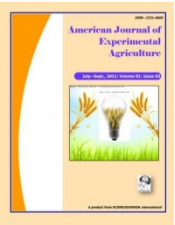

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Reproducible *Agrobacterium*-mediated Transformation of Nigerian Cultivars of Tomato (*Solanum lycopersicum* L.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOAAS designed the study, wrote the protocol, managed the literature searches, wrote the first draft of the manuscript and interpreted the statistical analysis with author OO. Author II gave technical guidance and support in the research and input on the manuscript. Author NRI and OO supervised the overall research and gave technical guidance and input on the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

This study was carried out to develop transformation protocol for the possible improvement of local cultivars of tomatoes in Nigeria using complete randomized design (CRD). The research was conducted at the Plant Biotechnology Centre, International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria between May 2009 and December 2009. Seeds of three promising farmer preferred varieties of cultivars of tomatoes namely Ibadan local, Ife and JM94/46 were selected and cultivated *in-vitro*. Sterile cotyledon and leaf explants were transformed using *Agrobacterium tumefaciens* strain LBA4404 with plasmid (pOYE153). Transformed plants were analyzed using *GUS* assay and PCR methods. Results showed that leaf explants had higher transformation efficiency than cotyledon explants in the three cultivars. Ife cultivar had the best transformation efficiency in both explant types - leaf 42.5% and cotyledon 8.89%. Histochemical *GUS* assay of transgenic plants showed blue coloration in leaves, stems and roots. PCR analysis showed amplification of 600 bp fragments of *GUS* and *nptII*

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genes in the transgenic plants on 1.0% agarose gel. The *GUS* and *nptII* genes were successfully integrated into the three cultivars of tomatoes thereby providing a reliable transformation protocol for the genetic improvement of local cultivars of tomatoes for desirable traits such as longer shelf-life, pest and disease resistance, enhanced nutrients, higher soluble solids, etc.

Keywords: Nigeria; tomatoes; Agrobacterium tumefaciens; genetic transformation; GUS gene.

1. INTRODUCTION

Nigeria is the largest producer of tomatoes in tropical Africa, with an annual production of 1,504,670 tons out of the estimated annual production of 16.55 million tons in Africa [1]. A total area of one million hectares is reportedly used for tomato cultivation in Nigeria [2,3]. The use of tomato is about 18 percent of the average daily consumption of vegetables in Nigeria [4] and is the most popular vegetable crop in Nigeria dominating the largest area under production among vegetable crops [5].

A substantial volume of the tomatoes in Nigeria are usually transported over long distances from the Northern part of the country to other parts and from the hinter lands to towns and cities. In Nigeria, as most other developing countries, efficient storage, packaging, transport and handling techniques are practically non-existent for perishable crops [6] resulting in considerable loss of produce. Also cultivated tomatoes suffer from a myriad of problems ranging from diseases caused by bacteria, fungi, viruses and nematodes to post harvest losses due to biochemical processes. Therefore improvements such as longer shelf-life, resistance to biotic and abiotic stresses, nutrient enhancement, higher soluble solids, etc are desirable in the local cultivars of tomato. However, the introduction of genes that confer these qualities to commercial cultivars by conventional breeding techniques often encounters serious difficulties due to high incompatibility barriers to hybridization [7]. To overcome these problems certain more recent approaches of gene manipulation might be required.

The cultivated tomato (*Solanum lycopersicum*) has been a subject of research because of the commercial value of the crop and its potential of amenability to further improvement through genetic engineering strategy [8]. Advances in agricultural biotechnology have provided the opportunity to expand the genetic resources available for tomato improvement. The goals of tomato genetic engineering have been to protect the tomato crop from environmental and biological assaults and to improve the quality of tomato fruit in order to deliver greater value in processed tomato products or more healthful and attractive fresh fruit.

There is however paucity of documented work on the genetic improvement of Nigerian cultivars of tomato; such work that would provide the background work for the application of genetic engineering in solving these problems. This study gives the report of the genetic transformation of the leaf and cotyledon explants from three Nigerian cultivars with *Agrobacterium* strain LBA4404 (pOYE513) containing *uidA* (GUS) and *nptII* genes using established in-vitro regeneration protocols for the three Nigerian cultivars of tomatoes [9,10]. This protocol can provide insight for the production of improved and more stress tolerant local cultivars and therefore reduce the importation and introduction of new varieties that

would have to be adapted to the local environment. There is therefore an urgent need to domesticate these technologies for the improvement of Nigerian indigenous cultivars of tomatoes that are already adapted to the local environment.

The choice of cultivars is based on agronomic studies carried out at the National Institute for Horticultural Research and Training (NIHORT). Ibadan local and Ife cultivars are farmer preferred varieties in the south-western part of Nigeria which are reported to be resistant to certain diseases and relatively high yielding [11,12]. ICS-Nigeria [13] also reported Ife cultivar to be high yielding with fruits and is a determinate bushy plant; and that other local cultivars are fairly resistant to virus, have round and irregularly shaped fruits but are soft and prone to cracking.

2. MATERIALS AND METHODS

2.1 *In-vitro* Cultivation of Tomato Seedling

Seeds of three promising farmer preferred varieties of cultivars of tomatoes namely Ibadan local, Ife and JM94/46 were obtained from the National Institute for Horticultural Research and Training (NIHORT), Ibadan, Nigeria. The seeds were sterilized in 3.5% (v/v) sodium hypochlorite (NaOCl) with a drop of Tween-20 for 20 min and were rinsed with sterile distilled water three times. Seeds were germinated on MS medium [14] with 30 gL^{-1} sucrose, 8 gL^{-1} agar gel at pH 5.8. Germination medium, 50-100 ml was dispensed into the culture jars and was autoclaved at 120ºC at 15psi for 15 min and then allowed to cool and solidify. Each culture jar containing the germination medium was inoculated with ten sterilized seeds and were placed in the dark at 25±2ºC for 3-5 d to germinate and then transferred to culture conditions of 16 h photoperiod with light intensity of 1500 lux for 10-13 d at the same temperature to produce the tomato seedlings.

2.2 Preparation of *Agrobacterium tumefaciens* Culture

Agrobacterium tumefaciens strain LBA4404 containing plasmid (pOYE153) consisting of kanamycin resistant gene (*nptII* -neomycin phosphotransferase II) as a selectable marker being controlled by the 35S Cauliflower mosaic virus (CaMV) promoter and *β-glucuronidase* (*GUS*) gene (*UidA*) as the reporter gene being controlled by the Cassava vein mosaic virus (CsVMV) promoter, with the *nopaline synthase (nos)* gene at the 3' terminator end (IITA, Ibadan) was used for the transformation of the three local tomato cultivars. *Agrobacterium tumefaciens* [LBA4404 (pOYE153)] (unpublished) culture was initiated from pure glycerol cultures of the *Agrobacterium* stored at -80ºC and grown in 5ml cultures in Luria broth (LB) medium with no selection for two (2) nights in 250 ml Erlenmeyer flasks at 29ºC, shaking on an orbit shaker at 250 rpm [15]. Single colonies from the 48 h cultures of *Agrobacterium tumefaciens* strain LBA4404 (pOYE153) were streaked on solid yeast extract broth (YEB) medium containing selective concentrations of rifampicin (100 mg/L) and kanamycin (100 mg/L) and grown for 24 h at 28ºC in petri dishes. Colonies from the plate were scraped and re-suspended in 1 ml of sterile inoculation medium (IM) containing MS medium with 30 g L^{-1} sucrose, 100 µM acetosyringone (AS) and 10 µM 2-mercaptoethanol. The optical density at 600 nm (OD_{600}) of the bacterial suspension was determined by spectrophotometry (DU 530 Beckman Coulter). The bacterial suspension had an OD_{600} = 3.8 and 1 ml was diluted with 3.8 ml of IM to obtain a concentration of OD_{600} = 1. This suspension was used for explants inoculation [16]. For genetic transformation experiments, the plasmid pOYE153 was utilized (unpublished).

2.3 *Agrobacterium*-mediated Transformation

Cotyledon and leaf pieces were excised from 10-13 d *in-vitro* seedling under aseptic conditions and the ends of each was cut off, sectioned into two halves at the mid-vein region and cut into 5x5 mm^2 pieces explants to allow it adsorb the bacterial suspension. The explants were inoculated with *Agrobacterium tumefaciens* strain LBA4404 (pOYE153) by dipping into the bacterial suspension and continuously shaken on shaker (Labnet-Orbit LS) for 45 min. The bacterial suspension was pipetted out and the explants were blotted dry on a sterilized paper towel. The explants were placed with the abaxial surface of the leaf in contact with the co-cultivation medium in petri-plates. The co-cultivation medium (CCM) contained MS salts, MS vitamins, MS iron, 30 g L^{-1} sucrose, 8 g L^{-1} agar gel, 1.5 mg L^{-1} zeatin and 100 µM acetosyringone, at pH 5.8. The plates were wrapped with aluminum foil and incubated for 3–4 d at 25±2ºC. After 4 days of co-cultivation, *GUS* assay was carried out on the explants to determine transient expression of the GUS gene.

2.4 Regeneration and Selection of Transformed Explants

After 3-4 d on the co-cultivation medium, the explants were washed in IM without acetosyringone and mercaptoethanol and then subcultured in callus induction medium (CIM) containing MS salts, MS vitamins, MS iron, 1.5 mg L^{-1} zeatin, 100 mg L^{-1} kanamycin, and 150 mg L^{-1} timetin (GlaxoSmithKline, Uxbridge, UK) at 25±2^{o}C for 16 h photoperiod with light intensity of 1500 lux in the growth room [17]. Explants commenced callus development at the edges about 4 weeks after *Agrobacterium* inoculation. The explants were subcultured onto fresh CIM every two weeks. After about 8 wks in culture, well-developed calli with mass of shoot buds were transferred to callus shooting medium (CSM) containing MS salts, MS vitamins, MS iron, 30 g L^{-1} sucrose, 8 g L^{-1} agar gel, 1.0 mg L^{-1} zeatin, 100 mg L^{-1} kanamycin, and 150 mg L^{-1} timetin at pH 5.8. Elongated multiple shoots were excised from the calli and transferred to non-selective rooting medium containing MS salts, MS vitamins, iron, 15 g L^{-1} sucrose, 8 g L^{-1} agar gel and 0.1 mg L^{-1} NAA [18] at pH 5.8. After 10 –14 d, rooted shoots were selected as putative transgenic plants and were transferred to peet pellet and vermiculite medium in humidity chamber for 2 weeks before planting in top soil, also under humidity chamber and gradually acclimatized.

2.5 *GUS* Histochemical Assay

Histochemical assay to determine *GUS* activity was carried out at the callus, shooting and rooting stages to determine the expression of the *GUS* gene in the plants. Histochemical staining to detect *β-glucuronidase* (*GUS*) gene expression in the explants was carried according to [19] using 0.33 g ferricyanide and 0.04 g ferrocyanide mixed in 10 ml sterile distilled water and the addition of 40 µl of triton in a solution. In separate vacuum tubes, 1.39 g NaH_2PO_4 and 2.42 g of Na_2HPO_4 respectively were dissolved in sterile distilled water to prepare 50 ml stock solutions each. 10 ml *GUS* assay buffer was prepared consisting of 5 ml sodium phosphate solution (2 ml of NaH_2PO_4 solution and 3 ml of Na_2HPO_4 solution) and 5 ml of ferrous solution. To carry out the assay, 0.0125 X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) was dissolved in 100 µl of Dimethyl sulfoxide (DMSO – used under fume hood) and added to 2.5 ml of *GUS* assay buffer solution.

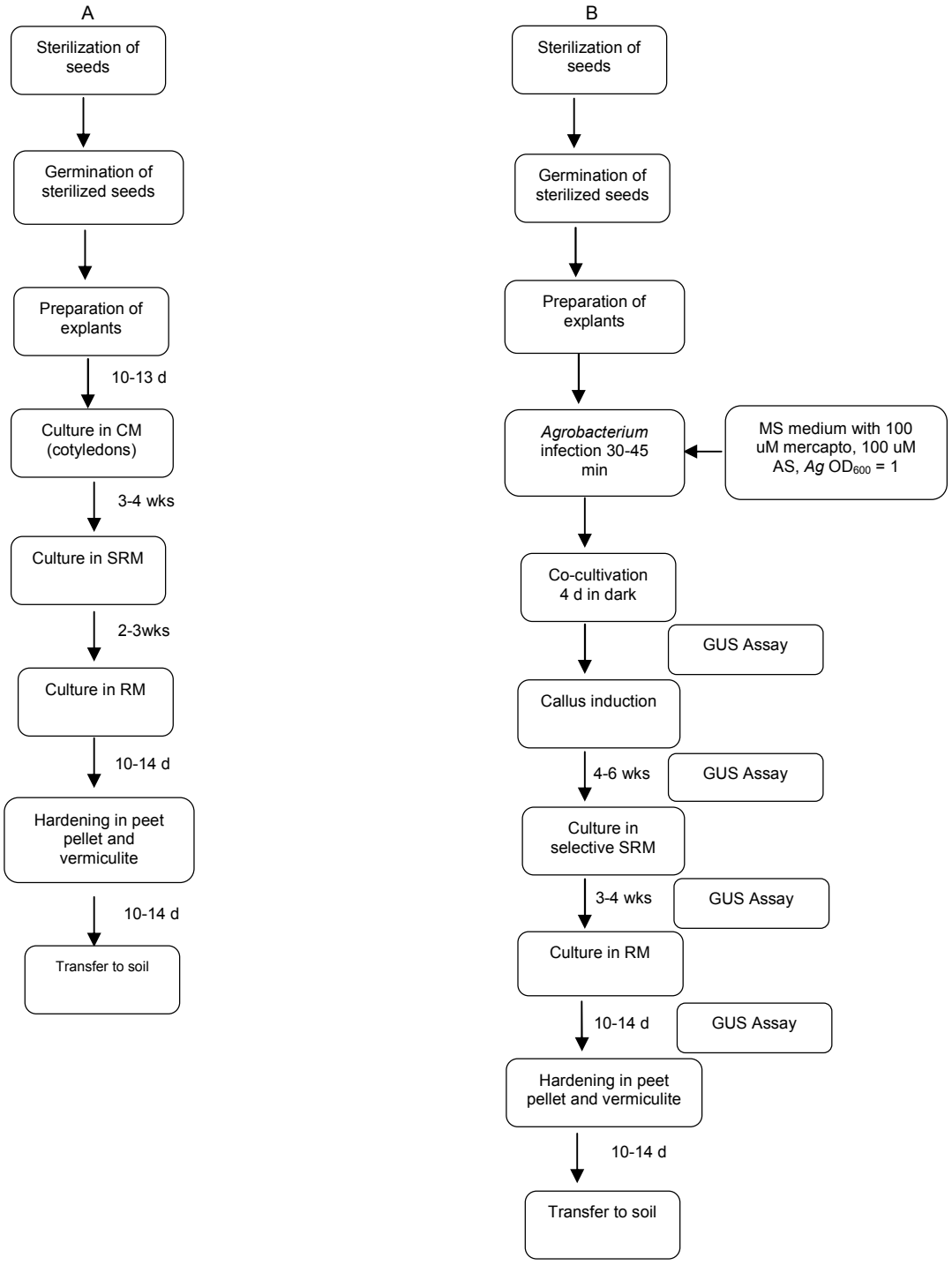

Fig. 1. (A) Schematic representation of tomato regeneration (B) Schematic representation of *Agrobacterium*-mediated transformation of Nigerian cultivars of tomato

Placing the explants in eppendorf tubes, the *GUS* assay solution was added and placed in the oven at 37ºC for 1-2 h. The solution was pipetted out and 70% ethanol was added to the explants and incubated in the ethanol for 4 d. The ethanol was replaced every day. The explants were observed under the microscope (Zeiss model Stemi 2000-C) for *GUS* activity. *GUS* assay was carried out on explants after co-cultivation with *Agrobacterium* for transient expression and on calli, shoots and roots of regenerated plants for putative expression.

2.6 PCR Analysis of Genomic DNA of Transgenic Plants

DNA was extracted from callus and different tomato tissues using established procedures [20]. Forward and reverse primers OLIV9 (5'-GGTGATCGGACGCGTCG-3') and OLIV13 (5'-CCGCTTCGCGTCGGCATC-3') and ARAJI1k (5'-ATGACTGGGCACAACAGACAATCGG-3') and ARAJI2k (5'-CGGGTAGCCAACGCTATGTCCTGATA-3') were used for the amplification of *UidA* and *nptII* genes respectively in PCR reactions. PCR was carried out using Peltier thermal cycler-PTC200. According to a modified procedure of [21], 10 µl of PCR mixes contained 1.0µl 10X reaction buffer (100 mM Tris pH 9, 15 mM $MgCl_2$, 500 M KCl and 0.1% Gelatin), 0.8 µl dNTPs 200 mM, 1 µl of each forward and reverse primers 5 µM *I* µl primer, 1µl of 20 ng/µl genomic DNA and 0.4 U/ ul red *Taq* polymerase (Sigma) and remaining water. Only one DNA sample and both forward and reverse primer were added to any single reaction. The thermocycling programme used for *UidA* was: one cycle (initial denaturing) at 96ºC for 3 min; 30 cycles at 95ºC for 1 min (denaturing); 60ºC for 1 min (annealing); 72ºC for 2 min and one cycle (final extension) at 72ºC for 5 min, kept at 4ºC. The *nptII* gene amplification program consists of 1 cycle at 94ºC for 4 minutes (initial denaturing), then 35 cycles at 94ºC for 30 seconds (denaturing), 60ºC for 30 seconds (annealing), then 72ºC for 30 seconds (extension) and kept at 4ºC. Bands were resolved on 1% agarose gel at 100 V for 3 h and the size of the band was determined by comparison with λPst ladder (Bioline) loaded on adjacent gel tracks.

2.7 Agarose Gel Electrophoresis of DNA of Transgenic Plants

Using modified procedures of [22], 1.0% agarose gel was prepared by weighing 1.5 g of agarose powder and melting in 150 ml 1% TBE buffer (10x -10.8 g Tris-base; 5.5 g boric acid; 20 mM EDTA in 1 L) in the micro-wave (100ºC) until completely dissolved. The gel was allowed to cool slightly to about 40ºC by continuous stirring on the magnetic stirrer (Thermolyne Cimarec 2) and then poured into the gel tank to set with the combs fixed. Then, 2 µl of gel loading dye (bromophenol blue) was added to 3 µl of PCR product and spun down in the centrifuge to mix thoroughly. The amplified DNA samples of the transgenic plants were loaded on the gel; with *λPst* (Bioline) loaded on adjacent gel tracks to determine the size of the bands by comparison and allowed to run for 2-3 hr at 100 volts (Voltmeter EC 105). The gels were dipped into ethidium bromide solution (10 mg/ml) for 1 min; de-stained for 5-10 min in tap water and then visualized at 302 nm on UV transilluminator and photographed with UVP bioimaging system (GDS-800).

2.8 Statistical Analysis

This study on the establishment of transformation protocol for three cultivars of Nigerian tomatoes was carried out using complete randomized design (CRD) via an intermediate callus phase from leaf explants. The average of pooled data from triplicate experiments was used to obtain the transformation frequency and Chi square test was used to determine the dependence of the transformation frequencies on the explant type (age of explant).

3. RESULTS AND DISCUSSION

3.1 *Agrobacterium*-mediated Transformation of Nigerian Tomato Cultivars

Results of the transformation experiment using cotyledon explants showed that JM had the highest number of explants forming calli (29); followed by Ife (20) and IbL (19). Ife however had the highest shooting frequency of 40.0%; IbL 15.79% and JM 8.16%. The highest value of 2.33 for average number of plants per callus was recorded for IbL; and 0.43 and 0.25 for Ife and JM respectively. Ife had the highest transformation frequency at 8.99% (Table 1). Using the true leaves, IbL had the highest number of explants forming calli, followed by Ife and JM with values of 59, 55 and 37 respectively. The value of 2.41 was recorded as the highest for average number of shoots per callus in Ife; IbL 1.52 and JM 1.10. Ife cultivar had the highest shooting frequency with the true leaves at 69.01%, with JM and Ife having 56.76% and 47.46% respectively (Table 2). Ife cultivar had the highest shooting frequency in both true leaves (69.01%) and in the cotyledon explants (8.99%). Highest transformation frequency was also recorded with Ife at 42.5%. Transformation frequencies of 3.33-8.89% were recorded from cotyledon explants while frequencies of 23.8-42.5 was recorded from secondary or leaf explants.

Table 1. Transformation frequency of tomato cultivars with **GUS* gene using cotyledon explants based on pooled data of three experiments

Cultivar	No. of explants	No. of explants forming calli	No. of shooting calli (rooted plants)	shooting frequency (%)	Av. plants/callus	transformation frequency (%)
IbL	90	19	3(7)	15.79	2.33	3.33
Ife	90	20	8(3)	40.00	0.43	8.89
JM	90	29	4(1)	8.16	0.25	4.44

Table 2. Transformation frequency of tomato cultivars with **GUS* gene in true leaves or secondary leaves explants based on pooled data of three experiments

Cultivar	No. of explants	No. of explants forming calli	No. of shooting calli (rooted plants)	shooting frequency (%)	Av. plants/callus	transformation frequency (%)
IbL	90	59	28(43)	47.46	1.52	31.3
Ife	90	55	38(92)	69.01	2.41	42.5
JM	90	37	21(23)	56.76	1.10	23.8

**GUS, β-glucuronidase gene*

Higher transformation efficiencies were recorded in true leaves than in cotyledon explants in these experiments, which agrees with the report of [23] that leaf explants showed higher organogenesis capacity (> 90%) than cotyledon explants. Higher transformation rates with tomato cotyledons than leaf was however reported by [15,24]. Rooted plants appeared morphologically true to type of tomato plants in the screen house and flowered

3.2 Regeneration of Transformed Plants

The responses recorded with the various cultivars and the two explant types (cotyledon and leaves) used in the experiments indicate that plant regeneration is often genotype

dependent. Other factors such as explants type, plant growth hormones and other environmental factors also play a critical role. This is the first report of the regeneration of transformed plants from these local cultivars and it would pave way for further research on these and other local tomato cultivars that requires improvement. Well developed calli were observed after about 6-8 wks on selective callus culture and Ife cultivar gave the highest shooting frequency in the two explants types used. Whole transformed plants were regenerated from the calli in about 4-5 months. [15] had opined that if there are no visible calluses or green bumps during the first three weeks of incubation, it was not worth continuing the experiment. In our experiments, visible callus growth was observed after about four to five weeks of incubation. Higher regeneration rate was obtained on the selective shoot regeneration medium containing 1 mg L-1 zeatin. The effectiveness of zeatin in the regeneration of kanamycin-resistant shoots was also reported by [25]. Although hypocotyls can be used for transformation, they are not as efficient in generating transgenic shoots and the shoots take longer to develop [15]. A regeneration frequency of 37-38% was reported [26] from six independent transformation experiments.

3.3 *GUS* Histochemical Assay

Both cotyledon and leaf explants were susceptible to transformation by *A. tumefaciens* strain LBA4404 (pOYE), however higher numbers of *GUS* positive explants were recorded from leaf explants than cotyledons. Blue coloration was observed in all tissues of assayed regenerated transformed plants i.e. leaves, stems and roots; which was absent in the control. This indicates that the transgene was constitutively expressed in all parts of the transformed plants.

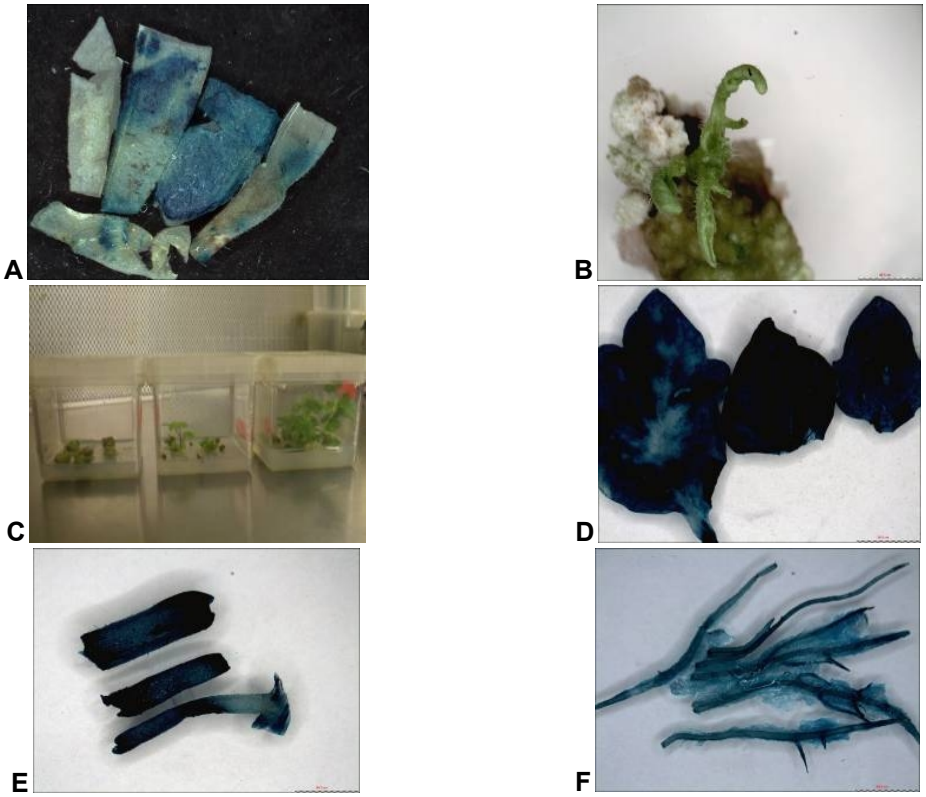

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Plate A. +ve *GUS* assay in leaf explant after 4 d co-cultivation
Plate B. Shoots from transgenic callus (3 wks in XSRM4) – lfe
Plate C. Seedlings from transgenic plants (6 wks in XSRM4)
Plate D. *GUS* expression in leaves of independent transgenic plants (jbf)
Plate E. *GUS* expression in stems - lbL
Plate F. *GUS* expression in roots – lfe
Plate G. Flowering transgenic tomato plant
Key: b- Ibadan local; j– JM/94/46; f-ife

3.4 PCR Analysis of Transgenic Plants

The presence of transgenes in the transformed plants was further confirmed by the amplification of genomic DNA from thirteen transformed plants; originating from the calli of seven independent transformation events, using specific *GUS* and *nptII* primers. The agarose gel electrophoresis of the amplified DNA products is shown in Plate 1 and Plate 2. For expression of the amplified *GUS* gene (Plate 1), distinct bands of about 600 bp fragments are seen in all thirteen transformants (lanes 1 – 13) and the positive control on lane 15. No band was seen in the negative control on lane 14 in Plate 1. A 600 bp fragment of the amplified *nptII* gene was also present in all the transformed plants (Plate 2). The band was however absent in the positive control despite several PCR runs, probably due to low GUS gene expression or silencing. The two genes were expressed about the same loci and their amplification and resolution on gel is an indication of the presence of the genes in the transgenic plants and is. These results are a further confirmation of the constitutive expression observed in the histochemical *GUS* assay that showed blue coloration in the leaves, stems and leaves of the transformed plants.

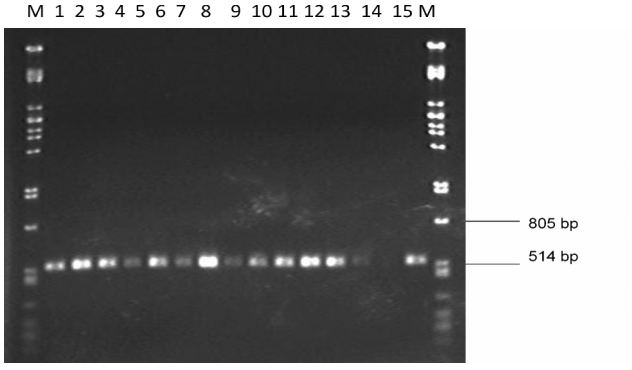

Plate 1. Amplified *GUS* gene in transformed plants
Lane M-Pst I Lambda marker; Lane 1-13-DNA of transgenic plants; Lane 14-ve control; Lane 15+ve control

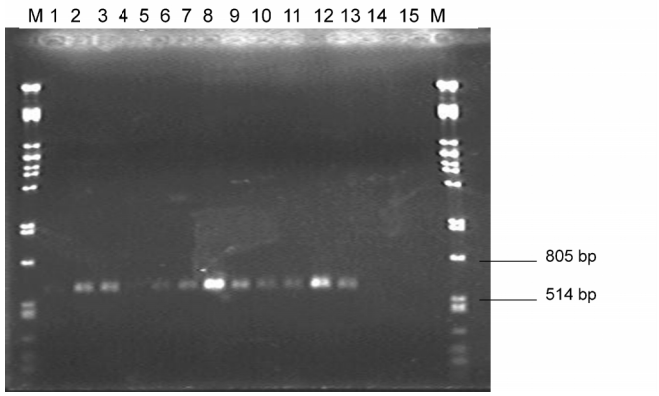

Plate 2. Amplified nptII gene in transformed plants
Lane M-Pst I Lambda marker; Lane 1-13-DNA of transgenic plants; Lane 14-ve control; Lane 15+ve control

4. CONCLUSION

The *GUS* and *nptII* genes were successfully integrated into the three cultivars of tomatoes thereby providing a reliable transformation protocol for the genetic improvement of local cultivars of tomatoes for desirable traits such as longer shelf-life, pest and disease resistance, enhanced nutrients, higher soluble solids, etc.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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