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Original Paper

An efficient *Agrobacterium*-mediated transformation method for switchgrass genotypes using Type I callus

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Abstract Switchgrass (*Panicum virgatum* L.) is an important bioenergy crop. A reliable and efficient transformation method is required to assist with molecular breeding of this crop. In this study, we established a simple and efficient *Agrobacterium*-mediated transformation method for caryopsis-derived Type I callus by optimizing the cocultivation (7 days at 22°C on medium supplemented with 10 g l^{-1} glucose and 100 μM acetosyringone) and preculture (2 weeks on medium supplemented with 5 g l^{-1} casamino acids after cell straining) conditions without the need for time-consuming treatments before and after cocultivation. The present transformation method was successfully applied to different genotypes of switchgrass including a recalcitrant lowland cultivar. The transformation efficiencies of callus lines of the lowland cultivars 'Alamo' and 'Kanlow' were 12.5–59% and 6.3–20%, respectively. An upland cultivar 'Trailblazer' formed Type I calli and one of three tested callus lines produced transgenic plants at relatively high efficiency (7.5%). In contrast, Type I callus formation was unsuccessful for two other upland cultivars, 'Blackwell' and 'Cave-in-Rock.' This simple and efficient transformation method is suitable for routine and large-scale experiments due to its ready availability of caryopses, prevalence of Type I callus formation, and longevity in the regeneration ability of the callus.

Key words: *Agrobacterium*-mediated transformation, cocultivation, embryogenic Type I callus, genotype, switchgrass.

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial C_4 grass native to North America and a promising crop for bioenergy (Parrish et al. 2012). With increasing interest in the breeding of this species, a variety of *Agrobacterium*-mediated transformation methods have been reported subsequent to that of Somleva et al. (2002). These methods differ in the explant source, callus type and procedure, and still require refinement to improve efficiency. The caryopsis is the most readily available explant source for embryogenic callus induction (Denchev and Conger 1994). However, switchgrass is a self-incompatible outcrossing species, so all cultivars are genotypically variable. Different genotypes within a cultivar affect the transformation efficiency (Ogawa et al. 2014; Somleva et al. 2002) and potentially affect characteristics of transgenic plants that carry the same transgene(s). Seedling-derived callus has been induced and used for transformation (Song et al. 2012), although these callus lines also comprise different genotypes. Inflorescences can be obtained from vegetatively propagated plants for callus induction, and thus are a suitable explant source from which to induce callus and transformants derived from a single genotype. However, because the inflorescence is a reproductive organ, explant collection is only possible during a limited developmental period. In addition, only elite genotypes of the cultivar 'Alamo' have been used previously for transformation of inflorescence-derived calli (Burris et al. 2009; Xi et al. 2009).

Compact embryogenic callus, termed Type I, is the most prevalent type generated in grass species including switchgrass (Denchev and Conger 1994; Vasil and Vasil 1994). Type I callus was used in the first successful *Agrobacterium*-mediated transformation of switchgrass by Somleva et al. (2002). Type I callus could be maintained for at least 2 years with relatively high regeneration ability and with low albino shoot formation rate (Ogawa 2015). Friable embryogenic callus, termed Type II, shows a high growth rate and regeneration ability and is used in more recent reports (Burris et al. 2009; Li and Qu 2011; Liu et al. 2015; Ramamoorthy and Kumar 2012). Li and Qu (2011) reported a high-throughput transformation protocol for Type II callus and achieved more than 44.6% transformation efficiency,

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; GA_3, gibberellic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MEPM, meropenem; MS, Murashige and Skoog.

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whereas 100% of long-term subcultured calli of 'Alamo' formed albino shoots. Albino shoot formation from Type II callus was also reported by Ramamoorthy and Kumar (2012). Very recently, Liu et al. (2015) reported efficient induction and transformation methods of Type II calli in coupled with the discovery of "shell-core" structure. They achieved 72.8% of transformation efficiency for a lowland cultivar 'Alamo', and successfully obtained first transgenic plants of an upland cultivar 'Dacotah'. However they used only freshly established (4-month-old) calli for transformation experiments and did not described for albino formation as well as efficiency on different genotypes within each cultivar.

Most of the reported transformation methods for Type I callus of switchgrass have achieved relatively low efficiencies (around 5%), involve laborious procedures and are limited to lowland cultivars (especially 'Alamo'). We previously found that the transformation efficiency of caryopsis-derived Type I calli could be increased by prolonged cocultivation (Ogawa et al. 2014). In the present study, we aimed to establish a simple and highly efficient *Agrobacterium*-mediated transformation method using caryopsis-derived Type I calli of switchgrass by focusing on the cocultivation conditions as well as the preculture conditions. Additionally, the applicability of the method to different genotypes of both upland cultivars was examined.

Materials and methods

Callus induction and maintenance

Five cultivars of switchgrass (*Panicum virgatum* L.), consisting of two lowland cultivars 'Alamo' and 'Kanlow' and three upland cultivars 'Blackwell', 'Cave-in-Rock' and 'Trailblazer', were used in the study. Embryogenic callus was induced from caryopses in accordance with the method of Denchev and Conger (1994) with modifications. All media used were based on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) and are listed in Table 1. Caryopses were dehusked with 60% H_2SO_4 for 30–45 min and rinsed with distilled water three times. Dehusked caryopses were treated with 0.5% (w/v) solution of the fungicide Benlate T20 (Sumitomo Chemical Company, Tokyo, Japan) overnight at room temperature with gentle shaking. The caryopses were then sterilized with 3.5% (w/v) $Ca(ClO)_2$ for 30 min. After rinsing with sterilized distilled water, the caryopses were placed on PVC medium and cultured at 28°C in the dark. Embryogenic Type I calli that showed high regeneration ability were subcultured every 4 weeks.

Agrobacterium-mediated transformation

Six- to twelve-month-old caryopsis-derived Type I calli were transformed using the *Agrobacterium*-mediated method of Ogawa et al. (2014) with modifications. One callus line of cultivar 'Alamo', A09-01, was used for optimization of transformation conditions. *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) carrying the binary vector pHKGB112 (Ogawa et al. 2014) was used for transformation. A glycerol stock of *Agrobacterium* was streaked onto a LB 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 for 2–3 days. Bacterial cells (10 μl) were collected using a loop and resuspended with 40 ml liquid PVC-CO medium ($A_{600}\approx0.2$). Embryogenic calli were strained with stainless sieve (Ogawa 2015) and cultured on either PVC, PVCc or PVCp medium for 2 weeks. Compact calli formed on PVC or PVCc medium (2- to 3-mm) and partially friable calli formed on PVCp medium (divided into ca. 3-mm clumps) were immersed in *Agrobacterium* suspension for 3 min with occasional agitation. Inoculated calli were blotted on paper towels, placed on PVC-based cocultivation medium, and cultured for 3–7 days at 19–28°C in the dark. The calli were then transferred to PVC-S medium without washing and cultured in the dark for 8 weeks with subculture at 2-week intervals. Herbicide-resistant calli were transferred to PVS-S medium and cultured under a 16-h photoperiod for 6 weeks with subculture at 2-week intervals. Selection and rooting of regenerated shoots were performed on PVR-S medium with a herbicide dip method (Ogawa et al. 2014) using 1% (v/v) solution of Basta

Table 1. Composition of media used in this study.

Medium	Composition	Reference
PVC	MS salts and vitamins, 30 g l^{-1} maltose, 22.6 μM 2,4-D, 4.4 μM BAP, 2.5 mM MES, 8 g l^{-1} agar, pH 5.7	Denchev and Conger (1994) Ogawa et al. (2014)
PVS	MS salts and vitamins, 30 g l^{-1} maltose, 1.4 μM GA_3, 2.5 mM MES, 8 g l^{-1} agar, pH 5.7	Denchev and Conger (1994) Ogawa et al. (2014)
PVR	half-strength MS salts and vitamins, 15 g l^{-1} sucrose, 2.5 mM MES, 7 g l^{-1} agar, pH 5.7	Ogawa et al. (2014)
PVC-CO	PVC with 10 g l^{-1} glucose, 100 μM acetosyringone	This work
PVCc	PVC with 5 g l^{-1} casamino acids	Ogawa (2015)
PVCp	PVC with 50 mM proline	Ogawa (2015)
PVC-S	PVC with 50 mg l^{-1} MEPM, 20 mg l^{-1} glufosinate	Ogawa et al. (2014)
PVS-S	PVS with 25 mg l^{-1} MEPM, 20 mg l^{-1} glufosinate	Ogawa et al. (2014)
PVR-S	PVR with 25 mg l^{-1} MEPM	Ogawa et al. (2014)

Heat labile elements (acetosyringone, glufosinate and MEPM) were added to media after autoclaving. Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; GA_3, gibberellic acid; MEPM, meropenem trihydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan); MES, 2-(*N*-morpholino)ethanesulfonic acid; MS, Murashige and Skoog (1962).

(Bayer CropScience, Monheim am Rhein, Germany). Putatively transformed plantlets were acclimatized and grown in hydroponic solution in a growth chamber or in potted soil in a greenhouse.

To assess the applicability of optimized transformation conditions (preculture on PVCc medium after cell straining and cocultivation on PVC-CO medium at 22°C for 7 days), three callus lines each for 'Alamo', 'Kanlow' and 'Trailblazer' were used for transformation.

Observation of GFP fluorescence

Expression of green fluorescent protein (GFP) in inoculated calli at the macroscopic level was monitored and recorded using a ProDoc-LED470GFP macro imaging system (OptoCode, Tokyo, Japan). Expression of GFP in calli and shoots at the microscopic level was observed using a Leica MZ FLIII fluorescence stereomicroscope with the filter sets 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).

Molecular analyses of transgenic plants

Molecular analyses of putatively transformed plants were performed as previously reported (Ogawa et al. 2014). All experiments were performed using standard molecular techniques and in accordance with the manufacturer's instructions. Leaf explants of putatively transformed plantlets were subjected to genomic polymerase chain reaction (PCR) using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) with primer sets to amplify the *virC*, *bar*, *GUSPlus* and *gfp* genes (Ogawa et al. 2014). Total RNA was extracted from leaves of PCR-positive plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and first-strand cDNA was synthesized using the PrimeScript II First Strand cDNA Synthesis Kit with the oligo-dT primer (Takara Bio, Otsu, Japan). Reverse transcription (RT) PCR was performed using primer sets for four genes: the *elf1* gene as an internal control and the *bar*, *GUSPlus* and *gfp* genes (Ogawa et al. 2014). Genomic DNA was extracted from leaf blades of PCR-positive plants using the DNeasy Plant Maxi Kit (Qiagen) and digested with *Xma*I. Digested DNA (15 μg for the tetraploid lowland cultivars 'Alamo' and 'Kanlow,' and 30 μg for the octoploid upland cultivar 'Trailblazer') were electrophoresed in a 0.7% agarose gel and transferred onto a nylon membrane. Hybridization and detection were performed with the DIG system (Roche Applied Science, Mannheim, Germany) using the *bar* gene as a specific probe.

Experimental design and statistical analysis

For optimization of transformation conditions, 20 calli were used for each transformation and the experiments were independently replicated three times. Forty calli of different genotypes were transformed in 2–3 replications. Transformation efficiency was defined as the percentage of inoculated calli that formed Bar-resistant plants. Frequencies of Bar-resistant calli and shoots per inoculated calli were also recorded. Calli that formed only albino shoot(s) were excluded from Bar-resistant ones. The data were presented as the mean±standard deviation (SD) of all replicates. Analysis of variance (ANOVA) was performed using a single-factor ANOVA or two-factor factorial ANOVA followed by the Tukey–Kramer test using Excel 2007 software (Microsoft, Redmond, WA, USA) with the add-in software Statcel3 (Yanai 2011).

Results

Optimization of cocultivation conditions

We previously showed that prolonged cocultivation improved the transformation efficiency of caryopsis-derived Type I callus of switchgrass (Ogawa et al. 2014), therefore further optimization of cocultivation conditions was undertaken. First, calli inoculated with *Agrobacterium* were cocultivated at four temperatures for 3, 5 or 7 days (Figure 1). Transformation efficiency was higher at lower temperatures (22 and 25°C) than the standard culture temperature (28°C), but was reduced at the lowest temperature tested (19°C). At all temperatures examined, transformation efficiencies increased with a prolonged cocultivation period. The optimal combination of cocultivation temperature and duration (22°C and 7 days) resulted in significantly higher transformation efficiency (40%) than the other combinations.

The induction of *Agrobacterium vir* genes on cocultivation medium of different compositions was compared (Figure 2). Unexpectedly, a lowered pH of 5.2, which is known as effective to induce *vir* genes, significantly reduced transformation efficiency compared with a pH of 5.7, which is the standard pH of the culture medium. In contrast, addition of 10 g l^{-1} glucose

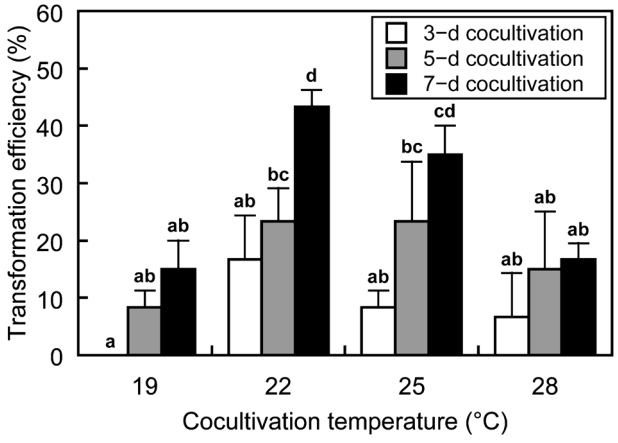

Figure 1. Effects of cocultivation temperature and duration on transformation of switchgrass 'Alamo' A09-01 callus line. Bars with the same letters are not significantly different according to the Tukey–Kramer test at the 5% significance level.

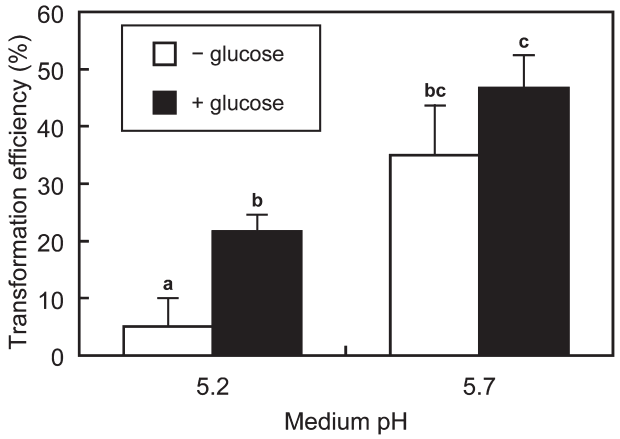

Figure 2. Effect of inclusion of glucose in the cocultivation medium on transformation of switchgrass 'Alamo' A09-01 callus line. Cocultivation was performed at 22°C for 7 days. Bars with the same letter are not significantly different according to the Tukey–Kramer test at the 5% significance level.

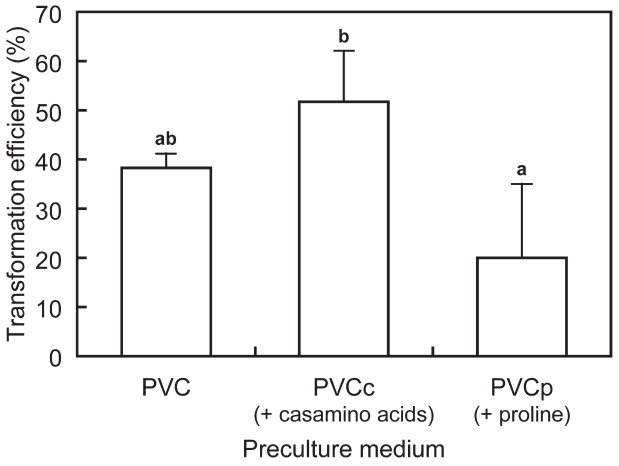

Figure 3. Effect of preculture medium composition on transformation of switchgrass 'Alamo' A09-01 callus line. Cocultivation was performed at 22°C for 7 days. Bars with the same letter are not significantly different according to the Tukey–Kramer test at the 5% significance level.

to cocultivation medium improved transformation efficiency regardless of the medium pH. On the basis of these results, PVC medium supplemented with 10 g l^{-1} glucose and of pH 5.7 was selected as the PVC-CO medium and used for further experiments.

Optimization of preculture conditions

The quality and type of callus strongly affect transformation efficiency. Caryopsis-derived calli were strained with stainless sieve and were then cultured for 2 weeks on PVC medium with and without additives and then used for transformation (Figure 3). Calli precultured on PVCc medium, which was supplemented with 5 g l^{-1} casamino acids, maintained the Type I callus morphology as previously reported (Ogawa 2015) but produced transgenic plants at higher efficiency than PVC medium without the additive. Calli precultured on PVCp medium, which was supplemented with 50 mM proline, were partially changed into friable Type II callus within 2 weeks of culture. These calli were capable of producing transgenic plants but the transformation efficiency was drastically decreased. These results suggested that the present transformation conditions were suitable for compact Type I callus but not for friable Type II callus.

Under the optimized transformation conditions (preculture on PVCc medium after cell straining and cocultivation on PVC-CO medium at 22°C for 7 days), 3.3% of inoculated calli and 5.1% of shoot-forming calli formed only albino shoots. Since albino formation rates were similar and negligibly low in all tested conditions, transformation efficiencies were calculated with the number of all-albino-forming calli excluded.

Applicability of optimized transformation conditions for different genotypes

To assess the applicability of the optimized conditions

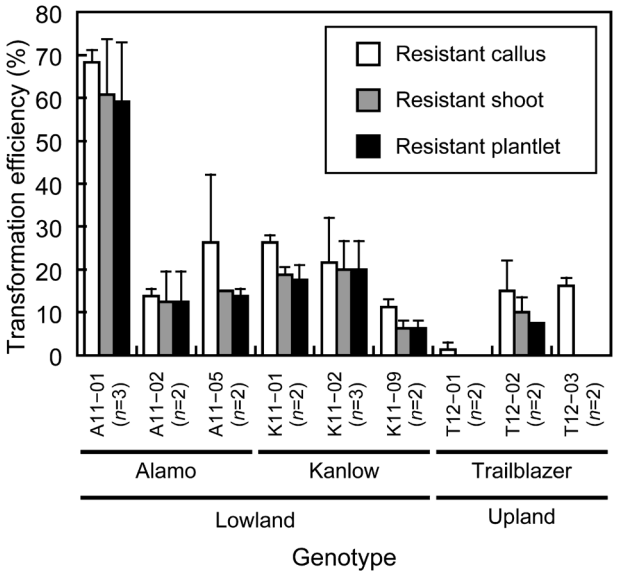

Figure 4. *Agrobacterium*-mediated transformation of embryogenic Type I calli of different switchgrass genotypes. Transformation experiments were replicated two or three times using 40 calli in each experiment. The number of replicates is indicated in parentheses below each cell line. The data are indicated as the mean±SD of replicates.

for different genotypes, caryopsis-derived Type I callus lines were induced for two lowland cultivars 'Alamo' and 'Kanlow' and three upland cultivars 'Blackwell', 'Cave-in-Rock' and 'Trailblazer' (Figure S1). Approximately 90% of caryopses germinated and formed calli on PVC medium in all cultivars. Fungal contamination was completely suppressed by fungicide treatment between dehusking and surface sterilization. More than 10% of caryopses formed Type I calli in both lowland cultivars and the remainder of caryopses formed non-embryogenic friable calli (Type III). In contrast, Type I calli formed on only

1.9% of caryopses of 'Trailblazer' (6/320 caryopses in duplicates). Compact callus was occasionally formed in 'Cave-in-Rock' (1/320 caryopses). However, this callus showed slow growth, mucoid texture, severe browning and low regeneration ability, and thus was not used further. All calli formed on 'Blackwell' caryopses were non-embryogenic friable calli.

Three callus lines each for 'Alamo', 'Kanlow' and 'Trailblazer' were subjected to the optimized transformation conditions in 2–3 independent experiments (Figure 4). Although transformation efficiencies varied among genotypes, all three callus lines of the lowland cultivar 'Alamo' showed vigorous growth and GFP expression and most of the lines regenerated Bar-resistant shoots (Figure 5). These shoots were rooted with herbicide dip selection and showed healthy growth without apparent abnormalities. Transformation efficiencies of the 'Alamo' callus lines were comparatively higher than other cultivars (12.5–59.2%). In the lowland cultivar 'Kanlow', slight overgrowth of *Agrobacterium* was observed below and around calli after 7 days of cocultivation, but it was completely controlled in subsequent cultures on medium containing meropenem, which is a highly active antibiotic against *Agrobacterium* (Ogawa and Mii 2007). Transgenic plants were recovered from all three callus lines of 'Kanlow' at relatively high efficiencies (6.3–20.0%). Some Bar-resistant calli of 'Kanlow' were smaller than those of 'Alamo', and an additional 1–2 cycles (2–4 weeks) of regeneration was required for shoot development sufficient for rooting.

One line of the upland cultivar 'Trailblazer', T12-01, produced occasionally Bar-resistant calli, but no resistant shoot was regenerated from these calli. Another 'Trailblazer' line T12-02 produced small Bar-resistant

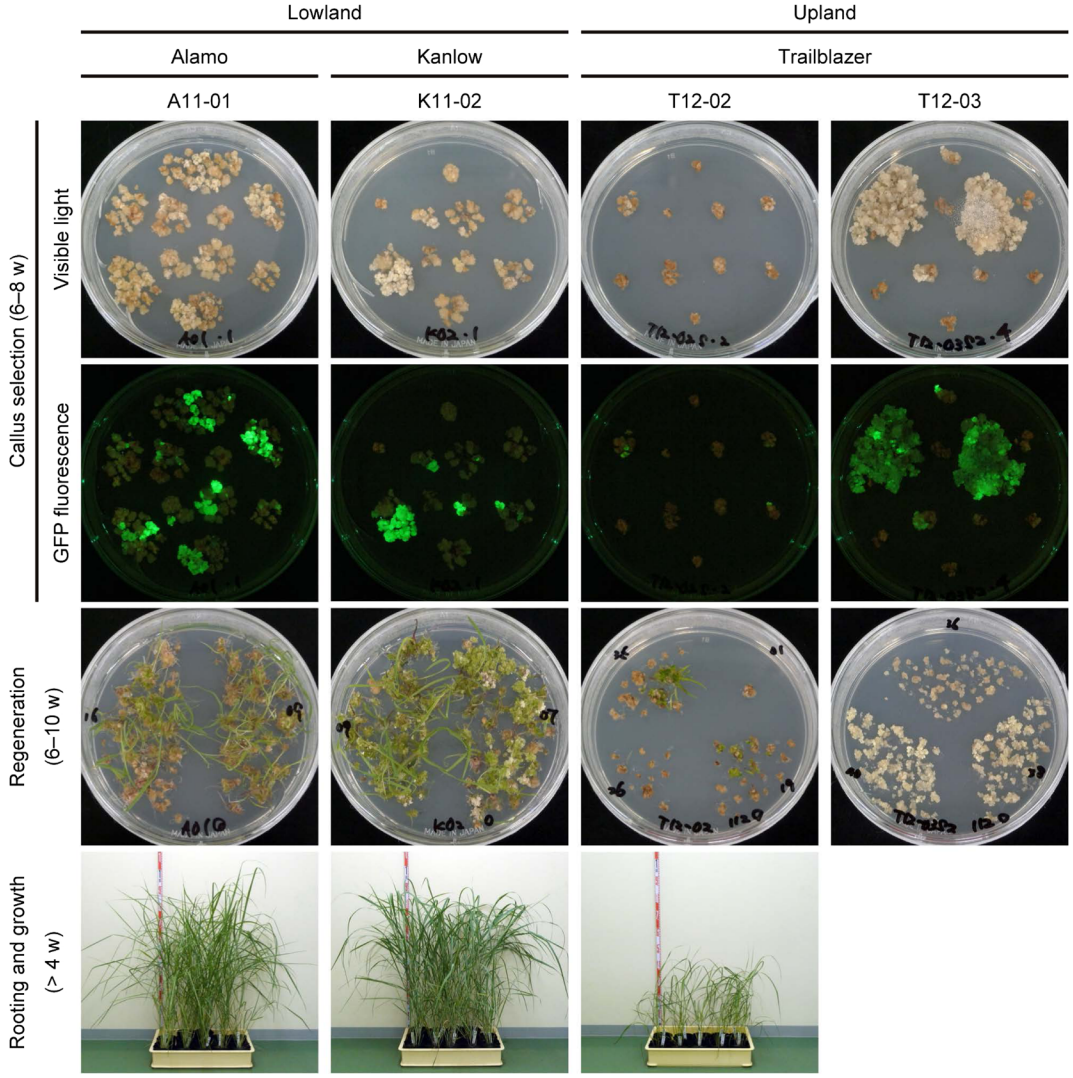

Figure 5. Stages in the *Agrobacterium*-mediated transformation of different switchgrass genotypes. Calli cocultivated with *Agrobacterium* carrying a pHKGB112 were cultured on PVC-S medium for 6–8 weeks and were observed under visible light and blue light for GFP fluorescence (upper). Bar-resistant calli were cultured on PVS-S medium for 6–10 weeks for regeneration (middle). Regenerated shoots were rooted and selected on PVR-S medium with a herbicide dip method, and then Bar-resistant plantlets were potted and acclimatized for further growth (lower).

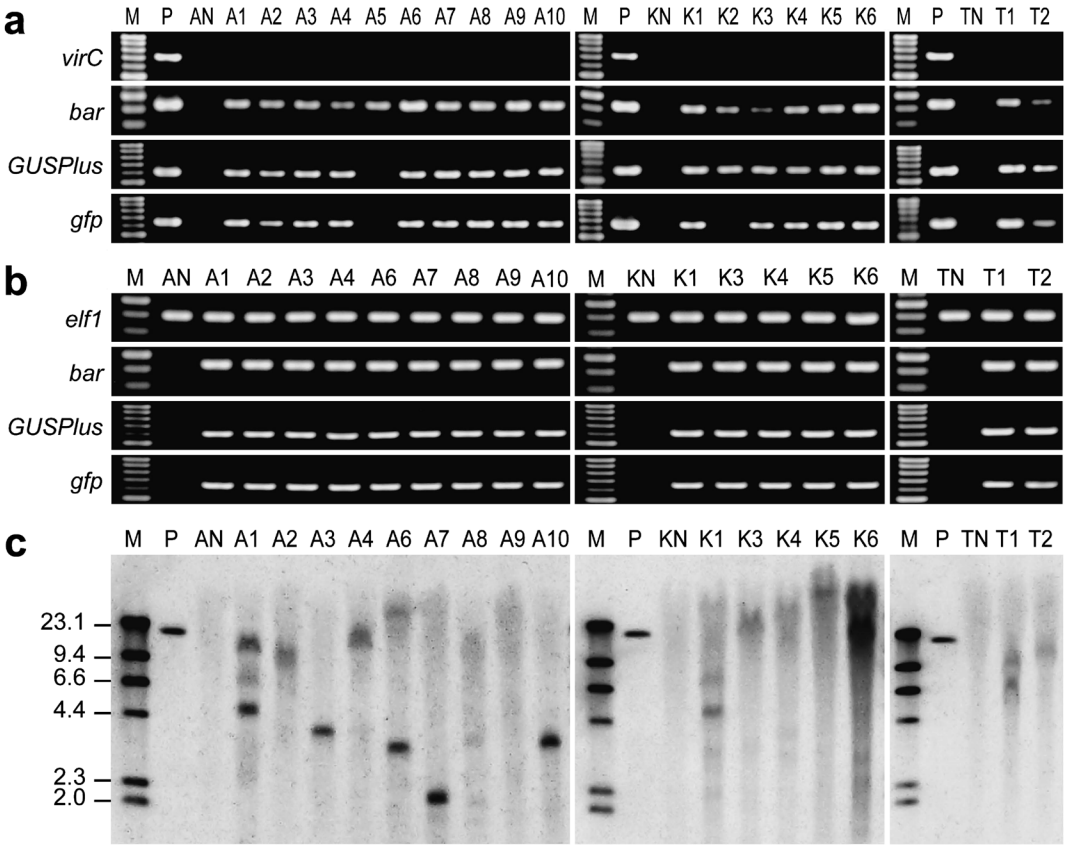

Figure 6. Genomic PCR (a), RT-PCR (b) and Southern blot hybridization (c) analyses of putatively transformed plants. Lane M, marker; lane P, plasmid pHKGB112 as the positive control; lane AN, non-transformed plants of 'Alamo'; lanes A1–10, putatively transformed plants of 'Alamo'; lane KN, non-transformed plants of 'Kanlow'; lanes K1–6, putatively transformed plants of 'Kanlow'; lane TN, non-transformed plants of 'Trailblazer'; lanes T1–2, putatively transformed plants of 'Trailblazer'.

GFP-expressing calli and, similar to 'Kanlow', resistant shoots were formed with an additional 1–2 cycles of regeneration. The transformation efficiency of this line was comparably high (7.5%) compared with previously reported efficiencies for 'Alamo' Type I callus. The callus line T12-03 was compact and partially friable after <4 months of induction, but changed to Type II-like friable callus during subculture and transformation (Figure S1). This line produced vigorously growing, friable Bar-resistant calli at relatively high efficiency (16.3%). Although these calli were cultured further on PVS-S medium and showed GFP-expressed fluorescence, no resistant shoot was regenerated (Figure 5). Given that shoot regeneration also failed from non-transformed control calli of this line on selective agent-free medium, it was concluded that the regeneration ability of this line was lost during subculture and transformation.

Molecular analyses of transgenic plants

In total 381 Bar-resistant plantlets, which were obtained from different calli as independent events after rooting with herbicide dip selection in this study, 346 plantlets (90.8%) showed distinct GFP fluorescence. In the genomic PCR analysis of 144 Bar-resistant plantlets, *bar*-specific fragments were amplified in all of the putative transformants, but *GUSPlus*- and *gfp*-specific bands were not amplified in some transformants (Figure 6a). Among 109 *bar*-positive and GFP-expressing plantlets, 103 plantlets (94.5%) showed *GUSPlus*-specific bands as well as *gfp*-specific bands. PCR-positive plants were subsequently subjected to RT-PCR analysis, and all tested plants showed *bar*, *GUSPlus* and *gfp* expression (Figure 6b). These results support the previous report, which demonstrated the reliability of herbicide dip selection for elimination of non-transformants and of a MultiRound Gateway vector for multigene transformation (Ogawa et al. 2014). Southern blot hybridization confirmed integration of a T-DNA fragment containing the *bar* gene in all transgenic lines tested (Figure 6c). Most of the transgenic lines carried the *bar* gene in a single copy or low (2–3) number of copies.

Discussion

We successfully established a simple and efficient transformation method for caryopsis-derived Type I callus of switchgrass genotypes in the present work. Transgenic plants were obtained in all tested callus

lines of two lowland cultivars, 'Alamo' and 'Kanlow', at comparably higher efficiency than that achieved in previous reports using Type I callus. The transformation efficiency of one 'Alamo' line, A11-01, was as high as 59.2%, which was comparable to the previously reported efficiency for Type II callus of 'Alamo' at 56.6% by Li and Qu (2011) and 72.8% by Liu et al. (2015). In addition, we successfully transformed the upland cultivar 'Trailblazer' using the present method. However, transformation of upland cultivars was still limited to one of three lines of 'Trailblazer' (line T12-02 at 7.5% of efficiency) and no transformants were obtained from calli of two additional upland cultivars, 'Blackwell' and 'Cave-in-Rock'. Seo et al. (2010) induced embryogenic callus of 'Blackwell GR63', although the frequency of shoot regeneration was low (10.9% of calli inoculated). Song et al. (2012) reported that herbicide- or hygromycin-resistant calli were induced from inoculated seedling segments of 'Cave-in-Rock' but no transgenic shoots were regenerated. Very recently, Liu et al. (2015) observed "shell-core" structure within a clump of caryopsis-derived callus of switchgrass cultivars. They induced Type II calli from pre-embryogenic core structures and successfully transformed one of the upland cultivar 'Dacotah' at the 8.0% of efficiency. However, transformation has not been tried in their work on another upland cultivar 'Blackwell', which was lower occurrence of a core structure and lower regeneration ability of Type II callus than 'Dacotah' and the lowland cultivars. Therefore, optimization of Type I callus induction, in similar to Type II callus induction, is a prerequisite for further efficient transformation of upland cultivars of switchgrass.

It should be noted that albino shoots were formed at a negligibly low frequency with the present method using 6- to 12-month-old caryopsis-derived Type I calli. Frequent albino formation was reported in transformation methods using Type II calli derived from caryopses (Li and Qu 2011) and seedlings (Ramamoorthy and Kumar 2012). A highly-efficient transformation method of Liu et al. (2015) has been used freshly established 4-month-old Type II calli for transformation experiments and relationships between subculture period and albino formation as well as transformation efficiency were not described. Considering the availability of explants and prevalence of formation, caryopsis-derived callus is the most useful material for switchgrass transformation. The disadvantage of caryopsis-derived callus is that the genotype of the callus line depends on that of the original caryopsis. Continuous subculturing of transformation-competent elite lines is required to successively obtain transformants possessing a specific genotype, although caryopsis-derived Type I calli can be maintained for at least 2 years without loss of regeneration ability and increase in albino formation rate (Ogawa 2015) and can be successfully used for transformation at least 1 year after induction in the previous (Ogawa et al. 2014) and the present studies. Such a requirement may be overcome by cryopreservation for embryogenic switchgrass calli. It is of interest to examine whether Type I callus derived from other explants, such as an inflorescence or seedling, and from other grass species can be transformed by the present method.

We established an efficient method by mainly modifying the cocultivation conditions, which is the essential period for transfer of T-DNA from an *Agrobacterium* cell to a plant cell, avoiding the need for additional time-consuming procedures before and after cocultivation. T-DNA transfer is mediated by *vir* genes on the Ti plasmid, and *vir* genes are induced by various factors (Gelvin 2006). It is reported that the optimum temperatures for *vir* gene induction and T-DNA transfer are below 25°C and 19–22°C, respectively (Alt-Moerbe et al. 1988; Dillen et al. 1997). A monosaccharide glucose induces *vir* genes (Ankenbauer and Nester 1990; Shimoda et al. 1990) and is used in the cocultivation medium for transformation of monocotyledonous species (Cheng et al. 2004). Low temperature and glucose also improved the transformation efficiency for Type I callus of switchgrass. Although an acidic pH also induced *vir* genes (Stachel et al. 1986), cocultivation at pH 5.2 resulted in a lower transformation efficiency than that at pH 5.7. This result suggested that pH 5.7 was sufficient to induce *vir* gene expression in *Agrobacterium* cells and pH 5.2 might have a physiologically negative effect on switchgrass cells and T-DNA integration in the plant genome (Gelvin 2010). Preculture conditions were also important to improve transformation efficiency. It has been suggested that cell straining treatment is useful to select embryogenic cells possessing high regeneration ability without the need for skillful judgment (Ogawa 2015). It has been also suggested that PVCc medium containing casamino acids is a better medium than proline containing medium to improve shoot regeneration without albino induction (Ogawa 2015).

We successfully established efficient *Agrobacterium*-mediated transformation method for Type I callus of switchgrass, both lowland and upland cultivars, in this study. Our transformation method is much simpler than most existing methods for switchgrass. Time-consuming procedures such as cold treatment, vacuum infiltration, desiccation, resting and washing before and after cocultivation are unnecessary in our method. Although Liu et al. (2015) established a method for induction of transformation-competent Type II calli, pre-embryogenic calli must be selected and separated from a core structure within a mature seed-derived callus clump using a sharp-tip tweezer under a stereomicroscope to induce and proliferate Type II calli. In contrast, transformation-competent small Type I clumps can be prepared by a

simple cell straining followed by 2-weeks preculture without laborious selection in our method. The simplicity of the method does not require especially skilled workers. Thus, the present transformation method is suitable for routine and high throughput transformation experiments in most laboratories and would be a potent protocol for compact embryogenic Type I callus of other grass species.

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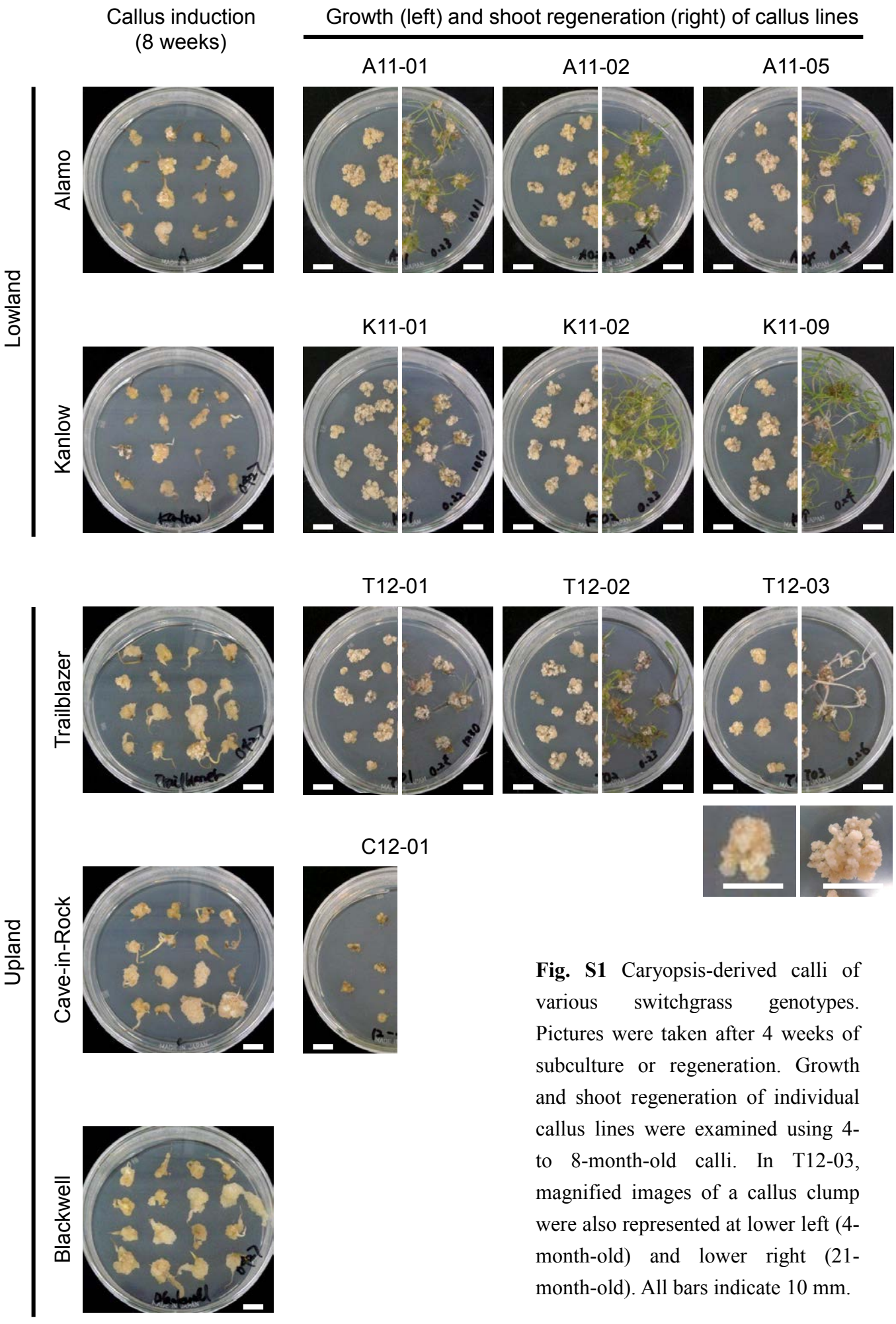

Fig. S1 Caryopsis-derived calli of various switchgrass genotypes. Pictures were taken after 4 weeks of subculture or regeneration. Growth and shoot regeneration of individual callus lines were examined using 4- to 8-month-old calli. In T12-03, magnified images of a callus clump were also represented at lower left (4-month-old) and lower right (21-month-old). All bars indicate 10 mm.