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Agrobacterium-mediated Transformation of Two Cassava Cultivars (Nwibibi and TMS 60444)

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Abstract

Cassava is an important crop in the tropics and subtropics for food security and income to some 500 million people. However, the ever-increasing human population especially in the developing countries, the low protein content, vitamins, minerals and various insects and virus diseases has posed a challenge to boost cassava production in limited cultivable land. Biotechnology has been identified as a powerful tool for genetic transformation. *Agrobacterium tumefaciens*, a ubiquitous soil borne pathogen employs a highly evolved and still incompletely understood gene transfer and integration system that appears optimized for efficient nuclear targeting and integration. A bacterial strain LBA 4404 containing a binary vector pB 2300 with the *npt11* gene as selectable marker and a *green fluorescent protein* (*GFP*) as a tracker was used for the experiments. Subsequent selection of transformed tissues with cefotaxime 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 use of *npt11*gene offers new possibilities to engineer transgenic cassava lines with T-DNA insertions and to produce transgenic cassava with improved agronomic traits. The positive response of this local Nigerian farmer preferred cassava cultivar to *Agrobacterium* transformation opens up possibilities of incorporating agronomically desirable traits for improved cassava root quality.

Keywords: Cassava, *Agrobacterium tumefaciens*, Transformation, Antibiotics, Tyrosine.

1. Introduction

Cassava (*Manihot esculenta* Crantz) ranks first amongst the root and tuber crops that are in contention for food security in Nigeria. It is an important food crop for some 500 million people in developing countries [1]. In Africa, the cultivation of cassava increased during the colonial times as a result of its recognition to prevent food shortages. This evolution reflects a series of structural changes in consumption and the use of this commodity that began several years ago. Thus, cassava is one of the most reliable and cheapest sources of food for fighting hunger, food sufficiency and income for rural and low-income farm households. In addition to its role as a local staple, cassava

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alleviates seasonal food shortages and fills food gaps caused by drought or famine [2]. The wide variety of food products that are made from cassava roots and its highly nutritious leaves widely consumed as a regular part of the diet are added reasons why cassava cultivation is expanding [3]. The crop's multiple uses have also facilitated greater consumption in various forms like gari, fufu, lafun etc. Other important uses of cassava include its use for animal feed, starch and glue production, ethanol and dried chips production for export. 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 diseases 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 [4]. Another advantage of cassava over other crops is its ability to be stored in the soil for over 36 months. Hence, cassava cultivation serves as a household food bank that can be drawn upon in times of need.

Cassava's vegetative propagation is a mixed blessing. Though the lignified stem cuttings used for planting can survive in spite of delayed rains while other consequences like build-up of systemic infections, especially of viruses and common bacterial blight in planting materials can cause production losses as high as 60% [5]. Another major constraints against development of cassava industry in Nigeria is low productivity resulting in an average of 12 tons/ha, compared to an average of 40-50 tons/ha in Thailand the world largest exporter of cassava-based products. Biotechnology interventions may offer exciting possibilities to overcome these limitations, not only provides an alternative approach, but also complements the efforts in traditional breeding [6]. Much of the support for plant transformation research as proposed by Birch [7] has been provided because of the expectations that this approach could: generate plants with useful phenotypes unachievable by conventional plant breeding, correct faults in cultivars more efficiently than conventional breeding and allow the commercial value of improved plant lines to be captured by those investing in the research more fully than is possible under intellectual property laws governing conventionally bred plants. Although successful transformation of some of our local farmer-preferred cultivars have been described by several authors through different approaches [8-10] some are until now recalcitrant to transformation techniques. Here a method for regenerating stably transformed Nwibibi cultivar after co-cultivation with *Agrobacterium* is described. Nwibibi cultivar was selected for transformation based on its outstanding high yield and resistance to pests and diseases while TMS 60444 is a model cultivar with good regeneration capacity of embryogenic tissues [11].

2. Materials and Methods

Young leaf lobes of cassava cultivars; TMS 60444 and Nwibibi, from National Root Crops Research Institute (NRCRI), Umudike, Umuahia, Nigeria were used for the induction of organized embryogenic structures (OES) on DKW2 50P for 3 weeks [11]. The medium pH was adjusted to 6.12 before autoclaving at 121^0C for 15 min. Filter sterilized picloram was added to the medium when the medium temperature was 42^0C before dispensing in sterile petri dishes. Ten leaf lobes were aseptically incubated on petri dishes containing 25 ml of solid DKW2 50P [12] 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. Friable embryogenic callus (FEC) was

generated on GD2 50P + 500 µM tyrosine and subcultured three times for callus proliferation for 9 weeks in a closed paper box [13].

2.1 Transformation and selection of transgenic tissues

Bacteria cultures were initiated by plating a frozen glycerol stock of the engineered *Agrobacterium* (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 visual marker) onto agar-solidified LB medium [14] 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 (LB medium + the antibiotics) were grown for 9 h. at room temperature (28^0C, in a shaker at 250 rpm). Iml 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 (YM medium + antibiotics) were further incubated overnight to an OD600 of 0.7 to 1.0 on a shaker at room temperature and at 250 rpm.

Enough FEC (approx. 0.6 ml) to cover the bottom of each well were aseptically transferred from plates to a sterile 12 well plate with sterile forceps. Two (2) ml of GD2 50P + 200µM AS was used to re-suspend the *Agrobacterium* before using to inoculate viable, moist, yellowish tissues of a three-times sub-cultured FEC selected under a stereo-microscope for a period of I hour. After 4 days of further co-cultivation 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) [12], supplemented with 20 g/l sucrose. The regenerated putative transgenic plantlets were screened for GFP expression under a UV microscope.

3. Results and Discussion

Since 1983 when the first transgenic plant was engineered, developments in crop improvement via biotechnology have revolutionized agricultural scene in a number ways that could solve specific problems in agricultural production. *Agrobacterium* is a natural soil borne bacterium that moderates the transfer of DNA and proteins into the nuclei of plant cells, thus providing one of the best-studied examples of response to the essential horizontal DNA transfer. Transformation via FEC production and co-cultivation with *Agrobacterium* is still the most efficient method for cassava transformation. The stages and the time-course adopted for the transformation and regeneration of the cassava plantlets is as summarized below in Figure 1.

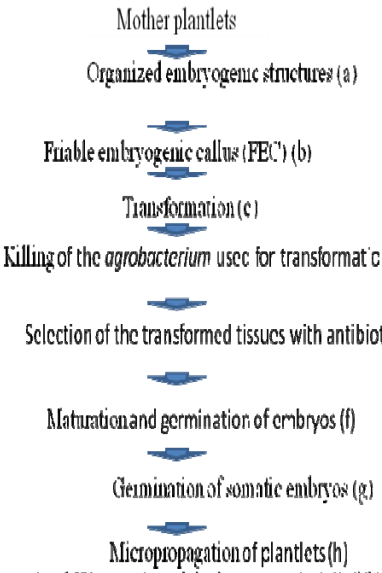

Figure 1: Schematic representation of FEC generation and plantlets regeneration in Nwibibi cassava cultivar.
(a) 3-4 weeks induction of OES on DKW2 50 µM picloram supplemented with 20 g/l sucrose
(b) Induction of FEC on GD2 50P + 500 µM tyrosine supplemented with 20 g/l sucrose (3-4 weeks). (C) Transformation of FEC with *agrobacterium* (1h.). (d) Killing of the *agrobacterium* with GD2 50 µM P + 200 µM tyrosine + 150 mg/l cef (after 15-18 days).
(e) Selection of the transformed tissues with GD2 50 µM P + 100 µM tyrosine + 150 mg/l cef. + 30 µM paro. (after 12 days). (f) Maturation and germination on regeneration medium (MS2 5 µM NAA + 75 mg/l cef. + 45 µM paramomycin (after 21 days).
(g) Germination of the somatic embryos on MS2 2BAP. (after 21 days). (h) Micropropagation of the plant ets on MS2 agar supplemented with 20 g/l sucrose (after 14-21 days).

In this study, young leaf lobes from the two cassava cultivars formed OES (Figure 2A) when cultivated on DKW2-based medium supplemented with auxin (picloram) within 3 weeks. Preliminary results showed that TMS 60444, a model cultivar produced 51.5% OES compared to the farmer-preferred cultivar, Nwibibi that produced 59.2%. Friable embryogenic callus (FEC) (Figure 2B & C) was successfully generated from both cultivars. They were maintained and multiplied by serial subculture every three weeks on semi-solid Gresshoff and Doy (GD) basal medium supplemented with 20 g/l sucrose, 50µM picloram and 500 µM tyrosine. Comparatively TMS 60444 produced more quality proliferating FECs (Figure 2B) than the Nwibibi cultivar (Figure 2C) and also responded more to the triggers of transformation by the *Agrobacterium* (Table 1).

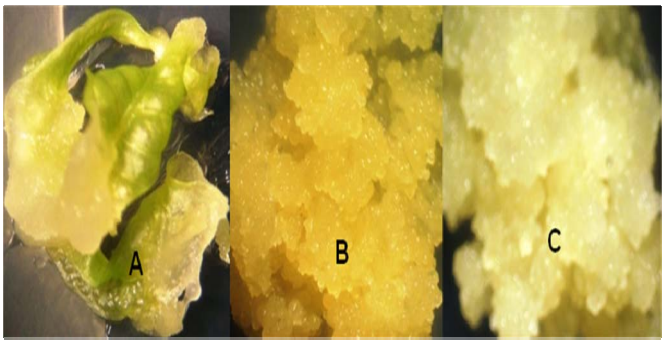

Fig.2: Developing organized embryogenic callus (OES) and friable embryogenic callus.
A, Developing OES
B, FEC produced from TMS 60444
C, FEC produced from Nwibibi cultivar.

Table 1. GFP expression of the cultivars under fluorescent microscope\

Sample	Nwibibi	TMS 60444
1	0	4
2	4	4
3	2	3
4	1	3
5	1	3
6	0	3
7	0	3
8	4	3
9	1	3
10	4	3

Scale: 1=poor, 2=fair, 3= good, 4=very good, 5= excellent

The transformed FEC under a UV microscope revealed a GFP expression (Figure 3) indicating that the FECs were amenable to transformation by the *Agrobacterium* strain. Out of the 169 Nwibibi lines, 50 germinated to produce putative transgenic plantlets (Figure 3B, C & D) suggesting a low recovery rate of the transgenics (Table 2). Efficient selections of the transformed tissues were achieved by increasing the selection pressure in a step wise manner from 150 to 75 mg/l and 30 to 45µM cefotaxime and paramomycin respectively. This stepwise process allows transformed tissues to express effectively the antibiotic-resistance gene and initiate cell division, thus improving regeneration of tissues to produce plants [8].

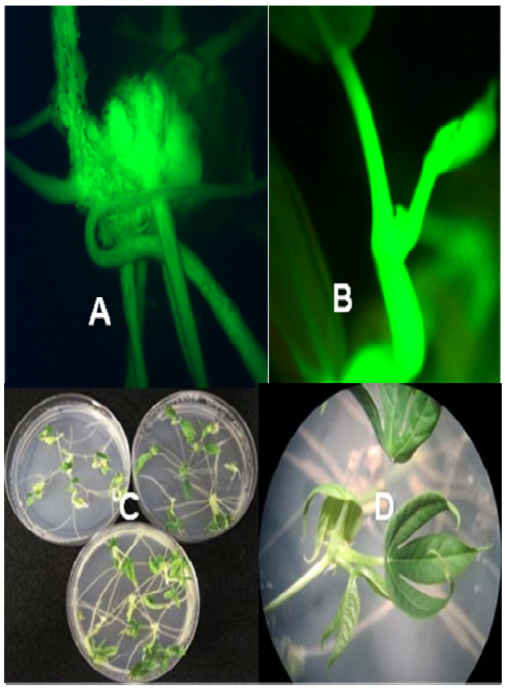

Fig.3: Developing transgenic plantles
A: Transgenic roots showing GFP marker fluorescence
B: Transgenic plantlet showing GFP marker fluorescence
C: Transgenic plantlets on MS2 agar plates
D: Growing plantlet

Table 2. Selection of the putative transgenic FEC lines of Nwibibi cultivar and TMS 60444

FEC	Number of lines generated	Plantlet recovery
TMS 60444 (GFP)	62	12
TMS 60444 (DsRed)	16	7
Nwibibi (GFP)	169	50
Nwibibi (GFP)	84	21

GFP, DsRed, and 631 are *Agrobacterium* gene construct

Thus, the GFP-positive tissues (Figure 3A & B) and the antibiotic–resistant nature of the tissues further confirmed that the transferred genes were active. Green fluorescent protein is a highly versatile reporter gene, because the GFP gene expression can be monitored any time in living cells under a fluorescence microscope in a non-destructive manner [15]. The same tissues may be used for regeneration of stable transformants which is not possible with other marker genes requiring destructive or toxic enzyme assays [16]. It has been extensively used in the transformation of many plant species such as *alfaafa* [17], *Petunia hybrida* [18] and pepper [19].

Agrobacterium-mediated transformation, our method of choice has been credited with the production of simpler integration patterns than direct gene transfer (gene gun), although both approaches may result in a similar range of integration events, including truncations, rearrangements, various copy numbers and insertion sites [7], though the frequency distributions of copy number and rearrangements vary with transformation parameters for both gene transfer

methods [20-22]. However, the major technical problem of transformation, regardless of the method used, is the low regeneration ability of the tissues but a drawback with *Agrobacterium* transformation is that the selection system based on antibiotics allows regeneration of escapes and that the antibiotics may also be deleterious to the regeneration process. Interestingly, the method of cassava transformation reported here is efficient and reproducible and it takes about 365 days to obtain a transformed cassava plant.

4. Conclusions

A simplified and efficient protocol for *Agrobacterium*-based transformation of two cassava cultivars, TMS 60444 and Nwibibi using young leaf lobes is established. Stable transgenic plantlets which showed GFP expression were successfully regenerated within 4-6 months. The GFP reporter gene was more strongly expressed in TMS 60444 compared to Nwibibi cultivar hence the transformation efficiency is sufficient for transgene expression studies and opens up possibilities of incorporating agronomically desirable traits for improved cassava root quality.

5. Acknowledgments

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