1). Other antibiotics at elevated concentration may suppress overgrowth of *Agrobacterium*, while chemical cost also elevates and transformation efficiency would be affected negatively by their phytotoxicity (Ogawa and Mii 2007). Using meropenem, cocultivation could be prolonged to 7 days without overgrowth of *Agrobacterium*, and this simple modification achieved 3.3- and 1.5-fold higher efficiency than 3 and 5 days of cocultivation, respectively (Table 2). Compact Type I calli comprise almost entirely tightly-packed meristematic cells (Vasil and Vasil 1994) and somatic embryogenesis of mature caryopsis-derived Type I calli of switchgrass was observed to take place in embedded areas of callus tissue (Denchev and Conger 1994). Therefore, the prolonged cocultivation period may facilitate the invasion and attachment of *Agrobacterium* to transformation-competent plant embryogenic cells existing in embedded callus areas.

When 150 mg l^{-1} Timentin was used in the present study, untransformed escape plants were frequently recovered (Table 1) at levels similar to those reported by Somleva et al. (2002). Because Timentin and other penicillin derivatives have been reported to have auxin-like effects on various plant species/tissues (Holford and Newbury 1992; Nauerby et al. 1997; Robert et al. 1989), the formation of untransformed escapes on switchgrass calli might have been caused by unexpected growth of untransformed cells by supra-optimal concentrations of auxin. It was reported that untransformed escapes were also formed using the *bar* gene as a selectable marker to show herbicide resistance, which is a useful trait for field-grown transgenic plants and is preferred to omit horizontal transfer of antibiotic resistance gene to microbes. To identify escapes of regenerated shoots, Somleva et al.

(2002) repeatedly applied a 0.1 % Basta solution to the leaf segments of in vitro- and greenhouse-grown putative transformants. To overcome the inconvenience of this method, we established a novel selection technique for complete elimination of untransformed escapes during the rooting stage (Fig. 5). The herbicide Basta contains glufosinate ammonium, which is a broad-spectrum, non-selective post-emergence herbicide that functions by inhibiting glutamine synthetase activity in plants, and is recommended for foliage application by spraying with a 100-fold dilution (final glufosinate concentration of 0.185 %) for perennial grass weeds (Bayer CropScience 2008). We succeeded in applying herbicide to foliage as follows: shoots of putative transformants were dipped in an herbicide solution and placed onto selective agent-free rooting medium. This technique, herbicide dipping selection, is simple and effective for the recovery of genuine transgenic switchgrass plants before acclimatization and cultivation, and could be used in the transformation of other plant species. Since Xi et al. (2009) used a hygromycin dipping test to detect *hph* expression in leaf blades of greenhouse-grown plants, we expect that the technique can be utilized in transformants carrying antibiotic resistance markers.

Using the present transformation method, independent transgenic switchgrass plants were recovered at a transformation efficiency of 2.0–17.4 % from all seven tested genotypes of the cultivar Alamo. These efficiencies were comparable to previous studies; 3.4 % by Somleva et al. (2002), 4.4 % by Burris et al. (2009) and 6 % by Ramamoorthy and Kumar (2012). Li and Qu (2011) have been reported a high-throughput transformation method achieving 56.6 % of transformation efficiency for Alamo, while this protocol is very laborious and tends to induce albino shoots. Because switchgrass is a self-incompatible, open-pollinated species, calli formed on an individual caryopsis can be of different genotypes. The present transformation method for Type I callus, which can be maintained long-term, would be suitable for obtaining a large set of transgenic plants carrying the same genetic background. In addition, a MultiRound Gateway-compatible vector would be useful to introduce multiple transgenes of interest in a single transformation. It would be interesting to determine whether the present transformation method could be applied to other cultivars and, moreover, could be improved to get the transformation efficiency to the maximum level reported previously.

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