Universidade Federal do Mato Grosso do Sul
Mestrado em Biotecnologia

Over-expression of morphogenic genes enhances plant regeneration in cassava (*Manihot esculenta*)

Aumento da regeneração de *Manihot esculenta* por genes morfogênicos após edição genética

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AUMENTO DA REGENERAÇÃO DE *MANIHOT ESCULENTA* POR GENES MORFOGÊNICOS APÓS EDIÇÃO GENÉTICA

Dissertação apresentada ao curso de mestrado acadêmico em Biotecnologia da Pró-Reitoria de Pos-graduação e pesquisa da Universidade Federal do Mato Grosso do Sul como requisito para obtenção do grau Mestre em Biotecnologia.

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Abstract

The Growth Regulating Factor (GRF) and its Interacting Factor (GIF) have been shown to stimulate regeneration of transgenic plants, with studies reporting increased transformation efficiency in multiple species including wheat, beet and citrus. The present work evaluated the effects of overexpressing *GRF4-GIF1* and *GRF5* on the regeneration of transgenic plants in cassava (*Manihot esculenta* Crantz). Effects of *GRF4-GIF1* and *GRF5* sequences derived from *Vitis vinifera* and *Arabidopsis thaliana* were assessed by cloning expression cassettes under control of strong constitutive promoters. Friable embryogenic callus from cassava varieties 60444 and NASE 13 were transformed with *Agrobacterium tumefaciens* strains LBA4404 and LBA4404 THY- and multiple independent transgenic plant lines recovered. Expression of the morphogenic genes did not enhance transformation efficiency above the GFP control, nor efficiency or timing of somatic embryo regeneration or whole plant recovery. Organogenesis experiments were carried out to observe effects of these genes on morphogenesis from petiole, leaf-petiole, and stem explants. Expression of *Vitis vinifera GRF4-GIF1* was found to stimulate rapid organogenesis and shoot regeneration from leaf-petiole explants with plant regeneration occurring within 3-4 weeks culture on medium containing the cytokinin meta-topolin. Effects at the whole plant level were accessed by establishing plants in the greenhouse, with *VviGRF4-GIF1* overexpression resulting in increased leaf size and total leaf area, and *AtGRF5* stimulating above average results for plant height.

Resumo

O Fator de Regulação do Crescimento (GRF) e seu Fator de Interação (GIF) demonstraram estimular a regeneração de plantas transgênicas, com estudos relatando maior eficiência de transformação em várias espécies, incluindo trigo, beterraba e frutas cítricas. O presente trabalho avaliou os efeitos da superexpressão de *GRF4-GIF1* e *GRF5* na regeneração de plantas transgênicas em mandioca (*Manihot esculenta* Crantz). Os efeitos das sequências *GRF4-GIF1* e *GRF5* derivadas de *Vitis vinifera* e *Arabidopsis thaliana* foram avaliados pela clonagem de cassetes de expressão sob o controle de fortes promotores constitutivos. Calos embriogênicos friáveis das variedades de mandioca 60444 e NASE 13 foram transformados com as cepas de Agrobacterium tumefaciens LBA4404 e LBA4404 THY- e várias linhas de plantas transgênicas independentes recuperadas. A expressão dos genes morfogênicos não aumentou a eficiência de transformação acima do controle GFP, nem a eficiência ou tempo de regeneração do embrião somático ou recuperação da planta inteira. Experimentos de organogênese foram conduzidos para observar os efeitos desses genes na morfogênese de explantes de pecíolo, folha-pecíolo e caule. Verificou-se que a expressão de *Vitis vinifera GRF4-GIF1* estimula a rápida organogênese e a regeneração de brotos de explantes de folha-pecíolos com a regeneração de brotos ocorrendo dentro de 3-4 semanas de cultura em meio contendo a citocinina meta-topolina. Os efeitos *in planta* foram acessados pelo estabelecimento de plantas em casa de vegetação, com a superexpressão de *VviGRF4-GIF1* resultando em aumento do tamanho da folha e da área foliar total, e *AtGRF5* apresentando resultados acima da média para a altura da planta.

Key words

Agrobacterium transformation, cassava, *GROWTH-REGULATING FACTOR*, morphogenesis, shoot regeneration.

Palavras-chave

Transformação por *Agrobacterium*, mandioca, FATOR REGULADOR DO CRESCIMENTO, morfogênese, regeneração de brotos.

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Índice

Literature review

The starchy root crop cassava (*Manihot esculenta* Crantz) is a major staple for hundreds of millions of people across the world's tropical regions, where it is surpassed only by rice and maize in its importance as a source of dietary carbohydrates (Clifton, 2016). Traditionally grown by smallholder farmers for on-farm consumption and sale in local markets, it is also becoming an important industrial crop, cultivated for its high-quality starch and as a source of biofuel, alcohol, and bioplastics (Wagaba et al., 2020).

Cassava is consumed either directly after boiling, or in the form of flour or starch in a great variety of ways. Brazil produced 18.5 million tons of cassava in 2022 cultivated on 1.36 million hectares, ranking third in the world behind Nigeria and Thailand (IBGE, 2022). Cassava starch is the main processed product, which in 2021 reached 636.21 thousand tons produced in Brazil (Alves and Isaias, 2022). Also cassava has been recognized as drought-tolerant crop and is therefore predicted to become increasingly important for future food, and economic security, and agricultural output in the tropics (Pushpalatha and Gangadharan, 2020). Genetic improvement of cassava is required to meet changing farmer and consumer needs, evolving pests and diseases, and challenges presented by climate change, population growth, and urbanization.

Conventional breeding in cassava is complicated by its high level of heterozygosity, inbreeding depression, and asynchronous flowering (Liu et al. 2011). Transgenic and genome-editing technologies offer important potential for introducing desired traits into farmer-preferred varieties and breeding lines and for studying the biology of this under-investigated crop species. Recovery of transgenic cassava plants was first reported in 1996 (Schopke et al. 1996). Multiple publications have subsequently described development and application of transgenic systems to produce plants genetically modified for disease resistance (Buyene et al. 2017; Gomez et al. 2018; Wagaba et al. 2017), modified starch (Bull et al. 2018), nutritional enhancement (Narayanan et al. 2019), and reduced cyanogenesis (Gomez et al. 2021), among other traits. The most frequently used protocol for genetic transformation of cassava relies on generation of embryogenic callus as target tissue for integration of transgenes and gene editing tools via *Agrobacterium tumefaciens*. Important progress has been made to increase efficiency and expand capacity into cassava genotypes of importance in Africa, Asia, and the Americas (Chavarriaga-Aguirre et al. 2016;

Narayanan et al. 2021; Taylor et al. 2012a; Utsumi et al. 2022). Nevertheless, production of transgenic cassava remains challenging for many researchers, with the capacity for reliable regeneration of modified plants restricted to only a few laboratories around the world.

Biotechnology is being applied to address constraints in cassava production to generate, for example, disease resistance (Wagaba et al. 2017), modified starch quality (Bull et al. 2018), enhanced nutritional content (Narayanan et al. 2019) and to generate plants expressing traits of agronomic interest such as resistance to cassava brown streak disease (CBSD), and cassava mosaic disease (CMD) (Taylor et al. 2012b). Use of transgenic and gene editing technologies relies on the production of morphogenic tissues into which transgenes and gene editing tools can be delivered and whole plants regenerated.

This process is possible due to hormones acting as growth regulators such as auxins and cytokinins, their manipulation in most plants can form a callus, an undifferentiated cell mass of dividing pluripotent stem cells (Taiz and Zeiger, 1997). Whole plants can be regenerated from callus explants if the correct conditions are in place to stimulate organogenesis (Altpeter et al. 2016). Although there's been considerable progress in recent years, regeneration efficiency in most plants remains low, which limits genome editing applications and transformation for crop improvement. In cassava plant recovery is also genotype specific with efficient plant regeneration limited to a relatively small subset of the many 100s of varieties grown by farmers across the tropics (Utsumi et al. 2022).

The tissue culture and gene transfer systems currently employed to produce transgenic, and gene edited cassava have improved significantly over the years. Well established systems for production of transgenic plants via somatic embryogenesis are in place (Taylor et al., 2012a; Chauhan et al., 2015), and organogenic systems for regeneration of shoots from petiole and stem tissues have been reported (Chauhan and Taylor, 2018). However, cassava genetic transformation remains a lengthy process, with four months necessary for production of FEC target tissues and after transformation up to six months to regenerate genetically modified plants, requiring skilled labor and abundant laboratory supplies (Segatto et al. 2022). Therefore, evaluation of other strategies for plant regeneration could lead to improvements to the current systems, especially regarding transformation efficiency of recalcitrant cultivars and speed of plant recovery after transformation which genes that control growth and development could benefit.

The *GROWTH-REGULATING FACTOR* (GRF) is a family of plant transcription factors defined by the presence of the WRC and QLQ protein domains. These factors have a role in promoting cell proliferation during leaf development and are required for the development and maintenance of the shoot apical meristem (Kim et al., 2003). GRFs interact with another family of transcription factors, the *GROWTH INTERACTING FACTOR* (GIF) forming a duo, in this functional unit GIF operates to recruit SWI/SNF chromatin remodeling complexes to their target genes so that they can be transcriptionally activated or repressed by GRF (Luo and Palmgren, 2021).

This functional unit also gives primordial cells of vegetative and reproductive organs a meristematic specification state, guaranteeing the supply of cells for organogenesis (Lee et al., 2009). Regarding age of the plant or organ, it has been shown the *AtGRF* expression levels decrease as the age of the organ increases, therefore GRFs are generally more expressed in actively growing tissues than in mature ones (Kim and Lee 2006; Rodriguez et al. 2010).

GRF expression is post-transcriptionally downregulated by microRNA396 (miR396). In *Arabidopsis*, the MIR396 gene family has two members (ath-MIR396a and ath-MIR396b). These can induce cleavage of seven *AtGRF* mRNA species, with the exception *AtGRF5* and *AtGRF6* transcripts which don't have the target site (Kim and Tsukaya, 2015). Overexpression of the microRNA396 causes post-transcriptional down-regulation of all target *AtGRF* genes, resulting in small plants and pistil abnormalities (Liang et al. 2014). In Qiu et al. 2022 an introduced mutation in the miR396 target site of *TaGRF4* resulted in a complex that outperformed the chimera without a miR396 mutation in wheat regeneration studies.

An important reference for the present work is the report of Debernardi and colleagues who combined *GRF4* and *GIF1* from wheat (*Triticum aestivum*) to generate a chimera, two or more genes that originally coded for separate proteins, and showed that the *GRF4-GIF1* chimera, was superior to either protein expressed separately and dramatically improved the regeneration efficiencies of monocotyledonous durum wheat, common wheat, and triticale, a hybrid between wheat and rye. Other *GRF4-GIF1* chimeras from Citrus and grape (*Vitis vinifera*), respectively, enhanced the regeneration ability of dicotyledonous citrus (Debernardi et al. 2020).

Another reference work is the report of Kong and colleagues who investigated the effects of transgenic expression of *AtGRF5* and its homologs, showing improved regeneration of dicot and monocot species, including canola, soybean, sunflower, maize and sugar beet, in which an

Arabidopsis GRF5 increased beet transformation efficiency twice as much as the beet *GRF5* ortholog (Kong et al. 2020).

According to the work of Vercruyssen et al. (2015), the transcription factor *GROWTH REGULATING FACTOR 5 (GRF5)* regulates duration of the cell proliferation period during leaf development in *Arabidopsis*. They have shown that overexpression of *GRF5* also stimulates chloroplast division, resulting in a higher chloroplast number per cell with increased chlorophyll levels in leaves which could maintain higher rates of photosynthesis. In addition, another characteristic of the *GRF5* plants is delayed leaf senescence and tolerance to nitrogen-depleted medium. The authors suggest these changes could potentially improve plant productivity.

Effectiveness of *GRF4-GIF1* in plant regeneration could be cultivar specific, Ryan, 2022 evaluated Citrus *GRF4-GIF1* in Populus and Eucalyptus transformation, with variable results, a few negative regeneration phenotypes were observed and a positive result with 37% increase in transformation efficiency for a recalcitrant *P. alba* genotype.

The studies that led the field into further investigation of plant genes that control growth and development started with *BABY BOOM (BBM)* and *WUSCHEL (WUS)* (Lowe et al. 2016; Gordon-Kamm et al. 2019), and more recently these growth regulators are being combined to further improve plant recovery, such as *GRF-GIF* and *BBM* in maize transformation (Chen et al. 2022). Use of morphogenic genes is a promising strategy to improve plant transformation with many interesting gene candidates and results are leading to more knowledge and advances at an impressive speed.

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Agrobacterium-mediated Genetic Transformation of Cassava

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The storage root crop cassava (*Manihot esculenta* Crantz) is predicted to remain central to future food and economic security for smallholder farming households and agricultural output in the tropics. Genetic improvement of cassava is required to meet changing farmer and consumer needs, evolving pests and diseases, and challenges presented by climate change. Transgenic and genome editing technologies offer significant potential for introducing desired traits into farmer-preferred varieties and breeding lines, and for studying the biology of this under-investigated crop species. A bottleneck in implementing genetic modification in this species has been access to robust methods for transformation of cassava cultivars and landraces. In this article, we provide a detailed protocol for *Agrobacterium*-mediated transformation of cassava and regeneration of genetically modified plants. Basic Protocol 1 describes how to establish and micropropagate *in vitro* cassava plantlets, and Alternate Protocol 1 details how to establish *in vitro* cultures from field or greenhouse cuttings. Basic Protocol 2 describes all steps necessary for genetic transformation in the model variety 60444, and Alternate Protocol 2 provides details for modifying this method for use with other cultivars. Finally, Basic Protocol 3 describes how to establish plants produced via Basic Protocol 2 and Alternate Protocol 2 in soil in a greenhouse. These methods have proven applications across more than a dozen genotypes and are capable of producing transgenic and gene-edited plants for experimental purposes, for testing under greenhouse and field conditions, and for development of plants suitable for subsequent regulatory approval and product deployment. © 2022 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Establishment and propagation of *in vitro* cassava plantlets
Alternate Protocol 1: Establishment of *in vitro* plants from field or greenhouse plants
Basic Protocol 2: Genetic transformation of cassava variety 60444
Alternate Protocol 2: Genetic transformation of additional cultivars
Basic Protocol 3: Establishment and growth of plants in the greenhouse

Keywords: cassava • friable embryogenic callus • genetic transformation • genome editing • somatic embryogenesis

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INTRODUCTION

The starchy root crop cassava (*Manihot esculenta* Crantz) is a major staple for hundreds of millions of people across the world's tropical regions, where it is surpassed only by rice and maize in its importance as a source of dietary carbohydrates. Traditionally grown by smallholder farmers for on-farm consumption and sale in local markets, it is also becoming an important industrial crop, cultivated for its high-quality starch and as a source of biofuel, alcohol, and bioplastics (Wagaba et al., 2020). Cassava has been recognized as drought-tolerant and is therefore predicted to become increasingly important for future food, and economic security, and agricultural output in the tropics (Pushpalatha & Gangadharan, 2020).

Genetic improvement of cassava is required to meet changing farmer and consumer needs, evolving pests and diseases, and challenges presented by climate change, population growth, and urbanization. Conventional breeding in cassava is complicated by its high level of heterozygosity, inbreeding depression, and asynchronous flowering (Liu, Zheng, Ma, Gadidasu, & Zhang, 2011). Transgenic and genome-editing technologies offer important potential for introducing desired traits into farmer-preferred varieties and breeding lines and for studying the biology of this under-investigated crop species. Recovery of transgenic cassava plants was first reported in 1996 (Li, Sautter, Potrykus, & Puonti-Kaerlas, 1996; Schöpke et al., 1996). Multiple publications have subsequently described development and application of transgenic systems to produce plants genetically modified for disease resistance (Gomez et al., 2018; Wagaba et al., 2017), modified starch (Bull et al., 2018), nutritional enhancement (Narayanan et al., 2019), and reduced cyanogenesis (Gomez et al., 2021), among other traits.

The most frequently used protocol for genetic transformation of cassava relies on generation of embryogenic callus as target tissue for integration of transgenes and gene-editing tools via *Agrobacterium tumefaciens*. Important progress has been made to increase efficiency and expand capacity into cassava genotypes of importance in Africa, Asia, and the Americas (Chauhan, Beyene, & Taylor, 2018; Chavarriaga-Aguirre et al., 2016; Narayanan et al., 2021; Taylor et al., 2012; Utsumi et al., 2022). Nevertheless, production of transgenic cassava remains challenging for many researchers, with the capacity for reliable regeneration of modified plants restricted to only a few laboratories around the world.

We describe here highly detailed methodologies to enable the production of transgenic cassava plants across a range of genotypes. The protocols presented provide details for all stages needed to initiate and maintain cassava plants *in vitro*, produce totipotent target tissues, perform *Agrobacterium*-mediated transformation, regenerate robust transgenic plants, and establish them in the greenhouse. Specifically, Basic Protocol 1 describes how to maintain micropropagated plantlets *in vitro*, while Alternate Protocol 1 details how to establish *in vitro* cultures from cuttings obtained from field- or greenhouse-grown plants. Basic Protocol 2 describes the steps necessary for genetic transformation in the model variety 60444, while Alternate Protocol 2 provides details for modifying this method for other farmer-preferred cultivars. Finally, Basic Protocol 3 describes how to establish regenerated plants in soil in the greenhouse.

STRATEGIC PLANNING

The protocols described below assume that the researcher has access to basic plant tissue culture equipment and reagents. These should be obtained before commencing work to produce genetically modified cassava plants. Equipment includes laminar flow hoods in which plant materials can be handled axenically, autoclaves for sterilization of plant media, a temperature-controlled growth room with lighting to maintain plant cultures, and an environmentally controlled greenhouse if establishment of whole plants in soil is desired.

To initiate transformation, the user will need viable cultures of *A. tumefaciens* LBA4404 or a similar strain harboring a plasmid with genes of interest and preferably a visual marker. We recommend the use of green fluorescent protein (GFP) to track transformation success and progress, but alternatives are available and function well (for details, see Background Information).

Only high-quality chemicals from reputable suppliers should be used in order to reliably replicate these protocols. For planning purposes, lists of media with their components and specific uses are provided in Tables 1 and 2 for cassava cultivar 60444 and other cultivars, respectively. Instructions for preparing media are provided in Reagents and Solutions.

If cultures of cassava plants established *in vitro* are already available, follow Basic Protocol 1 for micropropagation and multiplication, then proceed to Basic Protocol 2 for production of transgenic or gene-edited plants. If cassava plants need to be brought into culture from the greenhouse or field, start with Alternate Protocol 1, then multiply and maintain the *in vitro* plantlets prior to commencing Basic Protocol 2 or Alternate Protocol 2. Depending on the research goals, the recovered plants can be sent to the greenhouse for evaluation following details provided in Basic Protocol 3. If the researcher is inexperienced in cassava transformation, we recommend the use of the cassava cultivar 60444, as it offers ease of target tissue production, an amenable response to *Agrobacterium* transgene integration, and high regeneration efficiency.

NOTE: Before beginning work, regulatory approval from the relevant authorities must be in place to handle and produce transgenic bacteria and plants.

ESTABLISHMENT AND PROPAGATION OF *IN VITRO* CASSAVA PLANTLETS

BASIC PROTOCOL 1

Here we describe the steps required to establish *in vitro* cultures and how to micropropagate and multiply cassava plants. These mother plants generate the leaf explant material used to initiate the embryogenic target tissues for subsequent transgene integration. We provide directions to handle cassava plant material as *in vitro* plantlets obtained from germplasm collections such as those held by the International Center for Tropical Agriculture (CIAT) and the International Institute of Tropical Agriculture (IITA). Cassava genotypes obtained from germplasm collections are most often transported in glass or plastic test tubes. The steps below describe first how to establish mother plants from such cultures and then how to propagate them.

While growth and multiplication rates vary, this protocol has proven successful for micropropagation of many cassava varieties. Details are provided for establishing and growing mother plants in Petri dishes. Glass jars can also be used, but Petri dishes are preferred because they can be stacked three or four high, allowing large numbers of mother plants to be maintained in a relatively small space within growth chambers. Use of 100 × 25–mm Petri dishes is recommended for the MS2 agar plates to provide plants with maximum headspace for growth, although 100 × 15–mm sterile dishes also work well.

To provide sufficient numbers of explants for production of target tissues and recovery of transgenic plants, 20 Petri dishes carrying 7-8 mother plants each (140-160 *in vitro* plantlets) should be established. The established plantlets should be subcultured every 6-8 weeks to ensure continued healthy growth and production of shoot materials in optimal condition for induction of embryogenic tissues.

Materials

In vitro cassava cultures (e.g., from CIAT)
MS2 agar plates (see recipe)

Laminar flow hood
Sterile fine pointed forceps
Sterile 100 × 15–mm Petri dishes (VWR, 25384-342)
Miltex carbon steel no. 10 surgical blade (prod. no. 4-110)
Parafilm or Saran Wrap cut into 5-cm wide strips
28°C growth chamber

Establish mother plants from in vitro plantlets

1. Under a laminar flow hood and using sterilized forceps, carefully extract a plantlet from a test tube or other container and place on a sterile Petri dish lid.
2. Using sterilized forceps and scalpel blade, trim away and discard old leaves. Cut the plantlet transversely to generate 2-3 nodes per stem segment.
3. Transfer stem segments to MS2 agar plates, submerging 0.5 cm of the stem into the medium, and establishing 6-8 shoot explants per plate (20 plates per cultivar are ideal).
4. Seal and label plates appropriately and place in a 28°C growth chamber under bright light (90 µMol m^{-2} s^{-1}) with a 14-16 hr photoperiod.

Micropropagate mother plants

5. Open Petri dishes containing mother plants in the laminar flow hood.
6. Using sterile forceps and a scalpel blade, excise apical stem segments 2-3 cm in length. Trim away lower leaves and petioles from the stem and discard, leaving the apical meristem and the first mature leaf at the top of the micro cutting.
7. Place the cutting vertically into a new MS2 agar plate, submerging 0.5 cm of the stem into the medium, and placing 6-8 micro cuttings evenly spaced in each dish.
8. Seal and label plates appropriately and culture as above.

Mother plants can be harvested for leaf explants two to three times before they should be refreshed by subculturing as described above. After excision of new shoot growth, seal and return plates to the growth chamber and allow new shoots to grow. Four weeks later, new shoot growth can be harvested again from these mother plants. Mother plants should be renewed every 6-8 weeks by subculturing shoot cuttings onto fresh MS2 agar plates. If plants are not needed for establishing embryogenic cultures, the subculture period for maintenance of mother plants can be extended to 2-4 months.

ALTERNATE PROTOCOL 1

ESTABLISHMENT OF *IN VITRO* PLANTS FROM FIELD OR GREENHOUSE PLANTS

This protocol describes how to initiate sterile cassava *in vitro* if the researcher does not have access to previously established cultures of a desired genotype. Specifically, it provides a guide for surface sterilization and establishment of *in vitro* plantlets from greenhouse- or field-grown cassava plants. Only disease-free plants should be used to initiate cultures of a given variety. If stem cuttings are collected from the field, it is recommended that diagnostics be performed before proceeding in order to detect the presence of diseases such as cassava mosaic disease (CMD), cassava brown streak disease (CBSD), cassava bacterial blight (CBB), and cassava frogskin disease, as appropriate for the geographies where collection took place.

Materials

Field- or greenhouse-grown cassava plants
15% (v/v) bleach (sodium hypochlorite, 8.25%) in sterile diH_2O or reverse-osmosis (RO) water
Tween 20

Sterile RO water
MS2 Gelzan plates (see recipe)
MS2 agar plates (see recipe)

Straight single-edge razor blade
250- or 500-ml Erlenmeyer flasks
Aluminum foil
Rotary shaker (*optional*)
Laminar flow hood
100 × 15–mm sterile Petri dishes (VWR, 25384-342)
Waste bucket for bleach rinses
Fine pointed forceps
Miltex carbon steel no. 10 surgical blade (prod. no. 4-110)
Parafilm or Saran Wrap cut into 5-cm-wide strips
28°C growth chamber

NOTE: As in Basic Protocol 1, the use of 100 × 25–mm Petri dishes is recommended for MS2 Gelzan and MS2 agar plates to allow unrestricted growth of new shoots and mother plants.

Cut and surface sterilize stem nodes

1. Cut upper, green-colored stem material from field- or greenhouse-grown cassava plants.

   Two stems with 12-18 nodes should be adequate. Do not use woody or semi-woody stem segments, as these are problematic to surface sterilize.

2. Using a straight single-edge razor blade, cut off and discard all leaves and petioles to within 0.5 cm of the axillary buds.

3. Cut individual nodes from the stem, leaving ∼1.0 cm of stem above and below the axillary bud.

4. Place a maximum of 25 nodes in a 500-ml flask (15 nodes for a 250-ml flask; Fig. 1A) and cover with aluminum foil.

   Adding more than the specified number of nodes per flask will prevent proper contact of bleach solution with the stem surface and result in contaminated cultures.

5. Pour 100 ml of 15% bleach into each flask containing cut nodal segments and add two drops of Tween 20. Cover the tops tightly with foil and swirl.

6. Shake for 30 min at 150 rpm on a rotary shaker at room temperature, or swirl flasks by hand in a vigorous manner every 2 min for 30-40 min.

7. Transfer flasks to a laminar flow hood. Remove foil and invert a Petri dish on the top of the flask. Tilt the flask over a large beaker to let the bleach solution pour out, using the Petri dish to retain nodes within the flask.

8. To stop the sterilization process, add 150 ml sterile water to each flask, swirl, and drain the liquid into a waste bucket.

   Complete this process for all flasks before moving on to subsequent rinsing steps.

9. Rinse at least five more times with fresh sterile water until nodes are thoroughly rinsed and a bleach smell is no longer present.

10. Drain liquid and transfer nodes to new sterile Petri dish lids (one per flask).

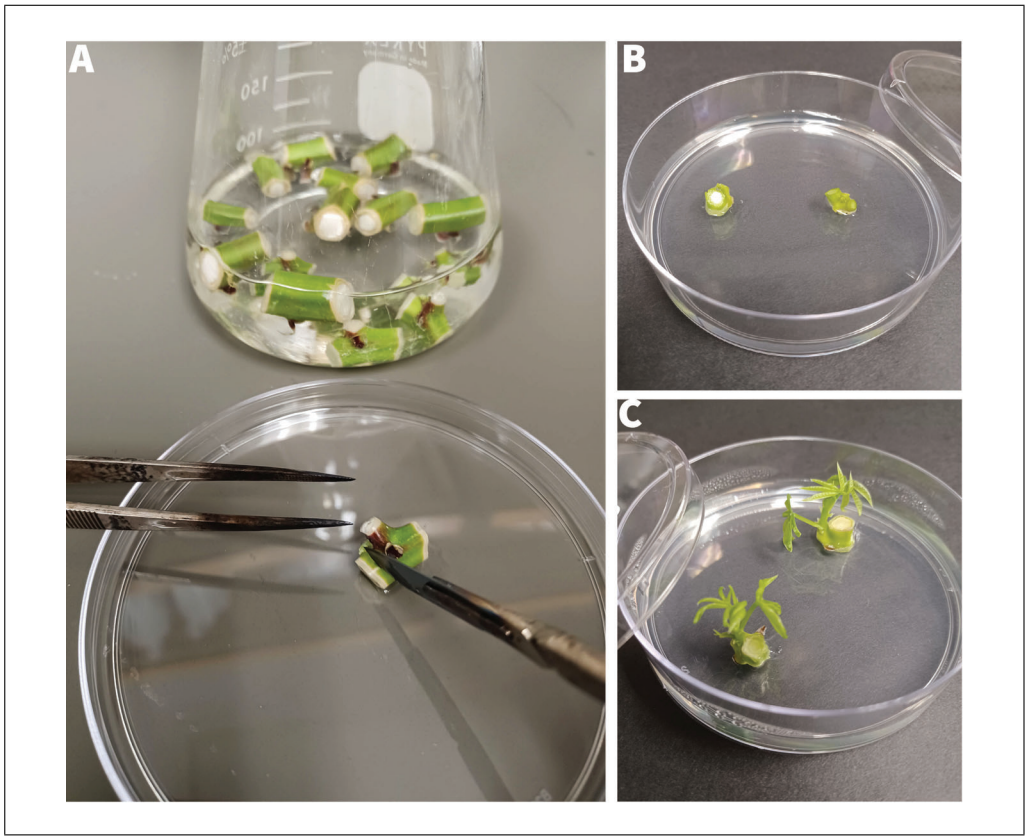

Figure 1 Establishment of cassava plantlets *in vitro* culture. (**A**) Surface sterilization of excised nodes. (**B**) Nodes placed on MS2 Gelzan medium. (**C**) Shoot regeneration from sterilized lateral buds after 8 days.

Grow shoots and establish mother plants

11. Under the laminar flow hood, use sterile forceps and scalpel blade to trim bleached tissues away from each nodal segment to expose green healthy tissue above and below the node, taking care not to damage the axillary bud (Fig. 1A).

12. Transfer trimmed nodes to an MS2 Gelzan plate, placing 2-3 nodes per dish, with the axillary buds facing up (Fig. 1B). Seal with Parafilm or Saran Wrap, label appropriately, and place in a 28°C growth chamber under bright light (90 µMol m^{-2} s^{-1}) with a light duration of 14-16 hr.

13. Observe plates every two days and allow shoots to grow to 2-3 cm in length. Discard if bacterial or fungal growth is observed.

    New shoots should emerge from the nodal explants 5-10 days after placing on Gelzan medium depending on the cultivar (Fig. 1C). If a nodal explant becomes contaminated, transfer the remaining axenic node(s) in the dish using sterile forceps to a fresh MS2 Gelzan plate.

14. Use sterile forceps and scalpel blade to cut new shoot growth away from the original nodes. Insert new stems 0.5 cm deep into MS2 agar plates, placing 6-8 micro cuttings per dish.

15. Culture new micro cuttings to generate roots and establish mother plants.

    Once 20 plates containing 6-7 plants each are established, grow for 4-6 weeks, then proceed to Basic Protocol 2. To maintain mother plants and multiply for future experiments, perform Basic Protocol 1 until ready for Basic Protocol 2.

GENETIC TRANSFORMATION OF CASSAVA VARIETY 60444

BASIC PROTOCOL 2

Here we describe steps required to produce transgenic plants in cassava variety 60444, a variety with high transformation efficiency. The process starts with production of organized embryogenic structures (OES) induced from immature leaf explants from *in vitro* plantlets produced in Basic Protocol 1. To begin production of target tissues, approximately 20 plates of 6- to 8-week-old mother plants should be available. After OES formation, friable embryogenic callus (FEC) tissues are produced in three cycles of 21 days. FEC tissues are amenable to transformation by *Agrobacterium* and subsequent selection and regeneration of somatic embryos. Finally, the latter are germinated to produce plantlets. The total time required 3-3.5 months from the time of inoculation with *Agrobacterium* to obtain transgenic plantlets.

Details are provided for use of *A. tumefaciens* LBA4404 carrying pCAMBIA2300-based gene constructs. pCAMBIA2300 has an *npt*I gene for bacterial selection and an *npt*II gene for plant selection. Table 1 lists the appropriate media for each step of the procedure, along with media composition, culture duration, and scale.

Materials

Twenty Petri dishes of micropropagated 60444 cassava plantlets (see Basic Protocol 1)

Table 1 Media and Culture Steps for Production of Transgenic Cassava Plants from Cultivar 60444

Medium	Purpose	Composition[a]	Culture duration (days)	Scale
MS2	Micropropagation	MS basal salts and vitamins	42-56	6-8 plants per plate, 12-20 plates
OES induction medium	Induction of OES from leaf explants	MS2, 50 μM picloram, 2 μM $CuSO_4$	28	10 explants per plate, 10- 20 plates
FEC induction medium	FEC induction and proliferation (3 cycles)	GD2, 50 μM picloram	21-28 per cycle	7-10 colonies per plate, 10- 20 plates
Liquid inoculation medium	*Agrobacterium* inoculation of FEC	GD2, 200 μM acetosyringone	30 min	3-7 samples per construct
Co-culture medium	FEC co-culture with *Agrobacterium*	GD2, 50 μM picloram, 200 μM acetosyringone	2	3-7 samples per construct
Resting medium	Resting phase	GD2, 50 μM picloram, 150 mg/L carbenicillin	8	3-7 samples per construct
Callus selection medium	Selection of transgenic callus	GD2, 50 μM picloram, 75 mg/L carbenicillin, 25 μM paromomycin	21	4 plates per sample
Stage 1 regeneration medium	Somatic embryo regeneration from FEC	MS2, 5 μM NAA, 75 mg/L carbenicillin, 45 μM paromomycin	18-21	10 colonies per plate
Stage 2 regeneration medium	Somatic embryo maturation	MS2, 0.5 μM NAA, 45 μM paromomycin	18-21	4-6 embryos per plate
Stage 3 regeneration medium (*optional*)[b]	Somatic embryo maturation	MS2, 0.05 μM NAA, 45 μM paromomycin	18-21	4-6 embryos per plate
Germination medium	Germination of somatic embryos	MS2, 2 μM BAP	18-21	4-6 embryos per plate
MS2	Plantlet establishment/ rooting	MS basal salts and vitamins	14-30	6-8 plants per plate

[a] All contain 20 g/L sucrose and 8 g/L Noble agar.
[b] See Basic Protocol 2, step 58.

OES induction plates (see recipe)
FEC induction plates (see recipe)
Glycerol stock of *A. tumefaciens* strain LBA4404
LB plates (see recipe) with antibiotics for *Agrobacterium* selection (e.g., 30 mg/L rifampicin, 30 mg/L streptomycin, and 50 mg/L kanamycin)
LB liquid medium (see recipe) with the same antibiotics
YM liquid medium (see recipe) with the same antibiotics
Liquid inoculation medium (see recipe)
Co-culture plates (see recipe)
GD2 liquid medium (see recipe)
250 mg/ml carbenicillin (see recipe)
Resting plates (see recipe) with carbenicillin
Callus selection plates (see recipe) with carbenicillin
Stage 1 regeneration plates (see recipe) with carbenicillin
Stage 2 regeneration plates (see recipe)
Stage 3 regeneration plates (see recipe)
Germination plates (see recipe)
MS2 agar plates (see recipe)

100 × 15–mm sterile Petri dishes (VWR, 25384-342)
Fine point forceps
Miltex carbon steel no. 10 surgical blade (prod. no. 4-110)
Laminar flow hood
Dissecting microscope
Sterile hypodermic needles (BD Precision Glide, 23-G × 1 IM TW, 0.6 × 25 mm)
60-ml sterile syringes
Sterile stainless steel mesh (Timesetl, 1-mm^2 pore size) cut into 36-cm^2 pieces
Sterile stainless steel spatula
Parafilm or Saran Wrap cut into 5-cm-wide strips
28°C growth chamber
Culture tubes (VWR, 60818-667)
28°C shaking incubator
250-ml Erlenmeyer flasks
Inoculating loops
Spectrophotometer
15- and 50-ml conical polystyrene tubes
Sterile 12-well plates (Corning Costar, 3513)
Sterile 100-µm nylon mesh (Sefar, 03-110/47) cut into 25-cm^2 squares
10-ml pipettes, regular and wide bore (Corning Costar, 4492)
Sterile 85-mm Whatman filter paper (1001-085, grade 1)

Induce OES

1. Select Petri dishes containing mother plants at 6-8 weeks since previous subculture onto MS2 agar plates.

   Do not use mother plants older than 10 weeks from previous subculture, as they will be stressed and the embryogenic response will be significantly reduced.

2. Open one Petri dish and use fine forceps and a scalpel blade to excise the topmost (apical) 1-2 cm portions from 3-5 individual shoots (Fig. 2A). Transfer to a sterile Petri dish lid or bottom.

3. In a laminar flow hood under a dissecting microscope, select cuttings in which the youngest leaves are immature, unopened, and 2-5 mm in length.

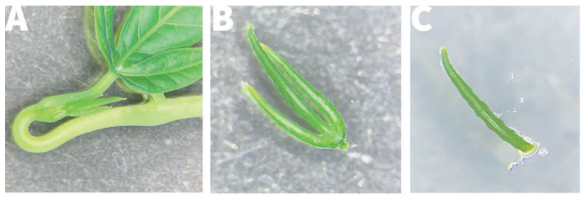

Figure 2 Preparation of leaf explants for induction of organized embryogenic structures (OES). (**A**) Shoot apical region excised from *in vitro* mother plants showing larger expanded tri-lobed cassava leaf and younger leaf possessing folded leaf lobes. The latter are the source of explants for production of OES. (**B**) Excised tri-lobed leaf explant with folded leaf lobes. (**C**) Individual excised leaf lobe explant placed correctly on OES induction medium with midrib in contact with the medium.

4. Using fine forceps to hold one cutting, use a 23-G hypodermic needle on a syringe to make incisions at the petiole/lamina junction to separate the three lobes of the leaf from the petiole (Fig. 2B) and from each other (Fig. 2C).

   Cut leaf lobes as close to the petiole junction as possible without including any petiole tissue.

5. Transfer 10-12 leaf lobe explants to an OES induction plate and orient to ensure that the midrib (abaxial side) is downwards and in contact with the medium (Fig. 2C).

   Correct orientation of the leaf explant on the OES induction medium is critical. Ensure that the midrib is in contact with the medium, but do not submerge explants into the medium.

6. Repeat steps 2-5 to obtain 100-200 leaf lobe explants in total.

7. Seal plates, label appropriately, and place in a 28°C growth chamber under low light (20 μMol m^{-2} s^{-1}) for 28 days.

   After 28 days, production of OES will be complete (Fig. 3A).

Induce and proliferate FEC

8. Open dishes and use fine forceps to transfer 2-3 explants to a sterile Petri dish lid.

9. Working under a dissecting microscope in a laminar flow hood, use a no. 10 scalpel blade and fine point forceps to excise the OES and separate them from the surrounding non-embryogenic, wet, mushy callus. Cut away all adhering callus tissue and place excised OES fragments onto an FEC induction plate. Cover with a lid to prevent desiccation.

   Work with 2-3 explants at a time until OES from all induction plates are collected.

10. Transfer all collected OES fragments to a sterile metal mesh (1-mm pore) placed on top of an empty sterile Petri dish (Fig. 3B).

11. Using a sterile stainless steel spatula, force the OES through the mesh, collecting the fragments below. Use a sterile needle to push adhering tissues through the mesh and add to tissue in the Petri dish.

12. Using sterile forceps, gently mix the meshed OES to homogenize the tissue mass.

13. Using fine forceps, transfer meshed OES to a new FEC induction plate and arrange fragments to form colonies of 10-15 fragments each (Fig. 3C). Establish 6-7 such colonies per FEC induction plate.

14. Seal plates with Parafilm or Saran Wrap, label appropriately, and place in a 28°C growth chamber under low light (20 μMol m^{-2} s^{-1}) for 28 days (1^{st} cycle).

15. Use fine forceps to select FEC tissues (Fig. 3D) from the colonies and transfer to fresh FEC induction plates. Do not subculture non-embryogenic tissues.

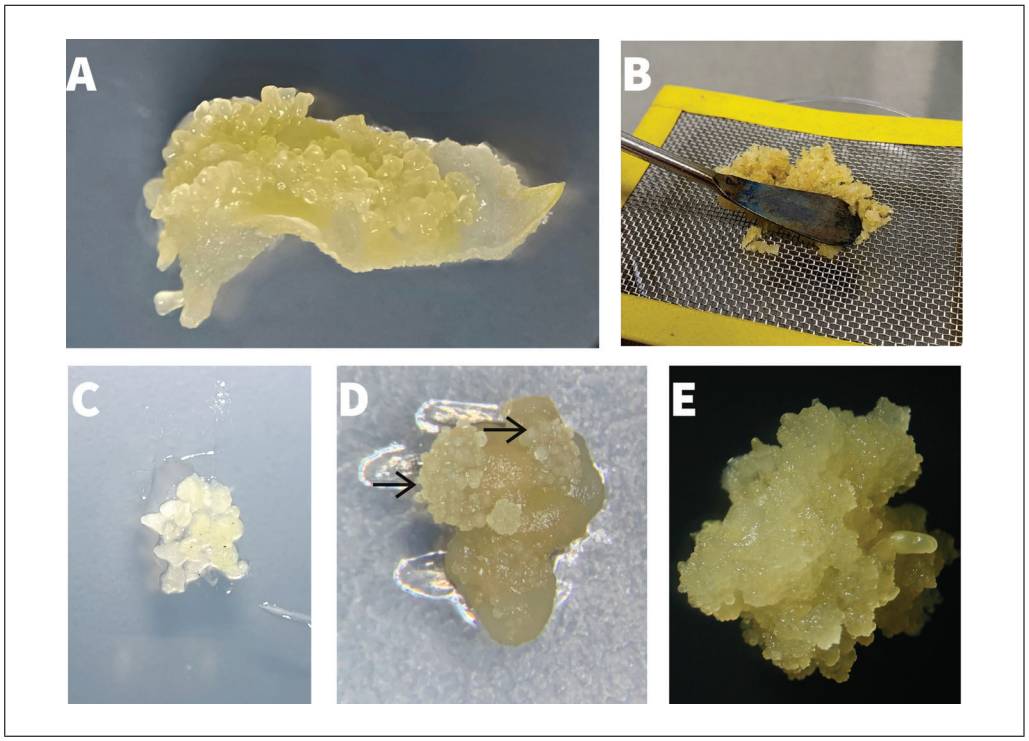

Figure 3 Production of friable embryogenic callus (FEC). (**A**) OES formed from leaf lobe explant after 28 days culture on OES induction medium. (**B**) Fragmentation of OES by forcing through a 1-mm^2 metal mesh. (**C**) Meshed OES fragments arranged into a colony and placed on FEC induction medium. (**D**) Production of FEC tissues (arrows) from meshed OES after 4 weeks culture in 1st cycle on FEC induction medium. (**E**) Homogeneous FEC colony after culture in 3rd cycle on FEC induction medium.

Place groups of FEC tissues in a monolayer to generate colonies ~0.5 cm in diameter, with 7-10 such colonies per dish, and incubate at 28°C in low light (20 µMol m^{-2} s^{-1}) for 21 days (2nd cycle).

FEC tissues can be identified as pale yellow, translucent spherical units 0.5-1.0 mm or smaller in diameter (Fig. 3D).

16. Use fine forceps to select FEC tissues from the colonies and transfer to fresh FEC induction plates to generate homogenous FEC tissues (Fig. 3E). Place FEC tissues to generate colonies ~0.5 cm in diameter, with 7-10 colonies per dish, and incubate at 28°C in low light (20 µMol m^{-2} s^{-1}) for 21 days (3rd cycle).

It is recommended to use FEC tissues after three culture cycles on FEC induction medium for transformation with Agrobacterium. Tissues can be used after two culture cycles, but transformation efficiencies are often reduced. Subculturing FEC tissues for more than three cycles on FEC induction medium is not recommended, as it increases the frequency of offtype plants recovered.

Prepare Agrobacterium suspension

17. Streak an *Agrobacterium* glycerol stock on an LB plate containing the appropriate antibiotics. Incubate inverted for 48 hr at 28°C.

For LBA4404 carrying a pCAMBIA2300-based vector, use LB medium containing 30 mg/L rifampicin, 30 mg/L streptomycin, and 50 mg/L kanamycin.

18. Take a single *Agrobacterium* colony and inoculate a culture tube containing 2 ml LB liquid medium with the same antibiotics. Incubate on a shaking incubator at 250 rpm and 28°C for 8-16 hr.

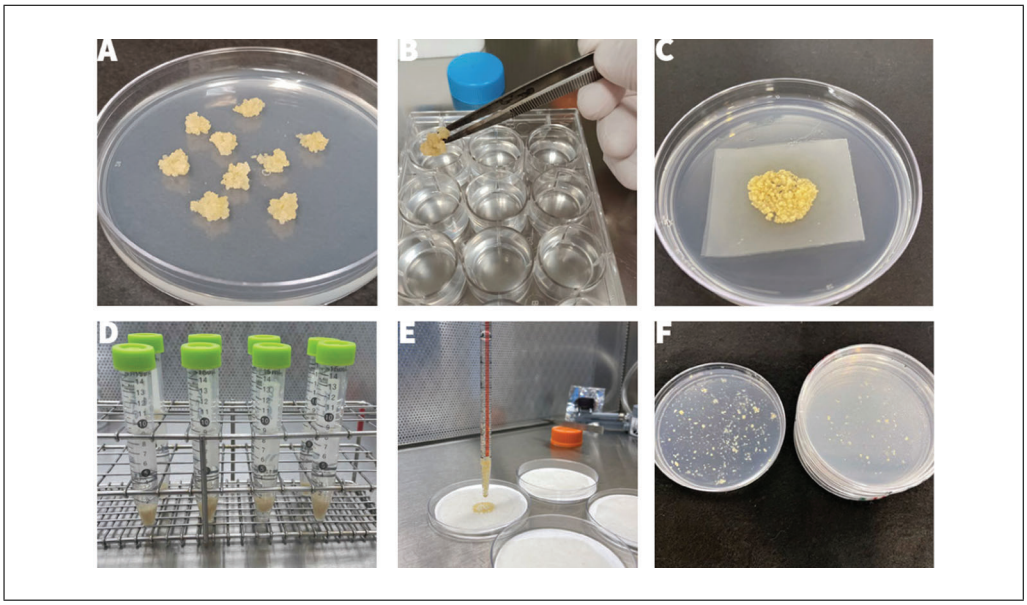

Figure 4 Transformation with *Agrobacterium* and early selection stages for transgenic FEC. (**A**) Plate of FEC ready for co-culture with *Agrobacterium*. (**B**) FEC tissue being placed in multiwell plate containing *Agrobacterium* suspension. (**C**) *Agrobacterium* co-culture with FEC tissues placed on a nylon mesh. (**D**) Washing FEC tissues and recording the settled cell volume after co-culture. (**E**) Pipetting FEC onto nylon mesh with sterile filter paper. (**F**) FEC tissues spread on multiple plates of callus selection medium.

19. Remove tube from shaker and allow any solids to settle. Take 0.5 ml and inoculate a 250-ml flask containing 20 ml YM liquid medium with the same antibiotics. Incubate overnight at 28°C with shaking at 250 rpm.

    YM medium is preferred for Agrobacterium LBA4404 liquid phases.

20. Determine the optical density (OD_{600}) using a spectrophotometer.

    A target OD_{600} of 0.5-1.0 should have been obtained. If an OD_{600} of 0.5 is not reached, continue growth and reassess at 2-hr intervals.

21. Once an OD_{600} of 0.5 has been reached, transfer bacterial suspension to a sterile 50-ml tube and centrifuge 5 min at 8000 × *g*. Pour off supernatant and resuspend bacteria in 20 ml liquid inoculation medium. Repeat centrifugation and discard supernatant.

    Use a fresh sterile acetosyringone stock in the inoculation medium for each transformation experiment.

22. Resuspend bacterial pellet in liquid inoculation medium at an OD_{600} of 0.5. Calculate the resuspension volume of medium (V_R) using the formula:

$$V_R = (V_S \times OD_M)/OD_T$$

    where V_S is the starting volume (in ml) and OD_M and OD_T are the measured and target OD_{600} values. For example:

$$V_R = (20\ ml \times 0.82)/0.5 = 32.8\ ml$$

Inoculate FEC with Agrobacterium

23. Remove 10-20 plates of FEC cultures from the growth chamber at the end of the 3rd cycle (Fig. 4A).

24. Using fine forceps and working under the dissection microscope, select and transfer good-quality FEC tissues to a fresh FEC induction plate.

Good-quality FEC is pale yellow, translucent, friable, and grows prolifically (Fig. 3E). Do not select callus tissue that is dark yellow or brown and non-friable in nature.

25. Use forceps to gently mix the FEC and homogenize the tissue mass.
26. Vortex the prepared *Agrobacterium* suspension for 10 s and dispense 2 ml to each well of a sterile 12-well plate.
27. Using sterile forceps, transfer samples of homogenized FEC to each well (Fig. 4B) until the bottom is covered (one sample is ~0.5-0.7 cm^3). Stir with a pipette tip.

    The FEC within a well is considered one sample. Establish 3-7 replicate samples for each gene construct to be transformed in a given experiment.

28. Place the lid on the plate and leave in the laminar flow hood for 30 min. Swirl gently by hand 2-3 times during this period to facilitate mixing.

Co-culture FEC and Agrobacterium

29. Place a piece of sterile nylon mesh on an empty sterile Petri dish lid.
30. Use a 10-ml wide-bore pipette to extract the FEC/*Agrobacterium* suspension from one well.
31. Touch the tip of the pipette to the mesh to prime it (so that moisture passes through the mesh), then transfer the tissue suspension onto the mesh.
32. Use sterile forceps to gently spread the sample to make a monolayer on the mesh.
33. Place the mesh with inoculated FEC on a co-culture plate (Fig. 4C) and label the plate accordingly.
34. Repeat steps 29-33 for each well individually.
35. Seal plates with Parafilm or Saran Wrap and incubate for 2 days at 22°C under bright light (90 μMol m^{-2} s^{-1}).

    We have found co-culture under light promotes more efficient transformation than co-culture in the dark.

    At the end of the co-culture period, transgenic single cells can be visualized under a fluorescence microscope if the gene construct carries a fluorescent marker such as GFP (Fig. 5A) or DsRed.

Wash FEC and culture on resting medium

36. Place one sterile 15-ml plastic tube per FEC sample/plate in a test tube rack and label accordingly.
37. Prepare a washing solution by adding 150 μl of 250 mg/ml carbenicillin to 250 ml GD2 liquid medium (final 150 mg/L carbenicillin). Pipette 8 ml to each 15-ml tube.
38. Using sterile forceps, transfer FEC from each plate/mesh to its corresponding tube and vortex 30 s to mix and disaggregate the FEC.
39. Allow tissues to settle for 10 min, then remove liquid using a sterile 10-ml pipette.
40. Add 8 ml fresh GD2 liquid medium containing 150 mg/L carbenicillin to each tube, vortex again, and allow to settle for 10 min (Fig. 4D).
41. Record the volume of FEC in cubic centimeters that has settled to the bottom of the tube.

    Recording the settled cell volume (SCV) for each sample of transformed FEC allows subsequent determination of transformation efficiency. A sample will typically consist of 0.5-0.7 cc SCV.

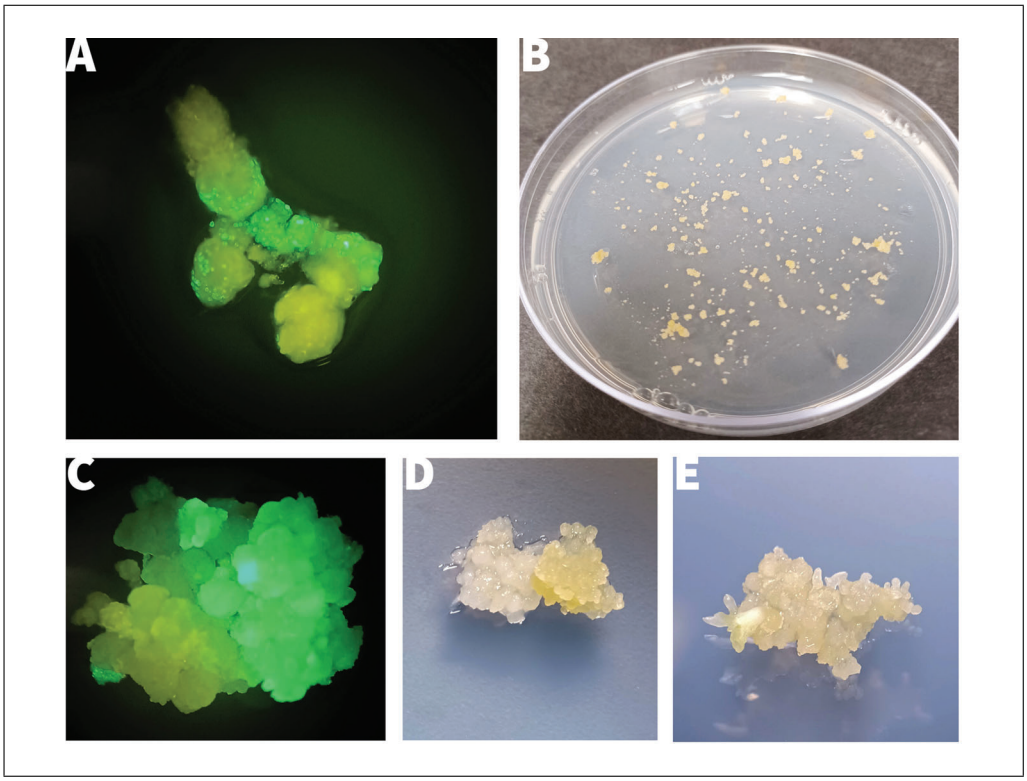

Figure 5 Assessment of transformation efficiency and recovery of transgenic tissues. (**A**) Individual cell transient GFP expression after 3-4 days co-culture with *Agrobacterium* (visual score 4). (**B**) Growing colonies on selection medium containing paromomycin. (**C**) Growing FEC colony with GFP-expressing tissues (right side). (**D**) Recovery of transgenic FEC on stage 1 regeneration medium. Tissue on right is healthy and growing; bleached tissue on left has died due to antibiotic selection. (**E**) Development of torpedo and very early cotyledon-stage somatic embryos on stage 1 regeneration medium.

42. Place a sheet of sterile filter paper on an empty sterile Petri dish and then place a piece of sterile 100-μm nylon mesh on top of the filter paper.

43. Use a 10-ml wide-bore pipette to extract 5 ml of medium from the first tube and discard, then transfer the remaining medium with FEC onto the nylon mesh, allowing the liquid medium to be absorbed into the filter paper (Fig. 4E).

44. Use the pipette or sterile fine forceps to spread the FEC in a monolayer on the mesh.

45. Transfer mesh with FEC to a fresh resting plate (Fig. 4F).

46. Repeat steps 42-45 for all samples.

47. Seal and label the plates appropriately and culture for 8 days at 28°C under low light (20 μMol m^{-2} s^{-1}).

    A period of 7-8 days on resting medium without antibiotic selection is beneficial to allow the tissues to recover from the stress of co-culture with Agrobacterium. By the end of this period, and if the gene construct carries a marker such as GFP or DsRed, cell division, with 2-16 cell stages, should be visible under a fluorescence microscope.

Recover transgenic tissues on callus selection medium

48. Transfer FEC from the mesh/plates to separate 15-ml tubes containing 8 ml GD2 liquid medium containing 150 mg/L carbenicillin as above (see steps 36-38).

49. Place four pieces of sterile nylon mesh on four separate sterile Petri dish lids. Use a 10-ml wide-bore pipette to extract the FEC from one tube and disperse equal portions across the four meshes.

50. Transfer FEC to callus selection plates by inverting the mesh onto the medium. Using sterile forceps, gently press on the mesh to transfer all of the FEC (Fig. 4C).

    Each FEC sample is spread evenly over four selection plates to ensure even exposure to antibiotic selection. This prevents the nursing effects of excessive tissue in a single location, which can result in recovery of non-transgenic "escape" plants.

51. Repeat for each sample.

52. Seal plates, label appropriately, and culture for 21 days at 28°C under low light (20 μMol m^{-2} s^{-1}).

Regenerate somatic embryos on stage 1 regeneration plates

53. Under a dissecting microscope, identify healthy growing colonies of FEC.

    Healthy FEC colonies will be pale yellow compared to non-growing white or brown callus.

54. Using sterile fine forceps, transfer about 10 healthy colonies to a stage 1 regeneration plate. Spread colonies evenly onto the medium to form distinct groups.

    Each FEC colony is considered to be a unique putative transgenic event. It should, therefore, be transferred separately and should not be mixed with other FEC colonies.

55. Seal plates and place in the 28°C growth chamber in stacks of not more than three plates. Culture for 18-21 days under bright light (90 μMol m^{-2} s^{-1}).

Mature somatic embryos on stage 2 regeneration plates

56. Use sterile forceps and a dissection microscope to select and transfer healthy, growing, and developmentally advanced colonies showing the presence of torpedo to early cotyledon-stage embryos (Fig. 5E) to stage 2 regeneration plates, establishing a maximum of 4-6 colonies per plate.

    If colonies do not show cotyledon-stage embryos, leave them for another 7-10 days before selecting and transferring.

    Each colony is now considered an independent transgenic event and should be assigned a unique tracking number. Colonies should be kept separated to avoid mixing putative transformation events. Placing more than six colonies per plate slows development of the somatic embryos and increases the risk of mixing the putative independent transgenic lines.

57. Seal plates and culture for another 18-21 days following step 55.

Germinate somatic embryos on germination plates

58. Using a dissection microscope, look for somatic embryos that have developed distinct green cotyledons (Fig. 6A-6C).

    If green cotyledon-stage embryos have not developed after 18-21 days on stage 2 regeneration medium, or if more embryos are required, subculture tissues onto stage 3 regeneration medium and culture for another 18-21 days to encourage embryo maturation and production of green cotyledon-stage embryos before proceeding to the next step.

59. Using fine forceps, pick individual cotyledon-stage embryos and trim away any adhering callus using a scalpel blade.

60. Transfer individual embryos to germination plates and gently press into the medium until the underside of the cotyledons is in contact with the medium. Do not submerge the embryo.

61. Place a maximum of 6 embryos from a given callus colony on a dish (Fig. 6D) and label with the line number.

    Placing more than 6 embryos per Petri dish will slow maturation and delay germination.

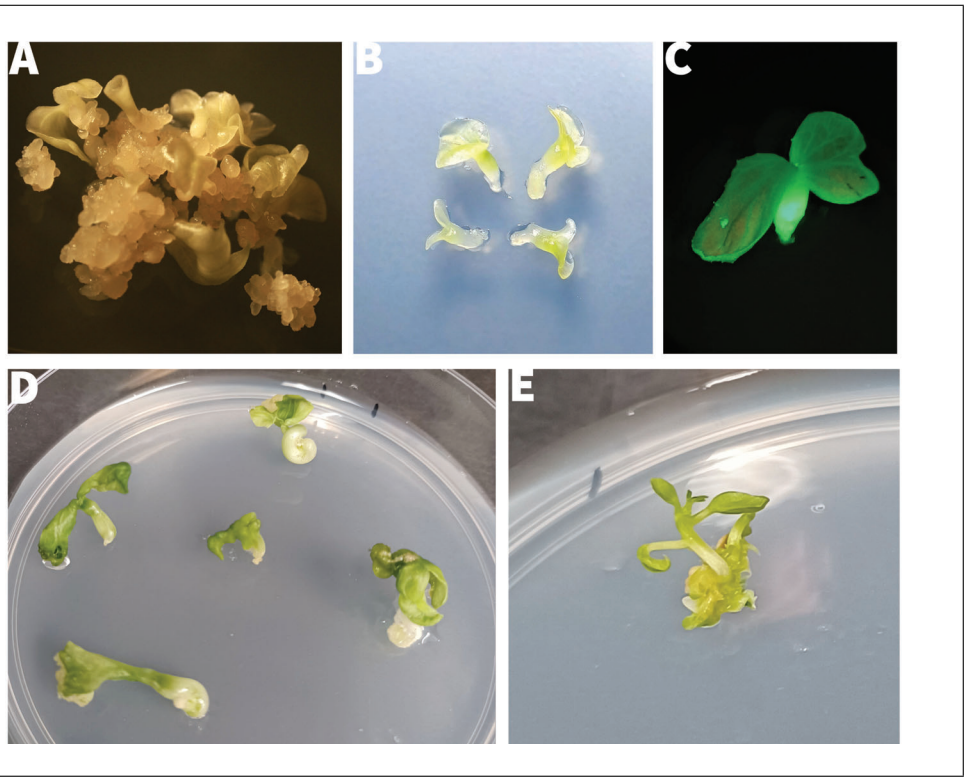

Figure 6 Somatic embryo maturation and regeneration of transgenic plants. (**A**) Cotyledon-stage embryos regenerating on stage 2 regeneration medium. (**B**) Individual cotyledon-stage embryos. (**C**) Cotyledon-stage embryo expressing GFP. (**D**) Matured cotyledon-stage embryos developing on germination medium. (**E**) Shoot regeneration from cotyledon-stage embryo.

62. Seal plates and culture 18-21 days as in step 55 for embryos to mature and germinate (Fig. 6D).

Establishing regenerated plants on rooting medium

63. Check for shoot production under a dissecting microscope.

    Shoot production from cotyledon-stage embryos should commence 18-21 days after subculture onto germination medium. The germination process is not synchronous across all somatic embryos. If germination has not occurred by 28 days, place embryos on an empty Petri dish lid and cut away green cotyledon tissues and adhering callus, taking care not to damage the hypocotyl or apical regions. Place the trimmed somatic embryo on fresh germination plates and check every seven days for shoot development.

64. Using sterile forceps and a scalpel blade, cut the stem of germinated plantlets just above the cotyledons (Fig. 6E). Transfer shoots onto MS2 agar plates, inserting stem ~0.5 cm into the medium to initiate rooting and plant establishment.

65. Allow plantlets to develop strong shoots and roots and then multiply by micropropagation as required (see Basic Protocol 1).

    After transgenic plant lines are recovered, molecular screening can be performed to confirm the presence and expression of transgenes, depending on the goals and needs. If phenotypic evaluation is required, follow Basic Protocol 3 for transfer of in vitro plants to soil and establishment in the greenhouse.

GENETIC TRANSFORMATION OF ADDITIONAL CULTIVARS

ALTERNATE PROTOCOL 2

Although Basic Protocol 2 is a robust method for production of plants from cassava variety 60444, it must be adapted to regenerate transgenic plants of other genotypes. Changes include the use of alternative media for production of FEC target tissues (e.g., inclusion of tyrosine to encourage FEC production), adjusted *Agrobacterium* ODs and co-culture,

and use of different antibiotics due to the toxicity of carbenicillin to cultivars other than 60444. Table 2 lists the appropriate media for each step of the procedure, along with media composition, culture duration, and scale. The adjusted protocols have proved effective for recovery of transgenic plants in NASE 13, NASE 14, TME 204, TME 419, TME 3, TME 7, TME 14, TMS 01/0040, TMS 01/1206, TMS 91/02324, TMS 92/0326, and TMS 98/0505, plus additional East African landraces.

Additional Materials *(also see Basic Protocol 2)*

FEC induction post-mesh plates (see recipe)
FEC proliferation plates (see recipe)
100 mg/ml cefotaxime (see recipe)
Resting plates (see recipe) with cefotaxime
Callus selection plates (see recipe) with cefotaxime
Stage 1 regeneration plates (see recipe) with timentin

Table 2 Media and Culture Steps for Production of Transgenic Cassava Plants from Other Cultivars

Medium	Purpose	Composition[a]	Culture duration (days)	Scale
MS2	Micropropagation	MS basal salts and vitamins	42-56	6-8 plants per plate, 12-20 plates
OES induction medium	Induction of OES from leaf explants	MS2, 50 μM picloram, 2 μM $CuSO_4$	28	10 explants per plate, 10-20 plates
FEC induction post-mesh medium	FEC induction	GD2, 50 μM picloram, 250 μM tyrosine	3-5	7-10 colonies per plate, 10-20 plates
FEC proliferation medium	FEC proliferation (3 cycles)	GD2, 50 μM picloram, 250 μM tyrosine, 50 mg/L moxalactam	21-28 per cycle	7-10 colonies per plate, 10- 20 plates
Liquid inoculation medium	*Agrobacterium* inoculation of FEC	GD2, 200 μM acetosyringone	45 min	3-7 samples per gene construct
Co-culture medium	FEC co-culture with *Agrobacterium*	GD2, 50 μM picloram, 200 μM acetosyringone	4	3-7 samples per gene construct
Resting medium	Resting phase	GD2, 50 μM picloram, 125 mg/L cefotaxime	10	3-7 samples per gene construct
Callus selection medium	Selection of transgenic callus	GD2, 50 μM picloram, 125 mg/L cefotaxime, 27.5 μM paromomycin	21	4 plates per sample
Stage 1 regeneration medium	Somatic embryo regeneration from FEC	MS2, 5 μM NAA, 30 mg/L timentin, 45 μM paromomycin	18-21	10 colonies per plate
Stage 2 regeneration medium	Somatic embryo maturation	MS2, 0.5 μM NAA, 45 μM paromomycin	18-21	4-6 embryos per plate
Stage 3 regeneration medium (*optional*)	Somatic embryo maturation	MS2, 0.05 μM NAA, 45 μM paromomycin	18-21	4-6 embryos per plate
Germination medium	Germination of somatic embryos	MS2, 2 μM BAP	18-21	4-6 embryos per plate
MS2	Plantlet establishment/ rooting	MS basal salts and vitamins	14-30	6-8 plants per plate

[a] All contain 20 g/L sucrose and 8 g/L Noble agar.

Prepare FEC for transformation

1. Induce OES and prepare a meshed and homogenized OES sample as described (see Basic Protocol 2, steps 1-12).

2. Using fine forceps, transfer meshed OES to an FEC induction post-mesh plate and arrange to form colonies of 10-15 fragments each. Establish 6-7 such colonies per plate.

   For some cultivars (e.g., TME 7), addition of a higher concentration of tyrosine is better (500 μM instead of 250 μM). The optimal level of tyrosine (100-500 μM) should be determined empirically when commencing work with a new variety.

3. Seal plates, label appropriately, and place in a 28°C chamber in the dark for 5 days.

   Dark conditions are most easily achieved by placing sealed Petri dishes in cardboard or black plastic boxes.

4. Using fine forceps, split each colony into two and subculture onto FEC proliferation plates, placing 6-7 colonies per plate. Seal plates and incubate for 28 days at 28°C in the dark (1st cycle).

5. Use fine forceps to select FEC tissues from the colonies and transfer to fresh FEC proliferation plates. Do not subculture non-embryogenic tissues. Place FEC tissues to generate colonies ∼0.5 cm in diameter, with 6-7 such colonies per plate, and incubate at 28°C in the dark for 21 days (2nd cycle).

   FEC tissues can be identified as pale yellow, translucent spherical units 0.5-1.0 mm or smaller in diameter (Fig. 3D).

6. Use fine forceps to select FEC tissues from the colonies and transfer to fresh FEC proliferation plates. Place FEC tissues to generate colonies ∼0.5 cm in diameter, with 6-7 such colonies per dish, and incubate in the dark for 18-22 days (3rd cycle).

Perform transformation

7. Prepare *Agrobacterium* strain LBA4404 as described (see Basic Protocol 2, steps 17-22).

8. Dilute *Agrobacterium* suspension by a factor of 10 with GD2 + 200 μM acetosyringone medium to reach an OD_{600} of 0.05.

   Use of Agrobacterium at this OD_{600} is highly effective for enhancing the efficiency of FEC transformation (Chauhan et al., 2015).

9. Inoculate and co-culture FEC with diluted *Agrobacterium* as described (see Basic Protocol 2, steps 23-35), but extend the inoculation period to 45 min and the co-culture period to 4 days.

Establish regenerated plants from transformed FEC

10. Wash FEC and culture on resting medium as described (see Basic Protocol 2, steps 36-47) with the following modifications:

    a. Replace carbenicillin in washing solution (steps 37 and 40) and resting plates (step 45) with 125 mg/L cefotaxime (final concentration).

    Inclusion of 50 mg/L moxalactam in the resting medium has been found to be highly effective for enhancing transformation efficiency in some cultivars. It is not routinely added but is worth testing.

    b. In step 47, increase the culture time from 8 days to 10 days.

11. Recover transgenic tissues on callus selection medium as described (see Basic Protocol 2, steps 48-52) with the following modifications:

a. In step 48, replace carbenicillin in the liquid medium with 125 mg/L cefotaxime (final).
b. In step 50, replace carbenicillin in callus selection plates with 125 mg/L cefotaxime and 27.5 μM paromomycin (final).

Sensitivity to paromomycin differs between cultivars. A lower concentration (25 μM) may be desirable at this stage for some cultivars (e.g., NASE 13) and should be tested empirically. Concentrations below 25 μM are not recommended due to the large proportion of non-transgenic escape plants that will be recovered.

12. Regenerate and germinate somatic embryos as described (see Basic Protocol 2, steps 53-62), but replace carbenicillin in stage 1 regeneration plates with 30 mg/L timentin (final).

13. Establish regenerated plants on rooting as described (see Basic Protocol 2, steps 63-65).

After transgenic plant lines are recovered, molecular screening can be performed to confirm gene presence. Other studies may include gene copy number and gene expression levels, depending on the research goals. If phenotypic evaluation is required, proceed to Basic Protocol 3 for establishment of in vitro cultures in the greenhouse.

BASIC PROTOCOL 3

ESTABLISHMENT AND GROWTH OF PLANTS IN THE GREENHOUSE

Once transgenic plants have been obtained (Basic Protocol 2 or Alternate Protocol 2), there is often a need to establish them in soil for subsequent studies. Plants are micropropagated onto MS2 Gelzan medium and cultured for 3-4 weeks. It has been found to be highly beneficial to propagate plants in Gelzan prior to soil transfer. The use of 2.2 g/L Gelzan provides sufficient support for the plants but is soft enough for easy removal with minimal damage to the roots. Plants are then potted in soil and maintained at high humidity. This protocol describes the steps necessary for high efficiency transfer of plants from *in vitro* conditions to soil for growth in a greenhouse or growth chamber.

Materials

In vitro cassava plantlets (see Basic Protocol 2 or Alternate Protocol 2)
MS2 Gelzan plates (see recipe)
Berger BM7 35% Bark HP (Hummert International, 10121500)
Gnatrol (Hummert International, 01-2035)
NPK 15-5-15 fertilizer (Hummert International, 07-5902)
MOST micronutrients (mix of soluble traces, Hummert International, 07-5990)
Sprint 330 chelated iron (Hummert International, 07-1511)
NPK 10-30-20 fertilizer (Hummert International, 07-593300)
NPK 15-16-17 fertilizer (*optional*; Hummert International, 07-592500)

28°C greenhouse
7.6-cm plastic pots (Hummert International, 11631100)
27.8 × 54.5 × 6.2–cm tray with holes (1020 flat, Hummert International, 11-3000)
27.8 × 54.5 × 6.2–cm tray without holes (NH flat, Hummert International, 11-3050)
8-L watering can
Low plastic dome (Hummert International, 11-3360)
Tall plastic dome (Humi-Dome, Hummert International, 14-3852-2)

Additional reagents and equipment for micropropagating *in vitro* plantlets (see Basic Protocol 1)

Establish plants in MS2 Gelzan

1. For each line to be propagated, micropropagate two ~3-cm-tall plants (see Basic Protocol 1), trimming older leaves, into one MS2 Gelzan plate, spacing them evenly apart.

   Do not place more than three plantlets per plate. Restricting to two to three plantlets per dish allows robust shoot development and growth of relatively large plantlets well suited to survive transfer to soil.

2. Label plates, seal, and place in a 28°C growth chamber under bright light (90 µMol m^{-2} s^{-1}) for 4 weeks, stacking no more than two plates high.

Establish plants in soil

3. Transfer plates to the potting area.

4. Set up and label one 7.6-cm plastic pot for each plantlet to be potted. Keep pots for different lines separate to avoid mislabeling.

5. Fill pots half full and at a slant with Berger BM7 35%, making sure that the soil is loose and has no large clumps. Place pots in a tray with holes.

6. Add the following to an 8-L watering can and mix to dissolve completely:

   7.6 L hand-warm tap water
   24 g Gnatrol
   12.5 g 15-5-15 fertilizer
   2.1 g micronutrients
   4.2 g Sprint 330

   Gnatrol is recommended to control insects such as fungus gnats that are present in many greenhouses. An 8-L watering provides sufficient mixture for three trays containing 21 pots each. Scale appropriately.

7. Pour mixture into a tray without holes until the liquid is just over the bottom of the pots and let soil become moist for ~20 min.

8. Remove lids from plates containing micropropagated plants and fill plates with warm water (33°-35°C).

   Use of warm water, not cold, prevents shocking of the delicate plantlets.

9. Gently remove plants from plates by loosening the gel. Break up gel in spots free of roots first. Swirl plates to see if plants are loose and thus ready to be transferred. Use fingers to hold the root ball, taking care not to squeeze or damage the stem.

   Plantlets must be handled gently to ensure that stems are not crushed and roots are not broken.

10. Lay each plant gently on the soil within a pot so that the apical meristem and 2-3 leaves are above the surface of the soil. Fill pot gently with Berger BM7 35%.

    Because roots will develop from portions of the stem buried below the soil, it is beneficial to place the plantlet deep into the pot and cover the lower stem with soil so the plant will be anchored firmly and more roots will be available for water and nutrient uptake. This also ensures that all plants established in soil at the same time will be of similar height and more uniform for use in experiments.

11. Water plants using a gentle stream from a hose. Place a low dome over pots to prevent wilting. Let tray sit in the Gnatrol mixture for 30 min until soil is saturated.

12. Remove the tray and drain away all water and Gnatrol mixture.

13. Replace low dome with a tall dome and place trays containing plants on a bench to provide 100% humidity. Allow plants to grow 7 days with at least 12 hr light, day temperature 26°C, and night temperature 25°C.

14. Remove trays containing plants from mist bench, puncture the tall dome, and place on the open greenhouse bench. Grow for 5-10 days with 12 hr light, day temperature 27°-32°C, night temperature 21°-26°C, relative humidity 60%-70%.

    Provide supplemental lighting if naturally available light drops below 500 W/m^2. This is provided by 1000-W metal halide fixtures generating light intensity of ∼145-165 W/m^2.

15. Remove dome gradually once leaves are fully expanded and plants look hardy. Arrange pots in the greenhouse with 10-15 cm spacing to allow vigorous growth.

16. Once established, water plants with micronutrients 28 days after transfer to soil following manufacturer instructions.

17. Apply 10-30-20 fertilizer (following manufacturer instructions) to hardened plants on the open bench as liquid feed each time a plant is watered until the plant has been in soil for 4-5 weeks. Then reduce fertilizer application to three times per week, watering on other occasions with regular water.

18. After 5 weeks, if production of storage roots is required, switch fertilizer to 15-16-17 fertilizer and apply three times per week (Taylor et al., 2012).

REAGENTS AND SOLUTIONS

For a direct comparison of media composition and uses, see Tables 1 and 2.

Growth Media for *Agrobacterium*

LB medium

For liquid medium: Place a 2-L graduated cylinder containing ∼1.5 L diH_2O on a stir plate and begin stirring at ∼200-250 rpm. Slowly add 40 g LB powder and stir until all powder is dissolved (∼10 min). Bring volume to 2 L with diH_2O. Set up six 500-ml orange-capped stock bottles with autoclave tape on the caps. Aliquot 340 ml medium to each bottle. Ensure that caps are threaded onto bottles but are still loose, then autoclave on liquid setting for 20 min. After cooling, add appropriate antibiotics and carefully tighten caps. Store up to 3 months at 4°C.

For agar plates: Add 4.5 g microbiology-grade agar to each bottle before autoclaving. After cooling, add appropriate antibiotics, carefully tighten caps, and gently swirl bottles. Dispense 30 ml per 100 × 15–mm plate. Store up to 3 months at 4°C.

LB is generally 20 g/L, but check product before measuring.

YM liquid medium

600 ml Milli-Q water
0.4 g yeast extract
10.0 g mannitol
0.2 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$)
0.5 g dipotassium phosphate (K_2HPO_4)
0.1 g NaCl

Combine reagents in the order listed and stir until dissolved. Bring to 1 L with Milli-Q water and adjust pH to 7.1. Dispense into 250-ml bottles and autoclave on liquid setting for 20 min. Store up to 3 months at room temperature.

Gresshoff and Doy (GD) and Derivative Media

The following are based on GD basal medium (Gresshoff & Doy, 1974). GD2 medium containing 50 μM picloram is commonly referred to as GD2 50P.

GD2 medium and agar plates

600 ml Milli-Q water
40 ml 25× GD macronutrients (see recipe)
5 ml 200× Fe-EDTA (see recipe)
1 ml 1000× GD micronutrients (see recipe)
1 ml 1000× GD vitamins (see recipe)
20 g sucrose (Fisher Chemical, S5-12 kg)

For liquid medium: Combine reagents in the order listed and stir vigorously until dissolved. Bring to 1 L with Milli-Q water and adjust pH to 6.12 with 1 M NaOH. Dispense into 250-ml autoclave bottles and autoclave. Store up to 3 months at room temperature.

For agar plates: Place 8 g Noble agar (Difco, 214230) in an autoclave bottle. Add 1 L prepared liquid medium, swirl, and autoclave. Cool to 44°C in a water bath, then dispense into 100 × 15–mm Petri dishes at 30 ml per dish. Store up to 1 month at room temperature or 4°C for medium containing antibiotics.

Callus selection plates (GD2 50P + antibiotics)

GD2 agar plates (see recipe)
For cultivar 60444, add after autoclaving and cooling:
5 ml/L picloram stock (see recipe)
300 μl/L carbenicillin stock (see recipe; final 75 mg/L)
250 μl/L paromomycin stock (see recipe; final 25 μM)
For other cultivars:
Replace carbenicillin with 1.25 ml/L cefotaxime stock (see recipe; final 125 mg/L)
Increase paromomycin to 275 μl/L (final 27.5 μM)

Co-culture plates (GD2 50P + 200 μM acetosyringone)

GD2 agar plates (see recipe)
After autoclaving and cooling, add:
5 ml/L picloram stock (see recipe)
2 ml/L acetosyringone stock (see recipe)

FEC induction plates (GD2 50P)

GD2 agar plates (see recipe)
After autoclaving and cooling, add 5 ml/L picloram (see recipe)

FEC induction post-mesh plates (GD2 50P + 250 μM tyrosine)

GD2 agar plates (see recipe)
After autoclaving and cooling, add:
5 ml/L picloram stock (see recipe)
25 ml/L l-tyrosine stock (see recipe)

FEC proliferation plates (GD2 50P + 250 μM tyrosine + 50 mg/L moxalactam)

GD2 agar plates (see recipe)
After autoclaving and cooling, add:
5 ml/L picloram stock (see recipe)
25 ml/L l-tyrosine stock (see recipe)
1 ml/L moxalactam stock (see recipe)

Liquid inoculation medium (GD2 + 200 μM acetosyringone)

GD2 liquid medium (see recipe)
After autoclaving, add 2 ml/L acetosyringone (see recipe)

Resting plates (GD2 50P + antibiotics)

GD2 agar plates (see recipe)
For cultivar 60444, add after autoclaving and cooling:
5 ml/L picloram stock (see recipe)
600 μl/L carbenicillin stock (see recipe; final 150 mg/L)
For other cultivars, replace carbenicillin with:
1.25 ml/L cefotaxime stock (see recipe; final 125 mg/L)

Murashige and Skoog (MS) and Derivative Media

The following are based on MS basal medium (Murashige & Skoog, 1962). MS2 medium containing 50 μM picloram is commonly referred to as MS2 50P.

MS2 agar plates

600 ml Milli-Q water
4.31 g MS basal salts (Sigma, M5524)
1 ml 1000× MS vitamins (see recipe)
20 g sucrose (Fisher Chemical, S5-12kg)

Combine reagents and stir vigorously until dissolved. Bring to 1 L with diH_2O and adjust pH to 6.15 with 1 M NaOH. Place 8 g Noble agar (Difco, 214230) in an autoclave bottle. Add prepared liquid medium, swirl, and autoclave. Cool to 44°C in a water bath, then dispense into 100 × 25–mm Petri dishes (VWR, 89107-632) at 40 ml per dish. Store up to 1 month at room temperature or 4°C for medium containing antibiotics.

In our hands, autoclaving this medium causes the pH to drop ∼0.3 points. Thus, the pH is adjusted to 6.1 prior to autoclaving, allowing it to reduce to the desired pH 5.7-5.8 in the poured medium. Users should test for the pH reduction caused by their own autoclaving equipment and adjust the pH during preparation accordingly.

Germination plates (MS2 + 2 μM BAP)

MS2 agar plates (see recipe)
Before autoclaving add 2 ml/L BAP stock (see recipe)

MS2 Gelzan plates

MS2 agar plates (see recipe) with 2.2 g/L Gelzan (Sigma, G1910) in place of agar

OES induction plates (MS2 50P + 2 μM $CuSO_4$)

MS2 agar plates (see recipe)
Before autoclaving, add 2 ml/L $CuSO_4$ stock (see recipe)
After autoclaving and cooling, add 5 ml/L picloram stock (see recipe)
Dispense into 100 × 15–mm Petri dishes at 30 ml per dish

Stage 1 regeneration plates (MS2 + 5 μM NAA + antibiotics)

MS2 agar plates (see recipe)
Before autoclaving, add 5 ml/L NAA stock (see recipe)
For cultivar 60444, add after autoclaving and cooling:
450 μl/L paromomycin stock (see recipe; final 45 μM)
300 μl/L carbenicillin stock (see recipe; final 75 mg/L)
For other cultivars, replace carbenicillin with:
150 μl/L timentin stock (see recipe; final 30 mg/L)

Stage 2 regeneration plates (MS2 + 0.5 μM NAA + 45 μM paromomycin)

MS2 agar plates
Before autoclaving, add 0.5 ml/L NAA stock
After autoclaving and cooling, add 450 μl/L paromomycin stock

Stage 3 regeneration plates (MS2 + 0.05 μM NAA + 45 μM paromomycin)

MS2 agar plates
Before autoclaving, add 0.05 ml/L NAA stock
After autoclaving and cooling, add 450 μl/L paromomycin stock

Stock Solutions

Acetosyringone, 100 mM

Place 196.2 mg acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone; Sigma, D134406) in a 15-ml conical tube. Add 5 ml of 100% ethanol to dissolve. Bring volume to 10 ml with Milli-Q water. Filter sterilize through a 0.22-μm syringe filter. Store up to 7 days at −20°C.

A new acetosyringone stock should be prepared for each transformation experiment.

6-Benzylaminopurine (BAP), 1 mM

Dissolve 11.26 mg BAP powder (Sigma, B3408) in a 50-ml tube by adding 1-2 ml of 1 M NaOH dropwise. Vortex to dissolve. Bring to 50 ml with Milli-Q water. Store up to 6 months at 4°C in a 50-ml conical tube.

Carbenicillin, 250 mg/ml

Dissolve 12.5 g carbenicillin (Phytotech, C346) in 35 ml Milli-Q water in a 50-ml conical tube. Vortex to dissolve. Bring to 50 ml with Milli-Q water. Filter sterilize using a 0.22-μm syringe filter and aliquot into sterile 2-ml microcentrifuge tubes. Store up to 1 year at −20°C.

Cefotaxime, 100 mg/ml

Dissolve 4.0 g cefotaxime powder (Phytotech, C381) in 30 ml of Milli-Q water. Vortex to dissolve. Bring to 40 ml with Milli-Q water. Filter sterilize using a 0.22-μm syringe filter and aliquot into sterile 2-ml microcentrifuge tubes. Store up to 1 year at −20°C.

Copper sulfate, 1 mM

Weigh out 12.49 mg $CuSO_4 \cdot 5H_2O$ (Sigma, C3036) on a 5-figure analytical balance. Dissolve in a clear 50-ml conical tube by adding 10 ml Milli-Q H_2O. Swirl or vortex to dissolve. Bring to 50 ml with Milli-Q water in a graduated cylinder. Store up to 6 months at 4°C in a clear 50-ml conical tube.

Fe-EDTA, 200×

Add 3.725 g $Na_2EDTA\ 2H_2O$ (Sigma, E5134) to a beaker containing 300 ml Milli-Q water and mix vigorously until dissolved. Slowly add 2.76 g iron sulfate $7H_2O$ and continue to mix vigorously until dissolved. Bring to 500 ml. Wrap with aluminum foil and store up to 6 months at 4°C.

Fe-EDTA may precipitate after storage. If this occurs, remove foil and microwave 5-6 min or until all the precipitate dissolves. This is only necessary once after the solution has been prepared.

GD macronutrients, 25×

25.0 g ammonium nitrate (Sigma, A3795)
1.625 g potassium chloride (Sigma, P9333)

8.675 g calcium nitrate $4H_2O$ (Sigma, C2786)
0.4275 g magnesium sulphate (anhydrous) (Sigma, M2643)
25.0 g potassium nitrate (Sigma, P8291)
7.5 g potassium phosphate monobasic (Sigma, P5655)

Add reagents in the order listed to a beaker containing 600 ml Milli-Q water. Mix vigorously until dissolved. Bring volume to 1 L with Milli-Q water. Store up to 6 months at 4°C.

GD micronutrients, 1000×

1000 mg manganese sulphate H_2O (Sigma, M7899)
25 mg cobalt (II) chloride $6H_2O$ (Sigma, C2911)
25 mg copper (II) sulfate $5H_2O$ (Sigma, C3036)
300 mg boric acid (Sigma, B6768)
25 mg molybdic acid (sodium salt) $2H_2O$ (Sigma, M1651)
800 mg potassium iodide (Sigma, P8166)
300 mg zinc sulphate $7H_2O$ (Sigma, Z1001)

Add reagents in the order listed to a beaker containing 400 ml Milli-Q water. Mix vigorously until dissolved. Bring to 1 L with Milli-Q water. Store in 50-ml conical tubes up to 6 months at 4°C.

GD vitamins, 1000×

0.1 g d-biotin (Sigma, B4639)
0.2 g glycine (free base, Sigma, G7403)
5 g myo-inositol (Sigma, I7508)
0.05 g nicotinic acid (free acid, Sigma, N4126)
0.05 g pyridoxine HCl (Sigma, P6280)
0.5 g thiamine HCl (Sigma, T1270)

Add reagents in the order listed to a beaker containing 400 ml Milli-Q water. Mix vigorously until dissolved. Bring to 500 ml with Milli-Q water. Filter sterilize using a 0.22-µm filter. Store in 50-ml conical tubes up to 6 months at 4°C.

Moxalactam, 50 mg/ml

Dissolve 2.0 g moxalactam (Sigma, M8158) in 30 ml Milli-Q water. Vortex to dissolve. Bring to 40 ml with Milli-Q water. Filter sterilize using a 0.22-µm syringe filter and aliquot into sterile 2-ml microcentrifuge tubes. Store up to 1 year at −20°C.

MS vitamins, 1000×

Dissolve MS vitamin powder (Sigma, M7150-100 ml) in 95 Milli-Q water in a beaker. Bring to 100 ml to a 1000× stock. Filter sterilize using a 0.22-µm filter. Store in 50-ml sterile conical tubes for up to 6 months at 4°C.

1-Naphthaleneacetic acid (NAA), 1 mM

Dissolve 46.5 mg NAA powder (Sigma, N0640) in a 50-ml conical tube using 3 ml of 1 M NaOH. Vortex to dissolve. Bring to 250 ml with Milli-Q water in a graduated cylinder. Store up to 6 months at 4°C in a 250-ml Pyrex bottle.

Paromomycin, 100 mM

Find the batch number on the paromomycin bottle (Sigma, P8692) and locate the Certificate of Analysis on the online Sigma product page. Find the purity of the batch by checking the µg paromomycin/mg anhydrous basis value. This should be greater than or equal to 675 (corresponding to 67.5% paromomycin). Calculate the amount needed for a 100 mM stock of 100% paromomycin and add this amount into a 50-ml conical tube. Bring to final volume with Milli-Q water and vortex.

Filter sterilize through a 0.22-µm syringe filter and aliquot into sterile 2-ml microcentrifuge tubes. Store up to 1 year at −20°C.

The purity of paromomycin varies by batch. To ensure the required amount of active paromomycin is present in GD- and MS-based selection media, the stock solution should be adjusted as described to compensate for batch variations.

Picloram, 10 mM

Dissolve 1.208 g picloram (Sigma, P5575) in a 50-ml conical tube using 3-5 ml of 1 M NaOH. If necessary, place tube in a 50°C water bath to dissolve. Bring to 50 ml Milli-Q water and vortex to mix. Transfer to a 500-ml cylinder and bring to 500 ml with Milli-Q water. Adjust pH to 5.8 by adding 1 M HCl dropwise. Filter sterilize though a 0.22-µm Stericup filter. Store up to 6 months at 4°C.

Timentin, 200 mg/ml

Dissolve 5.0 g timentin powder (Phytotech, T869) in 20 ml Milli-Q water and vortex to dissolve. Bring to 25 ml with Milli-Q water. Filter sterilize through a 0.22-µm syringe filter. Aliquot into sterile 2-ml microcentrifuge tubes and store up to 1 year at −20°C.

Tyrosine, 10 mM

Dissolve 2.18 g L-tyrosine HCl (Sigma, T2006) in a 50-ml conical tube by adding 2-3 ml of 5 M NaOH. Transfer to a graduated cylinder and add Milli-Q water to 990 ml (pH will be 11-12). Add concentrated HCl dropwise to adjust pH to 5.8, switching to 1 M HCl as you approach the desired pH (precipitation will occur). Heat using a heated stir plate or microwave until completely dissolved. Allow solution to cool to 45°-50°C. Filter sterilize using a 0.22-µm Stericup 2. Store up to 6 months at 4°C.

Before adding to medium, loosen the lid, swirl, and heat in a microwave until all crystals are redissolved (∼4 min).

COMMENTARY

Background Information

The genetic transformation systems described here are based on the production of totipotent embryogenic tissues that are amenable to T-DNA delivery by *Agrobacterium*. Selection of transgenic embryogenic callus is followed by regeneration of mature somatic embryos and their germination to produce genetically modified plantlets. Production of totipotent tissues commences by inducing somatic embryos from immature leaf explants cultured on MS-based medium supplemented with high levels of a potent auxin. In our hands, picloram is the most effective auxin for production of OES, but 2,4-dichlorophenoxyacetic acid (2,4-D) can be employed if the former is not available (Taylor et al., 1996). Totipotent FEC, used as the target tissue for transgene integration, is generated by subculture of the OES onto GD-based medium supplemented with picloram. Switching from MS-based to GD-based medium is critical for successful production of FEC, which is subsequently selected and proliferated to generate homogenous FEC target tissues for transformation with *Agrobacterium*. Transition back to MS-based medium with reduced auxin stimulates reorganization of the tissues to produce torpedo and cotyledon-stage embryos, which are then matured and germinated to recover plants.

The methods and technology described have proven to be robust for small- and large-scale recovery of transgenic and gene-edited cassava plants. The systems have been critical components of research programs in multiple laboratories investigating disease resistance (Beyene, Chauhan, et al., 2017; Gomez et al., 2018; Ogwok et al., 2012), nutritional enhancement (Beyene, Solomon, et al., 2017; Ihemere, Arias-Garzon, Lawrence, & Sayre, 2006; Narayanan, Beyene, Chauhan, Grusak, & Taylor, 2020), starch modification (Bull et al., 2018), post-harvest deterioration (Salcedo & Siritunga, 2011), herbicide tolerance (Hummel et al., 2018), and fundamental questions central to the biology of this under-investigated crop species. In some cases, these traits have been stacked to enhance potential value to farmers and consumers (Narayanan

et al., 2021). The methods described have proven suitable for large-scale plant production within product development programs (up to 500-1000 independent transgenic events per year). Performance of regenerated plants has been demonstrated in the field (Narayanan et al., 2020; Ogwok et al., 2012; Wagaba et al., 2017) and meets the quality required for regulatory approval (Wagaba et al., 2020). Transgenic cassava plants produced following Alternate Protocol 1 have been integrated into breeding programs in East Africa, proving the potential utility of these systems to impact cassava yields for farmers. Recently, the methods described have been successfully adapted for genome editing (Gomez et al., 2018; Odipio et al., 2017), being sufficiently powerful to support homologous recombination and allele replacement (Hummel et al., 2018).

These protocols were originally developed to produce transgenic plants in the West African cassava variety 60444 (Schöpke et al., 1996; Taylor et al., 1996). When applied as described in Basic Protocol 1, the method is powerful, with a trained laboratory technician able to produce more than 1000 independent transgenic events per year. Twenty to thirty independent events can be recovered per cm^3 SCV of starting material in a period of 4 months, from *Agrobacterium* transformation to plant recovery. Generation of transgenic plants in 60444 is especially effective because production of embryogenic target tissues and plant regeneration occur at high efficiencies, and because it is very amenable to *Agrobacterium* co-culture and T-DNA transfer (Bull et al., 2009; Taylor et al., 2012). Although no longer cultivated by farmers, 60444 remains a useful model cultivar for laboratory and greenhouse studies of transgene expression, cassava biology, and gene editing (Odipio et al., 2017; Taylor et al., 2012).

Cassava is a highly heterozygous species, with significant genetic variation between varieties. Many hundreds of cassava cultivars and landraces are cultivated across the tropics and have been selected to meet differing farmer, consumer, and processor needs. Expanding capacity for genetic transformation into a range of cassava varieties with relevance to cultivation in Africa, Asia, and Central/South America initially proved to be challenging. Over the last 10 years, significant success has been achieved in broadening the number of cassava cultivars that can be genetically transformed by the techniques described here. Alternate Protocol 1 and Table 2 provide adaptations to the culture media and transformation systems that are critical to this effort. The rationale behind these are detailed below.

Use of tyrosine for FEC induction

Supplementation of GD2 50P-based media with tyrosine is effective for encouraging production of FEC from fragmented OES. The amount of tyrosine required varies with the cultivar and should be assessed empirically when a new cultivar is brought into culture. For example, TME 7 responds well to 500 μM tyrosine, whereas TME 419 and NASE 13 require no more than 250 μM tyrosine to stimulate FEC production (Chauhan et al., 2015). Tyrosine is detrimental for induction of FEC in cv. 60444 and should not be used.

Moxalactam enhances transformation efficiency

Addition of moxalactam is highly effective for enhancing the quality of FEC produced and efficiency of T-DNA delivery when included in the later cycles of FEC production (cycles 2 and 3 on GD2 50P-based media), prior to inoculation with *Agrobacterium*. This response varies across cultivars. No effect is seen in 60444, but a 50-100× increase in transformation efficiency can be achieved in TME 204, TME 419, and other cultivars when this antibiotic is included in the GD2 50P FEC induction medium before and after transformation with *Agrobacterium* (Chauhan et al., 2015).

Reduction of Agrobacterium OD

In most plant transformation systems, *Agrobacterium* inoculations occur at bacterial densities of 0.3-0.7 OD_{600}. However, significant reduction in the OD_{600} was found to be beneficial for genetic transformation of cassava FEC. While OD_{600} 0.5 is effective in 60444, a reduction to OD_{600} 0.05 or even 0.01 is dramatically superior in other cultivars, especially when combined with a 4-day co-culture period (Chauhan et al., 2015).

Use of alternative antibiotics to control Agrobacterium

A critical discovery was that carbenicillin is toxic to most cassava cultivars (but not 60444) and prevents growth and recovery of transgenic callus after *Agrobacterium* co-culture. Use of cefotaxime in place of carbenicillin overcomes this problem and actively encourages proliferation of FEC tissues (Chauhan et al., 2015). Timentin is favored for use in stage 1 regeneration medium, where the goal is to suppress FEC production and stimulate regeneration of somatic embryos (Chauhan et al., 2015).

Co-culture in the light

While co-culture of FEC with *Agrobacterium* under dark conditions results in T-DNA transfer, especially in 60444, performing this step under light conditions has proven to enhance genetic transformation.

Elimination of antibiotics in later regeneration stages

Washing FEC tissues after co-culture as described is effective for eliminating *Agrobacterium* regrowth. This allows antibiotics to be omitted after stage 1 regeneration medium (embryo induction). We have confirmed by PCR that *Agrobacterium* is not detected in or associated with regenerated plantlets when the procedures described here are followed.

The paromomycin selection agent should not be included in the germination medium. It is not required and its presence suppresses shoot germination from mature cotyledon-stage somatic embryos.

Metatopolin as an alternative cytokinin for stimulating shoot regeneration

We have seen positive results with the use of 2 μM metatopolin for embryo germination in place of BAP. Shoot regeneration from mature cotyledon-stage embryos can be accomplished by culture on MS2 + 2 μM metatopolin + Gelzan just as effectively as on MS2 + 2 μM BAP + agar (Chauhan & Taylor, 2018).

Benefits of methods presented

The genetic transformation methods detailed here bring multiple benefits. Understanding them provides researchers with important opportunities to adapt the systems for their specific needs and laboratory capacities.

Suitability for use with a range of chemical and visual selectable markers. This wide adaptability is important for enabling recovery of transgenic plants (see further discussion below).

Compatibility with different Agrobacterium strains. While LBA4404 is the most effective strain in our hands, success has also been achieved with EHA105, GV3101, and AGL1. AGL1 is employed in our laboratory in cases where rearrangement of gene constructs takes place in LBA4404.

Production of nonchimeric regenerates. Plants regenerated via *Agrobacterium* transformation of FEC are reliably nonchimeric in nature. This contrasts with other cassava regeneration systems, in which transgenic plants are recovered from transformation of OES tissues or matured somatic embryo–derived cotyledons (Jørgensen et al., 2005, Li et al., 1996). In our hands, the latter systems produce a high frequency of chimeric plants, which complicates the production and analysis of quality events.

Adaptability to other gene transfer technologies. FEC tissues produced following the methods described here are adaptable for use with direct gene transfer technologies such as microparticle bombardment (Chellappan et al., 2004) and as a source of totipotent protoplasts for transgene integration and genome editing (Sofiari, Raemakers, Bergervoet, Jacobsen, & Visser, 1998).

Selectable and visual marker systems

An important benefit of the tissue culture and transformation system described here is its adaptability for use with different visual and selectable marker systems. The ability to employ alternative selectable markers provides flexibility to meet the preferences of different laboratories and allows retransformation to stack transgenes within the same cassava plant (Okwuonu, Achi, Egesi, & Taylor, 2015)

Paromomycin. The protocols detailed here are based on use of *npt*II as the selectable marker gene and utilize paromomycin at 25 or 27.5 μM as the selection agent in the GD2 50P–based callus selection medium. This is increased to 45 μM in the regeneration media. The presence of paromomycin causes non-transgenic tissues to become bleached and die, making visual selection for putative transgenic tissues straightforward. The use of paromomycin as described will result in recovery of less than 1%-2% of non-transgenic escape lines. Paromomycin should not be included in the germination medium, as it suppresses germination of somatic embryos to produce plants. Our standard plasmids use the 35S promoter to drive expression of *npt*II. The Nos promoter is also effective for driving expression of *npt*II and is preferred in some cases due to the tendency of the 35S promoter to act *in trans* on other promoters within the T-DNA (Mette, van der Winden, Matzke, & Matzke, 1999).

Hygromycin. The culture systems described here have been successfully adapted to use hygromycin (Bull et al., 2009). If employing the *hpt* selectable marker gene, paromomycin can be replaced with hygromycin at 20 mg/L in the callus selection medium and 40 mg/L in the regeneration media (Okwuonu et al., 2015).

Glyphosate. Cassava transformation systems have also been adapted to use the herbicide glyphosate as a selection agent. Expression of mutated versions of the *EPSPS* gene have been used for transgenic and allelic replacement technologies (Hummel et al., 2018). Use of glyphosate is ineffective in the callus selection medium, but its inclusion at 2.5 mM in the regeneration media strongly suppresses development of green cotyledon stage embryos in non-transgenic tissues to facilitate recovery of genetically modified and edited events.

GFP and DsRed for system development, quality control, and tracking transformation efficiency. The tissue systems detailed in these protocols are highly suited for use with non-lethal, visual marker genes such as GFP and DsRed. Both can be used to track transformation efficiency from the single-cell stages immediately after co-culture to whole plantlet regeneration. GFP is especially effective due to the non-photosynthetic nature of the embryogenic tissues and is included as an internal control in all experiments performed in our laboratory. Examining tissues using a UV dissecting microscope provides accurate assessment for success of each transformation experiment and its progress into stages of early cell division, establishment of FEC colonies, and regeneration of somatic embryos. Use of GFP or DsRed is also recommended when adapting the system for use with a new cultivar, attempting to develop enhanced processes, or establishing cassava transformation in a new laboratory. The ability to rapidly determine and understand transgene expression, and to do so in a non-lethal manner, is very effective for training researchers in the methods and for learning where success and bottlenecks are occurring at each stage of the transformation process. When performed under a fluorescence microscope in a laminar flow hood, visual identification, manual selection, and subculture of GFP- or DsRed-expressing callus lines and somatic embryos to the next culture stage is also an effective process for recovery of transgenic tissues and plants. This can be beneficial as it negates the need for use of a selectable marker transgene such as *npt*II and *hpt* and inclusion of chemical selection agents in the media.

Critical Parameters

Multiple aspects of the protocols have been found critical to success. These have been discovered over almost 20 years of experience with these systems and are discussed below.

General

Work under a dissecting microscope. Perform tissue manipulations under a good-quality dissecting microscope at all steps except whole plant establishment and micropropagation. In our laboratory, we use the Olympus SZ51. Working under the microscope is essential for accuracy of explant preparation, OES isolation, FEC identification, selection and subculture, isolation and subculture of healthy callus on antibiotic selection medium, identifying maturing somatic embryos, and handling cotyledon-stage embryos. Constant observations under the microscope are also central for learning to recognize the tissues being produced and gaining the knowledge required to optimize the culture systems for quality results.

Establish a tissue culture and transformation pipeline. The protocols detailed here can be used as one-off processes to produce a small number (5-20) of independent transgenic or gene-edited plants. It is recommended, however, to establish a pipeline that routinely produces FEC target tissues so that they are available as needed for use in transformation and gene-editing experiments. When FEC tissues are generated in this manner, only 3-5 months are required from the time gene constructs are available to recovery of modified plants of a given cassava variety. Establishing a pipeline requires micropropagation of *in vitro* mother plants every 6-8 weeks so that they remain in optimal condition. Leaf explants should be established on OES induction medium every alternate week and tissues moved through the GD2 50P–based media to ensure that FEC tissues are generated and available for transformation experiments on a continuous basis to support research programs.

Media

Use only high-quality agar. Cassava is susceptible to the impurities found in lower-grade agar products. Whole plants and embryogenic cultures will fail to develop, grow slowly, and/or be of poor quality if grown on low-grade, impure agar. Use only the highest quality agar, such as Noble agar (Difco, 214230). If high-grade agar cannot be obtained, Gelzan can be used in its place and is effective especially for whole plant culture at the beginning and end stages of the protocols.

Make basal media from stocks in-house. It is recommended not to rely on commercially sourced basal media unless they have been tested and proven to be effective. It is

preferable to make basal media from scratch using the individual chemical components to ensure quality and consistency. This is especially the case for Gresshoff and Doy basal media. It is recommended to not use GD basal salts medium sourced from Duchefa Biocheme, as we and several collaborating laboratories have found it to be ineffective for production of FEC from OES tissues.

Use deep Petri dishes to encourage somatic embryo development. Culture in deep Petri dishes (100 × 25 mm) encourages regeneration and maturation of somatic embryos. Use of deep dishes at stage 1 and 2 regeneration plates and germination plates is intentional, as we have found that the increased headspace significantly encourages somatic embryo development and shoot regeneration.

Use fresh media. We recommend keeping media for a maximum of 4 weeks. OES induction medium is optimal for up to 2 weeks. All media containing antibiotics should be kept refrigerated and warmed on the bench to room temperature before use.

Plant materials

Ensure high quality of mother plants. Mother plants for production of leaf explants must be kept in good condition by repeated subculture on fresh MS2 medium every 2-3 months. Propagate only strong, healthy plants. If a mother plant appears weak or slow-growing, do not micropropagate it to establish the next cycle of mother plants and do not use it as a source of leaf explants to initiate OES. In this manner, weak, slow-growing plants are eliminated in favor of strong, vigorously growing plants that provide the highest quality leaf explants.

Orient leaf explants correctly on the medium. Leaf lobe explants must be oriented so that the abaxial surface and midrib are in contact with the medium. Take care to orient each explant individually to ensure proper contact with the OES induction medium. Do not submerge explants into the medium. Failure to follow this process will result in little or no OES production.

Transfer only OES tissue onto FEC induction medium. Trim away and discard all non-embryogenic tissues from the OES before meshing and subculturing onto GD2 50P–based medium. It is important to take extra care and to perform this step under a dissecting microscope to be able to visualize the tissues accurately. Subculture of non-embryogenic tissues significantly reduces or negates FEC production.

Select FEC carefully and subculture to generate homogenous tissues lines. Use a dissecting microscope to visualize embryogenic tissues. Use fine forceps to select FEC and subculture to the next cycle of FEC induction. This ensures elimination of non-embryogenic tissues and production of the high-quality FEC tissues required for successful transformation.

Culture FEC for a maximum of three cycles on GD-based medium. It is highly recommended to not exceed three culture cycles of FEC on GD2 50P–based medium before transformation with *Agrobacterium*. Exceeding three cycles suppresses the regeneration potential of the embryogenic tissues and increases the frequency of offtype plants regenerated (Taylor et al., 2012).

Evenly spread tissues on callus selection medium. Ensure that FEC tissues are spread evenly on the callus selection medium and avoid clumping to ensure even exposure to the selection agent. This also allows transgenic events to be isolated early in the process to maximize the number of independent transgenic plant lines recovered in each experiment. If FEC tissues become clumped together when transferred onto the callus selection medium, use fine forceps to manually move FEC units apart and place arrange them on the same selection plate.

Do not overload plates with tissues. Statements for placing a specified number of callus tissues and somatic embryos per plate at each stage are intentional. Overloading the plates with tissues inhibits development, especially maturation of somatic embryos on stage 1 and regeneration media and shoot regeneration on germination medium. Exceeding the stated amounts will delay or prevent somatic embryo recovery and plant regeneration.

Subculture green cotyledon-stage embryos to germination medium. Allow somatic embryos to develop to the green cotyledon stage before subculture onto germination medium. Only distinct green embryos should be subcultured, as earlier stage embryos will not mature into the large green cotyledon-stage embryos capable of germinating to produce plantlets.

Trim mature embryos to encourage shoot regeneration. In some cases, mature cotyledon-stage embryos do not germinate on MS2 2BAP medium. Instead, large green cotyledon tissues develop. If this occurs, remove embryos and place on a sterile Petri dish lid. Use forceps and a scalpel blade to trim away excess foliose tissues to within 3-4 mm of the hypocotyl and apical region. Place trimmed embryos onto fresh germination

medium and return to the culture chamber. Observe weekly for shoot regeneration.

Troubleshooting

See Table 3 for a list of problems encountered with the protocols along with their causes and solutions.

Understanding Results

Using a visual marker to assess transformation efficiency

It is important to track the success of the transformation system within each experiment. This allows results to be understood, adjustments to be made, and experiments to be repeated, if required. The use of GFP or a similar non-lethal, visual marker is a powerful tool for understanding the genetic transformation system and assessing progress and efficiency of transgenic tissue and plant production. The non-photosynthetic nature of the FEC tissues facilitates easy visualization of fluorescent markers with minimal background interference, allowing visual tracking through all steps in the methodologies described in Basic Protocol 2 and Alternate Protocol 2. Transformation efficiencies can be determined at each stage of the process from co-culture with *Agrobacterium* to full plant recovery. Data can be collected as the number of GFP-expressing callus units and embryos per starting unit (cm^3) of FEC sample (Chauhan et al., 2015). This provides valuable information regarding success and failures within the transformation system, allowing them to be identified and addressed in a timely manner. GFP is used as an internal control in all experiments performed in our laboratory, allowing efficiency of the transformation process to be tracked across experiments, cultivars, and gene constructs. In our hands, the use of an ER-targeted GFP expression cassette driven by the constitutive 35S promoter allows optimal visualization of the transformation process from the single-cell to whole-plant stages (Taylor et al., 2012). Expected results when using GFP are shown in Table 4 and described below.

Calculating efficiency of genetic transformation

Efficiency of transformation for a given gene construct/cultivar/*Agrobacterium* interaction within and across experiments can be calculated and expressed at each stage of the method. At the end of the co-culture and resting stages, a subjective 0-5 scoring is most easily used because counting many hundreds of GFP-expressing single cells for multiple transformed FEC samples is time-consuming and inaccurate. A visual assessment from 0 for no expression to 5 for extremely high numbers (e.g., 1000s) of expressing cells per sample is routinely used in our lab (Chauhan et al., 2015; Fig. 5). A score of 0 at the end of the resting stage indicates that the transformation failed. In this case, it is recommended to stop the experiment for all gene constructs and repeat the transformation process as soon as possible. For possible reasons that FEC transformations can fail, see Table 3.

By the end of the callus selection stage, the number of putatively transgenic callus lines recovered per unit of starting material can be accurately assessed. The amount of starting material per sample can be calculated by determining the settled cell volume (SCV in cm^3) of FEC that was inoculated. SCV is determined by allowing the FEC tissues in a sample to settle to the bottom of a graduated tube (see Basic Protocol 2). This is best performed after co-culture in order not to complicate the inoculation stage, when multiple samples are handled at the same time. Once the SCV is known, the number of callus lines produced per starting sample can be determined, followed by the number of independent transgenic plants per SCV of starting material. In 60444 and TME 204, this is reliably as high as 20-30 plants/cm^3 SVC (Chauhan et al., 2015; Taylor et al., 2012).

In some laboratories, the use of GFP or alternative fluorescent markers may not be possible. In this case, an alternative visual marker gene such as *uid*A (GUS) can be used to assess and track efficacy of transformation from co-culture through plant recovery. However, the tissues being assessed will be lost due to the lethal nature of the transgene visualization process. Alternatively, the system can be tracked by counting the number of healthy yellow callus lines recovered at the end of the callus selection stage and similarly through the putative transgenic tissues recovered at the end of stages 1 and 2 regeneration, and the number of independent lines that produce plants by germination on germination medium.

Time Considerations

For a detailed description and timing of each cultivation step in the processes used to recover transgenic cassava plants, refer to Tables 1 and 2. Time from co-culture of FEC tissues with *Agrobacterium* is 3-3.5 months for 60444 and ~1 month longer for other varieties. As cassava is highly heterozygous and vegetatively propagated, the T0 generation is

Table 3 Troubleshooting Guide for Production of Transgenic Cassava Plants

Problem	Possible cause	Solution
In vitro plant establishment and micropropagation		
Contamination of nodal explants during culture on MS2 medium	Woody or semi-woody nodal sections used	Use only new growth with fresh green stem
	Too many nodes in bleach solution	Use a maximum of 12-15 green nodes per 250-ml flask or 25 per 500-ml flask
	Bleach stock solution too old	Use newly opened commercial bleach; ensure commercial bleach is at least 8% sodium hypochlorite
	Insufficient surfactant	Ensure that two drops of Tween-20 were added
	Insufficient agitation	Agitate at 150 rpm or by hand every 5 min
	Endophytic contamination	Eliminate endophytic contamination by propagating shoot tips from newly sprouted material and subculturing to new MS2 plate; flame sterilize instruments after handling each shoot, dipping in ethanol (bead sterilizers have not been effective in killing some endophytes)
Shoots fail to grow from nodal explants	Excessive bleach used or extended sterilization period	Do not add >15% (v/v) bleach; do not exceed recommended 30-40 min sterilization period
	Insufficient material retained above and below axillary bud	Leave at least 0.75-1.0 cm above and below node before sterilization to allow bleaching of tissues from the cut surface without bleaching and damaging of axillary bud
	Poor-quality stake material	Select only healthy new growth from sprouted stake cuttings
Poor health and growth of micropropagated mother plants	Poor quality gelling agent	Use highest-quality agar (Noble agar) or Gelzan
	Incorrect media composition	Ensure all media components were not expired and were added correctly
	Poor quality starting material	Use healthy starting material no more than 8-10 weeks old
Production of embryogenic target tissues		
No or very little OES production from leaf explants	Mother plants in poor condition	Use mother plants no more than 6-8 weeks from previous subculture
	Leaf explants not oriented on medium correctly	Ensure abaxial side of explant and midrib are in contact with medium, but do not submerge into the medium
	Medium too old or made incorrectly	Use freshly made induction medium or medium no older than 2 weeks; extend OES induction period from 4 to 5 weeks, if required (do not extend to point where distinct cotyledon-stage embryos start to form)
No or very little FEC produced from OES	Insufficient wounding of OES	Force OES tissues through 1-mm^2 mesh to ensure wounding
	OES too mature	Use developmentally young OES (before it reaches late torpedo embryo stage)
FEC is slow growing and/or unhealthy brown color	Media made incorrectly	Ensure all media components were not expired and were added correctly

(*Continued*)

Table 3 Troubleshooting Guide for Production of Transgenic Cassava Plants, *continued*

Problem	Possible cause	Solution
	Poorly sealed Petri dishes	Make sure Petri dishes are well sealed
	FEC left too long on GD2 50P induction medium	Subculture FEC to fresh medium every 21-28 days
	Too much tyrosine in GD2 50P induction medium	Reduce tyrosine in GD2 50P FEC induction medium
FEC cultures not homogenous by end of 3rd cycle, but a mix of tissue types	Did not select pure FEC when subculturing tissues	Select only distinct, pure FEC when subculturing, leaving hard brown callus and mushy, non-embryogenic callus tissues behind
Insufficient number of plates of FEC produced for transformation experiments	Too few leaf explants established	Establish sufficient leaf explants (e.g., for 60444, set up 200 leaf explants)
	Not enough FEC subcultured	Select pure FEC when subculturing
Agrobacterium culture and FEC inoculation and co-culture		
No or minimal growth of mini-culture	Excess or incorrect antibiotic	Check for correct antibiotic type(s) and concentrations
	Insufficient time at 28°C	Prolong mini-culture time for up to 24 hr
	Detrimental gene construct	Assess gene construct to determine if leaky expression may impact bacterial vitality and growth
No or minimal growth of overnight culture	Excess or incorrect antibiotics	Check for correct antibiotic type(s) and concentration
	Insufficient inoculum	Inoculate multiple flasks per gene construct with different inoculum amounts
	Insufficient time at 28°C	Prolong culture time; check culture status every additional 2 hr
	Detrimental gene construct	Assess gene construct to determine if leaky expression may impact bacterial vitality and growth
Recovery of transgenic tissues, regeneration of somatic embryos and plants		
No GFP expression at end of co-culture period	*Agrobacterium* delivery of T-DNA failed	Follow all proper *Agrobacterium* handling procedures and growth periods
	Poor-quality FEC tissue	Use FEC at 3rd cycle, 18-22 days since previous subculture
	Tissue/genotype recalcitrance to *Agrobacterium* infection	Adjust and test effects of co-culture time (2-4 days); perform co-culture in dark versus light conditions; test alternative *Agrobacterium* strains
All FEC tissue dies/does not grow on callus selection medium	Incorrect paromomycin concentration in selection medium	Check paromomycin concentration
	Incorrect medium composition	Ensure all media components were not expired and were added correctly
	No transgenic tissue present	Repeat transformation with fresh FEC target tissues
All FEC tissue grows on callus selection medium	Insufficient selection pressure	Check paromomycin concentration; prepare fresh antibiotic stock solutions and fresh selection medium
All callus lines die on stage 1 regeneration medium with 45 μM paromomycin	Excessive paromomycin	Check paromomycin concentration

(*Continued*)

Table 3 Troubleshooting Guide for Production of Transgenic Cassava Plants, *continued*

Problem	Possible cause	Solution
	Incorrect medium composition	Check medium composition
Cotyledon-stage embryos do not develop from healthy FEC callus on stage 1 and 2 regeneration medium	Insufficient culture time in reduced auxin conditions	Subculture tissues to stage 3 regeneration medium (MS2 + 0.05 μM NAA + 45 μM paromomycin)
Cotyledon-stage embryos do not germinate on MS2 + 2 μM BAP medium	Poor-quality embryos selected for transfer to germination medium	Select best-quality embryos for subculture. Embryos should have two distinct cotyledons and a hypocotyl with visible immature apical meristem. Avoid trumpet-shaped embryos or those with no cotyledon development.
	Excessive number of cotyledons on a plate	Place no more than 6 embryos per plate
	Some cultivars can be problematic at this stage, possibly because large foliose cotyledons inhibit shoot formation	Remove somatic embryos from germination medium and trim away excess cotyledon tissues within 2 mm of apical region; subculture cleaned embryos onto fresh germination medium
	BAP maybe insufficient for germination	If embryos refuse to germinate, trim away excess cotyledon tissues and subculture onto MS2 + 2 μM metatopolin + Gelzan
Regenerated plants are not transgenic	Insufficient selection pressure at callus selection and stage 1-2 regeneration stages	Check paromomycin concentration; use freshly made medium
		Do not exceed recommend tissue amounts per plate at these stages
Regenerated plants are offtype	Excess time in culture at FEC stages	Cycle FEC tissues for the appropriate times; do not exceed recommended number of FEC cycles (3 maximum) or time per cycle
	Transgenes impact phenotype	Research potential developmental impact of transgene(s) and modify expression cassetes as required
Transfer and establishment of in vitro plants in soil		
Failure of plantlets to establish in soil, plants die in 3-4 days; low-frequency plant establishment	Poor-quality or too-young material transferred to soil from tissue culture stage	Use plantlets 4-5 cm in height with well-developed root system
	Root or stem damage from improper handling of plants during transfer	Handle young plants gently, taking care not to damage the stem; use MS2 Gelzan medium for micropropagation to minimize root damage; place no more than 3 plants per plate of MS2 Gelzan medium
	Insufficient humidity for plant establishment	Make sure pots are thoroughly watered; maintain humidity close to 100%
Plants die after 4-10 days in soil	Fungal contamination causes damping off	Use good-quality sterile potting soil; water with fungicide (e.g., Mancozeb)
	Fungus gnats or similar insect pests	Water with Bt-based insecticidal solution (e.g., Gnatrol)

(*Continued*)

Table 3 Troubleshooting Guide for Production of Transgenic Cassava Plants, *continued*

Problem	Possible cause	Solution
	Insufficient humidity for plant establishment	Maintain humidity close to 100%
Plants die on open bench 10-28 days after transfer	Plastic domes removed too early	Follow recommended hardening period
	Over- or underwatering	Do not allow soil to dry out; do not allow plants to sit in water
Plants grow slowly or develop poorly	Insufficient fertilizer	Water with micronutrients (e.g., MOST) 28 days after transfer
Plants do not produce storage roots	Insufficient fertilizer; excessive nitrogen in fertilizer	Use a low-nitrogen fertilizer regime for storage root production (e.g., NPK 10-30-20)
	Pot too big	Use 7-9 cm pots; if the pot is too large, plants will produce shoot growth over storage root development

Table 4 Expected Results at Each Stage After *Agrobacterium* Co-culture

Culture stage	Expected result	Notes
End of co-culture	100s of single GFP-expressing cells per sample (Fig. 5A)	Large numbers of single GFP-expressing cells indicate successful T-DNA transfer
End of resting stage	Dozens of 2- to 8-cell-stage GFP-expressing cell units per sample	A large reduction in single-cell expression is expected. Cell division should be visible by this time, indicating healthy tissues and successful transformation.
End of callus selection stage	Growing GFP-expressing FEC colonies (Fig. 5C,D)	Rapid cell division indicates healthy tissues and resistance to antibiotic selection pressure.
End of stage 1 regeneration	Growing GFP-expressing FEC colonies with development of torpedo to early cotyledon-stage embryos (Fig. 5E)	Presence of some dead, white colonies is expected and indicates efficacy of the elevated antibiotic selection pressure at this stage.
End of stage 2 regeneration	GFP-expressing colonies consisting of torpedo and cotyledon-stage embryos (Fig. 6A-C)	Colonies displaying active growth and somatic embryo regeneration at this stage are considered transgenic and tracked onwards with a unique line number. If cotyledon-stage embryos have not developed, subculture to stage 3 regeneration medium (0.05 μM NAA).
Shoot regeneration stage	Development of somatic embryos with large green cotyledons, followed by production and growth of shoots (Fig. 6D,E)	

most often the product desired and upon which subsequent experimental procedures will be assessed. Recovery of transgenic plants within this timeframe therefore places cassava favorably compared to other crop species.

To prevent extended recovery time of transgenic cassava plants, it is highly recommended to establish a pipeline for production of FEC target tissues and to have them available for transformation every 2-3 weeks. In this manner extended times are not required between transformation and gene-editing experiments waiting for the next batch of FEC tissues to become available.

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Author Contributions

Rosana Segatto: Method development, writing (original draft and editing), photography; **Tira Jones**: Method development, photography; **Danielle Stretch**: Method development, photography; **Claire Albin**: Method development; **Raj Deepika Chauhan**: Method development; **Nigel Taylor**: Method development, writing (original draft and editing).

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Over-expression of morphogenic genes enhances plant regeneration in cassava (*Manihot esculenta*)

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Key words

Agrobacterium transformation, cassava, *GROWTH-REGULATING FACTOR*, morphogenesis, shoot regeneration.

Abstract

The Growth Regulating Factor (GRF) and its Interacting Factor (GIF) have been shown to stimulate regeneration of transgenic plants, with studies reporting increased transformation efficiency in multiple species including wheat, beet and citrus. The present work evaluated the effects of overexpressing *GRF4-GIF1* and *GRF5* on the regeneration of transgenic plants in cassava (*Manihot esculenta* Crantz). Effects of *GRF4-GIF1* and *GRF5* sequences derived from *Vitis vinifera* and *Arabidopsis thaliana* were assessed by cloning expression cassettes under control of strong constitutive promoters. Friable embryogenic callus from cassava varieties 60444 and NASE 13 were transformed with *Agrobacterium tumefaciens* strains LBA4404 and LBA4404 THY- and multiple independent transgenic plant lines recovered. Expression of the morphogenic genes did not enhance transformation efficiency above the GFP control, nor efficiency or timing of somatic embryo regeneration or whole plant recovery. Organogenesis experiments were carried out to observe effects of these genes on morphogenesis from petiole, leaf-petiole, and stem explants. Expression of *Vitis vinifera GRF4-GIF1* was found to stimulate rapid organogenesis and shoot regeneration from leaf-petiole explants with plant regeneration occurring within 3-4 weeks culture on medium containing the cytokinin meta-topolin. Effects at the whole plant level were accessed by establishing plants in the greenhouse, with *VviGRF4-GIF1* overexpression resulting in increased leaf size and total leaf area, and *AtGRF5* stimulating above average results for plant height.

Abbreviations

CaMV Cauliflower mosaic virus
CRISPR Clustered Regularly Interspaced Short Palindromic Repeats
CsVMV Cassava vein mosaic virus
FEC Friable embryogenic callus
GD Gresshoff and Doy basal medium
GFP Green fluorescent protein
GIF *GRF-INTERACTING FACTOR*
GRF *GROWTH-REGULATING FACTOR*
miR396 Micro RNA 396
mT Meta-topolin

NOS Nopaline synthase promoter
*npt*II Neomycin phosphotransferase II
OES Organized embryogenic structures
QLQ Glutamine-Leucine-Glutamine protein domain
RcbS Rubisco small subunit
ROI Region of interest
RT-PCR Reverse transcription-polymerase chain reaction
RT-qPCR Reverse transcription-quantitative polymerase chain reaction
SAM Shoot apical meristem
SCV Settled cell volume
WCR Tryptophan-Arginine-Cysteine protein domain

Introduction

The root crop cassava (*Manihot esculenta* Crantz) originated in the southwest Amazon in Brazil (Watling et al. 2018), and since has become an important staple food across the tropical regions of South America, sub-Saharan Africa, and Asia. Cassava is cultivated mostly by smallholder farmers for its large starchy storage roots, providing an important source of dietary calories and industrial starch (Chavarriaga-Aguirre et al. 2016). Transgenic and genome editing technologies have been utilized to address constraints in cassava production including disease resistance (Wagaba et al. 2017), modified starch quality (Bull et al. 2018), enhanced nutritional content (Narayanan et al. 2019) and herbicide tolerance (Hummel et al. 2018).

Application of transgenic and genome editing technologies relies on the production of morphogenic tissues in culture, into which transgenes and gene editing tools can be delivered, and whole plants regenerated. Regeneration of plants typically occurs through somatic embryogenesis or organogenesis, the latter via *de novo* formation of new meristems or rearrangement of pre-existing meristems. Traditionally this is achieved *in vitro* by manipulating auxin/cytokinin ratio in the culture medium. More recently, ectopic over-expression of plant genes that control growth and development has proven effective for stimulating plant regeneration (Gordon-Kamm et al. 2019). Debernardi and colleagues showed how the expression of *GROWTH-REGULATING FACTOR 4 (GRF4)* and its cofactor *GRF-INTERACTING FACTOR 1 (GIF1)* dramatically increased the efficiency and speed of plant regeneration in wheat and citrus (Debernardi et al. 2020), while the *GROWTH-REGULATING FACTOR 5 (GRF5)* increased transformation efficiency in beet, canola, soybean, and sunflower (Kong et al. 2020).

The *GROWTH-REGULATING FACTOR* (GFR) is a family of plant transcription factors defined by the presence of the WRC and QLQ protein domains. These factors have a role in

promoting cell proliferation during leaf development and are required for the development and maintenance of the shoot apical meristem (SAM) (Kim et al. 2003). GRFs interact with another family of transcription factors, the *GROWTH INTERACTING FACTOR* (GIF) forming a complex that gives the primordial cells of vegetative and reproductive organs a meristematic specification state, guaranteeing the supply of cells for organogenesis (Lee et al. 2009). GRF expression is post-transcriptionally downregulated by microRNA396 (miR396). In *Arabidopsis,* the miR396 gene family has two members (ath-mir396a and ath-mir396b). These can induce cleavage of *AtGRF* mRNA species, except for *AtGRF5* and *AtGRF6* transcripts which don't have the target site (Kim and Tsukaya 2015). According to Vercruyssen et al. (2015), the transcription factor *GRF5* regulates duration of the cell proliferation period during leaf development in *Arabidopsis*. They have shown that overexpression of *GRF5* also stimulates chloroplast division, resulting in a higher chloroplast number per cell with increased chlorophyll levels in leaves which could maintain higher rates of photosynthesis. Transgenic plants overexpressing *GRF5* showed delayed leaf senescence and enhanced tolerance to nitrogen-depleted medium. The authors suggest these changes could potentially improve plant productivity.

The tissue culture and gene transfer systems currently employed to produce transgenic and genome edited cassava have improved significantly over the years. Well established systems for production of transgenic plants via somatic embryogenesis are in place (Taylor et al. 2012; Chauhan et al. 2015), and organogenic systems for regeneration of shoots from petiole and stem tissues have been reported (Chauhan and Taylor, 2018). The production of genetically transformed cassava, however, remains a lengthy and skilled process. Four months is required to produce friable embryogenic callus (FEC) target tissues, followed by four to six months, to regenerate genetically modified plants after transformation by *Agrobacterium* (Segatto et al. 2022). Plant recovery is also genotype specific with efficient plant regeneration limited to a relatively small subset of the many 100s of varieties grown by farmers across the tropics (Utsumi et al. 2022). Continued evaluation of strategies for enhanced plant regeneration therefore remains important and could lead to improvements to the current systems, especially regarding transformation efficiency of recalcitrant cultivars and time to plant recovery after transformation. This work investigated the effects of over-expression of morphogenic gene regulators in cassava, and quantified regeneration rates to observe if these genes had a positive impact on transformation efficiency, organogenesis, and speed of plant recovery. The genes *VviGFR4-GIF1, AtGFR4-GIF1*

and *AtGFR5* were chosen based on previous reports of their demonstrated beneficial effects on recovery of genetically modified plants (Debernardi et al. 2020; Kong et al. 2020).

Objectives

Create gene constructs appropriate for cassava transformation containing the morphogenic genes *VviGFR4-GIF1*, *AtGFR4-GIF1* and *AtGFR5*.

Investigate the effects of the over-expression of such genes in cassava tissues, and quantify regeneration rates to observe if these genes have a positive impact on transformation efficiency, organogenesis, and speed of plant recovery.

Observe effects of morphogenic genes in plants grown in the greenhouse.

Material and Methods

Construction of binary plasmids

Plasmid cloning strategies were planned and designed using the SnapGene® version 5.3.2 software (Insightful Science; snapgene.com). The control vector (p8764) was prepared using a modified version of p6000 in which the *npt*II selectable marker is driven by the Nos promoter. This includes the visual marker green fluorescent protein (GFP) under the control of a 35S promotor and RbcS E-9 terminator.

Plasmid JD631 carrying the *Vitis vinifera GRF4-GIF1* gene sequences driven by the 35S promoter was obtained from Jorge Dubcovsky, University of California, Davis (Addgene plasmid #160399; http://n2t.net/addgene:160399; RRID:Addgene_160399). To produce the plasmid p8765, the donor *Vitis GRF4-GIF1* chimera was digested with SbfI and StuI and the *GRF4-GIF1* sequence purified and ligated into the GFP control plasmid (p8764) at the SmaI site.

A second set of morphogenic genes were prepared using gene sequences from *Arabidopsis thaliana*. *GRF4, GIF1* and *GRF5* were searched using The Arabidopsis Information Resource (TAIR), from which two copies of *GRF4* and *GRF5* plus one *GIF1* were identified. The protein coding sequences of *GRF4* accession number *(AT3G52910), GIF1 (AT5G28640),* and *GRF5 (AT3G13960)* were chosen based on description of their structure and activity according to published literature (TAIR, 2021). *GRF4* and *GIF1* sequences were fused together with an alanine linker and FASTA sequences sent to Genewiz® for synthesis and fusion to the Cassava vein mosaic virus (CsVMV) promotor (Verdaguer et al. 1998) and the NOS terminator.

The synthesized *AtGRF4-GIF1* in puc57Amp was digested with AhdI, EcoRI and KpnI and the top band gel purified. This fragment was cloned into p8764 in a 3-way ligation where p8764 was cut with KpnI and AscI to separate the plasmid backbone and GFP into two fragments. All three fragments were ligated to form the vector p8788. For *AtGRF5,* SpeI and PacI were used to remove *AtGRF4-GIF1* from p8788 replacing it by inserting *AtGRF5* to form the vector p8789. All plasmids were confirmed by restriction analysis and Sanger sequencing before transformation by electroporation into *Agrobacterium* strains LBA4404 and LBA4404 THY-.

Plant materials and *Agrobacterium* transformation of cassava

The African cassava varieties 60444 and NASE 13 were used for genetic transformation and plant regeneration studies. Plants were maintained by micropropagation on Murashige and Skoog (MS) basal medium, supplemented with 20 g/l sucrose (MS2), solidified with 8 g/l Noble agar (Segatto et al. 2022).

Production of friable embryogenic callus target tissues

Friable embryogenic callus (FEC) used for transformation was produced from organized embryogenic structures (OES) according to Segatto et al. (2022). Briefly, immature leaf explants approximately 2.5 mm in length were excised from micropropagated mother plants, placed onto MS2 medium supplemented with 50 μM picloram and 2 μM CuSO4, and cultured in low light (20 μMol m^{-2} s^{-1}) at 28°C. After four weeks, the OES that formed was excised, crushed through a 1 mm mesh, and placed onto Gresshoff and Doy (GD) medium supplemented with 20 g/l sucrose and 50 μM picloram, and solidified with 8 g/l Noble agar (GD2 50P). Tissues were cultured for three, 21-day cycles on GD2 50P medium under the same culture conditions to generate homogenous FEC tissues for co-culture with Agrobacterium.

Transformation with Agrobacterium and recovery of transgenic plants

All morphogenic gene constructs were transformed into FEC target tissues a minimum of four times with three samples each per transformation experiment. A GFP control and non-transformed control were also performed within each experiment.

A liquid *Agrobacterium* suspension with OD600 of 0.5 was prepared according to Segatto et al. (2022). When using LBA4404 THY- 50 mg/l thymidine was included in the suspension

medium. FEC tissues of 0.5–0.7 cc settled cell volume (SCV) per sample were placed in 12-well plates, and each sample inoculated with 2 ml of *Agrobacterium* suspension for 30 min. FEC tissues were then transferred onto GD2 50P medium supplemented with 200 mM acetosyringone and co-cultured for three days at 22°C under bright light (90 µMol m^{-2} s^{-1}). After co-culture, FEC tissues were washed with GD liquid medium containing 150 mg/l carbenicillin and cultured on GD2 50P medium supplemented with 150 mg/l carbenicillin under low light at 28°C for eight days. Tissue was then subcultured onto selection medium consisting of GD2 50P medium containing 150 mg/l carbenicillin and 27.5 µM paramomycin. For transformation of NASE 13 carbenicillin was replaced with 125 mg/l cefotaxime. Tissues were cultured for 21 days, followed by two subsequent regeneration stages on MS2 medium supplemented with 45 µM paramomycin and 5.0 µM and 0.5 µM NAA, respectively. Cotyledon-stage embryos were germinated on MS2 medium supplemented with 2 µM meta-topolin (mT), solidified with 2.2 g/l Gelzan (Segatto et al. 2022).

Tissues were monitored for expression of GFP using a Nikon C15304 dissecting microscope equipped with an excitation filter of 460–500 nm and barrier filter 510 LP. GPF visual scoring was performed according to Chauhan et al. (2015) where 0 = no visible signal; 1 = 1–10 GFP-expressing cells; 2 = 11–50 cells; 3 = 51–100 cells; 4 = 101–500 cells; and 5 = greater than 500 GFP-expressing cells visible. The number of growing colonies recovered on callus selection medium, those regenerating somatic embryos, and germinating plantlets expressing GFP were assessed for evaluation of transformation efficiency, by dividing the number at each stage by the initial settle cell volume (SCV) of FEC samples used (Segatto et al. 2022). Somatic embryos growing on germination medium were evaluated weekly to observe time required for germination. Regenerated shoots were rooted and micropropagated on MS2 solidified with 8 g/l Noble Agar.

Molecular confirmation of transgenic plants

*Reverse transcription-polymerase chain reaction (*RT-PCR) and RT-qPCR

GFP expressing plant lines were screened for *VviGRF4-GIF1, AtGRF4-GIF1,* and *AtGRF5* gene expression by RT-PCR and RT-qPCR. All primers were designed using the Primer3 program (Primer3web version 4.0.0) https://primer3.org/ and produced by Integrated DNA technologies – IDT (Coralville, Iowa, US), with details presented in Supplementary Table 1. Sequences selected for amplification started at the chimeric junction of the *GRF4-GIF1* fusion and were therefore not present in the cassava genome.

Young leaf tissues were collected from GFP expressing *in vitro* plants and RNA extraction performed using the Spectrum™ Plant total RNA kit (Sigma-Aldrich, Saint Louis, MO, USA). Samples were treated with DNAse (DNASE70 Sigma-Aldrich, Saint Louis, MO, USA) at room temperature for 15 min and run in 1% agarose gel to check RNA quality. Two micrograms of total RNA were reverse transcribed using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Waltham, MA, US). PCR cycling conditions comprised an initial denaturation holding stage at 94°C for 30 s, followed by 32 cycles of cycling stage at 94°C for 20 s, 55°C for 20 s, 68°C for 1:30 min, followed by final extension at 68°C for 5 min.

RT-qPCR was performed with SSO Advanced Universal SYBR® Green Supermix (Bio-Rad laboratories Inc., Hercules, CA, USA), with the endogenous cassava gene *PP2A* used as an internal control (Moreno et al. 2011). PCR cycling conditions comprised an initial denaturation holding stage at 95°C for 30 s, followed by 40 cycles of cycling stage at 95°C for 5 s, 61°C for 30 s, melt curve stage from 65°C to 95°C with 0.5 increments for 5 s, followed by final extension at 95°C for 5 min. Reactions were set up in triplicates for each sample. Quantification of the relative transcript levels was performed using the comparative C_T (threshold cycle) method (Livak and Schmittgen, 2001).

Organogenesis from tissues expressing *VviGFR4-GIF1*, *AtGRF4-GIF* and *AtGRF5*

Transgenic plant lines regenerated from variety 60444 and confirmed by RT-PCR to be expressing the morphogenic genes, were micropropagated on MS2 medium. Six-week old plants were selected and petioles, petioles with the leaf attached (leaf-petiole) and stems internodes (c. 1 cm in length) were excised and placed on MS2 medium supplemented with 2 µM mT, solidified with 2.2 g/l Gelzan and cultured under bright light (90 µMol m^{-2} s^{-1}) at 28°C for five weeks.

Tissues were visually scored under a dissection microscope and assessed for presence of callus, embryogenic structures, green foliose tissue, shoots, and roots. Callus production was scored using a 0-5 scale where 0 is absence of callus and 5 is the most amount of callus observed, at approximately 1 cm in diameter. Leaf-petiole regeneration studies were performed in triplicate and results expressed as mean score and percentage from the replicas.

Somatic embryo maturation

Transgenic lines of cv 60444 expressing *VviGRF4-GIF1* were assessed for regeneration of mature cotyledon-stage embryos from OES. OES was induced from immature leaf explants cultured on MS2 medium supplemented with 50 µM picloram and 2 µM CuSO4. After four weeks culture in low light (20 µMol m^{-2} s^{-1}) at 28°C the OES was excised, fragmented with a hypodermic needle and used to establish 2 mm diameter colonies. Twenty-five OES colonies were established per Petri dish containing MS2 Gelzan medium supplemented with 2 µM mT. After 10 days culture under bright light (90 µMol m^{-2} s^{-1}) at 28°C the number of green cotyledon-stage embryos developing from each OES unit was determined.

Plant establishment, growth, and assessment in the greenhouse

In vitro transgenic and control plantlets were planted in Berger BM7 35% Bark HP mixture potting compost (Hummert International, Earth city, MO, USA) in 7.6 cm pots and established in the greenhouse following Segatto et al. (2022). For all experiments, five independent transgenic plant lines were established with four biological replicas along with the GFP control. Plants were grown at 32°C/26 °C (day/night) with 60 –70 % relative humidity.

Plant height was measured manually, and mean height calculated. Leaf area was assessed by collecting the fourth expanded leaf below the shoot apical meristem after ten and fourteen weeks growth in the greenhouse. Leaf images were captured using a Nikon COOLPIX L830 camera mounted on a support set 40 cm above the leaf material. ImageJ (Schneider et al. 2012) web browser (https://ij.imjoy.io/) was used for leaf area calculation, where a known distance in cm was set as the scale, image threshold adjusted to default red, and then analyzed using ROI manager which selects, sums, and processes mean leaf area in centimeters squared.

After 14 weeks in the greenhouse a destructive harvest was performed. The stem was cut at the base and soil and fibrous roots removed from the storage roots. Stem height, number of nodes, fresh stem weight, number of storage roots, and fresh weight of storage roots were determined for each plant.

Experimental design and statistical analysis

Transformation experiments were performed four times, organogenesis studies were repeated three times. Data generated was subjected to ANOVA to determine significant differences and, where

appropriate mean separation was done with Dunnett test. Both ANOVA and mean separation were done using Minitab 17 statistical software package (Minitab Inc. State College, PA).

Results

Construction of binary plasmids

Three binary vectors were produced for integration of GRF morphogenic genes into cassava. Expression of genes derived from *Vitis vinifera* had not been previously attempted in cassava and had unknown functionality in this species. Therefore, a second version of the *GRF4-GIF1* and a *GRF5* expression vector were generated utilizing sequences from *Arabidopsis thaliana*. *A. thaliana* genes are known to function well when overexpressed in cassava (Narayanan et al. 2019). The GRF genes were driven by a strong constitutive promoter, in the case of *VviGFR4-GIF1* by the CaMV promoter and in *AtGRF4-GIF1, AtGRF5* by the CsVMV promoter. In all cases a GFP expression cassette driven by the 35S promoter was included within the T-DNA to enable real time, non-destructive observation of the transformation process from single cell expression immediately after co-culture, to recovery of fully developed plantlets. Details of all constructs plus the GFP-only control are shown in Table 1 and Figure 1. Gene constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 and the thymidine auxotrophic LBA4404 THY-, a modified version carrying extra virulence genes, previously reported for use with other morphogenic genes studies (Lowe et al. 2016). In our hands, LBA4404 THY- has been found to enhance efficiency of T-DNA transfer and transient GFP expression in cultivar NASE 13 (Supplementary Table 2).

Genetic transformation with morphogenic gene constructs

GFR4-GIF1 fusion gene constructs were used for Agrobacterium-mediated transformation of various explants including cotyledon tissues derived from somatic embryos. However, these resulted in very low efficiency of T-DNA delivery, as determined by few, to no, cells expressing GFP, and failure to recover transgenic tissues. Friable embryogenic callus was therefore utilized as the target tissue for transgene integration. The varieties 60444 and NASE 13 were selected to observe the effect of *GFR4-GIF1* and *GRF5* on transformation efficiency and plant regeneration. Variety 60444 has high transformation efficiency and potential of plant recovery (Taylor et al.

2012), while NASE 13, an East African farmer-preferred cultivar, possesses lower capacity for transformation and plant regeneration (Narayanan et al. 2021).

FEC tissues were transformed with the GFP-only control construct, *VviGRF4-GIF1*, *AtGRF-GIF1*, and *AtGRF5*. Transformed tissues were monitored for GFP expression, recovery of transgenic callus, and regeneration of somatic embryos and plants to assess if morphogenic genes affected transformation and/or plant regeneration efficiency. Data shown in Table 2 is averaged from four experiments.

Recovery of transgenic tissue and plants followed a pattern previously reported for 60444 (Taylor et al. 2012) with up to 250 callus lines, and as many as 29 independent transgenic plants recovered per cm^3 SCV of co-cultured FEC. As predicted, efficiencies were lower for NASE 13, with approximately 100 callus lines and 8-10 independent transgenic plants recovered per cm^3 SVC of transformed starting material. A difference was also seen in efficiency of recovery of plants from transgenic callus lines, with 14-23% GFP expressing callus lines regenerating to plants in 60444 compared to 7-10% in NASE 13 (Table 2). Time to germination of somatic embryos also differed, with shoots of 60444 appearing c. 28 days after being placed on germination medium, while cultivar NASE 13 required up to 70 days and 2-3 additional cycles on germination medium for plant recovery. Importantly, all morphogenic gene constructs behaved similarly during the stages of transgenic tissue recovery, somatic embryo formation and plant regeneration. At no stage did presence of the *GRF4-GIF1* or *GRF5* transgenes, whether derived from *Vitis* or *Arabidopsis* conferred measurable advantages for recovery of proliferating callus lines, regeneration of somatic embryos or germination of plants from transgenic somatic embryos (Table 2). With respect to time required for plant recovery from mature somatic embryos, there was also no difference observed between the morphogenic constructs and the GFP-only control.

Transgene expression confirmed by RT-PCR and RT-qPCR

Zero, or very low, transgene expression could explain why the morphogenic genes failed to simulate recovery of plants after transformation. Therefore, RT-PCR and RT-qPCR were performed on plants of 60444 regenerated from FEC transformed with morphogenic genes to confirm transgene expression. GFP expressing plant lines from each gene construct underwent initial screening by RT-PCR, with all found to be expressing the morphogenic transgenes (Supplementary Figure 2). In order to better determine morphogenic gene expression RT-qPCR

was performed. Figure 2 shows quantitative expression of *VviGRF4-GIF1, AtGRF4-GIF1* and *AtGRF5* from *in vitro* leaf tissue from ten different transgenic plant lines and GFP-only control. No expression was detected from plants of the GFP-only control and morphogenic genes were detected from very low to six-fold relative gene expression, most *VviGRF4-GIF1* lines expressed at 5-fold levels, *AtGRF4-GIF1* at 2-fold levels, while *AtGRF5* varied greatly across lines (Figure 2), such variable levels of expression are most likely due to position effect of the integrated transgenes.

Enhanced organogenesis observed from plants expressing morphogenic transgenes

Experiments were performed to determine if transgenic overexpression of *GRF4-GIFI* and *GRF5* affected morphogenic potential in cassava. Five mRNA expressing transgenic plant lines of 60444 regenerated from each morphogenic gene construct were selected for investigation of their organogenic potential by culturing explants on MS2 medium supplemented with 2 µM mT. Meta-topolin is a cytokinin produced from *Populus x robusta* and was previously found to stimulate the production of morphogenic tissues and plants in cassava (Chauhan and Taylor 2018). Initial evaluation was performed using two plant lines expressing *VviGRF4-GIF1* to determine comparative regeneration potential of petiole, leaf-petiole, and stem internode explants. Various tissues and morphogenic structures were produced from these explants over the five week observation period. These included non-morphogenic callus which was pale yellow in color, with a soft, watery consistency, embryogenic-like structures which were nodular in shape and cream to pale green in color, and green foliose tissues which developed from the embryogenic structures as soon as 10 days after explanting. The green foliose tissues proliferated to from more unorganized foliose tissues or regenerated to produce shoots (Figure 3, Supplementary Figure 1). Data revealed that all explant types were capable of generating non-morphogenic callus, with 75% of petiole and stem explants, and 95-100% of leaf-petiole explants producing this type of callus. Leaf-petioles were also superior for the production of morphogenic tissues including embryogenic structures, green foliose tissues, root and shoot regeneration compared to stem and petiole explants (Table 3). Additional experiments therefore focused on further characterizing the organogenic potential of leaf-petiole explants.

Table 4 shows data for callus formation, and production of embryogenic structures, green foliose tissues, roots and shoots formed by leaf-petiole explants derived from plants expressing

VviGRF4-GIF1, *AtGRF4-GIF1* and *AtGRF5* in comparison to the GFP-only control plants and non-transgenic 60444. Non-morphogenic callus developed from the basal, cut end of the petiole and to a lesser extend from the leaf tissue, starting approximately five days after placing on MS2 2 mT medium, and continued over the five-week culture period. The amount of callus produced was visually scored using a 0-5 scale, callus produced by *VviGRF4-GIF1* plant lines presented scores of up to 2.6, while controls averaged 2.0, and lines expressing *AtGRF4-GIF1* had the lowest callus formation with scores bellow 2.0 (Table 4).

A more complex response was seen for production of embryogenic structures and green foliose tissues (Figure 3 and Supplementary Figure 3), transgenic lines expressing *VviGRF4-GIF1* formed these tissues at up to 53% and 78% respectively, versus 12% and 3% for the GFP-only control. In contrast, production of these morphogenic tissues occurred at only 14% and 7% from plants transgenic for *AtGRF4-GIF*, and at 29% and 8% for plants expressing *AtGFR5*, values only slightly elevated from the GFP-only and the non-transgenic controls (Table 4). Rhizogenesis was observed in all plant lines, generally taking place within 15 days after explanting. Expression of morphogenic genes did not enhance this organogenic response, with similar root formation seen in the GFP-only and non-transgenic controls.

Shoot regeneration only occurred in transgenic lines which produced embryogenic structures, with between one and four shoots produced per responding explant. Shoot regeneration first became visible during the second week of culture on mT-containing medium, with clearly defined and recoverable shoots observed by the fourth week of culture. Shoot regeneration did not occur from explants derived from GFP-only controls and non-transgenic control plants. Nor was shoot regeneration observed in tissues produced by plant lines transgenic for *AtGRF4-GIF1*, while leaf-petiole explants expressing *AtGRF5* displayed low potential for shoot regeneration at 3-7%. In contrast, expression of *VviGRF4-GIF1* stimulated caulogenesis, with shoot regeneration occurring from all five of the lines transgenic for this construct. Maximum response, averaging 32%, was seen from event 65-6, which also showed high morphogenic potential for production of embryogenic structures and green foliose tissues (Table 4).

Stimulation of somatic embryo maturation

The effect of morphogenic gene expression on somatic embryo maturation was assessed using OES derived from plants expressing *VviGRF4-GIF1*. OES, induced from immature leaf

explants, were placed on MS2 2 µM mT medium and development of mature, green cotyledon-stage embryos assessed over a 10 day period. Figure 4 shows the results for three different *VviGRF4-GIF1* expressing lines plus controls, with an image illustrating the rapid maturity of these embryos compared to the GFP-only control. By the end of the 10-day observation period *VviGRF4-GIF1* expressing lines had developed significantly more mature cotyledon-stage somatic embryos and these lines were approximately 1.5 times larger than those produced by the control.

Growth and development of transgenic plants in the greenhouse

Transgenic plants expressing *VviGRF4-GIF1, AtGRF4-GIF1, AtGRF5,* and GFP-only were established in soil and grown under greenhouse conditions to assess effects of morphogenic gene overexpression on whole plants. All plant lines were robust and survived transfer to soil at the same rates. After 14 weeks growth in the greenhouse, plants were harvested and plant height, number of nodes, stem fresh weight, leaf area, storage root number fresh weight determined (Table 5). Calculation of leaf area was performed at 10 and 14 weeks after planting and is presented in Figure 5. By week 14 plants transgenic for *AtGRF5* were significantly taller averaging 63 cm in height, versus 55 cm for the controls. They also had the highest average for storage root weight at 70 g, although this was not statistically different from the control. Both *GRF-GIF* plant lines presented shorter stem height, with line 65-5 being significantly shorter at week 10 compared to the control. *VviGRF4-GIF1* lines were distinct in developing broader leaves that resulted in an increased leaf area, at levels significantly different in lines 65-1, 65-2, 65-5 and 65-6 compared to controls. This increased leaf area was accompanied by reduced storage root weight (Table 5).

Discussion

We report here that overexpression of *GRF4-GIF1* and *GRF5* stimulates morphogenesis in cassava. Our initial intent was to employ these morphogenic transcription factors to stimulate shoot regeneration from various explants, such as cotyledons from somatic embryos. However, T-DNA transfer to these tissues remained too inefficient to proceed. Agrobacterium-mediated transformation of FEC tissues is well documented in cassava and was therefore utilized to study the effects of these morphogenic transcription factors on the recovery of transgenic tissue and plants in this species. Transformation of the varieties 60444 and NASE 13 clearly showed that expression of these genes had no effect, beneficial of detrimental, on efficiency of the

transformation process or on regeneration of somatic embryos and plants. A lack of effect during the recovery of embryogenic tissues is perhaps not surprising and could be related to gene function since meristem formation and proliferation, for which these genes are known to function, was not occurring. Positive effects were expected, however, during the stages of somatic embryo maturation and germination when meristem formation does occur and foliose cotyledon tissues are being produced. Indeed, this lack of response across several hundred regeneration events in 60444 and NASE 13 contrasts for unknown reasons, with the significantly accelerated development and maturation which was observed from somatic embryos derived from plants transgenically expressing *VviGRF4-GIF1* (Figure 4).

We previously reported regeneration of transgenic plants in cv. NASE 13 (Narayanan et al. 2021), but did not describe the process or efficiencies for this Ugandan, farmer-preferred variety. As expected, NASE 13 responded at lower efficiencies at all stages of transformation and plant regeneration compared to 60444, but at levels which were still effective for recovery of transgenic plant lines. Data reported here reveals two stages where efforts should be focused to improve the transformation process for this cultivar. T-DNA transfer to the FEC target tissue is relatively efficient, especially when the LBA4404 THY- strain is used (Supplementary Table 2), but initiation of cell division from these transient events occurs at very low frequencies, thus constraining recovery of callus tissue that can then be cultured to regenerate plants. Likewise, germination of the matured cotyledon-stage embryos in NASE 13 is problematic, occurring at 30% compared to 70% in 60444, and requiring an extra 50-70 days for recovery of shoots compared to 60444. Focusing research efforts on these two areas would likely result in enhanced efficiencies for recovery of transgenic and genome edited events and generate knowledge applicable and valuable for improving these processes in additional cassava varieties.

RT-qPCR analysis confirmed expression of *GRF4-GIF1* and *GRF5* in all transgenic plants tested, although at varying levels (Figure 2). Three explant types were evaluated for their response to culture on MS2 medium supplemented with 2 μM meta-topolin, as this cytokinin was previously reported to stimulate organogenesis in cassava cultivars (Chauhan and Taylor, 2018). Data generated in the present study confirmed the petiole with leaf tissue attached, to be the most responsive for production of morphogenic tissues and shoot regeneration (Table 3). The superior organogenic potential of leaf-petioles could be due to an interaction between the cytokinin in the

growth medium and naturally occurring auxins in the leaf, since young leaves are known to be a primary origin of auxin (Taiz and Zeiger, 1998).

Investigations using leaf-petiole explants derived from plants transgenic for *GRF4-GIF1* and *GRF5* indicated a significant difference in morphogenic potential between gene constructs, with only *VviGRF4-GIF1* effective for stimulating shoot regeneration. While all *VviGRF4-GIF1* transgenic plant lines tested regenerated shoots, no correlation was observed between mRNA expression and organogenic response (Figure 2, Table 4). Transgenic lines tested expressing *Vitis*-derived *GRF4-GIF1* regenerated shoots at 20-30%, while no response was observed from plants expressing *Arabidopsis*-derived *GRF4-GIF1* (Table 4). This variance could be due to several reasons, such as amino acid sequence similarity, with *VviGRF4* protein sequence being 78.26% similar to the *Manihot esculenta GRF4,* while *AtGRF4* is 56.91% similar. Post-transcriptional regulation (Filipowicz et al. 2008; Furlan et al. 2021), ectopic expression (Siefers et al. 2009; Kerr et al. 2018) or functional orthologs (Das et al. 2016), could also play a role. It is worth noting that *GRF4* can be post-transcriptionally downregulated by microRNA396, while *GRF5* cannot, since the latter does not possess the binding site for microRNA396 (Kim and Tsukaya 2015). However, this did not result in an advantage for *GRF5* which was outperformed by *VviGRF4-GIF1* in leaf-petiole organogenesis. Different promoters were employed to drive the *GRF4-GIF* expression cassettes but this is not thought to explain why *VviGRF4-GIF1* was effective and *AtGRF4-GIF1* was not, as both of these strong constitutive promoters are known to be potent in cassava (Oyelakin et al. 2015). Indeed, *AtGRF5* under control of the CsVMV promoter did stimulate caulogenesis at low levels (Table 4).

Chauhan and Taylor (2018) reported the production of compact green nodular tissues, (here identified as embryogenic structures) produced when leaf-petiole explants were cultured on MS2 containing meta-topolin, and that shoots could be regenerated from these tissues but only after a series of sequential subcultures over a period of 10-14 weeks. This contrasts with the data reported here, where the nodular embryogenic-like tissues rapidly differentiated to form foliose tissues and shoots, with recoverable plantlets developing from *VviGRF4-GIF1* expressing tissues after only three to four weeks culture on the meta-topolin medium (Supplementary Figure 1). We postulate therefore that expression of *VviGRF4-GIF1* is stimulating organization and proliferation of the shoot meristems from these tissues.

Plants of 60444 transgenic for *GRF4-GIF1* and *GRF5* were established in soil and grown in the greenhouse to assess effects on whole plant development. Cassava plant lines overexpressing GRF5 were noticeably taller than the other transgenics and control (Table 5, Figure 6), while *VviGRF4-GIF1* showed larger leaf size and total leaf area compared to the control (Table 5, Figure 5). This is a not a totally unexpected result as *GIF1* is a known positive regulator of cell proliferation in leaves and flowers (Lee et al. 2009; Rodriguez et al. 2010; Debernardi et al. 2014). Increased leaf area, did not result in increased productivity in terms of storage root mass, but this may have been due to the relatively small pots used and short growing period of 14 weeks in this study. Longer cultivation in large pots is therefore recommended to better reveal interesting agronomic characteristics resulting from over expression of *GRF4-GIF1* and *GRF5*.

We have shown here that overexpression of heterologous *GRF4-GIF1* and *GRF5* can stimulate shoot regeneration in cassava. Further studies should be carried out to optimize this response and assess whether these and other morphogenic transcription factors can be utilized to promote recovery of transgenic tissues and plants from novel explant sources.

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Conflict of interest
The authors have no relevant financial or non-financial interests to disclose.

Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Rosana Segatto. The first draft of the manuscript was written by Rosana Segatto and all authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

Data availability
The raw data generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1 Binary vectors used for transformation of cassava cultivars

Construct #	Size (bp)	Genes of interest	Abbreviation
p8764	9829	*Green fluorescent protein*	GFP
p8765	12940	*Vitis vinifera GRF4-GIF1*	*VviGRF4-GIF1*
p8788	12797	*Arabidopsis thaliana GRF4-GIF1*	*AtGRF4-GIF1*
p8789	12206	*Arabidopsis thaliana GRF5*	*AtGRF5*

Table 2 Tissue and plant recovery from cultivars 60444 and NASE 13 transformed with morphogenic genes

Cultivar	Construct	SCV (cc)	# FEC samples per experiment	Average transient GFP Score (1-5)[a]	Average # callus lines recovered[b]	Average # callus lines forming cot stage embryos[c]	Average # independent transgenic plants recovered[d]	Average # callus lines recovered per cc SCV (±SE)[b]	Average # cotyledon stage per cc SCV (±SE)[c]	Average # rooted plantlets per cc SCV (±SE)[d]
60444	*GFP*	0.65	3	3.9	226.2	96.8	51.0	116.1±31.2[e]	49.6±8.5[f]	26.1±3.9[g]
	VviGRF4-GIF1	0.65	3	3.7	281.3	108.5	57.0	144.3±35.8[e]	55.6±6.6[f]	29.2±4[g]
	AtGFR4-GIF1	0.62	3	3.8	282.5	89.5	50.7	151.8±34.4[e]	45.9±13.2[f]	27.2±4.5[g]
	AtGRF5	0.62	3	3.8	255.7	94.5	34.5	137.5±34.9[e]	50.8±10.9[f]	18.5±3.9[g]
NASE 13	*GFP*	0.60	3	3.0	174.0	38.3	15.0	96.7±24[e]	21.3±6.1[f]	8.3±3[g]
	VviGRF4-GIF1	0.60	3	2.8	207.3	34.3	14.0	115.2±28.2[e]	19.1±1.6[f]	7.8±1.5[g]
	AtGFR4-GIF1	0.60	3	2.8	199.8	40.5	14.8	111.0±43.4[e]	22.5±7.4[f]	8.2±2.3[g]
	AtGRF5	0.60	3	2.9	186.3	51.3	19.5	103.5±25.5[e]	28.5±4.6[f]	10.8±3.1[g]

Friable embryogenic callus was transformed with Agrobacterium strain LBA4004 carrying morphogenic genes, then selected and regenerated following Segatto et al. (2022). Data shown is averaged from four independent transformation experiments.

[a] Average transient GFP score is a subjective visual score where 0 = no visible signal; 1 = 1–10 GFP-expressing cells; 2 = 11–50 cells; 3 = 51–100 cells; 4 = 101–500 cells; and 5 = greater than 500 GFP-expressing cells visible.

[b] Proliferating callus lines recovered after 21 days culture on GD2 50P medium supplemented with paromomycin

[c] Callus lines producing cotyledon-stage embryos after three weeks culture on MS2 medium supplemented with 0.5 μM NAA.

[d] Number of independent transgenic plants regenerated and established on MS2 medium

[e, f, g] same letters in a column are not statistically significant by Dunnett's method at 95% confidence.

Table 3 Organogenic potential of different explants derived from plants of cv 60444 expressing *VviGRF4-GIF1*

Response	**Petiole**			**Leaf-petiole**			**Stem**		
	NT[b]	Line 65-2	Line 65-4	NT	Line 65-2	Line 65-4	NT	Line 65-2	Line 65-4
Explants forming callus %	50	75	60	95	95	100	50	65	75
Av. callus size (0-5)[a]	0.5	1.1	0.8	1.0	1.6	1.8	0.6	1.4	1.9
Embryogenic structures %	2	10	2	2	15	28	0	2	0
Green foliose tissues %	2	5	10	2	25	23	2	5	5
Roots %	0	0	0	0	12	3	0	0	0
Shoots %	0	0	0	0	7	0	0	0	0

Transgenic plants of lines 65-2 and 65-4 known to express *VviGRF4-GIF1* were micropropagated on MS2 for six weeks. Explants were excised and placed on MS2 supplemented with 2 µM mT, cultured for five weeks and assessed for production of tissues and organs.

[a]Average callus size is a subjective visual score where 0 equates to absence of callus, 1 to minimal callus, to 5 for abundant callus growth reaching 1 cm in diameter.

[b] NT non-transgenic 60444 control, 65-2 and 65-4 are independent transgenic plant lines expressing *VviGRF4-GIF1*.

Table 4 Organogenesis from leaf-petiole explants derived from transgenic plants of cv 60444 expressing *VviGRF4-GIF1, AtGRF4-GIF1* or *AtGRF5*.

Morphogenic transgenes	**Response**	**NT**	**GFP[b]**	**65-1**	**65-2**	**65- 4**	**65-5**	**65-6**
VviGRF4-GIF1	Av. callus size (0-5)[a]	2.1±0.2	2.0±0.6	2.0±0.2	2.5±0.2	2.6±0.1	1.8±0.4	2.5±0.3
	Embryogenic structures %	5.3±4.2	12.6±3.1	44.6±15.5	34.6±5.0	38.3 ±7.6	53.0±13.5	43.6±16.8
	Green foliose tissue %	1.3±1.2	2.6±2.5	64.3±4	53.3±8.5	65.6±12.1	72.3±2.5	78.3 ±8.5
	Roots %	9.6±5.5	9.0 ±5.2	4.8±2.3	6.6±11.5	3.3 ±5.8	5.1±5.0	12.2±9.2
	Shoots %	0.0	0.0	24.3±3.8	14.6±4.5	26.0±12.5	21.6±2.9	31.6±4.7
	Response	**NT**	**GFP[c]**	**88-2**	**88-4**	**88-6**	**88-7**	**88-9**
AtGRF4-GIF1	Av. callus size (0-5) [a]	2.1±0.2	2.0±0.6	1.2±0.2	1.4±0.2	1.3±0.1	1.2±0.1	1.3±0.1
	Embryogenic structures %	5.3±4.2	12.6±3.1	14.0±6.9	12.0±3.0	8.3±3.5	7.3±2.3	7.0±4.6
	Green foliose tissue %	1.3±1.2	2.6±2.5	3.6±1.2	3.8±1.9	6.8 ±4.8	1.9 ±1.6	0
	Roots %	9.6±5.5	9.0 ±5.2	9.0 ±7.9	7.6±4.0	2.2±2.0	10.6±11.0	3.0 ±2.6
	Shoots %	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Response	**NT**	**GFP[d]**	**89-1**	**89-2**	**89-3**	**89-4**	**89-5**
AtGRF5	Av. callus size (0-5) [a]	2.1±0.2	2.0±0.6	2.0 ±0.1	1.6 ±0.4	2.1±0.4	2.0±0.6	2.3±0.2
	Embryogenic structures %	5.3±4.2	12.6±3.1	20.1±10.0	19.3±4.0	21.6±2.9	19.0±8.5	29.0± 8.5
	Green foliose tissue %	1.3±1.2	2.6±2.5	0.0	6.0 ±3.6	5.3±5	8.6±2.3	6.1±7.9
	Roots %	9.6±5.5	9.0±5.2	5.3 ±4.0	5.3±5.0	1.8±1.6	3.3±5.8	7.8±5.0
	Shoots %	0.0	0.0	0.0	0.8 ±1.4	1 ±1.7	1.6 ±2.9	0.0

Leaf-petioles explants of plant lines known to express *VviGRF4-GIF1, AtGRF4-GIF1* or *AtGRF5* were excised and placed on MS2 supplemented with 2 µM mT, cultured for five weeks and assessed for production of tissues and organs. Response is averaged from three replicas with 40 explants each ± SD.

[a]Average callus size is a subjective visual score where 0 equates to absence of callus, 1 to minimal callus, to 5 for abundant callus growth reaching 1 cm in diameter.

[b] GFP control, NT non-transgenic 60444 control, 65 are independent transgenic plant lines expressing *VviGRF4-GIF1*.

[c] GFP control, NT non-transgenic 60444 control, 88 are independent transgenic plant lines expressing *AtGRF4-GIF1*.

[d] GFP control, NT non-transgenic 60444 control, 89 are independent transgenic plant lines expressing *AtGRF5*.

Table 5 Growth of 60444 cassava plants expressing morphogenic genes in the greenhouse 14 weeks after planting

Transgenic plant line		Height (cm)	Leaf area (cm^2)	Average # nodes	Average stem weight (g)	Average # roots	Average storage root weight/plant (g)
GFP	1	55.7±3.5	101.5±15.9	35.50	27.8±4.4	7.25	64.0±4.0
	2	55.0±5.9	90.0±18.4	33.50	22.1±1.0	7.25	75.5±13.1
VviGRF4-GIF1	65-1	47.7±1.0	142.2±24.4	31.50	21.0±2.2	8.25	60.9±17.7
	65-2	54.5±1.9	143.9±27.0[a]	33.50	23.0±2.8	7.25	80.4±3.3
	65-4	50.2±3.2	130.8±30.8	32.25	23.4±3.4	6.25	51.6±8.5
	65-5	47.0±4.5	145.8±23.4[b]	27.75	18.5±2.1	5.50	31.8±6.0
	65-6	49.0±1.2	129.4±18.6	30.75	19.3±2.5	7.25	63.1±9.6
AtGRF4-GIF1	88-2	52.5±3.9	107.7±11.0	32.25	18.2±1.5	5.75	54.8±15.8
	88-4	58.2±2.5	130.6±13.3	32.75	22.0±2.6	6.00	50.8±6.5
	88-6	49.2±2.2	112.8±10.5	35.50	24.6±2.1	7.25	63.1±25.0
	88-7	49.5±3.5	98.5±19.2	31.75	19.1±4.2	6.75	46.1±19.0
	88-9	51.7±5.0	112.0±12.1	32.25	23.5±2.6	5.25	64.8±6.1
AtGRF5	89-1	64.0±4.1[a]	102.5±20.0	36.75	27.2±2.3	7.00	79.0±12.7
	89-2	68.0±3.3[b]	113.1±18.8	36.75	32.0±4.4	5.00	44.2±7.3
	89-3	59.5±4.0	131.3±29.1	36.50	25.7±3.9	6.00	75.6±5.8
	89-4	63.2±4.0[c]	110.3±10.2	34.00	28.8±3.3	6.50	77.7±11.3
	89-5	60.5±1.3	91.2±8.8	35.50	26.7±3.1	6.00	77.1±14.7

Cassava plants expressing morphogenic genes and GFP-only control were established in soil and grown in the greenhouse for 14 weeks. Results shown are averages from four biological replicas ±SD.
[a, b, c] different letters in the column are significant different $p < 0.05$

Figure legends

Fig. 1 Schematic representation of gene constructs used to generate transgenic plants in cassava varieties 60444 and NASE 13. GFP-only control plasmid p8764. *Vitis vinifera GRF4-GIF1* plasmid p8765. *Arabidopsis thaliana GRF4-GIF1* plasmid p8788. *A. thaliana GRF5* plasmid p8789. Organization of the T-DNA, arrows represent promoters and coding sequences. Rectangles represent terminators. Restriction sites used for the construction are indicated

Fig. 2 Quantitative expression of morphogenic genes from *in vitro* cassava leaf tissues. Expression was compared and normalized to protein phosphatase 2 (*pp2A*). Expression values of 65-3, 88-8, 89-6 were adjusted to a value of 1 with other values expressed relative to these. Values are means of three technical replicates. Error bars represent SD. **a** *VviGRF4-GIF1* expression. **b** *AtGRFR4-GIF1* expression. **c** *AtGRF5* expression

Fig. 3 Structures and organs regenerated from leaf-petiole explants after culture on MS2 medium supplemented with 2 μM mT. **a** Initial explant excised from micopropagated mother plant. **b** Non-morphogenic callus developing from basal, cut end of the petiole, assessed as a visual score of score 3 (0-5 scale). **c** Embryogenic structure developing from basal end of the leaf-petiole explant. **d** Development of green foliose tissues with putative bud-like structures. **e** Regeneration of a fully formed shoot from the basal end of the leaf-petiole explant after 30 days culture. **f** Root regenerated from the basal end of the leaf-petiole explant after 20 days culture

Fig. 4 Stimulation of somatic embryo maturation in variety 60444. **a** Average number of embryos matured to green cotyledon-stage per OES fragment. **b** Maturation of somatic embryos from GFP-only control. **c** Development of many mature, green cotyledon-stage embryos from OES expressing *VviGRF4-GIF1*

Fig. 5 Increased leaf area in greenhouse-grown plants transgenic for *VviGRF4-GIF1*. **a** Leaf area per plant line * statistically significant $p < 0.05$

Fig. 6 Height difference in plant lines expressing morphogenic genes. **a** GFP control and *VviGRF4-GIF1*. **b** GFP control and *AtGRF5*.

Figures

Figure 1

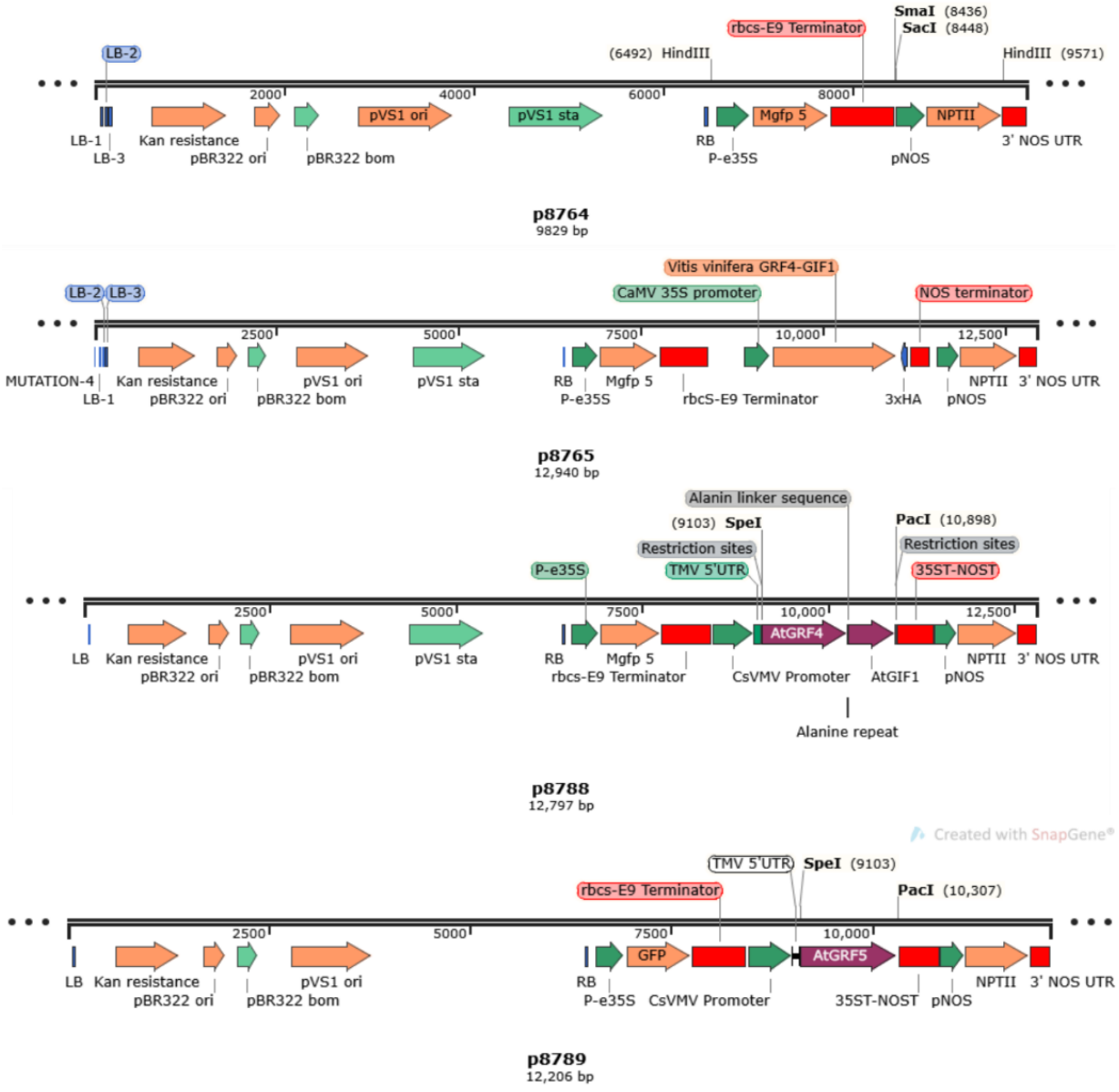
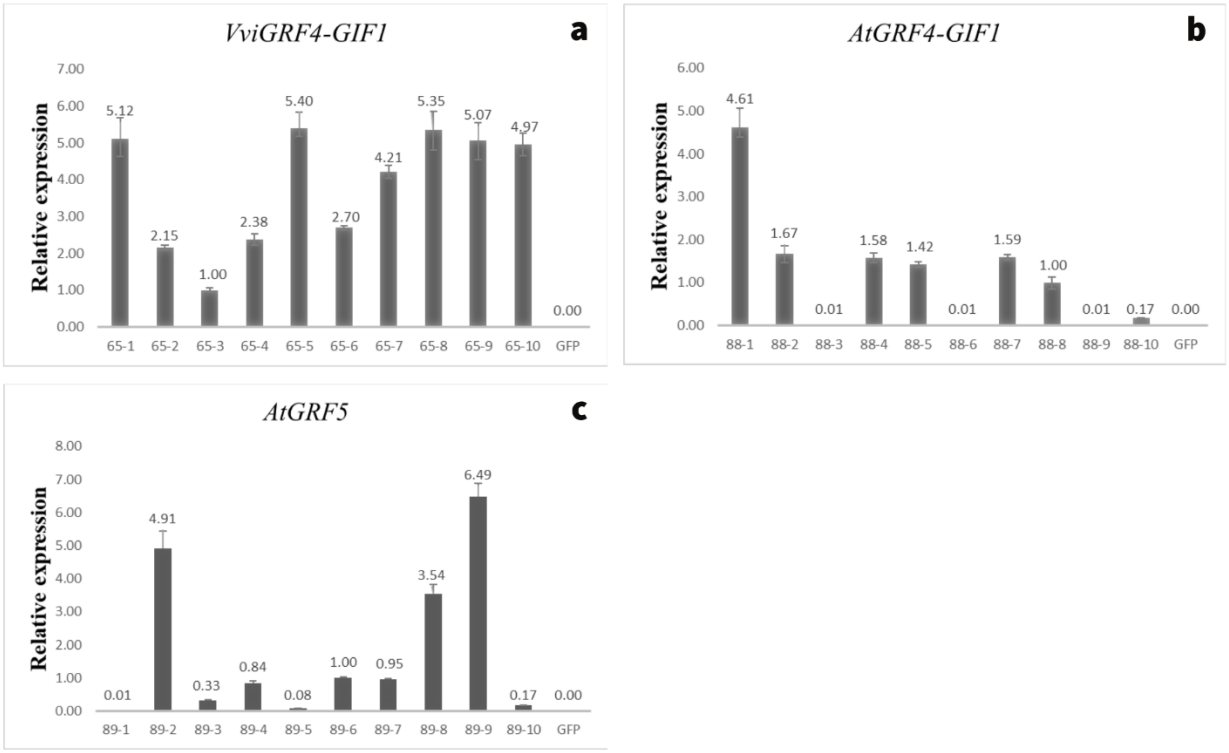


Figure 2


VviGRF4-GIF1
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Relative expression
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5.00
4.00
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2.00
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0.00
5.12
2.15
1.00
2.38
5.40
2.70
4.21
5.35
5.07
4.97
0.00
65-1
65-2
65-3
65-4
65-5
65-6
65-7
65-8
65-9
65-10
GFP
AtGRF4-GIF1
b
Relative expression
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3.00
2.00
1.00
0.00
4.61
1.67
0.01
1.58
1.42
0.01
1.59
1.00
0.01
0.17
0.00
88-1
88-2
88-3
88-4
88-5
88-6
88-7
88-8
88-9
88-10
GFP
AtGRF5
c
Relative expression
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5.00
4.00
3.00
2.00
1.00
0.00
0.01
4.91
0.33
0.84
0.08
1.00
0.95
3.54
6.49
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89-1
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89-10
GFP

Figure 3

Figure 4

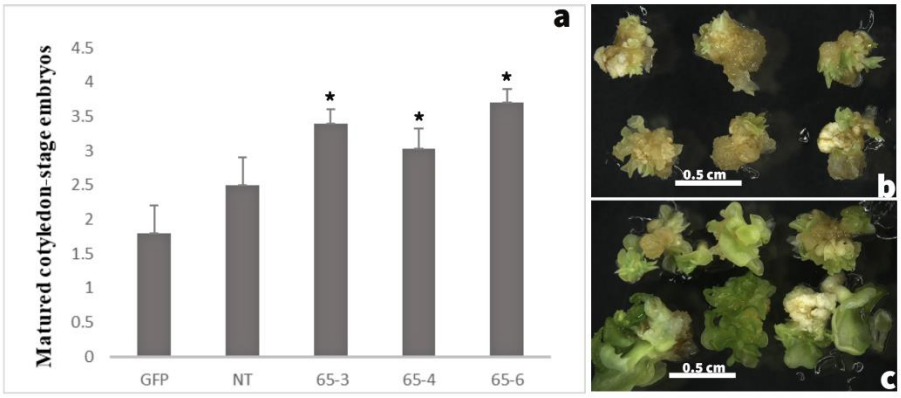


Figure 5

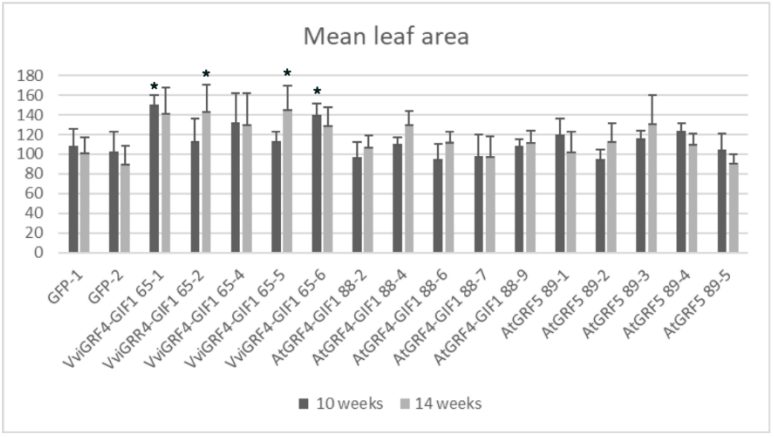
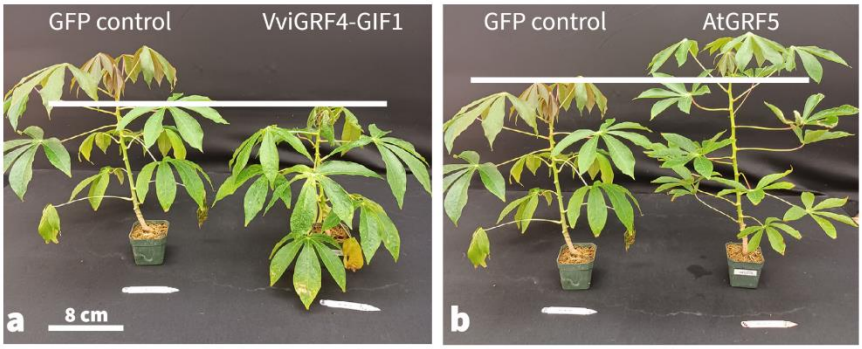
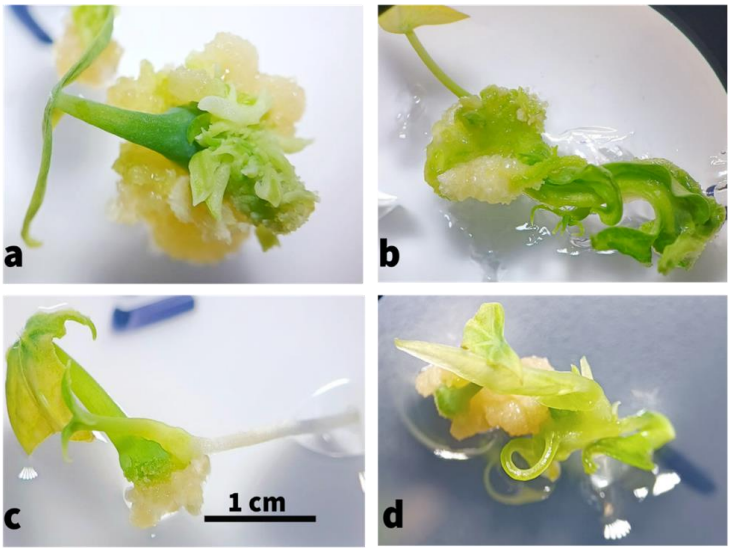


Figure 6

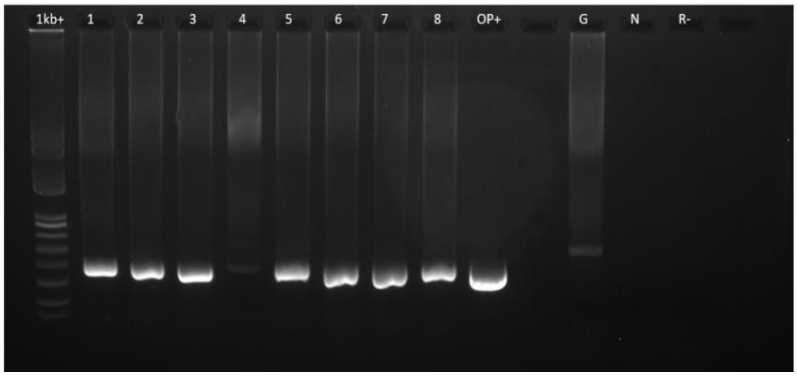


Annex

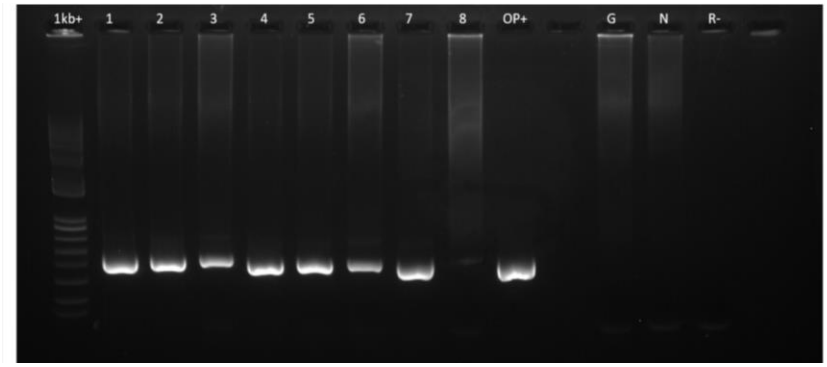
Supplementary material



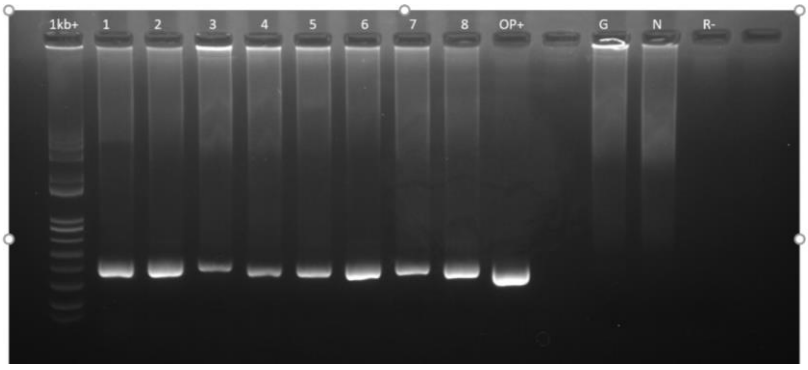
Supplementary Figure 1. Morphogenesis and shoot regeneration from leaf-petiole explants of 60444 plants transgenic for *VviGRF4*-GIF1 after 4 weeks culture on MS2 medium supplemented with 2 µM meta-topolin. **a** non-morphogenic yellow colored callus and green foliose tissues developing from basal end of leaf-petiole explant. **b** proliferation of green foliose tissues. **c** formation of shoot and root from embryogenic-like structures. **d** development of fully formed shoot



Supplementary Figure 2a. RT-PCR of plants transgenic for *VviGRF4-GIF1* RT-PCR. *VviGRF4-GIF1* RT-PCR loading order: 1 kb plus DNA ladder, c-DNA samples 1 – 8, original plasmid positive control, GFP control, non-transformed control, negative reaction control. Amplicon ~ 400 bp.



Supplementary Figure 2b. RT-PCR of plants transgenic for *AtGRF4-GIF1* RT-PCR. *AtGRF4-GIF1* RT-PCR loading order: 1 kb plus DNA ladder, c-DNA samples 1 – 8, original plasmid positive control, GFP control, non-transformed control, negative reaction control. Amplicon ~ 500 bp.



Supplementary Figure 2c. RT-PCR of plants transgenic for *AtGRF5*. *AtGRF5* RT-PCR loading order: 1 kb plus DNA ladder, c-DNA samples 1 – 8, original plasmid positive control, GFP control, non-transformed control, negative reaction control. Amplicon ~ 430 bp.

Supplementary Table 1. Primers used for RT-PCR and RT-qPCR amplification of *VviGRF4-GIF1*, *AtGRF4-GIF1*, *AtGRF5* transgenes.

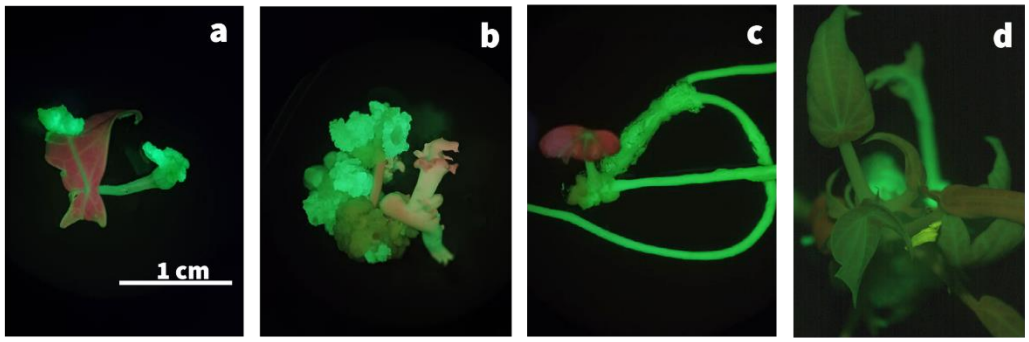
Primer name	Primer sequence	Amplicon size (bp)	Purpose	Hybridization temperature (°C)
VviGRF4-GIF1 Fw	CGTCGGGAGAGGTTGTGAAA	403	RT-PCR	55
VviGRF4-GIF1 Rv	GAATCAGCGATTGCAGCCAG			
AtGRF4-GIF1 Fw	ACGATTGGCCACGATCATCA	496	RT-PCR	55
AtGRF4-GIF1 Rv	TTGCGTTGAAGCCTTGCTTG			
AtGRF5 Fw	GCCTCTGCTACTGACCACAA	431	RT-PCR	55
AtGRF5 Rv	TTGTTCTGTGCCCAGTCCTC			
VviGRF4-GIF1 Fw	CCTCCTAGTGGCATTGTTCAG	114	RT-qPCR	61
VviGRF4-GIF1 Rv	TTGTTGGGAGTACAGCATGG			
AtGRF4-GIF1 Fw	ATTGCACCATAGCCAGCTT	127	RT-qPCR	61
AtGRF4-GIF1 Rv	ACTTCCCATTTCCGGCTTC			
AtGRF5 Fw	CAAGAAGTGTCCTTGCAAACTC	109	RT-qPCR	61
AtGRF5 Rv	TCCTCACCAAAGAAGTGATGTAG			

Supplementary Table 2. Effect of Agrobacterium LBA4404 and LBA4404 THY- on recovery of GFP expressing tissues in cultivar NASE 13.

Agrobacterium	Transient score (0-5) [a]	# GFP expressing cells [b]	# GFP callus lines recovered [c]
LBA4404	0.6 ± 0.6	8 ± 4.4	10 ± 3
LBA4404 THY-	2.8 ± 0.7	14.7 ± 3.5	22.3 ± 8.4

Friable embryogenic tissues of NASE 13 were co-cultured with Agrobacterium strains LBA4404 or LBA4404 THY- and tissues assessed for expression of GFP. Data shown are averages from three independent transformations each with five FEC samples per experiment.

[a] Transient expression score determined after three days co-culture with Agrobacterium using a 0-5 scale as described in Materials and Methods. [b] GFP expressing cells after 10 days culture on GD2 50P medium supplemented with 125 mg/l cefotaxime. [c] Independent callus lines growing after 21 days culture on GD2 50P medium supplemented with 125 mg/l cefotaxime and 27.5 µM paromomycin.



Supplementary Figure 3. Expression of GFP across tissue types. **a** non-morphogenic callus developing on leaf and petiole with initial root development**. b** early-stage shoot and foliose tissues. **c** different root structures. **d** whole plantlet