Plant Biotechnology 34, 143–150 (2017)
DOI: 10.5511/plantbiotechnology.17.0623a

Original Paper

Plant regeneration from embryogenic callus derived from shoot apices and production of transgenic plants by particle inflow gun in dwarf napier grass (*Pennisetum purpureum* Schumach.)

Takahiro Gondo[1,†], Nafiatul Umami[2,†], Melody Muguerza[3], Ryo Akashi[3]*

[1]Frontier Science Research Center, University of Miyazaki, 1-1 Gakuenkibanadai-Nishi, Miyazaki 889-2192, Japan; [2]Faculty of Animal Science, Gadjah Mada University, Jl. Fauna 01 Sleman Yogyakarta 55281, Indonesia; [3]Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-Nishi, Miyazaki 889-2192, Japan
*E-mail: rakashi@cc.miyazaki-u.ac.jp Tel: +81-985-58-7257 Fax: +81-985-58-7761

Received February 3, 2017; accepted June 23, 2017 (Edited by S. Nonaka)

Abstract Napier grass (*Pennisetum purpureum* Schumach.) is a highly productive C4 tropical forage grass that has been targeted as a potential bioenergy crop. To further increase the efficiency of bioethanol production by molecular breeding, a reliable protocol for genetically transforming napier grass is essential. In this study, we report the creation of transgenic napier grass plants derived from embryogenic callus cultures of shoot apices. Embryogenic callus was initiated in three accessions of napier grass and a napier grass×pearl millet hybrid using Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg L^{-1} 6-benzylaminopurine (BAP) and 50 μM copper sulfate ($CuSO_4$). Of the accessions tested, a dwarf type with late-heading (DL line) had the best response for embryogenic callus formation. Highly regenerative calli that formed dense polyembryogenic clusters were selected as target tissues for transformation. A plasmid vector, pAHC25, containing an herbicide-resistance gene (*bar*) and the β-glucuronidase (GUS) reporter gene was used in particle bombardment experiments. Target tissues treated with 0.6 M osmoticum were bombarded, and transgenic plants were selected under 5.0 mg L^{-1} bialaphos selection. Although a total of 1400 target tissues yielded nine GUS-positive bialaphos-resistant calli, only one transgenic line that was derived from target tissue with the shortest culture term produced four transgenic plants. Thus, the length of time that the target tissue is in callus culture was one of the most important factors for acquiring transgenic plants in napier grass. This is the first report of successfully producing transgenic napier grass plants.

Key words: bioenergy plant, C4 tropical grass, dwarf napier grass, genetic transformation, particle inflow gun.

Introduction

Bioenergy refers to renewable energy from biological sources and its global production has increased from 45,933 million L in 2006 to 117,715 million L in 2013 (Koizumi 2015). Production of first-generation biofuels such as starch- and sugar-derived ethanol and plant oil-derived biodiesel is unlikely to increase significantly, whereas the use of lignocellulosic ethanol in biofuel production is expected to expand (Wang et al. 2016). Lignocellulosic ethanol, a second-generation biofuel, has the potential to fill most global transportation fuel needs and does not present a conflict between energy demands and the food supply (Sims et al. 2010). More importantly, grass biomass is one of the world's most productive and sustainable lignocellulosic bioenergy sources (Jørgensen 2011).

Napier grass (*Pennisetum purpureum* Schumach.) is a C4 tropical grass and an important forage crop in tropical and subtropical areas of the world. Notable for its high biomass production of 67–93 t DM ha^{-1} yr^{-1} (McLaughlin and Kszos 2005; Takara and Khanal 2011), napier grass is the most productive of forage crops. Therefore, the species has recently been targeted as a bioenergy crop, especially as a source for biofuel production. Feedstocks based on lignocellulosic biomass such as grasses and woods do not compete with biofuel production and food supplies such as starch (maize) or sugar (sugarcane and sugar beet) crops (Sims et al. 2010). Moreover, many C4 tropical grasses, including napier grass, are perennials and do not need to be reseeded each growing season; therefore, cultivation costs are lower. However,

[†]These authors contributed equally to this work.
This article can be found at http://www.jspcmb.jp/
Published online September 15, 2017

these grasses have a high lignin content that limits the saccharification of cellulose and hemicellulose for biofuel production (van der Weijde et al. 2013). Lignin content is also a limiting factor in forage utilization; a major problem for cattle feeding on C4 tropical grasses is poor digestibility. Hence, modification of cell wall components in tropical grasses is a breeding objective for both biofuel production and forage improvement.

Napier grass is a cross-pollinating allotetraploid with a chromosome number of 2n=4*x*=28 (Dos Reis et al. 2014; Harris et al. 2010; Negawo et al. 2017) and occurs as two different plant types: the normal type includes cultivars such as 'Merkeron' (Burton 1989) and 'Wruk wona' (Mukhtar et al. 2003), and there is also a dwarf type (Hanna and Monson 1988). Seed production is low and the seeds are normally very small, light, of poor quality, weak seedlings and the spikelets are prone to shattering in this grass (Negawo et al. 2017; Singh et al. 2013). Therefore, seeds are considered inappropriate for the propagation of napier grass and vegetative propagation through stem cuttings is commonly used (Negawo et al. 2017; Singh et al. 2013). An in vitro propagation system was recognized as a powerful tool for producing high quality seedlings (Pongtongkam et al. 2006) and was developed by using multiple-shoot clumps from shoot apices of dwarf napier grass (Umami et al. 2012). This protocol is considered suitable for nursery plant production of dwarf napier grass seedlings with identical morphological characteristics.

Embryogenic callus cultures of napier grass were described in many reports more than three decades ago (Bajaj and Dhanju 1981; Chandler and Vasil 1984; Haydu and Vasil 1981; Wang and Vasil 1982). Regeneration from suspension cultures and protoplasts was also established in the 1980's (Karlsson and Vasil 1986; Vasil et al. 1983). Although genetic transformation has been recently perfected in many C4 grasses (Giri and Praveena 2015; Wang and Ge 2006), there have been no reports of successfully generating transgenic plants in napier grass. Previously, we established efficient transformation systems in other C4 grasses, including bahiagrass (*Paspalum notatum*) (Gondo et al. 2005; Himuro et al. 2009), rhodesgrass (*Chloris gayana*) (Gondo et al. 2009), and ruzigrass (*Brachiaria ruziziensis*) (Ishigaki et al. 2012).

In this study, we report the procedures for callus induction from shoot apices of three napier grass accessions and a napier grass×pearl millet hybrid. Efficient tissue culture system has been established in a dwarf type with late-heading (DL line). In addition, we produced transgenic plants by particle bombardment in the dwarf type napier grass.

Materials and methods

Plant materials and induction of embryogenic callus

The napier grass (*Pennisetum purpureum* Schumach.) accessions used in this study were two normal types, 'Wruk wona' (Mukhtar et al. 2003) and 'Merkeron' (Burton 1989), a dwarf type with late-heading, DL (Mukhtar et al. 2003), and a hybrid of pearl millet (*Pennisetum glaucum* [L.] R. Br.) and napier grass, HY (Table 1). These plant materials were maintained and cultivated by vegetative propagation in the experimental field at the University of Miyazaki. 'Wruk wone' and 'Merkeron' were brought from Kyushu National Agricultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries (MAFF) (present: Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization [NARO]), Japan in the 1980's. DL line was provided by Kasetsart University, Thailand in 1996, although it was originally selected in Florida, USA and introduced to Thailand. Hybrid Napiergrass (HY) is a hexaploid line (2n=6*x*=42) from a cross between napier grass (2n=4*x*=28) and pearl millet (2n=2*x*=14) in Florida University (Schank and Chynoweth, 1993) from which the seeds were obtained but maintained by vegetative propagation in Miyazaki University.

Shoot-tillers used as explants were collected from the experimental field at the University of Miyazaki, Japan. Shoot-tillers washed with running tap water were sterilized with 70% (v/v) ethanol for 2 min (min) and a 2% (v/v) sodium hypochlorite solution for 15 min, followed by three washes with sterile water. Shoot apices were excised from shoot-tillers and cultured on MS medium (Murashige and Skoog 1962) containing 3.0% (w/v) sucrose, 0.3% (w/v) Phytagel (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (v/v) PPM™ (Plant Preservative Mixture, Plant Cell Technology, Inc., Washington DC, USA) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 2.0 mg L^{-1}) and 6-benzylaminopurine (BAP; 0, 0.01, 0.1, 0.5 mg L^{-1}).

Embryogenic callus culture and plant regeneration

Embryogenic calli of DL were transferred onto MS medium with 2.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 50 μM copper sulfate

Table 1. Formation of embryogenic callus from shoot apices in three napier grass accessions and a hybrid napier grass.

Genotype[†]	No. of shoot apices	No. of embryogenic calli formed	% of embryogenic calli formed
WK	60	0	0.0
ME	60	10	16.7[b]
DL	60	21	35.0[a]
HY	60	5	8.3[b]

Values are the means of three replications. Letters indicate a significant difference by Tukey's test ($p<0.05$).[†] WK, a normal type 'Wruk wona'; ME, a normal type 'Merkeron'; DL, a dwarf type with late-heading; HY, a hybrid of pearl millet and napier grass.

($CuSO_4$) (MS-DBC medium). Single shoot apices, compact calli and dividing clumps were sub-cultured every 14 days onto the same medium. For plant regeneration, embryogenic calli were transferred to MS medium containing combinations of BAP (0, 2.0 mg L^{-1}) and napthaleneacetic acid (NAA) (0, 0.01, 0.1 mg L^{-1}). Regenerated shoots were transferred to 1/2 MS medium for root induction.

All media were adjusted to pH 5.6–5.8 prior to being autoclaved at 121°C for 15 min. All cultures were incubated at 31°C in constant fluorescent light (2000 lux).

Bombardment with a particle inflow gun

The self-built particle bombardment apparatus (spray gun) was constructed as described previously (Akashi et al. 2002). Four to 12-month old embryogenic calli (2–3 mm in diameter), approximately 30 clumps per bombardment, were used as the target tissues. Four hours (h) prior to bombardment, embryogenic calli were transferred to MS-DBC medium supplemented with equimolar amounts of mannitol and sorbitol to yield 0–1.2 M and were left on this medium for 16 h after bombardment as an osmotic post-treatment.

The plasmid pAHC25 (Christensen and Quail 1996), containing the β-glucuronidase (GUS) gene and the bialaphos resistance gene (*bar*) under the control of separate maize *polyubiquitin-1* (*ubi-1*) promoters and its first intron, was used in the transformation experiments. Plasmid DNA was precipitated onto gold particles (1.5–3.0 μm diameter; Aldrich, USA) as described by Gondo et al. (2005). Bombardment was carried out at a reduced air pressure of −0.1 MPa, a target distance of 9.6 cm, a helium pressure of 5 kg/cm^2 and single shots per plate.

The histochemical GUS assay followed a modified Jefferson (1987) method. To assay transient GUS activity 16 h after bombardment, tissues were incubated for 18–24 h at 37°C in 1.9 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), 50 mM phosphate-buffered saline (PBS) pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 20% (v/v) methanol.

Selection and regeneration of transgenic plants

Following bombardment and an osmotic post-treatment, embryogenic calli were placed on MS-DBC medium for 3 days and subsequently sub-cultured several times for 14 days periods on the same medium containing 5 mg L^{-1} bialaphos. After 60–70 day of subculture under selective conditions, bialaphos-resistant clumps were transferred to MS medium supplemented with 2.0 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 5 mg L^{-1} bialaphos and cultured for approximately 30 days to regenerate plants. All regenerated shoots were transferred to hormone-free 1/2 MS medium containing 10 mg L^{-1} bialaphos. After 2–3 weeks, rooted plants were analyzed for GUS expression and by PCR for the presence of foreign DNA. Subsequently, PCR-positive plants were transferred to soil and grown to maturity in the greenhouse.

DNA isolation, polymerase chain reaction (PCR) and DNA gel blot analysis

For PCR, plant genomic DNA was extracted from leaf tissue (0.1–0.5 g) of rooted plantlets using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Primers for the internal 1.1 kb GUS gene fragment were 5′-AAC TGG ACA AGG CAC TAG CGG-3′ and 5′-AAG TTC ATG CCA GTC CAG CGG T-3′ (Jefferson et al. 1986), and primers for the internal 0.34 kb *bar* gene fragment were 5′-CTT CGA GAC AAG CAC GGT CAA CTT C-3′ and 5′-ATA TCC GAG CGC CTC GTG CAT GCG-3′ (Cho et al. 1998). PCR was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA) in a 10 μl reaction that included 0.5 μM of each primer and 25 ng of genomic DNA. Samples were first heated to 95°C for 10 min and then subjected to 35 cycles of the following sequence: 30 s at 95°C, 30 s at 56°C and 1 min at 72°C. Finally, samples were subjected to 72°C for an additional 7 min and stored at 4°C until analyzed. PCR products were separated by 1% agarose gel electrophoresis and visualized with Midori Green (Nippon Genetics, Japan).

Genomic DNA (4 μg) for DNA gel blot hybridization was extracted from leaf tissue by the CTAB method (Murray and Thompson 1980). The DNA was *Sac*I-digested, separated on a 0.8% agarose gel and transferred to Hybond nylon membranes (Roche, Germany). Membranes were hybridized using a 1.1 kb GUS gene fragment labeled with a PCR DIG Probe Synthesis Kit (Roche, Germany). Hybridization signals were visualized on FUJI X-ray Film (Fuji, Japan).

Results

Embryogenic callus formation and plant regeneration

Initial explants of shoot apices were isolated from the shoot tillers of three napier grass accessions and one napier grass×pearl millet hybrid. Shoot apices were cultured on MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. After 10 days of culture, callus formation from the apical meristems was evident (Figure 1A). Embryogenic callus was visible after 40 days of culture (Figure 1B). The frequency of embryogenic callus formation varied with the genotype of the accessions. Table 1 summarizes the results after 50 days of culturing the shoot apices from the four accessions. Embryogenic callus formation was found to be best from 'DL' with 35% of the shoot apices producing embryogenic calli. The addition of 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP to MS medium was the most effective hormone combination for embryogenic callus formation (Table 2). After 50 days in culture, high quality, compact embryogenic callus was selected and repeatedly subcultured on MS medium supplemented with 2.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 50 μM $CuSO_4$ (MS-DBC medium) at 14-day intervals. These callus cultures resulted in a higher frequency of pre-embryo formation and more compact callus clusters

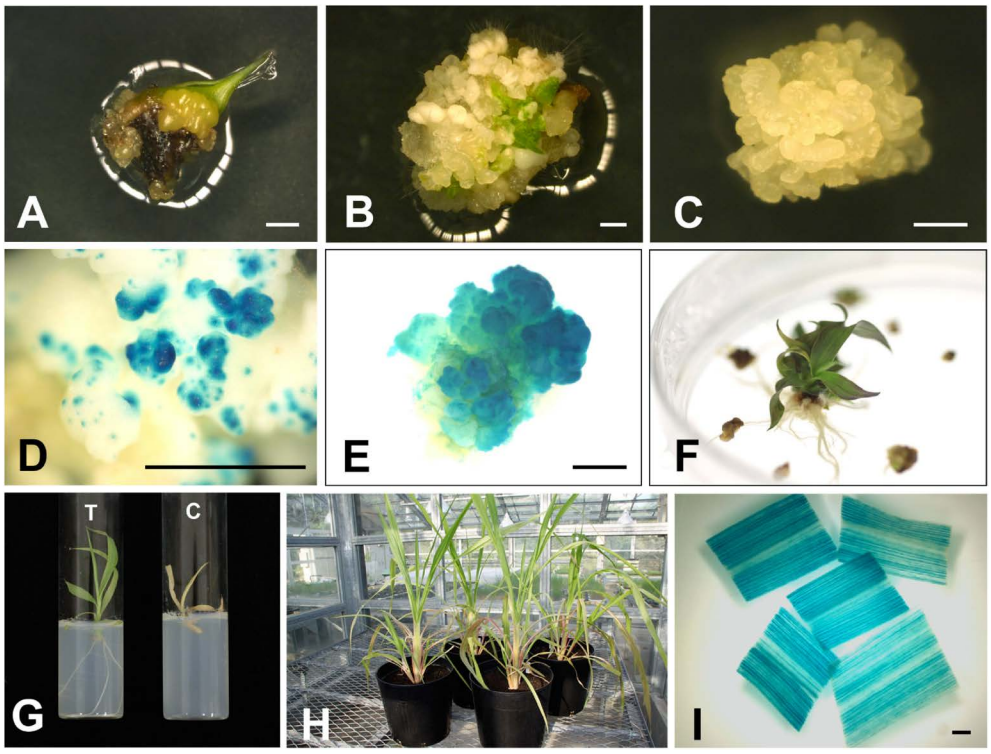

Figure 1. Formation of embryogenic callus and genetic transformation in dwarf napier grass line (DL) mediated by particle inflow gun transformation. (A) An excised shoot apex from a shoot tiller served as the initial explant. (B) Primary embryogenic callus after 45 day of culture on MS medium containing 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. (C) Compact and proliferating uniform embryogenic callus (target tissue). (D) Transient GUS expression 16 h after bombardment. (E) Stable GUS expression on bialaphos-resistant callus. (F) Plant regeneration from transformed callus in MS medium containing 2.0 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 5.0 mg L^{-1} bialaphos. (G) Rooting of transgenic plants on half-strength MS medium with 10.0 mg L^{-1} bialaphos (T, transgenic plant; C, non-transgenic plant). (H) Potted and growing transgenic plants. (I) GUS expression in leaves of transgenic plants. Bars represent 1 mm.

Table 2. Effect of hormone concentration on embryogenic callus formation from shoot apices of dwarf napier grass DL.

Hormone concentration (mg L^{-1})		No. of shoot apices	No. of embryogenic calli formed	% of embryogenic calli formed
2,4-D	BAP			
0	0	60	0	0
2	0	60	0	0
2	0.01	60	5	8.3[b]
2	0.1	60	12	20.0[a]
2	0.5	60	15	25.0[a]

Values are the means of three replications. Letters indicate a significant difference by Tukey's test ($p<0.05$).

Table 3. Effect of hormone concentration on plant regeneration from embryogenic calli of dwarf napier grass DL.

Hormone concentration (mg L^{-1})		No. of calli	No. of regenerated calli (%)	No. of regenerated plants
BAP	NAA			
0	0	60	13 (20.6)[b]	26[b]
2	0	60	9 (14.3)[b]	28[b]
2	0.01	60	7 (11.1)[b]	16[b]
2	0.1	60	25 (39.7)[a]	55[a]

Values are the means of three replications. Letters indicate a significant difference by Tukey's test ($p<0.05$).

(Figure 1C). The percentage of plants regenerated from embryogenic calli ranged from 11.1 to 39.7% in some hormone combinations; 2.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA was the most effective hormone combination/concentration for plant regeneration (Table 3).

Transient GUS expression

Osmotic stress (equimolar amounts of mannitol and sorbitol) affected the transient GUS expression rate. The highest expression of GUS in embryogenic calli was found at 0.6 M osmoticum, 0.3 M mannitol and 0.3 M sorbitol (Figure 1D, Figure 2). Higher concentrations of the osmotic treatment decreased GUS expression and induced callus necrosis. Thus, we chose to use 0.6 M osmoticum as the best concentration for bombarding embryogenic callus without causing cell damage.

Selection and recovery of transgenic plants

Bombarded tissues were placed on MS-DBC medium for three days, and subsequently subcultured several times for 14-day periods on the same medium containing 5 mg L^{-1} bialaphos. During selection on bialaphos-containing medium, almost all bombarded calli gradually

turned brown, but bialaphos-resistant cells proliferated and were identified within 60–70 days. Further, these newly formed calli had stable GUS expression (Figure 1E). A total of nine transgenic calli were obtained from 1400 pieces of target tissues in six transformation experiments for a transformation efficiency of 0.64% (Table 4). However, only one callus line produced transgenic plants with the remaining lines unable to regenerate (Figure 1F). Transgenic plants were selected on 1/2 MS medium with 10 mg L^{-1} bialaphos (Figure 1G), and the presence of GUS expression and the *bar* gene by PCR were confirmed in all rooted plants.

Analysis of the transgene

Four months after bombardment, transgenic plants were potted in soil and grown in the greenhouse (Figure 1H). The presence of the GUS gene in the genomic DNA of a transgenic napier grass line was confirmed by DNA gel blot hybridization analysis (Figure 3). Four transgenic plants were regenerated from a single cell line and were characterized as having the same band pattern resulting from the identical transformation event. *Sac*I-digested DNA samples were used in this analysis, and three GUS gene copies integration were identified in each of the four transgenic plants and the GUS gene expression were confirmed in the leaves (Figure 1I).

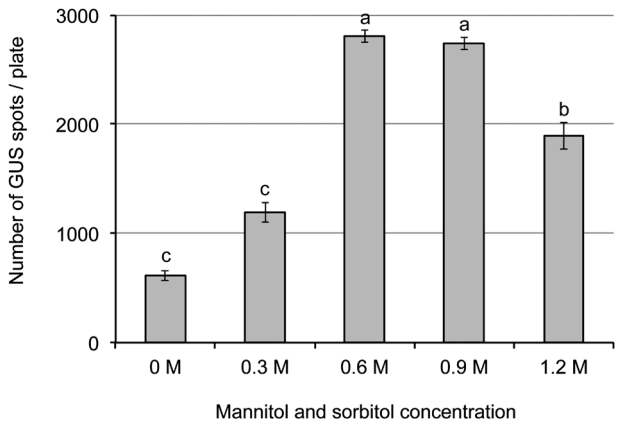

Figure 2. Effect of osmotic treatment with different mannitol and sorbitol concentrations on transient GUS expression in embryogenic calli of dwarf napier grass DL. Values are the means of three replications, and vertical bars represent standard deviations. Letters indicate a significant difference by Tukey's test ($p<0.05$).

Discussion

In previous reports, a single napier grass cultivar, 'Merkeron', was primarily used in tissue culture experiments (Haydu and Vasil 1981; Wang and Vasil 1982). The tissue culture system that was used in the 1980's was not sufficient for producing transgenic plants due to the low quality of embryogenic callus and the low efficiency of plant regeneration. In this report, we have produced transgenic napier grass plants for the first time through the selection and adaptation of a genotype that leads to efficient embryogenic callus formation. 'DL' is the best accession for callus induction, and high quality callus lines were screened on MS medium with 2.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 50 μM $CuSO_4$. This highly regenerative callus formed dense polyembryogenic clusters (Figure 1C). A key factor for this success was likely the specific quality of an embryogenic callus for the target tissue used for transformation. We previously established a genetic transformation

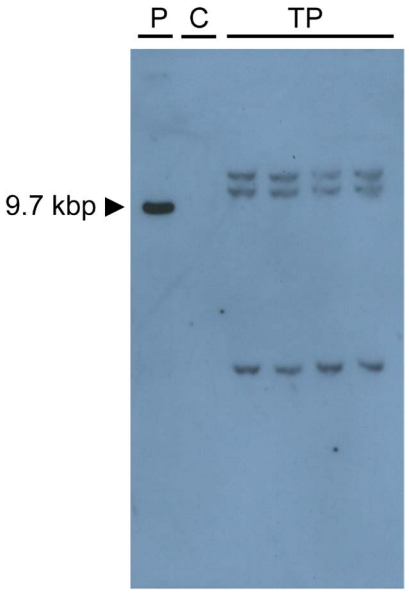

Figure 3. DNA gel blot analysis of transgenic napier grass. Four μg genomic DNA isolated from leaf tissue of a non-transgenic plant (C) and four transgenic plants from a single line (TP) was digested with *Sac*I. Hybridization was carried out with a DIG-dUTP-labeled GUS gene probe. P is a positive control of 5 pg *Sac*I-digested plasmid pAHC25 DNA.

Table 4. Effect of embryogenic callus culture term on regeneration from transformed callus in dwarf napier grass DL.

Experiment	Culture term (days)	No. of bombarded calli	No. of bialaphos resistance calli	No. of GUS-positive calli (%)	No. of regenerated calli	No. of GUS-positive transgenic lines
1	140	200	6	1 (0.5)	1	1
2	156	200	6	0	1	0
3	198	200	12	0	1	0
4	223	200	6	3 (1.5)	0	0
5	293	200	6	1 (0.5)	0	0
6	356	400	12	4 (1.0)	0	0

system for bahiagrass (Gondo et al. 2005) and ruzigrass (Ishigaki et al. 2012), both C4 grasses. This optimized callus culture system was applied to napier grass in this report. Although nine transformed calli were produced under bialaphos selection, only one transgenic line was recovered (Table 4). We hypothesize that this low recovery of transgenic lines is due to a decrease in plant regeneration potential after a long culture period. Long-term cultures are often reported to lead to decreased plant regeneration efficiency and increases in the number of albino plant regenerants (Cho et al. 1998; Gondo et al. 2005; Lambé et al. 1998). Similar phenomenon has occurred within a 3-month culture term in napier grass (Chandler and Vasil 1984) and our data showed that we obtained a transgenic plant which was recovered from callus with the shortest culture term of 140 days (Table 4). Therefore, we recommend to either limit the culture term for 4 to 5 months or devise a different tissue culture system.

The multiple-shoot culture system offers a high potential for plant regeneration, and the capacity is maintained for a longer time than for embryogenic cultures (Gondo et al. 2007). Usually shoot formation is induced by increasing the cytokinin to auxin ratio of the culture medium. The culture system can be proliferated continuously by producing secondary shoot tips maintained as a differentiated tissue structure unlike embryogenic cultures. Multiple-shoot clumps (MSCs) consist of a dense and large primary meristem that is a dome shape, making MSCs a reliable structural characteristic for genetic transformation (Gondo et al. 2009). Previously, we established an in vitro regeneration system via formation of MSCs from shoot apical meristems of napier grass (Umami et al. 2012). This alternative culture system has a high potential for plant regeneration and production of a continuous supply of high-quality target tissue for genetic transformation over long periods of time. Therefore, we hypothesize that the MSC culture system can solve the transformation problems experienced in this study.

Napier grass is targeted as a bioenergy plant because of its highly productive and perennial nature without competing as a human food supply. Down-regulation of the lignin content of grass cell walls directly impacts enzymatic saccharification and creates an efficient bioethanol production system (Chen and Dixon 2007). There are a few reports of transgenic forage grasses expressing modified lignin biosynthesis genes (Zhao and Dixon 2014). Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme involved in the last step of lignin biosynthesis (Tsuruta et al. 2007), and down-regulation of CAD has been reported as an effective way to increase bioethanol production (Fu et al. 2011; Saathoff et al. 2011). Increasing the soluble carbohydrate levels of grasses would strategically improve yield for bioethanol production, too (Farrar et al. 2012). There are some reports that transgenic plants overexpressing a fructan biosynthesis gene produced fructan and increased the total sugar content of rice (Kawakami et al. 2008) and bahiagrass (Muguerza et al. 2013), two plants which otherwise have no system for accumulating fructan. We have already confirmed a decrease in lignin and an increase in soluble carbohydrates by down-regulation of CAD and the overexpression of a fructan biosynthesis gene, respectively, in transgenic bahiagrass (Muguerza et al. 2013; Muguerza et al. 2014). We intend to apply the same strategy to napier grass as a bioenergy crop and will evaluate the utility of the resulting genetic changes.

Now, genome editing is advertised as a new breeding technology in plants and is proposed as the next breakthrough for agriculture (Bortesi and Fischer 2015; Schaeffer and Nakata 2015). This technique induces mutations in targeted genes by genetic transformation. Editing plant genomes without using transgenes may relieve the regulatory concerns associated with genetically modified plants (Waltz 2016). Clearly, the establishment of an effective transformation system is the first step in exploiting the genome editing method in napier grass.

Acknowledgements

We are grateful to Professor Yasuyuki Ishii (University of Miyazaki, Japan) for providing the napier grass accessions and a napier grass×pearl millet hybrid for our plant materials. This work was supported in part by a cooperative Research Grant of the Plant Transgenic Design Initiative, Gene Research Center, the University of Tsukuba

References

Akashi R, Yuge C, Gondo T, Kawamura O, Hoffmann F (2002) Bialaphos-resistant cells of dallisgrass (*Paspalum dilatatum* Poir.) through particle bombardment with a simple self-built inflow gun. *Grassl Sci* 47: 588–593

Bajaj YPS, Dhanju MS (1981) Regeneration of plants from callus-cultures of napiergrass (*Pennisetum purpureum*). *Plant Sci Lett* 20: 343–345

Bortesi L, Fischer R (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol Adv* 33: 41–52

Burton GW (1989) Registration of 'Merkeron' napiergrass. *Crop Sci* 29: 1327

Chandler SF, Vasil IK (1984) Optimization of plant-regeneration from long term embryogenic callus-culture of *Pennisetum purpureum* Schum (napier grass). *J Plant Physiol* 117: 147–156

Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nat Biotech* 25: 759–761

Cho MJ, Jiang W, Lemaux PG (1998) Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Sci* 138: 229–244

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res* 5:

213–218
Dos Reis GB, Mesquita AT, Torres GA, Andrade-Vieira LF, Pereira AV, Davide LC (2014) Genomic homeology between *Pennisetum purpureum* and *Pennisetum glaucum* (Poaceae). *Comp Cytogenet* 8: 199–209
Farrar K, Bryant DN, Turner L, Gallagher JA, Thomas A, Farrell M, Humphreys MO, Donnison IS (2012) Breeding for bioethanol production in *Lolium perenne* L.: Association of allelic variation with high water-soluble carbohydrate content. *Bioenerg Res* 5: 149–157
Fu C, Xiao X, Xi Y, Ge Y, Chen F, Bouton J, Dixon RA, Wang ZY (2011) Downregulation of cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. *Bionerg Res* 4: 153–164
Giri CC, Praveena M (2015) In vitro regeneration, somatic hybridization and genetic transformation studies: An appraisal on biotechnological interventions in grasses. *Plant Cell Tissue Organ Cult* 120: 843–860
Gondo T, Tsuruta S, Akashi R, Kawamura O, Hoffmann F (2005) Green, herbicide-resistant plants by particle inflow gun-mediated gene transfer to diploid bahiagrass (*Paspalum notatum*). *J Plant Physiol* 162: 1367–1375
Gondo T, Matsumoto J, Yamakawa K, Tsuruta S, Ebina M, Akashi R (2007) Somatic embryogenesis and multiple-shoot formation from seed-derived shoot apical meristems of rhodesgrass (*Chloris gayana* Kunth). *Grassl Sci* 53: 138–142
Gondo T, Matsumoto J, Tsuruta S, Yoshida M, Kawakami A, Terami F, Ebina M, Yamada T, Akashi R (2009) Particle inflow gun-mediated transformation of multiple-shoot clumps in rhodes grass (*Chloris gayana*). *J Plant Physiol* 166: 435–441
Hanna WW, Monson WG (1988) Registration of Dwarf N 75 napiergrass germplasm. *Crop Sci* 28: 870–871
Harris K, Anderson W, Malik R (2010) Genetic relationships among napiergrass (*Pennisetum purpureum* Schum.) nursery accessions using AFLP markers. *Plant Genet Resour* 8: 63–70
Haydu Z, Vasil IK (1981) Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* Schum. *Theor Appl Genet* 59: 269–273
Himuro Y, Gondo T, Yamakawa K, Akashi R (2009) Genetic transformation of bahiagrass (*Paspalum notatum* Flügge) by visually screening cells expressing green fluorescent protein. *Grassl Sci* 55: 216–220
Ishigaki G, Gondo T, Suenaga K, Akashi R (2012) Fertile transgenic *Brachiaria ruziziensis* (ruzigrass) plants by particle bombardment of tetraploidized callus. *J Plant Physiol* 169: 546–549
Jefferson RA, Burgess SM, Hirsh D (1986) β-Glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc Natl Acad Sci USA* 83: 8447–8451
Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol Biol* 5: 387–405
Jørgensen U (2011) Benefits versus risks of growing biofuel crops: The case of *Miscanthus*. *Curr Opin Environ Sustain* 3: 24–30
Karlsson SB, Vasil IK (1986) Morphology and ultrastructure of embryogenic cell suspension cultures of *Panicum maximum* (Guinea Grass) and *Pennisetum purpureum* (Napier Grass). *Am J Bot* 73: 894–901
Kawakami A, Sato Y, Yoshida M (2008) Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. *J Exp Bot* 59: 793–802
Koizumi T (2015) Biofuels and food security. *Renew Sustain Energy Rev* 52: 829–841
Lambé P, Mutambel HSN, Deltour R, Dinant M (1998) Somatic embryogenesis in pearl millet (*Pennisetum glaucum*): Strategies to reduce genotype limitation and to maintain long-term totipotency. *Plant Cell Tissue Organ Cult* 55: 23–29
McLaughlin SB, Kszos LA (2005) Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biom Bioen* 28: 515–535
Muguerza M, Gondo T, Yoshida M, Kawakami A, Terami F, Yamada T, Akashi R (2013) Modification of the total soluble sugar content of the C4 grass *Paspalum notatum* expressing the wheat-derived sucrose: Sucrose 1-fructosyltransferase and sucrose:fructan 6-fructosyltransferase genes. *Glassl Sci* 59: 196–204
Muguerza M, Gondo T, Ishigaki G, Akashi R (2014) Lignin content and digestibility in transgenic bahiagrass (*Paspalum notatum* Flügge) obtained by genetic manipulation of cinnamyl alcohol dehydrogenase gene. *Asian J Plant Sci* 13: 8–17
Mukhtar M, Ishii Y, Tudsri S, Idota S, Sonoda T (2003) Dry matter productivity and overwintering ability of the dwarf and normal napiergrasses as affected by the planting density and cutting frequency. *Plant Prod Sci* 6: 65–73
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assay with tabacco tissue culture. *Physiol Plant* 15: 473–497
Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Res* 8: 4321–4326
Negawo A, Teshome A, Kumar A, Hanson J, Jones CS (2017) Opportunities for napier grass (*Pennisetum purpureum*) improvement using molecular genetics. *Agronomy* 7: 28
Pongtongkam P, Peyachoknagul S, Arananant J, Thongpan A, Tudsri S (2006) Production of salt tolerance dwarf napier grass (*Pennisetum purpureum* cv. Mott) using tissue culture and gamma irradiation. *Kasetsart J-Nat Sci* 40: 625–633
Saathoff AJ, Sarath G, Chow EK, Dien BS, Tobias CM (2011) Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PLoS One* 6: e16416
Schaeffer SM, Nakata PA (2015) CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field. *Plant Sci* 240: 130–142
Schank SC, Chynoweth DP (1993) The value of triploid, tetraploid and hexaploid napier grass derivatives as biomass and (or) forage. *Trop Agric (St Augustine)* 70: 83–87
Sims REH, Mabee W, Saddler JN, Taylor M (2010) An overview of second generation biofuel technologies. *Bioresour Technol* 101: 1570–1580
Singh BP, Singh HP, Obeng E (2013) Elephant grass. In: Singh BP (ed) *Biofuel Crops: Production, Physiology and Genetics*. CAB International, Fort Valley State University, Fort Valley, GA, USA, pp 271–291
Takara D, Khanal SK (2011) Green processing of tropical banagrass into biofuel and biobased products: An innovative biorefinery approach. *Bioresour Technol* 102: 1587–1592
Tsuruta S, Ebina M, Nakagawa H, Kawamura O, Akashi R (2007) Isolation and characterization of cDNA encoding cinnamyl alcohol dehydrogenase (CAD) in sorghum (*Sorghum bicolor* (L.) Moench). *Glassl Sci* 53: 103–109
Umami N, Gondo T, Ishigaki G, Rahman MM, Akashi R (2012) Efficient nursery production and multiple-shoot clumps formation from shoot tiller-derived shoot apices of dwarf napiergrass (*Pennisetum purpureum* Schumach.). *J Warm Regional Soci Ani Sci, Japan* 55: 121–127

van der Weijde T, Alvim Kamei CL, Torres AF, Vermerris W, Dolstra O, Visser RG, Trindade LM (2013) The potential of C4 grasses for cellulosic biofuel production. *Front Plant Sci* 4: 1–18

Vasil V, Wang DY, Vasil IK (1983) Plant regeneration from protoplasts of Napier Grass (*Pennisetum purpureum* Schum.). *Z Pflanzenphysiol* 111: 233–239

Waltz E (2016) Gene-edited CRISPR mushroom escapes US regulation. *Nature* 532: 293

Wang DY, Vasil IK (1982) Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpureum Schum* (Napier or elephant grass). *Plant Sci Lett* 25: 147–154

Wang Y, Fan C, Hu H, Li Y, Sun D, Wang Y, Peng L (2016) Genetic modification of plant cell walls to enhance biomass yield and biofuel production in bioenergy crops. *Biotechnol Adv* 34: 997–1017

Wang ZY, Ge Y (2006) Recent Advances in genetic transformation of forage and turf grasses. *In Vitro Cell Dev Biol Plant* 42: 1–18

Zhao Q, Dixon RA (2014) Altering the cell wall and its impact on plant disease: From forage to bioenergy. *Annu Rev Phytopathol* 52: 69–91