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***Short Communication***

# Improvement of *Agrobacterium*-mediated transformation for tannin-producing sorghum

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**Abstract** Sorghum (*Sorghum bicolor* L.) ranks as the fifth most widely planted cereal in the world and is used for food as well as a biomass plant for ethanol production. Use of the TX430 non-tannin sorghum variety has enhanced *Agrobacterium*-mediated sorghum transformation. These protocols could not be applied, however, to other tannin producing sorghum varieties such as the BTx623 model cultivar for sorghum with full genome information of sorghum. Here we report an improved protocol for *Agrobacterium*-mediated genetic transformation of tannin-producing sorghum variety BTx623. We successfully developed modification of root regeneration condition for generation of transgenic plant of BTx623. We inoculated immature embryos with *Agrobacterium tumefaciens* strain EHA105 harboring pMDC32-35S-GFP to generate transgenic plants. In the root regeneration step, we found that regeneration from transformed calli was affected by tannin. For root regeneration, shoots that appeared were not transferred to agar plate, but instead transferred to vermiculite in a plastic pod. Direct planting of regenerated shoots into vermiculite prevented the toxic effect of tannin. Root regeneration efficiency from calli emerged shoots in vermiculite was 78.57%. Presence of *sGFP* transgene in the genome of transgenic plants was confirmed by PCR and *sGFP* expression was confirmed in transgenic plants. This improved protocol of *Agrobacterium*-mediated transformation for tannin-producing sorghum BTx623 could be a useful tool for functional genomics using this plant.

**Key words:** *Agrobacterium*, BTx623, Sorghum, tannin, transformation.

Sorghum is the fifth largest crop cultivated in the world. It is used for food in Africa (Maunder 2003) and recently studied as a source of sugar and starch for starting material in production of several materials such as plastics and fibers (Mathur et al. 2017). The first transgenic plant was developed by biolistic gene transfer method (Casas et al. 1993). *Agrobacterium*-mediated sorghum transformation procedure was first reported by Zhao et al. in 2000. *Agrobacterium*-mediated sorghum transformation procedures were reported using non-tannin sorghum variety, TX430 (Gao et al. 2005; Jeoung et al. 2002; Wu and Zhao 2017; Wu et al. 2014). However, a large variety of sorghum produces tannin. Sorghum varieties that produce tannin in the grain are more resistant to molds and herbivores than are low-tannin varieties (Cai and Butler 1990). But one main problem in transformation of sorghum is production of phenolic compounds at the regeneration step, especially in varieties producing tannin (Cai and Butler 1990; Nguyen et al. 2007). Reported *Agrobacterium*-mediated sorghum transformation procedures do not apply well to sorghum varieties producing tannin, such as BTx623. BTx623 was used recently to complete the genomic sequence, (Paterson et al. 2009). Because of the need for full genome sequence information of plants modified using recent advances in genetic modification such as genome editing (Altpeter et al. 2016), improvement of transformation protocol for sorghum variety BTx623 could serve as a powerful tool for functional genomics in sorghum. Using the particle bombardment method was reported for generation of genetically transformed BTx623 (Girijashankar et al. 2005). *Agrobacterium*-mediated transformation, however, is more acceptable for low copy number and single-copy insertion of DNA sequences than for transformation using a direct DNA delivery system. We optimized an *Agrobacterium*-mediated transformation procedure for BTx623 to accelerate genetic modification for functional genomics.

The Genetic Resources Center of the National Agriculture and Food Research Organization provided BTx623 seeds. *Agrobacterium*-mediated transformation procedures for sorghum variety BTx623 in this study were modified from procedures of Zhao et al. (2000) and Wu et al. (2014). The main point of modification was changing the conditions in root regeneration to generate transgenic plants of BTx623. We used *Agrobacterium*

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Table 1. Culture media used for callus induction and plantlet regeneration.

| Medium | |
|---|---|
| mPHI-I | 4.3 g l$^{-1}$ MS Salt (DUCHEFA Biochemie, Netherlands), MS Vitamins (100 mg l$^{-1}$ Myo-inositol, 0.5 mg l$^{-1}$ Nicotinic acid, 0.5 mg l$^{-1}$ Pyridoxine–HCl, 0.1 mg l$^{-1}$ Thiamin–HCl, 2 mg l$^{-1}$ L-glycine), 68.5 g l$^{-1}$ Sucrose, 36 g l$^{-1}$ Gulcose, 1 g l$^{-1}$ Casamino acid, 2 mg l$^{-1}$ 2,4-D, pH 5.2 with 40 mg l$^{-1}$ Acetosyringone added before using. |
| PHI-T | 4.3 g l$^{-1}$ MS Salt, 250 mg l$^{-1}$ Myo-inositol, 1 mg l$^{-1}$ Thiamine–HCL, 2 mg l$^{-1}$ 2,4-D, 20 g l$^{-1}$ Sucrose, 10 g l$^{-1}$ Gulcose, 690 mg l$^{-1}$ L-proline, 1.22 mg l$^{-1}$ $CuSO_4$, 40 mg l$^{-1}$ Acetosyringone, 2 g l$^{-1}$ Agarose, pH 5.8. |
| mDBC3 | 4.3 g l$^{-1}$ MS Salt, 250 mg l$^{-1}$ myo-inositol, 1 mg l$^{-1}$ Thiamine–HCl, 2 mg l$^{-1}$ 2,4-D, 30 g l$^{-1}$ Maltose, 690 mg l$^{-1}$ L-proline, 1.22 mg l$^{-1}$ $CuSO_4$, 2 g l$^{-1}$ Gellan Gum, pH 5.8 with 25 mg l$^{-1}$ Meropenem trihydrate added before using. |
| mDBC3-Hyg1 | mDBC3 containing 2 mg l$^{-1}$ Hygromycin. |
| mDBC3-Hyg2 | mDBC3 containing 5 mg l$^{-1}$ Hygromycin. |
| PHI-XM-Hyg | 4.3 g l$^{-1}$ MS Salt, MS Vitamins (100 mg l$^{-1}$ Myo-inositol, 0.5 mg l$^{-1}$ Nicotinic acid, 0.5 mg l$^{-1}$ Pyridoxine–HCl, 0.1 mg l$^{-1}$ Thiamin-HCl, 2 mg l$^{-1}$ L-glycine), 60 g l$^{-1}$ Sucrose, 0.5 mg l$^{-1}$ Zeatin, 1 mg l$^{-1}$ IAA, 0.1 $\mu$M ABA, 0.1 mg l$^{-1}$ Thidiazuron, 1.25 mg l$^{-1}$ $CuSO_4$, 8 g l$^{-1}$ Agarose, pH 5.6 containing 5 mg l$^{-1}$ Hygromycin. |

PHI-T, PHI-XM media from Zhao et al. (2000). mPHI-I, mDBC3 media were modified from media by Zhao et al. (2000) and Wu et al. (2014).

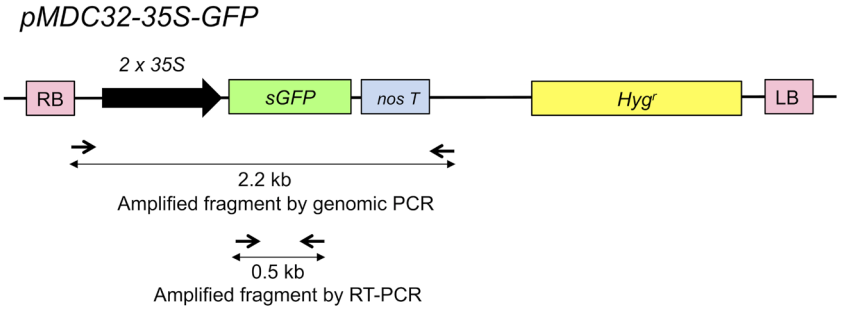


Figure 1. Map of T-DNA region of pMDC32-35S-GFP vector. RB, right border of T-DNA; 2×35S, double Cauliflower mosaic virus 35S promoter; sGFP, synthetic GFP gene; nos T, nos terminator; Hyg$^r$, Hygromycin Phosphotransferase gene; LB, left border of T-DNA. Arrows show the regions of amplified fragments forming genomic PCR and RT-PCR respectively.

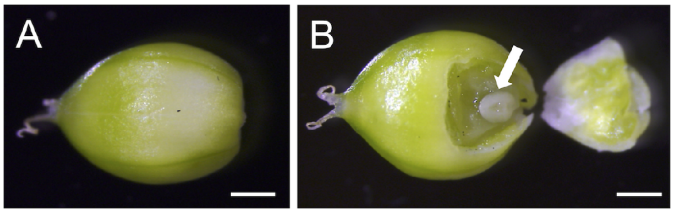


Figure 2. Preparation of immature embryos. A) Immature seed. B) Immature embryo appeared after removing seed shell. White arrow indicates immature embryo. Scale bars represent 0.5 mm. Pictures were taken by stereoscopic microscopes OLYMPUS BX60 (OLYNPUS, Japan, Tokyo) and camera cyber-shot DSC-RX100 (Sony, Japan, Tokyo).

*tumefaciens* strain EHA105 (Hood et al. 1993) and binary vector pMDC32-35S-GFP harboring synthetic green fluorescent protein *(sGFP*) gene (Niwa 2003) driven by Cauliflower mosaic virus (CaMV) 35S promoter and hygromycin resistance gene (Figure 1; Curtis and Grossniklaus 2003). Treatment by heating immature embryos (Gurel et al. 2009) was applied in our procedure. We used basal media developed by Zhao et al. (2000) and Wu et al. (2014). Media used in this study are listed in Table 1. We modified a concentration of 2,4-D in mPHI-I medium and we used meropenem trihydrate as antibiotic to remove *Agrobacterium* instead of carbenicillin in mDBC3 plate.

We constructed a pMDC32-35S-GFP vector for transformation. *sGFP* gene amplified from pGWB5 vector (Nakagawa et al. 2007) was inserted downstream of CaMV 35S promoter in pMDC32 vector (Figure 1).

We isolated immature embryos as the first step in preparing calli. We harvested immature seeds within 2 weeks after pollination (Figure 2A) and sterilized them with 10% (v/v) Heiter (Kao, Japan, Tokyo) for 10 min. Seeds were rinsed with sterile water three times. We obtained immature embryos with longitudinal axis of 0.5 to 1.5 mm by using forceps to peel off the seed surface (Figure 2B). Embryo size was very critical for callus formation. The tips of the forceps should be treated to make a flat surface to prevent damage to the embryo. Collected immature embryos were kept

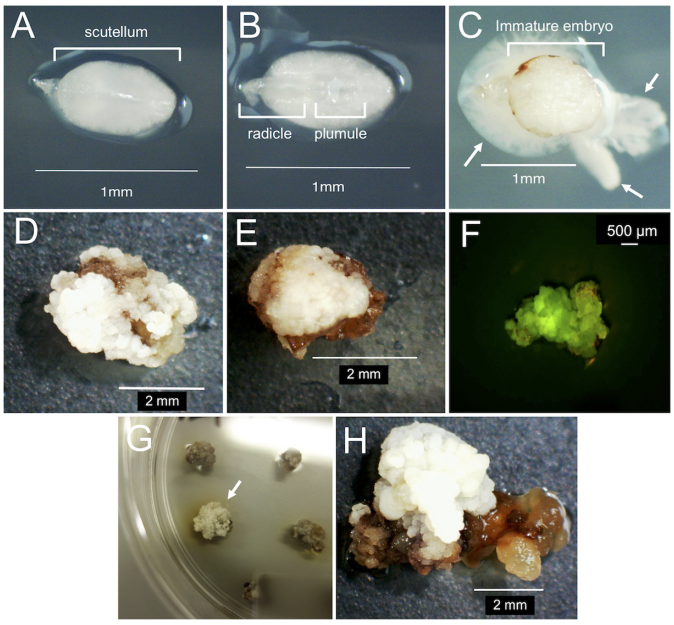


Figure 3. *Agrobacterium* infection and callus induction. A) Right arrangement of immature embryo. Parenthesis indicates scutellum. Scutellum is facing upward on agar plate. B) Wrong arrangement. Parentheses indicate radicle and plumule. These are facing upward. C) Immature embryo after a week co-cultivation with *Agrobacterium*. *Agrobacterium* covered all of immature embryo. Parenthesis indicates immature embryo. Arrows indicate growing *Agrobacterium*. D) Newly propagated callus appeared on surface of embryo after three weeks on mDBC3-Hyg1 plate. E) Callus without new cell outgrowth. They did not grow further. F) GFP fluorescence was detected in calli after 20 days of incubation on mDBC3-Hyg1. Pictures were taken by fluorescence stereoscopic microscope OLYMPUS SZ61 (OLYMPUS, Japan, Tokyo). G) Hygromycin-resistant callus (arrow) and non-resistant callus grown on mDBC3-Hyg2 plate. H) Growth of hygromycin-resistant callus after 20 days on mDBC3-Hyg2 plate.

in mPHI-I medium in a test tube. Gurel et al. (2009) reported heating immature embryos to enhance sorghum transformation frequency. We incubated embryos in hot water at 43°C for 3 min, and then cooled them at 25°C for 2 min. The following *Agrobacterium*-mediated transformation steps were applied to these heat-treated embryos.

(1) Preparation of *Agrobacterium* suspension: Suspension was prepared of *Agrobacterium* strain EHA105 harboring pMDC32-35S-GFP plasmid with mPHI-I medium ($OD_{550}$=0.7).

(2) *Agrobacterium* infection: Just after heat-treatment, embryos were incubated with the *Agrobacterium* suspension for 5 min. At this point, *Agrobacterium* suspension should be poured to just cover embryos.

(3) Co-cultivation and callus induction: *Agrobacterium*-treated embryos were placed on PHI-T plate with adaxial side facing upward (Figure 3A). At this step if the embryo was placed with abaxial side facing upward (Figure 3B) it did not succeed in callus induction (Supplementary table 1). Embryos were incubated for a week at 25°C in darkness until the embryos were covered with *Agrobacterium* (Figure 3C).

(4) Removal of *Agrobacterium*: Embryos covered by *Agrobacterium* were transferred to a plate of mDBC3 containing 25 mg $l^{-1}$ meropenem trihydrate (FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation, Japan) as antibiotic to remove *Agrobacterium*. The antibacterial antibiotic meropenem trihydrate prevents bacterial cell wall formation. Embryos were incubated in darkness for a week at 28°C.

(5) Selection of calli containing transgene: Embryos were incubated on an mDBC3-Hyg1 plate that contained 2 mg $l^{-1}$ hygromycin and 25 mg $l^{-1}$ meropenem trihydrate at 28°C in darkness for 3 weeks while changing to a new plate every week. At this stage, calli were formed from embryos. Some calli could be formed with white newly emerging cells (Figure 3D). Calli with no production of newly emerging cells did not grow (Figure 3E). GFP fluorescence could be detected from transformed calli (Figure 3F). Calli were incubated on a mDBC3-Hyg2 plate which contained 5 mg $l^{-1}$ hygromycin at 28°C in darkness for 3 weeks while changing to a new plate every week. Calli with newly emerging cells can be distinguished easily from those without new cells (Figure 3G). Newly emerging cells grew further to cover original callus (Figure 3H).

(6) Shoot induction and regeneration: Calli were moved to a PHI-XM-Hyg plate containing 5 mg $l^{-1}$ hygromycin and incubated at 25°C for four weeks in darkness while replacing the plate every week. At this stage, nascent etiolated shoots emerged from calli (Figure 4A). When calli were grown under white light they started to produce tannin and could not grow further (Figure 4B).

Shoots that appeared were transferred to sterile vermiculite in a plastic bottle and incubated at 25°C under 16 h light/8 h dark cycle for root regeneration

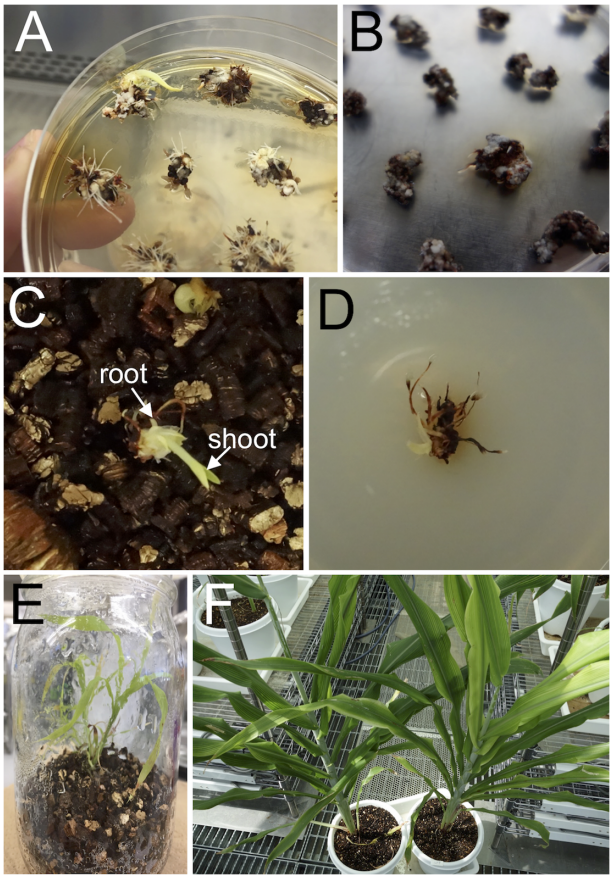


Figure 4. Regeneration of shoot and root to mature plant. A) Shoot emerging from hygromycin-resistant calli after 3 weeks of incubation on shoot inducing PHI-XM-Hyg plate in dark condition. B) Hygromycin-resistant calli incubated under 16 h light/8 h dark at 25°C on shoot-inducible PHI-XN-Hyg plate for 2 weeks. Most calli stopped development. C) Regeneration of shoot and roots after transfer of etiolated shoot on vermiculite bottle for 5 days. The arrows indicate shoot and root. D) Regeneration of plantlet 3 weeks after transfer to vermiculite bottle. E) Mature plants two month after transfer to pots.

(Figure 4C). At this stage, a plantlet was produced with de-etiolated shoot and nascent roots. The most important point of this procedure was to change the conditions for root regeneration from agar with root-inducing medium to vermiculite. In all cases when shoots were moved to root-inducing agar plate, they started to accumulate tannin and stopped development (Figure 4D, Table 2). After growth in a bottle for 3 weeks (Figure 4E), plants were transferred to a plant pot and grown in a greenhouse (Figure 4F).

To monitor the transformation, we performed PCR using total genomic DNA prepared from leaves of two independent regenerated sorghum $T_0$ plants. By the procedure described above, we successfully amplified 2.2 kb PCR fragments derived from the region covered with whole 2×35S promoter and s*GFP* gene (Figure 1, Figure 5A). We examined GFP fluorescent analysis by

Table 2. Effect of culture condition on root regeneration frequency.

| Root regeneration condition | Total No. of immature embryos[a] | Total No. of calli with regenerated shoots | Total No. of calli with regenerated roots | Root regeneration (%) |
|---|---|---|---|---|
| Agar[b] | 1658 | 22 | 0 | 0.00 |
| Vermiculate | 572 | 14 | 11 | 78.57 |

[a]Total No. of immature embryos used for ten experiments. [b]PHI-Z medium (Wu et al. 2014) was used in agar condition.

Table 3. Copy number and transgene (*sGFP*) segregation analysis of $T_1$ plants.

| Transgenic line | Copy number by real-time PCR | Total $T_1$ plants analyzed | $T_1$ segregation (*GFP* positives: *GFP* negatives) | Chi square value | *p* value |
|---|---|---|---|---|---|
| #1 | 1.29 | 15 | 11:4 | 0.89 | 0.65 |
| #2 | 2.29 | 16 | 12:4 | 0.00 | 1.00 |

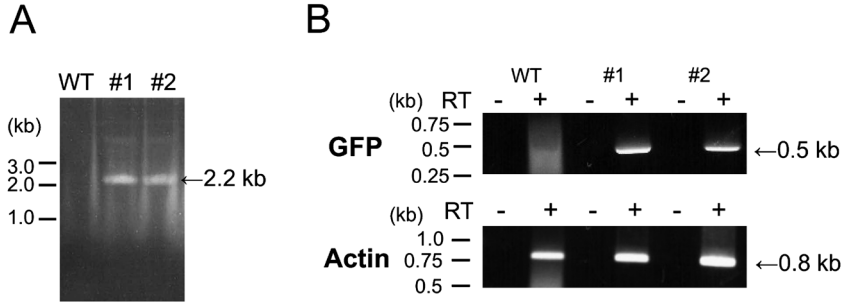


Figure 5. PCR detection and RT-PCR analysis of transgene *sGFP*. A) Detection of 2.2 kb of PCR fragment of transgene (*sGFP*) introduced into genomic DNA of $T_0$ transgenic plants. To detect integration of *sGFP* gene, total genomic DNAs were extracted from leaves of WT and transgenic plants and genomic-PCR were performed with primers (5′-GGAAACAGCTATGACCATGATT-3′/5′-CCAGTCACGACGTTGTAAAACG-3′). From left, WT: wild type plant of sorghum BTx623; #1, #2: two independent $T_0$ transgenic plants. B) RT-PCR amplification of 0.5-kb of *sGFP* and 0.8-kb of *Actin* fragments in $T_1$ plants from two independent $T_0$ plants. To detect expression of *sGFP* gene, total RNAs were extracted from leaves of WT and transgenic plants. After reverse transcription (RT) PCR was performed with primer set of (5′-GACCGTAAAGAAAAATAAGCACAAG-3′/5′-TGTTGTAATTCATTAAGCATTCTGC-3′) for *sGFP* gene and (5′-GAC AAT GGA ACC GGT ATG GTC-3′/5′-TGG AGT TGT AGG TAG TCT CGT GAA-3′) for *Actin* gene respectively. For control PCR was performed without RT.

Table 4. Transformation efficiency.

| Experiment | No. of starting immature embryo | No. of calli survived after co-cultivation on PHI-T | Survival ratio (%) | No. of calli survived after incubation on mDBC3 | Survival ratio (%) | No. of calli survived after selection by mDBC3-Hyg1 | Survival ratio (%) | No. of calli survived after selection by mDBC3-Hyg2 | Survival ratio (%) | No. of calli with regenerated shoots | Shoot regeneration ratio | No. of calli with regenerated roots | Transformation efficiency (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 149 | 101 | 67.79 | 52 | 34.90 | 6 | 4.03 | 5 | 3.36 | 1 | 0.67 | 1 | 0.67 |
| 2 | 244 | 235 | 96.31 | 92 | 37.70 | 20 | 8.20 | 10 | 4.10 | 1 | 0.41 | 1 | 0.41 |
| 3 | 215 | 204 | 94.88 | 68 | 31.63 | 15 | 6.98 | 4 | 1.86 | 2 | 0.93 | 1 | 0.47 |
| 4 | 104 | 104 | 100.00 | 14 | 13.46 | 4 | 3.85 | 3 | 2.88 | 0 | 0.00 | 0 | 0.00 |
| 5 | 61 | 60 | 98.36 | 30 | 49.18 | 12 | 19.67 | 4 | 6.56 | 2 | 3.28 | 2 | 3.28 |
| 6 | 90 | 89 | 98.89 | 22 | 24.44 | 7 | 7.78 | 4 | 4.44 | 0 | 0.00 | 0 | 0.00 |
| 7 | 254 | 248 | 97.64 | 112 | 44.09 | 36 | 14.17 | 23 | 9.06 | 4 | 1.57 | 2 | 0.79 |
| 8 | 230 | 219 | 95.22 | 109 | 47.39 | 35 | 15.22 | 27 | 11.74 | 3 | 1.30 | 3 | 1.30 |
| 9 | 102 | 50 | 49.02 | 28 | 27.45 | 9 | 8.82 | 3 | 2.94 | 0 | 0.00 | 0 | 0.00 |
| 10 | 65 | 58 | 89.23 | 45 | 69.23 | 16 | 24.62 | 9 | 13.85 | 1 | 1.54 | 1 | 1.54 |
| Total | 1514 | 1368 | | 572 | | 160 | | 92 | | 14 | | 11 | |

using transgenic plant. We could not observe strong fluorescence as observed in callus. This may be due to the overlapping of auto-fluorescence of chlorophyll or due to gene silencing in adult plants. To confirm expression of *sGFP* transgene, we performed RT-PCR using total RNAs from adult $T_1$ plants derived from two independent transgenic $T_0$ plants. As shown in Figure 5B, we could observe PCR band corresponding to *sGFP* only from RNA treated with reverse transcriptase.

We determined the transgene copy number by quantitative real-time PCR method (Song et al. 2002; Wu et al. 2014). DNA from $T_1$ plants derived from two independent transgenic $T_0$ plants was subjected to quantitative real-time PCR analysis using primers (5′-ATG TGG CGT GTT ACG GTG AA-3′/5′-GGG ATT GGC TGA GAC GAA AA-3′) specific for the *sGFP* gene. Quantitative real-time PCR was performed using TUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Japan, Osaka) and Mx3000p system (Agilent, USA, CA). A standard curve was obtained by mixing pMDC32-35S-GFP plasmid with wild-type (non-transgenic) genomic DNA based on calculated ratio of transgene copy number to size of sorghum genome. The two independent transgenic plants displayed single-copy and two-copies, respectively (Table 3). To confirm that transgene was transmitted to the next generation, PCR was used on $T_1$ plants to confirm inheritance *sGFP* transgene. The Chi square test was used to confirm Mendelian inheritance ratios by screening of *sGFP* transgene. Transgenic $T_1$ plants from two independent $T_0$ plants have shown no significant deviation from Mendelian 3 : 1 ratio (Table 3).

The survival rate and regeneration rate for each step is summarized in Table 4. We infected a total of 1,514 immature embryos with *Agrobacterium*. Survival rate for callus induction after *Agrobacterium* infection was from 49.02 to 100.00% (88.73% on average). After *Agrobacterium* infection and selection with hygromycin containing plate, the survival rate of hygromycin-resistant calli was 1.86 to 13.85% (6.07% on average). The rate of shoot regeneration from hygromycin-resistant calli was from 0 to 3.28% (0.97% on average). After shoot regeneration in darkness, we immediately moved the calli to sterile vermiculite bottle for root regeneration. The success rate for root regeneration was from 0 to 3.28% (0.85% on average). Transformation efficiency showed a large variation between each experiment. A possible explanation for this large variation was the slight difference in stage and quality of the immature embryos used for each experiment. By our procedure for *Agrobacterium*-mediated genetic transformation of BTx623, we obtained 11 transgenic plants.

Incubation of calli in dark condition (Zhao et al. 2000) was applied in our procedure. We examined the effect of light in shoot regeneration using tannin-producing BTx623 (Table 5). Calli incubated under light condition

Table 5. Effect of light on shoot regeneration frequency.

| Shoot regeneration condition | Total No. of survived calli by selection[a] | Total No. of survived calli with regenerated shoots | Shoot regeneration (%) |
|---|---|---|---|
| Light[b] | 269 | 0 | 0.00 |
| Dark | 92 | 14 | 15.22 |

[a]Total No. of survived calli used for ten experiments. [b]Light condition means 16 h light/ 8 h dark cycle.

could not survive (Figure 4B). It has been reported that production of phenolic material from calli causes necrosis in tannin-producing sorghum varieties (Cai and Butler 1990; Nguyen et al. 2007). The most important point of the procedure in this study was that direct planting of regenerated shoots into vermiculite prevented the toxic effect of tannin in the generation of sorghum BTx623 transgenic plants (Figure 4C, Table 2).

The full genome sequence has been determined using BTx623 (Paterson et al. 2009). Our protocol for *Agrobacterium*-mediated genetic transformation of BTx623 could be a useful tool for functional genomics in sorghum. In several crop-transformation cases, such as in maize and in sorghum, tannin-less cultivars have been used to prevent toxic effects of tannin on transformation (Yadava et al. 2017). On the other hand, a variety of cultivars with useful traits have been reported in sorghum and in maize (Dwivedi et al. 2016). We hope that our method can be helpful for transformation of such useful cultivars.

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