Chapter 10

Agrobacterium-Mediated Transformation of *Sorghum bicolor* Using Immature Embryos

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Abstract

Successful efforts describing in vitro culturing, regeneration, and transformation of grain sorghum were reported, using particle bombardment, as early as 1993, and with *Agrobacterium tumefaciens* in 2000. Reported transformation efficiencies via *Agrobacterium* routinely range from 1 to 2%. Recently, such efficiencies via *Agrobacterium* in several plant species were improved with the use of heat and centrifugation treatments of explants prior to infection. Here, we describe the successful use of heat pretreatment of immature embryos (IEs) prior to *Agrobacterium* inoculation to increase routine transformation frequencies of a single genotype, P898012, to greater than 7%. This reproducible frequency was calculated as numbers of independently transformed IEs, confirmed by PCR, western, and DNA hybridization analysis, that produced fertile transgenic plants, divided by total numbers of infected IEs.

Key words: *Agrobacterium tumefaciens*, GFP, Heat treatment, Immature embryos, Phosphomannose isomerase, Sorghum, Transformation

1. Introduction

Worldwide sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal crop, ranking fifth in production. Its relative tolerance to drought and heat makes it an ideal grain for human and animal consumption in areas with extreme temperatures and minimal precipitation, like the semiarid regions of Asia and Africa. Use of dryland crops, such as sorghum, will surely increase elsewhere as global climate changes lead to dramatic changes in temperatures and water availability. With these likely changes, efforts to increase soghum's yield and quality will be of critical importance.

Application of genetic engineering and genomic technologies is one means to improve sorghum in the future. Successful in vitro culturing, regeneration, and transformation of grain sorghum have been reported (1–15). The first fertile transgenic sorghum plants

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were obtained in 1993, using particle bombardment (3), and in 2000 by *Agrobacterium*-mediated transformation (14). Several studies have focused on improving transformation and regeneration frequencies via either strategy (6, 7, 10, 16, 17); reported transformation efficiencies via *Agrobacterium* were in the range of 1–2%. In a 2006 study (10), a 4.5% frequency (4 events from 89 explants) was achieved in 1 of 17 experiments; however, frequencies in the other 16 ranged from 0.3 to 1.9%. Results like these underscore the recalcitrance and lack of reproducibility of such approaches with sorghum (18, 19).

Transformation efficiencies via *Agrobacterium* were improved recently in several plant species using heat and centrifugation treatments of transformation explants. For example, Khanna et al. (20) reported heat shocking banana suspension cells before *Agrobacterium* infection, doubling postinfection viability and increasing transformation efficiency; centrifuging suspension cells with *Agrobacterium* resulted in quadrupled efficiencies. In 2006 rice and maize transformation frequencies were increased severalfold by heat treatment and centrifugation of immature embryos (IEs) before *Agrobacterium* infection (21). The increased transformation frequencies reported in Gurel et al. (8) suggest that heat pretreatment of IEs prior to infection of IEs from sorghum variety P89012 induces a stress response that allows cells to survive cocultivation with *Agrobacterium*. In addition, according to Carvalho et al. (16), as well as from our efforts aimed at transforming several sorghum varieites, one primary factor affecting success of *Agrobacterium*-mediated transformation is the cocultivation method used to infect IEs.

Using a selectable marker gene for phosphomannose isomerase (*pmi*) and an *sgfp* reporter gene, a relatively efficient, reproducible protocol for *Agrobacterium*-mediated transformation of P898012 was developed using heat treatment of IEs prior to *Agrobacterium* infection (8). Evaluation of effects of heat treatment and centrifugation on transient GFP expression and stable transformation frequency is described here.

2. Materials

2.1. Growth of Donor Plants

1. Sorghum seeds of P898012 can be obtained from the USDA ARS Germplasm Resources Information Network (http://www.ars-grin.gov/npgs/orders.html).
2. Use 4-gallon pots containing Supersoil potting soil (Rod McClellan Co., South San Francisco, CA, USA).
3. Grow either in walk-in growth chamber (Kolpak, River Falls, WI, USA) with 16 h day/8 h night temperatures of 28/22°C

and sodium vapor and metal halide lights at ~700 μmol/s/m^2 or in the greenhouse under comparable natural and artificial lighting and day/night temperatures.

4. Use Jack's Professional Fertilizer 20-20-20 (J.R. Peters Inc., Allentown, PA, USA).

2.2. Sterilization of Plant Seeds: Isolation of Immature Embryos

1. Panicles ~12–14-day postpollination.
2. 70% ethanol.
3. 20% (v/v) bleach solution (5.25% sodium hypoclorite).
4. Sterile distilled water.
5. Sterile 7 cm diameter Whatman #1 filter disks.
6. Liquid inoculation medium (see Table 1).

2.3. Pretreatment with Heat

1. Carefully controlled 43°C water bath.
2. Water bath at 25°C.

2.4. Introduction of Plasmid Vectors into Agrobacterium

1. Binary vector, pPZP201-GFP-PMI (pGFP-PMI; see Fig. 1) (7), containing *sgfp* encoding the green fluorescence protein (GFP); (22) and *pmi* encoding phosphomannose isomerase (23).
2. *A. tumefaciens* strains, EHA101 (24) and LBA4404 (25).

2.5. Preparation of Agrobacterium Suspension and Infection of IEs

1. Master plate of *Agrobacterium* with pPZP201-GFP-PMI on YEP agar medium (see Note 1) plus 100 mg/L spectinomycin (Sigma S4014) for EHA101 and LBA4404.
2. Liquid inoculation medium (see Table 1).
3. Liquid culture of *Agrobacterium* at $OD_{600} = 0.4$ (see Subheading 3.4).
4. Stock of 200 mM acetosyringone (filter sterilized; Sigma D134406).

2.6. Culturing of Tissue, Selection, and Regeneration

1. Various tissue culture media (see Table 1).
2. Full-strength Murashige and Skoog (MS) basal salts (Pytotech Labs: M419) and MS micronutrient solution (Phytotech Labs: M654) are used except in rooting medium.
3. Phytagel (Sigma: P8169) at 2.0 g/L is added to all media before autoclaving at 121°C.
4. 1% (w/v) polyvinylpyrrolidone (PVP) (Sigma: 234257) added to medium before autoclaving to reduce tissue blackening of high-phenolic cultivars, e.g., P898012.
5. Mannose (Sigma: M6020) is added at 2%, 1.5%, and 1.0% to selection media.
6. After autoclaving, cool media to 55°C before adding carbenicillin (Sigma: C9231), acetosyringone (Sigma: D134406), and ascorbic acid (Sigma:A4544).

Table 1 Chemical composition of different culture media used for tissue culture and transformation studies of sorghum.

Components (stock concentrations)	Inoculation medium (liquid)[a]	Cocultivation medium[a]	Callus induction medium[a]	Selection medium I[a]	Selection medium II[a]	Selection medium III[a]	Regeneration medium[a]	Rooting medium[a]
Murashige and Skoog basal salts	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	2.2 g
Nicotinic acid (5 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Pyridoxin HCl (5 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Thiamine HCl (10 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Myo-innostiol	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.05 g
2,4-Dichlorophenoxy acetic acid (2,4-D) (0.5 mg/mL)	4 mL	4 mL	4 mL	4 mL	4 mL	4 mL	–	–
Sucrose	68.5 g	20 g	30 g	30 g	20 g	15 g	–	–
Glucose	36 g	10 g	–	–	–	–	–	–
Mannose	–	–	–	20 g	15 g	10 g	10 g	10 g
2-(*N*-morpholino) ethane-sulfonic acid (MES)	0.6 g	0.6 g	–	–	–	–	–	–
Proline	–	0.7 g	–	–	–	–	0.7 g	–

Polyvinylpyrrolidone (PVP)	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g
Indole acetic acid (IAA) (1 mg/mL)	–	–	–	–	–	–	200 µL	200 µL
Kinetin (1 mg/mL)	–	–	–	–	–	–	500 µL	–
1-Naphthaleneacetic acid (NAA) (1 mg/mL)	–	–	–	–	–	–	–	200 µL
Indole-3-butyric acid (IBA) (1 mg/mL)	–	–	–	–	–	–	–	200 µL
MS Micronutrient solution	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	–	–
pH[b]	5.6	5.2	5.8	5.8	5.8	5.8	5.8	5.8
Phytagel	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g
Autoclave for	22 min	22 min	22 min	22 min	22 min	22 min	22 min	22 min
Acetosyringone (200 mM)	500 µL	500 µL	–	–	–	–	–	–
Carbenicillin (100 mg/L)	–	–	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Ascorbic acid (100 mg/mL)	–	100 µL	–	–	–	–	–	–

[a]Amounts listed are for 1 L of medium
[b]For all media, pH adjusted with 1 N KOH

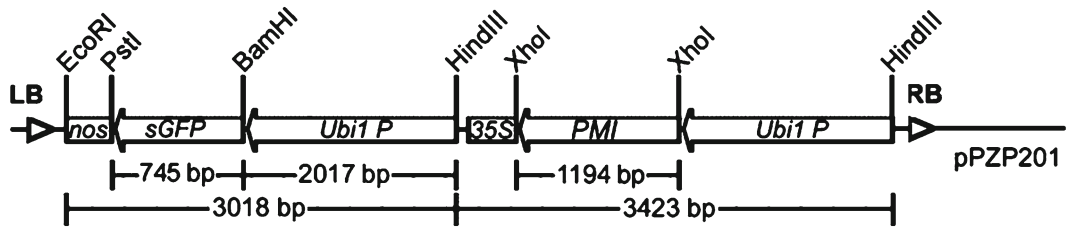

Fig. 1. pGFP-PMI Map (29). *Ubi1 P* ubiquitin promoter and first intron, *35S* and *nos* 3′ termination regions, *PMI* phosphomannose isomerase, *sGFP* synthetic green fluorescence protein, *physical distances* in base pairs (bp), *Eco*RI, *Pst*I, *Bam*HI, *Hin*dIII, *Xho*I restriction sites for respective enzymes, *LB, RB* left and right borders, respectively, of *Agrobacterium tumefaciens* T-DNA; *arrows*: direction of transcription of PMI and GFP genes; pPZP201 vector backbone (30).

7. Stocks of nicotinic acid (Sigma: N0765), pyridoxine HCl (Sigma: P8666), thiamine HCl (Sigma: T3902), and ascorbic acid (Sigma: A4544) are prepared in water. Store at 4°C in 10 mL aliquots.
8. Dissolve 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma: D7299) in 200-proof ethanol; add water for 0.5 mg/mL stock; add to medium before autoclaving. Store at 4°C.
9. Dissolve 3-indole-acetic acid (IAA) (Pytotech Labs: I885) in 200-proof ethanol; add water for 1 mg/mL stock; filter-sterilize. Store at –20°C.
10. Dissolve 1-naphthaleneacetic acid (NAA) (Phytotech Labs: N600) in 1 N KOH; add water for 1 mg/mL stock; filter-sterilize. Store at –20°C.
11. Dissolve indole-3-butyric acid (IBA) (Phytotech Labs: I538) in 200-proof ethanol; add water for 1 mg/mL stock; filter-sterilize. Store at –20°C.
12. Store N^6-furfuryladenine (kinetin) (Sigma: K3253) in sterile 1 mL aliquots at –20°C. Add to medium after autoclaving.
13. Dissolve 3′,5′-dimethoxy-3′-hydroxyacetophenone (acetosyringone) (Sigma: D134406) in dimethylsulphoxide (DMSO) (390 mg/10 mL DMSO); filter-sterilize. Store at 4°C.
14. Dissolve carbenicillin (Sigma: C3416) in water for 100 mg/L stock; filter-sterilize. Store at –20°C in aliquots.

3. Methods

3.1. Growth of Donor Plants

1. Plant four seeds per 4-gallon pot and thin to three plants following germination.
2. Keep plants well-watered to avoid water deficit, especially during seed set, and fertilize twice weekly with Jack's Professional Fertilizer 20-20-20 (see Note 2).

3.2. Sterilization of Plant Seeds: Isolation of Immature Embryos

(Steps 3–6 should be done in a laminar flow hood)

1. At ~12–14-day post-pollination, IEs should be ~1.5 mm, just turning opaque (see Notes 3 and 4).
2. Remove seeds from spikes; place in sterile 100 mm × 20 mm petri plate.
3. Sterilize in 70% ethanol for 5 min; rinse twice with ddH_2O (see Note 5).
4. Place seeds in sterile 50 mL centrifuge tube; add 40 mL 20% v/v bleach (5.25% hypochlorite). Place on rotary shaker for 20 min to agitate seeds.
5. Rinse seeds in hood in sterile ddH_2O four times for 1 min each; pour off excess liquid; place seeds in sterile petri plate with two sterile, 7 cm Whatman #1 filter papers.
6. Excise IEs (see Note 6) and place in 1.5 mL microfuge tube with room temperature liquid inoculation medium, ~25–30 IEs per tube.

3.3. Pretreatment with Heat

1. Remove inoculation medium leaving only enough to cover IEs.
2. Incubate tube with IEs in carefully controlled 43°C water bath for 3 min.
3. After heat treatment, cool tubes for 2 min in 25°C water bath (see Notes 7 and 8).
4. Remove excess liquid medium from heat-treated IEs.

3.4. Preparation of Agrobacterium Suspension

1. Dissolve spectinomycin (Sigma: S4014) in water for 100 mg/mL stock; filter sterilize; store at −20°C in aliquots. Add stock to achieve 100 mg/L final concentration.
2. For glycerol stocks of *Agrobacterium*, grow bacteria from single colony in sterile 14 mL snap cap tube with 5 mL YEP medium (see Note 1) plus 100 mg/L spectinomycin for EHA101 and LBA4404 for 8–12 h at 28°C, shaking at 225 rpm. Following incubation, place 0.75 mL bacterial culture and 0.75 mL sterile 80% glycerol in 1.5 mL microfuge tube, invert to mix and store for up to 1 year at −80°C.
3. Master plate of *A. tumefaciens* EHA101 or LBA4404 with pPMI-GFP is made by streaking from glycerol stock with a sterile inoculating loop onto YEP agar medium + 100 mg/L spectinomycin for EHA101 and LBA4404 and cultured at 28°C for 2 days. Master plate can be kept for 2 weeks at 4°C and used to streak additional plates for transformation.
4. One day prior to transformation, use a sterile inoculating loop to streak a single EHA101 or LBA4404 *Agrobacterium* colony

from the master plate onto a fresh YEP plus 100 mg/L spectinomycin agar plate. Incubate ON at 28°C.

5. For transformation, use a sterile metal spatula to resuspend two pencil-eraser-sized portions of the culture from the ON plate in 5 mL liquid inoculation medium (Table 1). Homogenize culture by gentle pipetting; remove 1 mL and place in 1.5 mL spectrophotometer cuvette.
6. Measure optical density; adjust OD_{600} to 0.4 by adding liquid inoculation medium according to the following formula:

$$\frac{OD_{600}\text{value}}{0.4} \times \text{mL remaining culture} = \text{final total volume.}$$

7. Add appropriate volume of 200 mM acetosyringone stock to achieve 100 μM final concentration.
8. Use culture immediately or keep at 4°C for short periods until used for transformation.

3.5. Agrobacterium Cocultivation with IEs (see Note 9)

1. Place 1 mL bacterial culture (see Subheading 2.5) in 1.5 mL microfuge tube containing IEs; gently invert tubes and incubate at RT 15 min.
2. Place 25 IEs scutellum side-up on solid cocultivation medium (see Note 10, Table 1).
3. Seal petri plates with parafilm to reduce culture contamination and dessication; incubate in darkness for 2 days at 24°C.
4. Transfer IEs to callus-induction medium (CIM; see Table 1, Note 11) with 100 mg/L carbenicillin for 4 weeks (see Note 12) to inhibit *Agrobacterium* growth before selection. Visible coleoptiles should be removed as soon as observed.
5. After 10 days on CIM, screen IEs for GFP expression (see Note 13, Fig. 2). Keep those IEs expressing GFP on CIM; eliminate IEs with no GFP expression, no callus or those which die during culture.

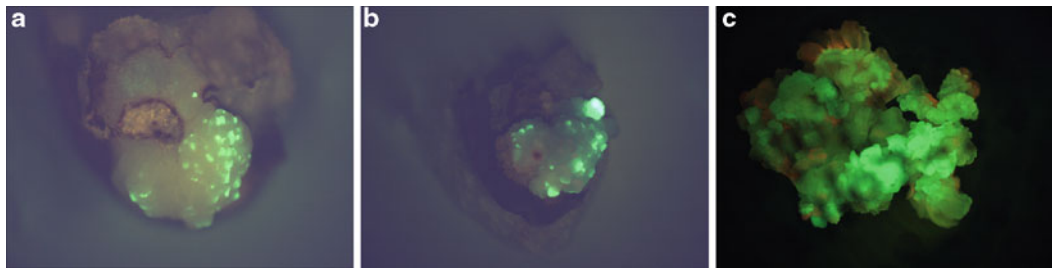

Fig. 2. P898012 immature embryos infected with *Agrobacterium tumefaciens* with the transformation vector, pPZP201-pGFP-PMI, cultured on callus induction medium (see Table 1) for (**a**) 10 days and on mannose selection medium (see Table 1) for (**b**) 30 days and (**c**) 60 days and visualized for GFP fluorescence. Magnification = ×20.

6. During culture on CIM, subculture IEs every other week, or sooner if heavy phenolic production is observed (see Note 14).
7. Continue to transfer and track GFP-expressing calli (see Note 15) on selection medium (see Subheading 3.6).

3.6. Culturing of Tissue, Selection, and Regeneration

Three-stage mannose selection involves successively lowering levels of mannose and sucrose (see Note 16).

1. Culture GFP-expressing calli (see Subheading 4.1) in the dark for 2 weeks on Selection Medium I (see Table 1).
2. Transfer GFP-expressing calli to Selection Medium II (see Table 1) for 2 weeks in the dark.
3. For the final 2 weeks, culture in the dark on Selection Medium III (see Table 1).
4. For slower growing calli, third stage of selection can be extended for two additional weeks.
5. Transfer selected calli to Regeneration Medium (see Table 1); incubate at low-light intensity (85 μmol/s/m^2) with 16 h/8 h light/dark for ~4 weeks. For first 5 days of regeneration, cover petri plates with white paper to acclimate calli to light (see Note 17).
6. Transfer ~3–5 cm shoots to Rooting Medium (see Table 1); place at 100 μmol/s/m^2 with 16 h/8 h light/dark for 4–6 weeks.
7. All regeneration and rooting media contain only 1% mannose plus 100 mg/L carbenicillin.
8. After 4–6 weeks on Rooting Medium, transfer shoots with ≥1 long root (see Note 18) to small pots with Supersoil; transfer to larger pots as plants grow.
9. Plants are grown initially in the Conviron E-15 growth chamber with a 16 h/8 h light/dark period under fluorescent and incandescent lighting (250 μmol/s/m^2) and day/night temperatures of 25/20°C.
10. When plants are 8–10″ tall, transfer to Kolpak walk-in growth chamber under same growth conditions or to greenhouse; water and fertilize as for donor plants (see Subheading 2.1).

4. Analysis

4.1. Visual Screening for GFP Expression

1. Observe IEs using epifluorescence stereomicroscope [Zeiss Lumar V12 fluorescence dissecting scope: filter set (Chroma Endow): excitation filter, BP470-490; emission filter, BP 505–550]

attached to Q Imaging Megapix camera with ivision 4.0.15 imaging software.

2. Track GFP-expressing calli by circling with a marker on the bottom of the petri plate.

4.2. Genomic DNA and Protein Expression Analyses

1. Although the technical aspects of analyzing genomic DNA and protein expression are not described in this review, confirming stable transformation and independence of transgenic events is necessary to determine accurate stable transformation frequencies.

5. Notes

1. YEP medium contains 10 g/L yeast extract (Becton Dickinson Difco: 212750), 10 g/L Bacto Peptone (Becton Dickinson Difco: 211677), 5 g/L NaCl (Fisher: BP 358–212); adjust pH to 7.2 with 1 N NaOH. Solid medium contains 18 g/L Bacto-agar (Becton Dickinson Difco: 214010). Autoclave for 22 min at 121°C. Store at 4°C.
2. Plants should be grown under optimal conditions as quality of donor plants affects capacity of IEs to produce embryogenic callus. During winter months in colder climates, prewarmed water should be used to water donor plants.
3. Collect spikes from plants ~12–14 days postpollination when IEs are ~1.5 mm; they are not transparent but just turning cloudy due to starch accumulation. Younger IEs, being nearly transparent, are difficult to locate and do not give the appropriate culture response. The developmental stage that is ideal for successful transformation varies among genotypes, but for P898012 the optimal size range is 1.5–2.5 mm.
4. For optimum response, use IEs taken from spikes immediately or within 1 day after collection, as callus response declines with time.
5. Prolonged periods of sterilization may damage IEs; insufficient sterilization may result in microbial contamination.
6. IE excision can be performed in several ways; the most important aspect being to minimize explant damage. To excise IEs locate notched side of the seed as the side opposite the notch is the location of the embryo. Firmly hold the seed with forceps so the notched side faces down; with a scalpel peel back the pericarp and seed coat around the tip. Use the scalpel to carefully lift out the IE without damaging it and place it in liquid inoculation medium.

7. Heating P898012 IEs at varying temperatures for 3 min before *Agrobacterium* infection increases to varying degrees frequencies of GFP-expressing calli, of mannose-selected calli and of transformed calli. The most optimal treatment for this variety is 43°C for 3 min with cooling at 25°C. When this optimal heat pretreatment was done prior to inoculating IEs of three other varieties, Tx430, Tx623, and N247, frequency of calli expressing GFP after 10 days also increased compared to non-heat-treated controls; however, variation in temperature and time of heat treatment were not attempted in order to optimize the response, and stable transformation efforts were not completed. That heat treatment increased transformation frequencies is perhaps due to the fact that short periods of heat shock reverse cell death. For P898012, longer 43°C heat treatments (5, 10, and 30 min) decrease numbers of GFP-expressing IEs and tissues surviving selection.
8. Centrifuging P898012 IEs before inoculation either without heat or with heat at various temperatures decreases frequencies of all tissue responses. Using both heat and centrifugation, however, increases tissue de-differentiation of P898012.
9. Methods used for inoculation and cocultivation of IEs are critical for successful callus induction and all other tissue culture steps. Other varieties in which we have attempted certain aspects of this procedure are as follows: Tx430, an elite variety with robust in vitro performance (26); Tx623, the variety for which the genome was recently sequenced (27); and N247, a short-season variety (28). It is well known that IEs of some sorghum varieities respond more negatively to the stress of *Agrobacterium* inoculation than others (16); this response is manifested by tissue blackening after cocultivation and secretion of phenolics.
10. It is advisable to culture only 25 IEs spread out on each petri plate to encourage callus formation; examine cultures daily.
11. Callus induction from IEs and growth rate of tissues is genotype-dependent.
12. The length of callus induction can be decreased to 1 week, depending on the genotype. When calli have visible white, nodular, embryogenic structures and phenolic secretion subsides, tissues can be transferred to selection medium. Because recovery from cocultivation varies by genotype, resting period lengths vary.
13. Although low-level GFP expression can be observed at 2–3-day postinfection, transient GFP expression is better scored at 10-day postinfection (see Fig. 2a). Visual monitoring of fluorescence can continue during selection and regeneration as expression levels increase over time (see Fig. 2b, c).

GFP expression at 10-day postinoculation is distinct and notable and can be used to compare different treatments. Nearly all GFP-expressing cells develop from the scutellum; this side grows rapidly, forming large masses of callus. During regeneration, most shoots developing from callus that survives selection express GFP.

14. If the sorghum variety produces noticeable amounts of phenolics, 1% PVP can be added to all media to reduce tissue blackening. Despite adding PVP, heavy phenolic production can still occur. Close monitoring of culture materials and transfer, sometimes daily, to fresh medium is necessary, if blackened or dead tissues exist; transfer only nonblackened tissues. Most sorghum varieties produce phenolics immediately following *Agrobacterium* inoculation with the amount increasing during cocultivation, but decreasing after the cocultivation period.
15. To facilitate tracking GFP-expressing tissues, circle tissues on the bottom of the petri plate with a permanent marker. During selection and regeneration steps, calli from a single embryo are tracked by drawing sectors around the tissues on the bottom of the petri plate or etching a furrow in the agar around the tissue.
16. For optimal mannose selection, sucrose is used in combination with mannose because it is believed that sucrose significantly decreases the phytotoxicity of mannose. Nontransgenic cells turn dark brown and then black, failing to survive; putative transgenic cells do not discolor, and maintain an opaque, white color and continue to express GFP.
17. Regenerability of transformed calli can vary between varieties owing to the fact that many cultivars lose the ability to re-differentiate into plants after many weeks in culture.
18. For shoots with at least one long root, gently loosen agar with forceps, pull shoots from agar slowly, rinse with luke-warm tap water to remove excess agar and plant in soil.

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