AGROBACTERIUM-MEDIATED TRANSFORMATION OF A MODEL CASSAVA CULTIVAR (TMS 60444)

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Abstract
Cassava is a major staple food crop in Nigeria. Recent developments in biotechnology offers useful alternative to the breeding constraints of this highly heterozygous crop. *Agrobacterium tumefaciens*, a ubiquitous soil borne pathogen was used for the transformation. A bacterial strain LBA 4404 containing a binary vector pB 2300 with npt11 gene as selectable marker and a green fluorescent protein (GFP) as a tracer was used for the experiments. Two cassava cultivars (TMS 60444 and Sandpaper) were amenable to the antibiotics carbenicillin and paramomycin and responded optimally to tyrosine. Selection of the transformed tissues with cefotaxine and paramomycin resulted in the recovery of antibiotic-resistant GFP-expressing lines of friable embryogenic callus from which embryos and subsequent plants were regenerated. The positive response of these two cassava cultivars to *Agrobacterium*-mediated transformation signals the possibilities for modification of the cassava cultivars and also opens up approaches to solve specific needs through biotechnology.
Key words: Cassava, *Agrobacterium tumefaciens*, transformation, antibiotics, tyrosine.

Introduction

Cassava (*Manihot esculenta* Crantz) is a major staple food crop in Nigeria. It ranks first amongst the root and tuber crops that are in contention for food security. It is an important food crop for some 500 million people in developing countries. Increased production of cassava is largely a consequence of the crop's low labour input requirements, ability to produce good yield on degraded soils, drought tolerance, and its resistance to pests and disease makes it a crop of choice for farmers. A unique advantage of cassava over other crops for example cereals, is its flexible harvesting time that makes it excellent famine food stuff. Moreover, cassava's starchy roots produce more calories per unit of land than any other crop except sugarcane (Henry *et al*., 1995). Another advantage of cassava over other crops is its ability to be stored in the ground for over 36 months. Hence, cassava cultivation serves as a household food bank that can be drawn upon in times of need.

Regrettably, lack of resistance genes in the available germplasm, high heterozygosity, allopolyploidy, low fertility, and unsynchronized flowering make cassava improvement by conventional breeding a long and tedious process (Ceballos *et al*., 2004 and Rudi *et al*., 2010). The emergence of genetic transformation of plants as a viable alternative technique to complement conventional breeding (Ubalua *et al*., 2010) has opened up fascinating opportunities in improving the agronomic properties of cassava (Sayre *et al*., 2011; Liu *et al*., 2011). The body of literature is replete with protocols using either cotyledons or embryogenic cultures as target tissues and particle bombardment procedures respectively (Liu *et al*., 2011). We now report on the use of *agrobacterium*-mediated transformation of friable embryogenic callus. The method is efficient and reproducible compared to cotyledon-based protocol and particle bombardment technique.

Materials and Methods

Apical cuttings (1cm) were excised from two cassava cultivars (Sandpaper and TMS 60444). The cuttings were immediately immersed in water and subsequently rinsed with sterile water. They were further immersed in 70% ethanol for 5 min and surface sterilized for 20 min in 5% bleach and rinsed three times with sterile distilled water in a laminar air flow cabinet. The pH of the Murashige and Skoog (MS) medium was previously adjusted to 5.8 before sterilizing by autoclaving at 121^0C for 15 min. Nodal cuttings of the apical shoots were cultured in the same medium in test tubes containing 10 ml of the sterile medium. The tubes were sealed properly and labeled before incubating at a temperature of 26 ± 2^0C under light. Subculture was done every two months.

Induction of organized embryogenic structures (OES)

Young leaf lobes of the *in vitro*-grown cassava plantlets of the two cultivars were excised and used for the induction of organized embryogenic structures (OES) on DKW2 50P medium for 3 weeks. The medium pH was adjusted to 6.12 before autoclaving at 121^0C for 15 min. Filter sterilized picloram (5ml/L) was added to the medium when the medium temperature was 42^0C before dispensing in sterile petri dishes. Ten leaf lobes were aseptically inoculated onto petri dishes containing 25 ml of solid DKW2 50P medium solidified with 8 g/l of agar and supplemented with 20 g/l of sucrose. A stereo dissecting microscope, sterile hypodermic needle and forceps were used for the inoculation. The petri dishes were sealed with parafilm and after 3 weeks of incubation under dimmed light conditions at 26 ± 2^0C, the cultures were scored for the presence of organized embryogenic structures. The promising yellowish structures were pooled together and the whitish mucus discarded. The pooled yellowish structures were meshed with a sterile spatula on a sterile mesh. The method as

described by Taylor et al., (1996) was adopted for the incubation, maturation and subsequent generation of friable embryogenic callus (FEC) as follows. The meshed OES were placed in small dots on freshly prepared GD2 50P + 500 µM tyrosine plates and incubated in a closed paper box. Friable embryogenic callus (FEC) was generated on GD2 50P + 500 µM tyrosine and recycled 3 times for callus proliferation for 9 weeks in a closed paper box. The experiments were conducted using completely randomized design. The treatments were repeated three times (40 explants per treatment) and data were taken 3 weeks after each treatment and analyzed using analysis of variance (ANOVA).

Transformation and selection of transgenic tissues:
Bacterial cultures were initiated by plating a frozen glycerol stock of the engineered *Agrobacterium tumefaciens* (strain LBA 4404 containing a Pcambia 2300-based binary vector carrying the npt11 gene as selectable marker and the green fluorescent protein (GFP) as a tracer) onto agar-solidified LB medium containing the antibiotics rifampicin (30 mg/ml), streptomycin (30 mg/ml) and kanamycin (50mg/ml). After a 2-day growth, single colonies were inoculated into 2 ml liquid LB medium containing the above antibiotics. The bacterial cultures and the standard were grown from morning till evening at room temperature (28^0C, in a shaker at 250 rpm). I milliliter of the *Agrobacterium* cultures was inoculated into a flask containing 20 ml of YM medium with antibiotics (rifampicin, 20 mg/ml, streptomycin, 30mg/ml and kanamycin 50mg/ml). The inoculated flask and a blank were further incubated overnight to an OD600 of 0.7 to 1.0 on a shaker at room temperature and at 250 rpm.

Selection of the transgenics:
A 0.6 ml of FEC was aseptically transferred from plates to a sterile 12 well plate with sterile forceps. Two (2) ml of the *Agrobacterium* suspension were used to inoculate viable, moist, yellowish tissues of a three-time recycled FEC selected under a stereo-microscope for a period of 1 hour. After 4 days of coculture with the *Agrobacterium* in a 22^0C Percival incubator, the tissues were cleaned-up with GD2 50P + 200 µM tyrosine +150 mg/l cefotaxime liquid and grown on GD2 50P + 200 µM tyrosine +150 mg/l cefotaxime (solid medium) for about 15-18 days in a dimly lit growth chamber, depending on the observation of cell division under a UV microscope.

The tissues were further cultured on selection media (GD2 50P + 100 µM tyrosine + 150 cefotaxime + 30 µM paramomycin and MS2 5NAA + 75 mg/l Cefotaxime + 45 µM paramomycin. The emerging secondary somatic embryos were harvested and transferred to regeneration medium (MS2 0.5 µM NAA + 45 µM paramomycin). After 21 days development under light at 28^0C in the growth chamber, the emerging embryos (with green cotyledons, swollen hypocotyls and meristematic region) were transferred to elongation germination medium (MS2 2 µM BAP). Plantlets regeneration were induced by transferring the putative embryogenic tissues to hormone-free medium (MS2 agar), supplemented with 20 g/l sucrose. The regenerated putative transgenic plantlets were screened for GFP expression under a UV microscope.

Results and Discussion
Induction of somatic embryos and subsequent regeneration of plants represents one of the most exciting models for studies of plant morphogenesis (May and Trigiano, 1991; Samaj *et al*., 1999). The present study presents interesting aspects of callus initiation and somatic embryo induction from young leaf lobes of the cassava cultivars. Young leaf lobes from *in vitro* mother plants of the cassava cultivars sandpaper and TMS 60444 were used to induce organized embryogenic structures in the induction medium (DKW2 50P). The two cultivars produced OES at varying frequencies, although some of the explants did not respond to the treatment. A colour change of the lobes from greenish to pale yellow within ten days of incubation in the induction medium was observed. Organized embryogenic structures were observed between 14 and 21 days of incubation. Table 1 presents the developmental trend of OES in the induction medium containing increasing concentrations of picloram. The explants formed OES regardless of the concentration of picloram (Table 1) in the medium, although induction was promoted by continuous incubation in dark. However, there are reports of some species that forms embryos in light as well as in darkness (Gingas and Lineberger, 1989). Data as shown in Table 1 reveals that OES induction was enhanced by increasing the concentration of picloram in the induction medium for both cultivars. The optimum level of picloram at which maximum stable frequencies (53.1±17.6; 51.5±14.6) of OES induction was obtained was 5 ml/l (Table 1). Percentage decline was observed at a concentration of 7 ml/l picloram which is higher than 5 ml/l. While sandpaper cultivar produced a relatively higher positive response of 53.1±17.6% compared to 51.5±14.6 from TMS 60444 at 5 ml/l, 44.3±15.8 and 40.8±9.2% were produced at 12 ml/l concentration respectively (Table 1). Similar results were obtained by Takahashi *et al*., (1999) and Li *et al*., (1998) on solid medium supplemented with 36 µM 2,4-D, where approximately 50% of the explants were able to form embryos.

Friable embryogenic callus (FEC) (Fig. 1a and b) was successfully generated from both cultivars. They were then maintained and multiplied by serial subculture every 3 weeks on semi-solid Gresshoff and Doy (GD) basal medium supplemented with 20 g/l sucrose, 50 µM picloram and 500 µM tyrosine. Comparatively TMS 60444 produced more

quality proliferating FECs (Fig.1a) at a higher frequency than the Sandpaper cultivar and also responded more to the triggers of transformation by the *Agrobacterium*.

Cocultivation of the FECs with the *Agrobacterium* strain LBA 4404 revealed on observation under a UV microscope, a GFP expression indicating that the FECs were amenable to transformation by the *Agrobacterium* strain. Selection of the putative transgenic FEC lines of TMS 60444 and Sandpaper were more successful with carbenicillin and paramomycin. Out of the 182 TMS 60444 and 143 Sandpaper lines, 68 and 47 putative transgenic embryos emerged into normal cassava plantlets on MS2 agar respectively. Sixty two and thirty eight out of the 68 and 47 TMS 60444 and Sandpaper plantlets respectively were seen to express fluorescence protein under the UV microscope. The successful production of organized embryogenic structures and friable embryogenic callus used for the transformation and the use of acetosyringone for the induction of the *Agrobacterium tumefaciens* during co-cultivation were important for the optimal transformation frequencies.

Agrobacterium-mediated transformation, our method of choice has been credited with the production of simpler integration patterns than direct gene transfer, although both approaches may result in a similar range of integration events. However, the major technical problem of transformation, regardless of the method used, is the low regeneration ability of the tissues. This present study therefore opens up the possibilities of incorporating agronomically desirable traits for improved cassava root quality. Interestingly, the method of cassava transformation reported here is efficient and reproducible and it takes about 365 days to produce a transformed cassava plant.

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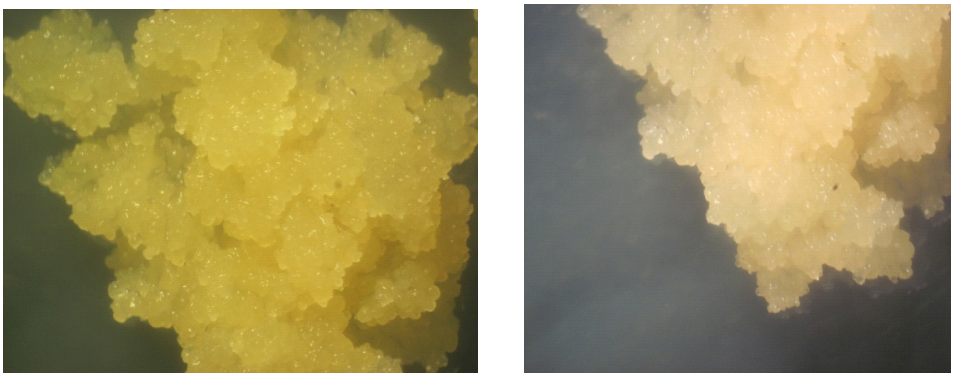
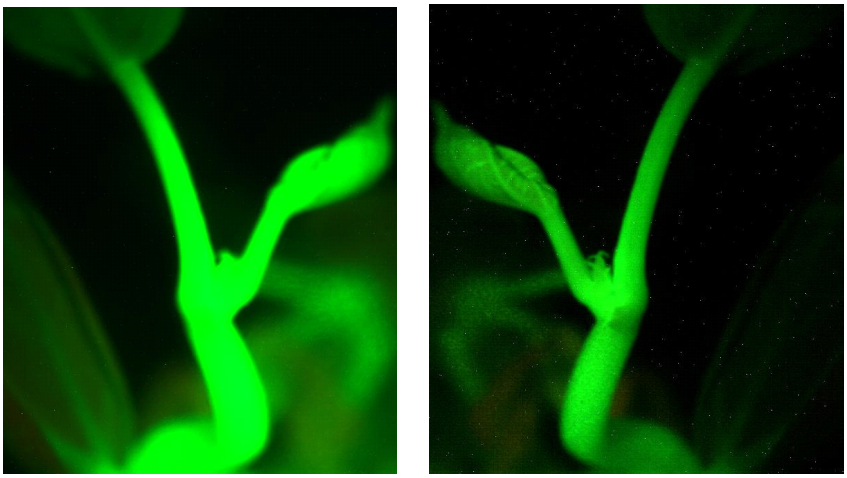
Table 1: Effect of picloram concentration on the induction of OES from in vitro young leaf lobes of TMS 60444 and Sandpaper cassava cultivars.

Picloram (ml/l)	TMS 60444		Sandpaper	
	% positive	% Negative	% Positive	% Negative
1	9.4±2.6	90.6±20.5	12.3±4.2	87.7±6.8
3	34.1±9.4	65.9±13.6	39.2±13.8	60.8±11.4
5	51.5±14.6	48.5±10.5	53.1±17.6	46.9±9.5
7	44.6±12.7	55.4±28.1	46.4±11.2	53.6±14.6
9	41.2±21.4	58.8±13.2	43.5±12.9	56.5±13.5
12	40.8±9.2	59.2±12.9	44.3±15.8	55.7±10.4



a **b**

Figure 1: a = FEC from TMS 60444, b = FEC from sandpaper



a **b**

Figure 2: a = Plantlet from TMS 60444 showing GFP marker fluorescence
b = Plantlet from sandpaper showing GFP marker fluorescence.