

Cassava (*Manihot esculenta* Crantz)

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Summary

During the last three years the generation of stably transformed cassava plants having value-added traits has become a reality. Currently, two *Agrobacterium*-mediated transformation systems are routinely used to engineer cassava. These systems use either somatic embryos or friable embryogenic calli. This paper presents detailed protocols for the transformation of cassava using primary somatic embryos. The effects of explant types, tissue culture conditions, and bacterial and plasmid related factors on transformation efficiency are discussed.

Key Words: *Agrobacterium tumefaciens*; apical leaves; auxiliary buds; cassava; direct embryogenesis; explant; friable embryogenic callus; genetic transformation; germinating somatic embryos; *Manihot esculenta* Crantz; shoot regeneration; somaclonal variation.

1. Introduction

The agronomic improvement of cassava (*Manihot esculenta* Crantz) using conventional breeding approaches has been hampered by a variety of factors including low production of flowers, apomixis (1,2), a long reproduction cycle, limited seed set (3), variability in ploidy number, and inbreeding depression (4). Furthermore, cassava is most commonly propagated as genetically uniform, clonal stem cuttings. These factors make cassava an ideal target for genetic improvement via transgenic approaches involving clonal propagation. The recent development of reliable and, to some degree, genotype-independent methodologies for the transformation of cassava now makes it possible to consider directed strategies for the genetic improvement of cassava via transgenic approaches.

Recently, advancements in cassava transformation technology and its applications have been described in several review articles (5–7). Both particle gun-mediated bombardment (8) and *Agrobacterium tumefaciens*-mediated gene transfer (9–11)

have been used to engineer cassava. To date, *Agrobacterium*-mediated transformation of cassava has proven to be more successful than particle gun-mediated transformation (6,9–15). This may be because Ti plasmid integration commonly occurs in transcriptionally active domains of the chromatin as well as to a reduced number of gene copies integrated relative to particle gun-mediated transformation (6,14). To date, however, these hypotheses are only speculative as a result of the limited numbers of experiments reported using both systems.

The plant tissue types used for *Agrobacterium*-mediated transformation include shoots induced by organogenesis (15) and germinating somatic embryos (GSE) (9,16). Direct shoot induction from cotyledons of somatic embryos (somatic cotyledons) has been used in both particle bombardment (14) and in *Agrobacterium*-mediated transformation (17). However, plant regeneration efficiency is highly variable (5–70%) and genotypic dependent (14). As a result of these cultivar-dependent differences a variety of tissues including auxiliary buds (17–19), apical leaves (10), and floral meristems (20) have been used for *Agrobacterium*-mediated transformation of cassava. Briefly, embryos are induced from callus tissues and directly inoculated with *Agrobacterium* for transformation. When using friable embryogenic callus (FEC), embryos are initiated and then transferred from Murashige and Skoog (MS)-based medium to Greshoff and Doy (21) basal medium supplemented with a high concentration of the auxins 4-amino-3,5,6-trichloropicolinic acid (picloram) or 2,4-dichlorophenoxyacetic acid (2,4-D) to initiate massive secondary embryo production. A disadvantage of the FEC system (compared to the direct somatic embryogenic system), however, is that it results in a high frequency of somaclonal variants (22,23). In this paper we describe detailed protocols for the *Agrobacterium*-mediated transformation of cassava using GSEs. Embryos are produced from explant tissues cultured on semi-solid MS-based callus induction medium (24). Embryos are then induced from callus tissues and directly inoculated with *Agrobacterium* for transformation. Selection for transformants is typically based on antibiotic resistance conferred by a gene present in the T-DNA. The transformation efficiency using our system ranges between 1 and 5% [defined as the frequency of polymerase chain reaction (PCR) transgene-positive plants per 100 cassava GSEs infected].

2. Materials

2.1. Plant Materials

Greenhouse grown cassava (MCol 2215, TMS 71173) plants are introduced in vitro by sterilizing the nodal segments of 4- to 6-mo-old plants with 20% (v/v) household bleach (sodium hypochlorite solution), 0.05% (v/v) Tween-20 (polyoxyethylenesorbitan monolaurate) (Sigma-Aldrich, St. Louis, MO; cat. no. 63178), or 0.05% (v/v) Silwet L-77 (Polyalkyleneoxide) (Setre Chemical Company, 6075 Poplar Memphis, TN, cat. no. 38119).

2.2. *Agrobacterium* Strain and Constructs

1. *Agrobacterium* LBA4404 (Invitrogen) was used in cassava transformation.
2. The main vector backbone used in our laboratory is pKYLX (*see* **Note 1**).

2.3. Stock Solutions

All prepared media stock solutions are filter sterilized using a 0.45 μm Cameo 25ES filter (Fisher Scientific) attached to a 60 mL BD syringe (Fisher Scientific) and are stored at 4°C. Plant hormones and antibiotics are supplied by Sigma-Aldrich.

1. α -Naphthaleneacetic acid (NAA, 0.02 mg/L) solution: 0.02 mg NAA in 1 L of ddH₂O. The NAA stock is made in 20% (v/v) 1 N NaOH. The stock can be stored at 4°C for 6 mo.
2. 6-Benzylaminopurine (BAP, 10 mg/L) solution: The BAP stock solution is made by dissolving in 20% (v/v) 1N NaOH and can be stored at 4°C for 6 mo.
3. Gibberelic acid solution (GA, 10 mg/L): The stock solution for GA is made by dissolving in 20% (v/v) 95% ethanol and can be stored at 4°C for 6 mo.
4. Thiamine (100 mg/L) solution: The thiamine-HCl (100 mg/L) stock is made by dissolving in distilled water. The stock is stored at 4°C for 6 mo.
5. 2,4-D (2,4-dichlorophenoxyacetic acid) solution: 0.5 mg/mL stock. The 2,4-D (0.5 mg/mL) stock is made by dissolving appropriate concentrations in 20% (v/v) 1 N NaOH. The stock is stored at 4°C for 6 mo.
6. 100X B5 vitamin solution: The 100X B5 vitamin stock solution was made by dissolving 10 g myo-inositol, 100 mg nicotinic acid, 100 mg pyridoxine-HCl, and 1 g thiamine-HCl in 1 L distilled water. The stock is stored at 4°C for a maximum of 6 mo.
7. Peters nutrient solution: 10% (w/v) nitrogen (ammonium sulfate), 50% (w/v) phosphorus (ammonium phosphate sulfite), and 10% (w/v) potassium (potassium sulfate) (J.R. Peters, Inc., Allentown Way, PA).
8. Acetosyringone solution: 50 mM acetosyringone (3, 5-dimethoxy-4 -hydroxy-acetophenone) is made by dissolving 98 mg of acetosyringone with 10 mL of 95% ethanol. The stock solution is stored at -20°C.
9. Antibiotic solutions: The antibiotic (e.g., paromomycin and streptomycin) stock solutions are made by dissolving 20 mg antibiotic/mL in distilled water and filter sterilized using a 0.45 μm syringe filter. They are stored at -20°C. The antibiotics in the plant tissue culture media are added fresh to the media once it's cooled to 50°C each time media is made.

2.4. Plant Media

Four types of media are used in cassava transformation and in the subsequent recovery of putative transformants. With the exception of the rooting medium, all the media contain equivalent concentrations of MS salts and sucrose but differ in the concentrations and types of hormones included (*see* **Table 1**). All

Table 1

Reagent	Stock concentration	Final concentration	4E shoot multiplication/L	MS8 callus/somatic embryos induction/L	RM1 embryo maturation/L	17N rooting medium/L
MS salts			4.3 g	4.3 g	4.3 g	1.44 g
B5 Vitamins	100X	1X	–	10 mL	–	–
Sucrose		20 g/L	20 g	20 g	20 g	20 g
myo-inositol	8 g/L	100 mg/L	100 mg		100 mg	
BAP	10 mg/L	0.04 mg/L	4 mL	–	–	–
2,4-D	0.5 mg/ml	8 mg/L	–	16 mL	–	–
GA	10 mg/L	4E-0.05 mg/L RM1-1.0 mg/L 17N-0.01mg/L 4E-10 mg/L				
Thiamine-HCl	1 g/L		5 mL	–	10 mL	1 mL
NAA	10 mg/L		10 mL	–	10 mL	6 mL
CuSO ₄	1 mM		2 mL	–	1 mL	1 mL
Casein hydrolysate		1.88 µM	–	1.88 mL	–	–
Peters nutrient	5 g/L	50 mg/L 25 mg/L	–	50 mg	–	5 mL
pH		5.7	5.7	5.7	5.7	5.7
Agar		8 g/L	8 g	–	–	8 g
Phytigel		2 g/L	–	2 g	2 g	–

media are adjusted to pH 5.7, autoclaved (20 min at 1.06 kg/cm² pressure), cooled to 50°C and dispensed into 100 × 15-mm Petri dishes. Prepared media are stored at 4°C. Final concentrations for all components are listed in Table 1.

1. 4E-Micropropagation medium: 1X MS salts (4.3 g), sucrose (20 g), myo-inositol (100 mg), and 2.0 mL of NAA solution are dissolved in 800 mL de-ionized water. The pH is then adjusted to 5.7 using 1 N potassium hydroxide (KOH) or 1 N hydrochloric acid (HCl) and the final volume is brought to 1 L. Add 8 g of agar (Fisher Scientific) after pH adjustment. The media is then autoclaved and 4 mL of BAP solution, 5 mL of GA solution, and 10 mL of thiamine-HCl solution are added after the medium has cooled to 50°C (see **Note 2**).
2. MS8-callus/somatic embryo induction media: 1X MS salts (4.3 g) and sucrose (20 g) are dissolved in 800 mL of de-ionized water. Add 1.88 mL of copper sulfate solution, 16 mL of 2,4-D solution, and 50 mg of casein hydrolysate. The pH is adjusted to 5.7 using 1 N KOH or 1 N HCl, and the final volume is brought to one liter before adding 2.0 g of Phytigel prior to autoclaving. After autoclaving, add 10 mL of B5 vitamin solution when the medium cools down to about 50°C (see **Note 3**).
3. RM1-embryo maturation medium: 1X MS salts (4.3 g), sucrose (20 g), myo-inositol (100 mg), and 1.0 mL of NAA solution are dissolved in 800 mL of de-ionized water. The pH is adjusted to 5.7 as described above and the final volume is brought to 1L. Add 2 g of phytigel and autoclave. Add 10 mL of GA solution and 10 mL of thiamine solution after autoclaving when the medium has cooled to 50°C (final concentrations listed in Table 1) (see **Note 4**).
4. 17N-rooting medium: Add $\frac{1}{3}$ MS salts (1.44 g), sucrose (20 g), 1.0 mL of NAA solution and 5 mL of Peters Nutrient fertilizer to 800 mL of deionized water. Adjust the pH to 5.7 as described above and bring to a final volume of 1 L. Add 8.0 g of agar prior to autoclaving. Add 1.0 mL of GA solution and 6.0 mL of thiamine solution after autoclaving when the medium cools down to 50°C (final concentrations listed in **Table 1**). Fifty milliliters of the medium is prepared and dispensed in magenta boxes. Alternatively, 4E media supplemented with 0.5 g charcoal/L can be used for rooting.

2.5. Agrobacterium Media

1. YM-*Agrobacterium* medium: One liter contains 0.4 g yeast extract and 10 g mannitol. Salts are present in the following final concentrations: 1.7 mM NaCl, 0.8 mM MgSO₄, and 2.2 mM K₂HPO₄, pH 7.0. For solid medium, add 15 g /L agar before autoclaving. Appropriate antibiotics are added as required after autoclaving and cooling to 50°C.
2. Co-culture medium: 1X MS basal salt medium devoid of nitrogen (see **Table 1**), but supplemented with 10 g/L glucose and 10 g/L galactose and the pH is adjusted to 5.5.

2.6. Other Reagents and Supplies

1. Sterilization solution: 20% (v/v) household bleach (sodium hypochlorite solution), 0.05% (v/v) Tween-20 (Polyoxethylenesorbitan monolaurate) (Sigma), or 0.05% (v/v) Silwet L-77 (Polyalkyleneoxide) (Setre Chemical Company).

2. No. 1 Whatman filter paper (Fisher Scientific).
3. Magenta box: 77 × 77 × 97 mm (Sigma).
4. BD1M needle: 38-mm long (Fisher Scientific).
5. No. 11 surgical blade (Fisher Scientific).

3. Methods

Various types of meristematic tissues are used to initiate callus, which is subsequently transferred to embryo-induction medium, and to transform developing embryos with *A. tumefaciens*. The explants have included apical leaves, undifferentiated callus tissue, germinating somatic embryos, auxiliary buds, and floral tissues (*see Fig. 1*). Because cassava rarely flowers, floral parts are not frequently used for cassava transformation. To date, only a few cassava cultivars have been reported to have been transformed. Below, we provide detailed descriptions of the explant tissues commonly used in cassava transformation. A flow diagram describing the overall scheme for *Agrobacterium*-mediated transformation of cassava is presented in **Fig. 2**.

3.1. Preparation of Explants for Transformation

3.1.1. Surface Sterilization, Preparation of Apical Leaf and Initiation of In Vitro Plants

1. Sterile in vitro cassava plants are initiated from immature stem cuttings (located 2–10 cm from the shoot apex) obtained from 4- to 6-mo-old greenhouse grown plants.
2. Stems are excised, and divided into pieces with two to three nodes.
3. The pieces are then surface sterilized using sterilization solution, and maintained on a shaker (250 rpm) for 10 min.
4. The stem pieces are then rinsed three times with sterilized distilled water and blot-dried on sterile No. 1 Whatman filter paper.
5. The stem pieces are cultured by inserting the basal stem portion into Magenta boxes containing 50 mL semi-solid MS shoot multiplication medium (4E).
6. Cultures are incubated at 26°C under a 12-h light/dark regime provided by cool-white fluorescent lamps (40 $\mu\text{mole/m}^2/\text{s}$) until young actively growing apical leaves emerge (about 3 wk after culture).
7. At this stage the apical leaves are ready for initiating embryogenic cultures (*see Subheading 3.1.2.*) or direct transformation (*see Subheading 3.3.*). The in vitro plants can supply apical leaves for up to 3 mo.

3.1.2. Preparation of Undifferentiated Callus Tissue

1. Young actively growing apical leaves (*see Fig. 1A*) are excised from clean (non-contaminated) cultures, placed onto a sterile filter paper and wounded (5–7 pin holes) using a sterile 38- mm long BD1M needle.
2. Wounded apical leaves are then placed with the adaxial surface on MS8 medium to initiate callus cultures.

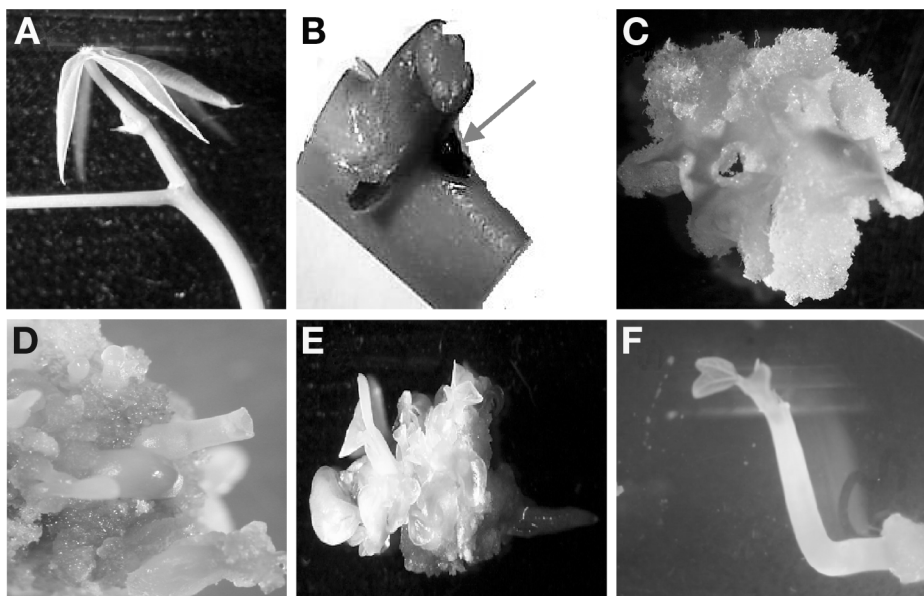


Fig. 1. In vitro and in vivo cassava tissues: (A) apical leaf, (B) axillary bud, (C) callus tissue, (D) embryogenic callus tissue, (E) germinating somatic embryos, (F) a developing plantlet. Arrow indicates axillary bud.

3. Small embryos will typically develop from 3- to 4-wk old callus cultures (see **Fig. 1D**) and may be used directly for transformation or transferred to embryo maturation (see **Fig. 2**) (RM1) medium to develop germinating somatic embryos (Section 3.1.3).

3.1.3. Preparation of Germinating Somatic Embryos

1. Three- to four-wk old embryoid callus tissues (see **Subheading 3.1.2.**) are transferred to embryo maturation medium (RM1) and incubated at 26°C under a 12-hr light/dark regime.
2. After 3- to 4-wk, germinated somatic embryos are excised and briefly washed in MS media containing 0.05% (v/v) Tween-20.
3. The germinated somatic embryos are then cut into pieces approxi 25–50 mm² and are wounded using a sterile 38-mm long BD1M needle prior to inoculation with *Agrobacterium* (see **Fig. 1**).

3.1.4. Preparation of Auxiliary Buds

1. Nodal stem cuttings with two to three auxiliary buds are excised from immature stem portions and surface sterilized as described in **Subheading 3.1.1.**
2. Buds are excised using a No 10 surgical blade. A small incision is made on the surface of the excised bud.

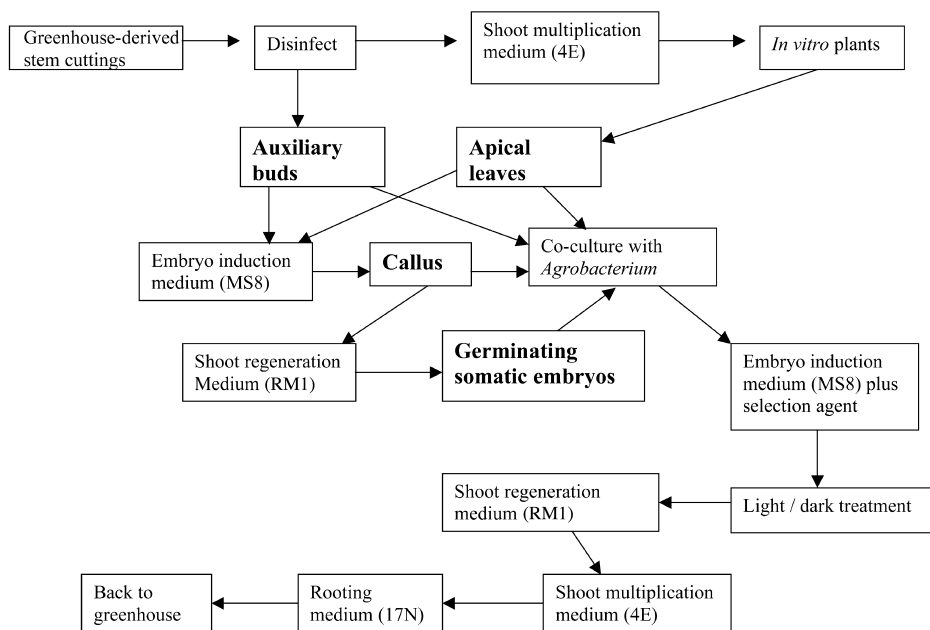


Fig. 2. Overall scheme of *Agrobacterium*-mediated transformation of cassava. Explant types are shown in bold.

3. At this stage the buds may be used to initiate callus cultures (**Fig. 2**). To do so, the buds are cultured on MS8 medium supplemented with 16 mg/L (MS16, two times the regular amount) 2,4-D.
4. Alternatively, the buds may be directly inoculated with *Agrobacterium* and inoculated on solid MS16 medium (*see Subheading 3.3.*).

3.2. *Agrobacterium* Preparation

1. *Agrobacterium* strain LBA4404 (*see Note 5*) containing the vector of interest is streaked and grown for 2 d on solid YM medium supplemented with 100 mg/L streptomycin and 6 mg/L tetracycline (or relevant antibiotic according to the plasmid of choice). Cultures are incubated at 28°C.
2. After 2 d, single colonies are used to inoculate 20 mL of liquid YM medium supplemented with 200 μ M acetosyringone, 100 mg/L streptomycin, 6 mg/L tetracycline (or the antibiotics used depend on the plasmid used), and incubated at 28°C on a shaker at 250 rpm for 2 d.
3. After 2 d growth, the bacteria are pelleted by centrifugation (10,500g) for 7 min, and resuspended in 20 mL of MS basal salt (co-culture) medium devoid of nitrogen, but supplemented with 1% (w/v) of glucose and 1% (w/v) of galactose, and the pH adjusted to 5.5.
4. The optical density (OD₆₀₀) of the bacterial suspension is measured and adjusted to 0.5 (approx 0.5×10^9 cfu/mL) using a spectrophotometer.
5. At this stage the bacteria are ready for inoculating the explants.

3.3. Explant Inoculation and Cocultivation

1. Following wounding explants are transferred onto solid MS8 medium supplemented with 200 μ M acetosyringone.
2. Two drops of the bacterial suspension in liquid MS [to which is added 0.05% (v/v) Tween-20] are applied to the explant tissue, and the tissue is incubated in the dark for 2 d at 24–28°C. Subsequently, the explants are transferred to a new MS8 plate with 200 μ M acetosyringone for another 3 d co-cultivation (*see Note 6*).
3. Following co-cultivation with *A. tumefaciens*, explants are transferred to selection media (MS8 supplemented appropriate selective agent according to the vector used and 500 mg/L of carbenicillin) (*see Note 7*).
4. To induce embryogenesis explants are incubated in the dark for 4 wk. For germplasm maintenance explants are transferred directly to a 12-h light/dark regime under 40 μ mol/m²/s lighting intensity after 1 wk dark incubation (*see Note 8*).
5. Explants are maintained on selection medium for 4 to 6 wk. Somatic embryos produced on selection media are transferred to selective RM1 media (RM1 supplemented appropriate selective agent according to the vector used and 500 mg/L of carbenicillin) on which the embryos convert to plants.
6. Plantlets are regenerated following successive transfers of the inoculated nodal segment explants onto shoot induction (4 wk), shoot regeneration (4 wk), and shoot multiplication media (4 wk) (*see Note 9*).

3.4. Selection and Regeneration of Putative Transformants

1. Selection of putative transformants follows successive transfers of the inoculated explants onto somatic embryo induction (MS8), and embryo maturation (RM1) media supplemented with appropriate selection agents plus carbenicillin (500 mg/L) to kill *A. tumefaciens*.
2. Cultures are examined biweekly under a dissecting microscope to check for presence of embryos (*see Fig. 1D*) ready for transfer to maturation medium (RM1).
3. To transfer the embryos, a sterile needle is used to pick the embryos which are aseptically placed on maturation medium.
4. Sequentially, embryos are transferred from selection media to media free of the selection agent until shoots have elongated to about 1 cm.
5. Elongated shoots are excised and transferred to shoot multiplication medium (4E). Cultures are incubated at 26°C under a 12-h light/dark regime provided by cool-white fluorescent lamps (40 μ mol/m²/s) for 4 wk to allow the plants to grow to sufficient size for root induction.
6. From 4E medium, shoots are excised and cultured on rooting medium (17N).
7. Root formation takes approx 2–3 wk after which the plants are transplanted to soil. In vitro grown plants maintained on 4E medium are used for transgene analysis.

3.5. Greenhouse Care of Transgenic Plants

1. Transgenic plants that have been grown in vitro for 1 mo are transferred to sterile soil (3:1 soil/vermiculite) and grown in the greenhouse under natural lighting conditions and a temperature range of 22–30°C. The transferred plants are covered with transparent plastic bags to maintain high humidity.

2. The bags are removed after 2–4 wk to expose the plants to normal environment.
3. The transformed plants are now treated like the wild-type cassava plants by watering three times a week.

4. Notes

1. We have typically used the binary plasmids pKYLX or pBI121 in our laboratory for cassava transformation (**9–11**). Using *Agrobacterium* strains harboring the binary plasmids having T-DNA inserts ranging from 3.5 to 5.5 kb, we achieved transformation rates between 0.5 and 1.0% when using cassava apical leaves and between 2.4 and 5.0% when using germinated somatic embryos. The T-DNA included the *nptII* selectable marker driven by the NOS promoter. Transformants were selected using 75 mg/L paromomycin and confirmed by PCR analysis of the integrated transgene.
2. Micropropagation medium (4E) is designed not only to quickly multiply the plantlets but also to maintain the explants for a long period of time prior to subculture.
3. A prominent feature of the callus induction medium (MS8) is the addition of B5 vitamins (7), copper sulfate and casein hydrolysate (*see Table 1*). Copper sulfate is reported to increase the number of primary and secondary embryos (7).
4. The embryo maturation medium (RM1) contains no auxins or copper sulfate.
5. Other *Agrobacterium* strains such as C58 and EHA101 could be used for transformation. However, we found that LBA4404 worked better for our transformation system.
6. Co-culture of *A. tumefaciens* with the explant in liquid medium (*see Subheading 3.2.1.*) works well for large sized explants such as callus tissues and clumps of germinating somatic embryos. Small sized, and fragile explants such as wounded apical leaves and auxiliary buds respond better when co-cultured with *A. tumefaciens* on solid MS medium (*see Subheading 3.2.2.*).
7. The type of selection agent used in the medium is determined by the marker gene in the plasmid construct. Marker genes used in our plasmid constructs have included neomycin phosphotransferase II (*npt II*) which confers resistance to aminoglycoside antibiotics such as kanamycin and paromomycin (25), and phosphomannose isomerase (PMI) which catalyzes the reversible inter-conversion of mannose 6-phosphate to fructose 6-phosphate (26). Plant cells lacking the PMI gene are incapable of surviving on synthetic medium containing mannose as a carbon source (26). The embryo induction and embryo maturation selection media used contain paromomycin (75 mg/L), an analog of kanamycin or mannose (10 g/L, w/v) as selection agents, and carbenicillin (500 mg/L) to kill *A. tumefaciens*.
8. Light is reported to have a significant effect on cassava plant regeneration and to promote gene transfer from *A. tumefaciens* to plant cells (27).
9. Transformation efficiency (TE) is a quantitative measurement of the integration of the gene of interest into the host plant genome. TE may be measured by the number of desirable events (trait under selection, e.g., herbicide resistance) recovered per 100 explant pieces inoculated (5). In this paper, transformation efficiency refers to the number of PCR amplified transgene-confirmed positive plants per total number of explants inoculated (23).

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Weston Msikita and Uzoma Ihemere contributed equally to this document.

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