Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 4(11) pp. 761-768, November, 2015.
Available online http://garj.org/garjas/home

Full Length Research Paper

Enhanced efficiency of *Agrobacterium*-mediated transformation by sulfamethazine treatment in ravenna grass, *Erianthus ravennae* (L.) Beauv.

Kazuki Shimomae[1)], So Makabe[1)], Tanaphol Boriboonkaset[1)], Dong Poh Chin[1)],Tomoko Igawa[1)], Raham Sher Khan[2)], Masahiro Mii[1), 3)] and Ikuo Nakamura[1)]

[1)] Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo, Chiba 271-8510, Japan.
[2)] Department of Biotechnology, Abdul Wali Khan University, Mardan 23200, Pakistan
[3)] Center for Environment, Health and Field Sciences, Chiba University, 6-2-1 Kashiwanoha, Kashiwa, Chiba 277-0882, Japan.

Accepted 06 November, 2015

***Agrobacterium*-mediated transformation system was developed in ravenna grass [*Erianthus* ravennae (L.) Beauv.], a potential source of bioenergy. *Agrobacterium tumefaciens* EHA101 harboring pGWB-UbiGFP, which contains the maize *ubiquitin* promoter (Ubi)-driven green fluorescent protein (*GFP*) gene and cauliflower mosaic virus (CaMV) 35S promoter-driven hygromycin phosphotransferase (*hpt*) gene, was used for transformation. Calli cultured in liquid medium were infected and co-cultivated for 3 days on medium with all MS-salts removed. The transformed calli were selected on MS media containing 50 mg l^{-1} hygromycin. After 6 months of the selection, 43 hygromycin-resistant callus lines (8.5%) were obtained from 500 infected calli. The GFP florescence was weak in the all hygromycin-resitant calli, likely due to the silencing of *GFP* transgene. These calli were treated with 30 µ M sulfamethazine (SMZ) for 2 months, resulting that SMZ-treated calli exhibited stronger GFP florescence than non-treated calli. Finally, seven shoots (16.3%) were regenerated from the SMZ-treated callus lines, while only one shoot (2.3%) was obtained from the non-treated callus lines. GFP florescence was also observed in leaves, roots, and shoot apex of plants regenerated from the SMZ-treated calli. Integrations of *GFP* and *hpt* transgenes into the genome of regenerated plants were confirmed by PCR and Sothern blot analyses. The expressions of these transgenes were confirmed by RT-PCR.**

Keywords: *Agrobacterium tumefaciens*, *Erianthus*, transformation, sulfamethazine, MS-free medium.

INTRODUCTION

Ravenna grass [*Erianthus ravennae* (L.) Beauv.] is C_4-type perennial grass native to South Europe and West Asia. *Erianthus* spp. can also produce large amount of biomass (40-60 t/ha·yr), which is higher than that of *Miscanthus*

spp. (12–40 t/ha·yr) and switchgrass (7–35 t/ha·yr) (Hattori *et al*. 2010). It is considered as biomass plant for production of cellulosic ethanol, because *Erianthus* species also have beneficial traits such as tolerance against abiotic and biotic stresses, and high-growth under low input conditions of water and nitrogen (Deren *et al*. 1991; Samson et al. 2005; Thetford et al. 2009).

In economical aspect, cellulosic bioethanol production by current technologies is very expensive. For instance, enzyme costs of cellulosic and maize grain bioethanol production are $79.3 and $2.6 - 5.3 per 1000L, respectively (Sainz 2009). Therefore, to solve these problems, it is meaningful to establish *Agrobacterium*-mediated transformation system in *Erianthus* spp. to confer useful traits, such as autolysis of their cellulose fibers to small sugars after the maturation, faster vegetative growth and increased biomass etc. *Agrobacterium*-mediated transformation has been most widely used for genetic engineering of various plants. However, drawbacks such as low efficiency of the transformation and DNA silencing of the transgene were frequently observed in genetic transformation of commercial and industrial plants (Matzke et al. 2002, Baulcombe 2004).

It has been reported that the transformation efficiency was improved by additions of acetosyringone (3', 5'-Dimethoxy-4'-hydroxyacetophenone) (Hiei *et al*. 1997) and 3-amino glutarimide (Sandal *et al*. 2011) into the co-cultivation media, and use of MES (2-morpholinoethane-sulfonic acid) to stabilize pH (Ogaki *et al*. 2008). Azadi *et al*. (2010) found that removal of most MS salts from the co-cultivation medium improved transformation efficiency of *Lilium* × *formolongi*. Therefore, we test the influence of the MS salts-free (MS-free) infection and co-cultivation media on the transformation efficiency. In addition, when we introduced cauliflower mosaic virus 35S promoter (CaMV 35SP) driven green fluorescent protein (*GFP*) gene to confirm transgene expression (Chiu *et al.* 1996), GFP fluorescence was found very weak in all transformed calii. Thus, we tested the effect of sulfamethazine (SMZ), because it has been reported that SMZ recovered the expression of the silenced *GFP* transgene in *Arabidopsis* (Zhang *et al*. 2012). Several papers have been reported on *Agrobacterium*-mediated transformation of grasses, such as *Miscanthus sinensis* (Hwang 2013), switchgrass (Li and Qu 2011), tall fescue (Wang and Ge 2005), creeping bentgrass (Yu *et al*. 2000). In *Erianthus ravennae*, transformation of calli have been reported (Someya *et al*. 2013), however, there has been no report on the production of transgenic plants.

In the present study, we found that SMZ treatment increased *GFP* expression in the transgenic calli, suggesting the suppression of transgene silencing was effective for *E. ravennae* transformation. Here, we report first successful production of transgenic *E. ravennae* plants through *Agrobacterium*-mediated transformation, which will contribute to breed to high biomass yielding cultivars of ravenna grass through the improvement of resistance against biotic and abiotic stresses.

*Corresponding Author's E-mail: inakamur@faculty.chiba-u.ac.jp; Tel & Fax: +81-47-308-8852

MATERIALS AND METHODS

Plant materials and culture condition

Calli (*ca*. 3 g) derived mature seeds of ravenna grass maintained for 8 months on the agar-solidified medium (Shimomae *et al*. 2013) were transferred to the liquid MS medium (Murashige and Skoog 1962) supplemented with 2 mg l^{-1} 2,4-D, 0.2 mg l^{-1} BAP and 30 g l^{-1} maltose, at 25 ± 1°C. For regeneration, calli were transferred onto the shoot regeneration medium comprised of MS medium containing 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, and 8 g l^{-1} agar (Wako Pure Chemical, Tokyo, Japan), as reported by Shimomae *et al.* (2013). The cultures were incubated at 25 ± 1°C under a 16 h light - 8h dark photoperiod at 35 µmol m^{-2} s^{-1}. After 5 weeks of culture, shoot regeneration frequency was evaluated as the percentage of the number of calli with regenerated shoots to total number of calli tested.

Construction of binary vector

A promoter sequence (1.8 kb) of the maize *ubiquitin* (Ubi) gene (Christensen *et al*. 1992) was linked to *GFP* gene within pGWB4 vector by using LR clonase reaction (Nakagawa *et al*. 2007) (Figure. 1). The obtained plasmid pGWB-UbiGFP, which also contains kanamycin (*nptII*: Datla *et al*. 1991) and hygromycin resistance (*hpt*: Van den Elzen *et al*. 1985) cassettes, was transferred into *Agrobacterium tumefaciens* strain EHA101 (Hood *et al*. 1986) by freeze-thaw method (Wise *et al*. 2006).

Infection and co-cultivation of calli with Agrobacterium

The liquid-cultured calli at 10 days from subculture were used for *Agrobacterium*-mediated transformation. The *Agrobacterium* cultured in LB liquid medium (OD_{600}=1.0) was diluted to 1:10 with MS or MS-free infection medium, which are comprised of 30 g l^{-1} maltose, 2 mg l^{-1} 2,4-D, 0.2 mgl^{-1} BAP, 100 µM acetosyringone (3,5-dimethoxy-4-hydroxy- acetophenone; Sigma-Aldrich, St Louis, MO, USA) and 10 mM MES (2-morpholinoethane-sulfonic acid) with and without adding MS salts, respectively. The calli (*ca*. 30 g) were steeped in MS or MS-free infection medium containing the *Agrobacterium* for 15 min. Each infected calli were co-cultivated for 3 days on the MS or MS-free co-cultivation medium solidified with 8 g l^{-1} agar at 25 ± 1°C in the dark.

Plant regeneration from transgenic calli

After 3 days of co-cultivation, the calli were washed with liquid MS medium containing 30 g l^{-1} maltose and 20 mg l^{-1} meropenem (Meropen; Sumitomo Pharmaceuticals, Osaka, Japan) as bactericide to remove *Agrobacterium* (Ogawa and Mii 2007). Then, the calli were then transferred onto selection medium comprised of MS medium containing 30 g l^{-1} maltose, 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, 20 mg l^{-1} meropenem, 50 mg l^{-1} hygromycin, and 8 g l^{-1} agar. After 6 months of the selection, hygromycin-resistant calli were transferred to the shoot regeneration medium supplemented with 20 mg l^{-1} meropenem and 50 mg l^{-1} hygromycin. After 2 months, the regenerated shoots were transferred to rooting medium comprised of half-strength MS medium containing 30 g l^{-1} sucrose, 20 mg l^{-1} meropenem, 50 mg l^{-1} hygromycin, and 8 g l^{-1} agar).

Effect of SMZ treatment on the expression of GFP *gene*

After 6 months from *Agrobacterium*-infection, the 43 hygromycin-resistant callus lines were selected and transferred to the selection medium supplemented with or without 30 µM SMZ (4,6-Dimethysulfadizine; Tokyo Chemical Industry, Tokyo, Japan).

After 2 months of the treatment, *GFP*-expressing callus lines were transferred to the shoot regeneration medium.

The GFP fluorescence was captured with an Olympus SZX9 StereoZoom Microscope (Olympus, Tokyo, Japan) equipped with a GFP detection filter set (excitation filter; 460-490 nm, dichromatic mirror; 505 nm, emission filter; 510 nm) by using an Olympus DP-72 digital camera and accompanying software (celluSens; Olympus). After 2 months of SMZ treatment, GFP fluorescence of the treated and non-treated callus lines was captured at the same exposure time under the fixed sensitivity.

DNA isolation and molecular analyses

Total genomic DNA was extracted from leaves (0.5 g) of transgenic plants using the CTAB methods (Murray and Thompson 1980). *GFP* and *hpt* transgenes were amplified using *gfp* primers (5'-CGGTACCATGGTGAGCAAGGGCGAGGA-3' and 5'-CGGTACCACTTGTACAGCTCGTCCATG-3') and *hpt* primers (5'-AGTCAATG ACCGCTGTTATGCG-3' and 5'-ACAGCGTCTCCGACCTGATGCA-3') (Hamill *et al*. 1991), respectively. PCR was performed as follow: 30 cycles of 94°C for 1 min, 62°C (*gfp*) or 59°C (*hpt*) for 1 min, and 72°C for 1.5 min. Amplified products were separated on a 1.0 % agarose gel and visualized by ethidium bromide staining. For Southern blot analysis, 10 µg of genomic DNA digested with *Hin*dIII, which cuts a single site within the T-DNA, was separated on a 0.8 % agarose gel and transferred to a nylon membrane (Immobilon-Ny + Transfer Membrane; Millipore Co, Billerica, MA, USA). The probe (720 bp) generated using a pair of *gfp* primers by a PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany) was hybridized with the membrane. The hybridized membrane was finally exposed to a detection film (Lumi-Film Chemiluminescent Detection film; Roche Diagnostics) for 1 hour.

For RT-PCR, total RNA was extracted from leaves of transformed *E. ravennae* plants using PurLink™ Plant RNA Reagent (Invitrogen, California, USA). DNAs were removed with RNase-free DNase (TaKaRa, Tokyo, Japan) at 37°C for 30 min.

DNase-treated RNAs samples were reverse-transcribed using a SuperScriptIII Reverse Trascriptase kit (Life technologies, CA, USA) and the resultant cDNA was used as template to amplify expressed *GFP* and *hpt*. *Actin* mRNA of *E. ravennae* was also amplified using primers ′for rice actin gene, 5 -TCCATCTTGGCATCTCTCAG-3′and 5′ -CATGGGAGTAGTCCGTAGAC-3′.

Data analysis

Analysis of variance (ANOVA) was performed using the SPSS statistical package (SPSS Statistics 17.0; IBM, NY, USA). Student's t-test was performed to identify significant differences ($P<0.05$).

RESULTS AND DISCUSSION

Establishment of liquid callus culture in *E. ravennae*

Efficient propagation system of calli, which have high shoot regeneration ability, is prerequisite for the successful *Agrobacterium*-mediated transformation. Growth rate of calli cultured in liquid medium was 5-fold for 2 months while the weight of calli cultured on agar-solidified medium increased only twice. Thus, growth rate of calli in the liquid medium was 2.5-fold higher than that on the agar medium. Although shoot regeneration efficiencies from the calli cultured in liquid (88.5 ± 0.3%) and on agar-solidified medium (89.6 ± 0.2%) were almost same (Table 1), liquid culture system was effective in plant regeneration of *E. ravennae.*

Effect of MS salts on the transformation efficiency

The calli of *E. ravennae* were infected and co-cultivated for 3 days with *A. tumefaciens* EHA101 harboring pGWB-UbiGFP plasmid (Figure. 1). Six months after transferring of the calli to the selection medium containing 50 mgl^{-1} hygromycin, overall 43 lines of hygromycin-resistant calli were obtained from 500 infected-calli (8.5 ± 0.9 % from 3 independent experiments) using the MS-free infection and co-cultivation media. On the other hand, application of

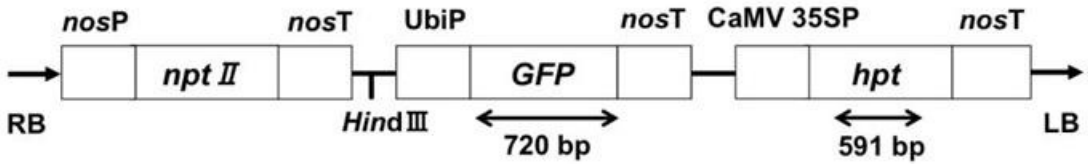

Figure 1 Structure of T-DNA region of pGWB-UbiGFP used in *E. ravennae* transformation. RB and LB: right and left border of the T-DNA region, respectively.
CaMV35SP: Cauliflower mosaic virus 35S promoter, UbiP: ubiquitin promoter, *nos*P: promoter of the nopaline synthase gene (Depicker *et al*. 1882), *nos*T: terminater of the nopaline synthase gene, *GFP*: green fluorescent protein gene, *hpt*: hygromycin phosphotransferase gene, *nptII*: neomycin phosphotransferase II gene. Double arrows: PCR-amplified region used to confirm the existence and expression of the transgene in transgenic plants.

Table 1: Effect of medium type on callus growth and shoot regeneration of *E. ravenane*

Medium type	Callus growth rate (fold) [a]	Shoot regeneration efficiency (%) [b]
Liquid	5.0	87.5 ± 0.3
Agar-solidified	2.0	89.6 ± 0.2

[a] Callus growth rate was calculated as (final callus fresh weight/initial callus fresh weight after 2 weeks of culture). Data were expressed as the average of 3 repeated subcultures.
[b] Each value represents a mean ± SE of three independent experiments. The data are not significantly different at the 5 % level according to student's t-test.

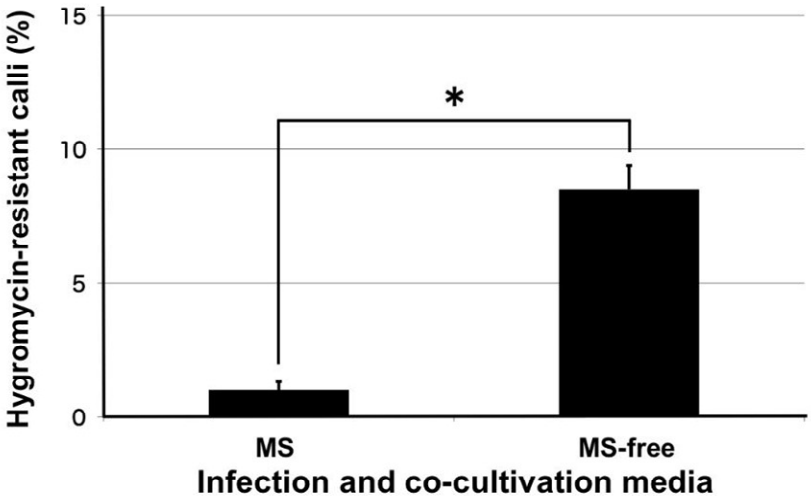

Figure 2 Effect of the type of medium used for infection and co-cultivation on transformation frequency. The percentage of hygromycin-resistant calli per total infected 500 calli of three independent experiments was calculated after 6 months of the selection. Each value with vertical bar represents a mean ± SE of three independent experiments. Asterisk indicates that the data are significantly different at 5 % level according to the student's t-test.

MS media for the infection and co-cultivation resulted in significantly decreased frequency of hygromycin-resistant calli production (5 lines, 1.0 ± 0.3 %) (Figure. 2). These results were similar to the finding reported by Azadi *et al*. (2010). It was suggested that most of the major mineral salts in MS medium reduced the efficiency of *Agrobacterium*-mediated transformation in lily (Azadi *et al*. 2010), although the mechanism has still unknown.

Effect of SMZ on the GFP *expression*

In genetic transformation studies, DNA silencing of

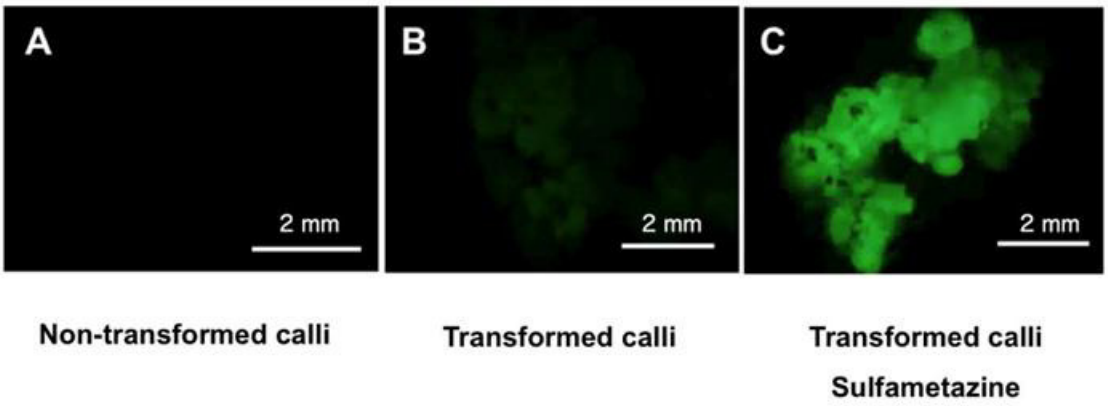

Figure 3 Effect of SMZ treatment on *GFP* expression in the transgenic calli. The hygromycin-resistant calli were cultured on the selection medium supplemented with 30 µM SMZ, and images were captured after 2 months of treatment. (a) non-trasnsformed calli, (b) hygromycin-resistant calli without SMZ treatment, (c) hygromycin-resistant calli treated with 30 µM SMZ. Bars = 2 mm.

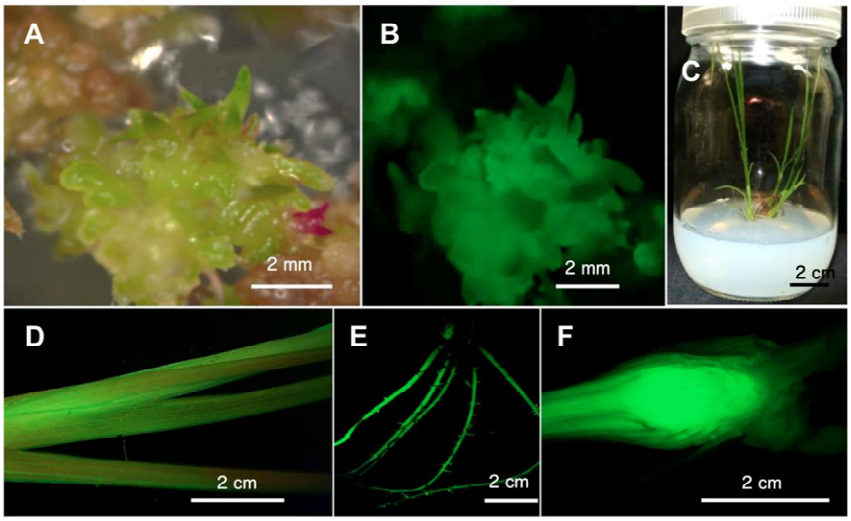

Figure 4 Production of transgenic plants of *E. ravennae*. Regenerated shoot from hygromycin-resistant calli (a) showed *GFP* expression (b) after 1 month of shoot induction. (c) Hygromycin-resistant transgenic plantlet derived from SMZ-treated calli.
GFP expression was detected in leaves (d), roots (e) and shoot apex (f) of transgenic plants.

transgene is one of general problems (Mishiba *et al*. 2005; Domínguez *et al*. 2002; Elmayan and Vaucheret 1996).

All 43 lines of hygromycin-resistant calli obtained in this study showed weak *GFP* expressions after 6 months of culture on the selection medium (Figure. 3b) because *GFP* transgene was probably silenced by post-transcriptinal mechanism (Baulcombe 2004).

Since SMZ was reported as a suppressive reagent of DNA silencing (Patra and Bettuzzi 2009; Monneret 2005, Zhang *et al*. 2012), the hygromycin-resistant calli were treated with SMZ to test the recovery of *GFP* expression.

After 2 months of culture on the selection medium supplemented with 30 µM SMZ, all 43 callus lines grew and increased the GFP fluorescence (Figure. 3c). With higher concentration (50-100 µM) of SMZ, calli were turned brown and retarded the growth (Data not shown). As Zhang *et al*. (2012) reported that SMZ had cytotoxic effects such as growth retardation in *Arabidopsis*, 30 µM of SMZ treatment was suitable to increase the expression of *GFP* transgene in hygromycin-resistant calli of ravenna grass.

Regeneration of transgenic plants from GFP-*expressing calli*

A month after transferring to the regeneration medium, the SMZ-treated calli showed higher shoot regeneration efficiency (7 lines, 16.3%) compared to the non-treated calli (1 line, 2.3%). The regenerated shoots showed strong GFP fluorescence (Figure. 4) and produced roots after 2 months of culture in the rooting medium (Figure. 4c). These results indicated that SMZ treatment was effective to increase the expression of silenced *GFP* gene

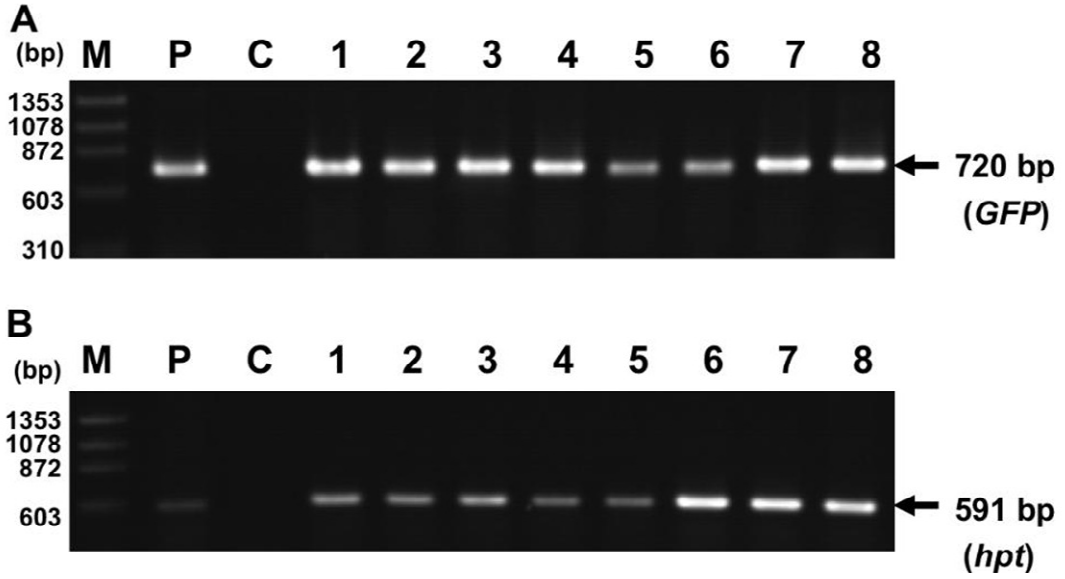

Figure 5 Genomic PCR analysis for *GFP* (a) and *hpt* (b) genes with transgenic plants.
Lane M: molecular size marker (λ/*Hin*dIII,φ×174/*Hae*III), Lane P: positive control (pGWB-UbiGFP), Lane C: negative control (non-transformed control plant), Lanes 1-7: transformed plants regenerated from SMZ-treated calli (#1-7) and Lane 8: non-treated calli (#8).

as well as *hpt* gene because the shoot regeneration frequency was also improved. *GFP* expression was detected in leaves (Figure. 4d), roots (Figure. 4e) and shoot apex (Figure. 4f) of the regenerated plants from SMZ-treated calli (line #3 in Figure. 5 and 6), while regenerated plants from non-treated calli (line #8 in Figure. 5 and 6) did not show GFP florescence (data not shown).

Molecular analyses of transgenic plants

Among the 8 transgenic plants obtained in this study, 7 lines (#1-7) and 1 line (#8) were regenerated from SMZ-treated and non-treated calli, respectively. Genomic PCR analysis of all the 8 transgenic plants showed 720 and 591 bp fragments corresponding to *GFP* and *hpt* genes, respectively (Figure. 5). These two bands were not amplified in genomic DNA from non-transformed plant. Southern blot analysis using *GFP* as a probe showed hybridization signals from one to three copies of the transgenes in all the 8 transgenic plants (Figure. 6, lanes 1-8), whereas no signal was detected in the non-transformed plant (Figure. 6, lane C). When RT-PCR analysis was performed to confirm the expression of *GFP* and *hpt*, the results showed that similar expression levels of *GFP* and *hpt* were exhibited among all transgenic plants but no band was detected in the non-transformed plant (Figure. 7) and in the control reactions without adding reverse transcriptase (data not shown).

SMZ is sulfonamide antibiotics, which inhibits an enzyme activities involved in the folate synthesis. Thus, SMZ treatment probably reduces a pool size of folate in plant cells. As folate is an essential water-soluble vitamin required for enzymes that are responsible for DNA methylation as well as DNA synthesis and cell proliferation, SMZ treatment reduced DNA methylation level in plant cells (Zhang *et al*. 2012). They also reported that SMZ treatment increased expression of silenced *nptII and luciferase* transgenes in *Arabidopsis*. We also confirmed that expression of *GFP* transgene was increased by SMZ treatment (Figure. 3, 4), moreover,μM)treatmentSMZ(30of the transgenic calli enhanced the transformation efficiency of ravenna grass. It was considered that both *hpt* and *GFP* transgenes were silenced in the transgenic calli and their expression was recovered by reduction of *de novo* DNA methylation. The SMZ treatment will be useful in the case that transgene silencing via DNA methylation is obstacle for production of transgenic plants.

In this study, we first produced transgenic plants of ravenna grass by *Agrobacterium*-mediated genetic transformation. We found that calli of ravenna grass were effectively transformed with *Agrobaterium* by removal of

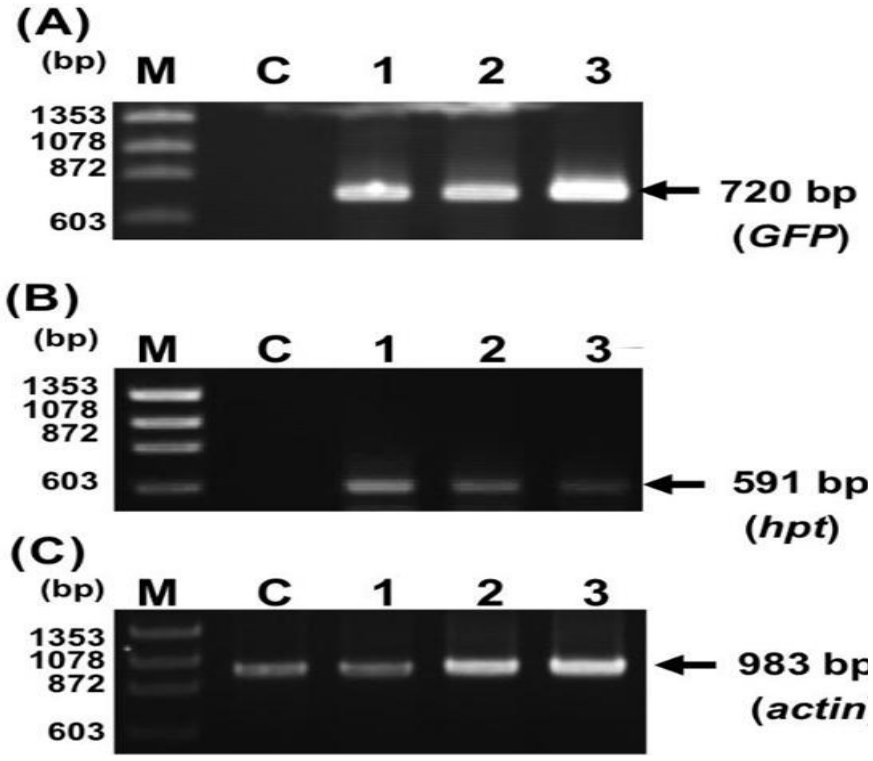

Figure 7 RT-PCR analysis of transgenic plants for (a) *GFP*, (b) *hpt* and (c) *actin* genes. Lane M: molecular size marker (λ/ *Hin*dIII,φ×174/*Hae*III), Lane C: negative control (non-trasnsformed control plant), Lanes 1-3: transformed plants regenerated from SMZ-treated calli (lines #3, 4, 7 in Fig. 5 and 6).

MS salts from the medium during infection and co-cultivation period, and that SMZ treatment was useful to increase expression of transgenes resulting in improvement of transformation frequency. This protocol reported here will contribute to produce transgenic *E. ravennae* carrying practical important traits, such as resistances against the environmental stresses, creation of male sterile line for hybrid vigor breeding, and enhancement of biomass production for reducing the emission of carbon dioxide.

REFERENCES

Azadi P, Chin DP, Kuroda K, Khan RS, Mii M (2010). Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium.* Plant Cell Tiss Organ Cult 101, 201-209.

Baulcombe D (2004). RNA silencing in plants. Nature 431, 356-363.

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6, 325-330.

Christensen AH, Sharrock RA, Quail PH (1992). Maize polyubiquitin genes; structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18, 675-689.

Datla RS, Hammerlindl JK, Pelcher LE, Crosby WL, Selvaraj G (1991). A bifunctional fusion between beta-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. Gene 101, 239-246.

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM (1982). Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1, 561-573.

Deren CW, Snyder GH, Tai PYP, Turick CE, Chynoweth DP (1991). Biomass production and biochemical methane potential of seasonally flooded inter-generic and inter-specific *Saccharum* hydrids. Bioresour Technol 36, 179-184.

Domínguez A, Fagoaga C, Navarro L, Moreno P, Peña L (2002). Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. Mol Gen Genet 267, 544-556.

Elmayan T, Vaucheret H (1996). Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. Plant J 9, 787-797.

from seed-derived callus of ravenna grass [*Erianthus ravennae* (L.) Beauv.]. Plant Biotech 30, 473-478. Someya T, Nonaka S, Nakamura K, Ezura H (2013). Increased 1-aminocyclopropane-1-carboxylate deaminase activity enhances *Agrogacterium tumefasience*-mediatedgene] delivery into plant cells. Microbiologyopen 2,873-880.

Hattori T, Shiotsu F, Doi T, Morita S (2010). Suppression of tillering in *Erianthus ravennae* (L.) Beauv. due to drought stress at establishment. Plant Prod Sci 13, 252-255.

Hiei Y, Komari T, Kubo T (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 35, 205-218.

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986). The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168, 1291-1301.

Hwang OJ, Cho MA, Han YJ, Kim YM, Lim SH, Kim DS, Hwang I, Kim JI (2014). *Agrobacterium*-mediated genetic transformation of *Miscanthus sinensis*. Plant Cell Tiss Organ Cult 117, 51-63.

Li R, Qu R (2011). High throughput *Agrobacterium*-mediated switchgrass transformation. Biomass Bioenerg 35, 1046-1054.

Metzke MA, Aufsatz W, Kanno T, Mette MF, Metzke (2002). Homology-dependent gene silencing and host defense in plants. Adv Genet 46, 235-275.

Mishiba K, Nishihara M, Nakatsuka T, Abe Y, Hirano H, Yokoi T, Kikuchi A, Yamamura S (2005). Consistent transcriptional silencing of 35S-driven transgenes in gentian. Plant J 44:541-556.

Monneret C (2005). Histone deacetylase inhibitors. Eur J Med Chem 40:1-13.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473-497.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8, 4321-4325.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104, 34-41.

Ogaki M, Furuichi Y, Kuroda K, Chin DP, Ogawa Y, Mii M (2008). Importance of co-cultivation medium pH for successful *Agrobacterium*-mediated transformation of *Lilium* × *formolongi*. Plant Cell Rep 27, 699-705.

Ogawa Y, Mii M (2007). Meropenem and moxalactam: Novel *β*-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. Plant Sci 172, 564-572.

Patra SK, Bettuzzi S (2009). Epigenetic DNA-(cytosine-5-carbon) modifications: 5-aza-2'-deoxycytidine and DNA-demethylation. Biochemistry 74, 613-619.

Sainz MB (2009) Commercial cellulosic ethanol: The role of plant-expressed enzymes. In Vitro Cell Dev Biol-Plant 45, 314-329.

Samson R, Mani S, Boddey R, Sokhansanj S, Quesada D, Urquiaga S, Reis V, Lem CH (2005). The potential of C_4 perennial grasses for developing a global BIOHEAT industry. Crit Rev Plant Sci 24, 461-495.

Sandal I, Bhattacharya A, Saini U, Kaur D, Sharma S, Gulati A, Kumar JK, Kumar N, Dayma J, Das P, Singh B, Ahuja PS (2011) Chemical modification of L-glutamine to alpha-amino glutarimide on autoclaving facilitates *Agrobacterium* infection of host and non-host plants: A new use of a known compound. BMC Chem Biol 11, 1.

Shimomae K, Chin DP, Khan RS, Mii M (2013). Efficient plant regeneration system

Thetford M, Norcini JG, Ballard B, Aldrich JH (2009) Ornamental landscape performance of native and nonnative grasses underlow-input conditions. Horttechnology 19, 267-285.

Van den Elzen PJ, Townsend J, Lee KY, Bedbrook JR (1985). A chimaeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol. Biol 5, 299-302.

Wang ZY, Ge Y (2005). *Agrobacterium*-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). J Plant Physiol 162, 103-113.

Wise AA, Liz Z, Binns AN (2006). Three methods for the introduction of foreign DNA into *Agrobacterium*. Methods Mol Biol 343, 43-53.

Yu TT, Skinner DZ, Liang GH, Trick HN, Huang B, Muthukrishan S (2000). *Agrobacterium*-mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas 133, 229-233.

Zhang H, Deng X, Miki D, Cutler S, La H, Hou YJ, Oh J, Zhu JK (2012). Sulfamethazine suppresses epigenetic silencing in *Arabidopsis* by impairing folate synthesis. Plant Cell 24, 1230-1241.