

Reproducible *Agrobacterium*-mediated transformation of Nigerian cultivars of tomato (*Solanum lycopersicum* L.)

Abstract

Transformation of the leaf and cotyledon explants from three local cultivars of Nigerian tomatoes – Ibadan Local (IbL), Ife and JM94/46 (JM) was carried out with *Agrobacterium* strain LBA4404 (pOYE513) containing *GUS* and *nptII* genes. Co-cultivation medium (CCM): MS + 30 g L⁻¹ sucrose + 8 g L⁻¹ agar gel + 1.5 mg L⁻¹ zeatin + 100 µM acetosyringone and callus induction medium (XCM): MS + 30 g L⁻¹ sucrose + 8 g L⁻¹ agar gel + 1.5 mg L⁻¹ zeatin + 100 mg L⁻¹ kanamycin + 150 mg L⁻¹ timentin revealed that leaf explants had higher transformation efficiency than cotyledon explants in the three cultivars. Ife cultivar had the best regeneration efficiency in both explant types - leaf 42.5 % and cotyledon 8.89 %. Histochemical GUS assay of transgenic plants showed blue colouration in leaves, stems and roots. PCR analysis of transgenic plants with *GUS* and *nptII* primers showed amplification at 514-805 bp sizes in 1.0 % agarose gel.

Key words: genetic transformation, GUS gene, Nigeria, tomatoes

INTRODUCTION

Nigeria is the largest producer of tomatoes in tropical Africa, with an annual production of 1,504,670 tons (FAOSTAT, 2011) out of the estimated annual production of 16.55 million tons in Africa (FAOSTAT, 2011). A total area of one million hectares is reportedly used for tomato cultivation in Nigeria (Anonymous, 1989; Bodunde, Erinle, Eruotor, & Amans, 1993). The use of tomato is about 18 percent of the average daily consumption of vegetables in Nigeria (Olayide, Olatunbosun, Idusogie, & Abiagom, 1972); and is the most popular vegetable crop in Nigeria dominating the largest area under production among vegetable crops (Ramalan, 1994).

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 (Babalola, Makinde, Omonona, & Oyekanmi, 2010) 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 (Kaul, 1991). 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 (Evans, 1989). 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 *GUS* and *nptII* genes using established *in-vitro* regeneration protocols for the three Nigerian cultivars of tomatoes (Ajenifujah-Solebo *et al.*, 2012; Ajenifujah-Solebo *et al.*, 2013). 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 (Badra *et al.*, 1984; Anno-Nyako and Ladunni, 1984). ICS-Nigeria (Anon., 2000) 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.

MATERIALS AND METHODS

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 (Moorashige and Skoog, 1962) 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 120 °C at 15psi for 15 min and then allowed to cool and solidify. Each culture jar containing the germination medium was inoculated with ten sterilised seeds and were placed in the dark at 25±2 °C 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.

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)] (Oyelakin *et al.*, manuscript in preparation) culture was initiated from pure glycerol cultures of the *Agrobacterium* stored at -80 °C and grown in 5ml cultures in Luria broth (LB) medium with no selection for two (2) nights in 250 ml Erlenmeyer flasks at 29 °C, shaking on an orbit shaker at 250 rpm (McCormick *et al.*, 1991). 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 28 °C in petri dishes. Colonies from the plate were scraped and resuspended in 1 ml of sterile inoculation medium (IM) containing MS medium with 30 g L⁻¹ sucrose, 100 μ M acetosyringone (AS), and 10 μ M 2-mercaptoethanol. The optical density at 600 nm (OD₆₀₀) of the bacterial suspension was determined by spectrophotometry (DU 530 Beckman Coulter). The bacterial suspension had an OD₆₀₀ = 3.8 and 1 ml was diluted with 3.8 ml of IM to obtain a concentration of OD₆₀₀ = 1. This suspension was used for explants inoculation (Soma and Sikdar, 1999). For genetic transformation experiments, the plasmid pOYE153 was utilized (Oyelakin *et al.*, manuscript in preparation).

***Agrobacterium*-mediated transformation**

Cotyledon explants were excised from 10-13 d *in-vitro* seedling under aseptic conditions and the ends of each cotyledon was cut off, sectioned into two halves at the mid-vein region and cut into 5x5 mm² pieces 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 μ M acetosyringone, at pH 5.8. The plates were wrapped with aluminum foil and incubated for 3–4 d at 25 $\pm$ 2 °C. After 4 days of co-cultivation, GUS assay was carried out on the explants to determine transient expression of the GUS gene.

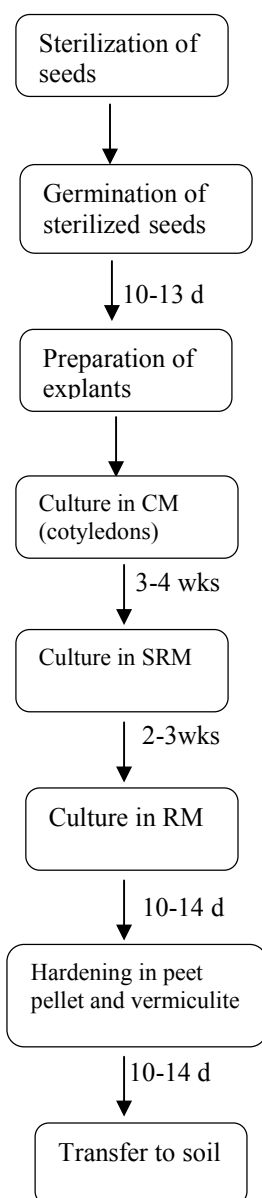
Regeneration and selection of transformed explants

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 °C for 16 h photoperiod with light intensity of 1500 lux in the growth room (Sun *et al.*, 2006). 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, 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 (Davis *et al.*, 1994) at pH 5.8. After 10 –14 d, rooted shoots were selected as putative transgenic plants and were transferred to peat pellet and vermiculite medium in humidity chamber for 2 weeks before planting in top soil, also under humidity chamber and gradually acclimatized. 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.

GUS histochemical assay

Histochemical staining to detect β -glucuronidase (GUS) gene expression in the explants was carried out at various stages of the experiment according to Jefferson *et al.*, (1987) using 0.33 g ferricyanide and 0.04 g ferrocyanide mixed in 10 ml sterile distilled water and the addition of 40 μ l of triton in a solution. In separate vacuum tubes, 1.39 g NaH₂PO₄ and 2.42 g of Na₂HPO₄ 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 NaH₂PO₄ solution and 3 ml of Na₂HPO₄ 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 μ l of Dimethyl sulfoxide (DMSO – used under fume hood) and added to 2.5 ml of GUS assay buffer solution. Placing the explants in eppendorf tubes, the GUS assay solution was added and placed in the oven at 37 °C for 1-2 h. The solution pipetted out and 70% ethanol was added to the explants and incubated in the ethanol for 4 d. The ethanol was replaced everyday. 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 stable expression.

A



B

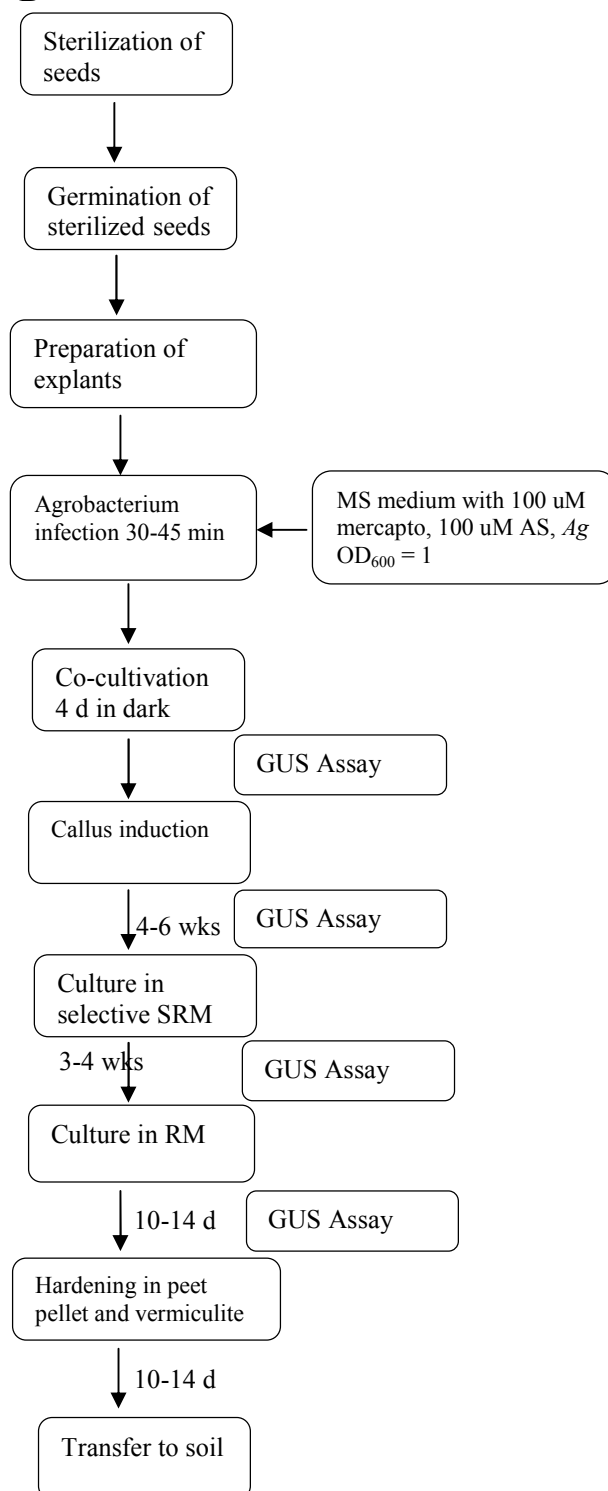


Fig 1: (A) Schematic representation of tomato regeneration (B) Schematic representation of *Agrobacterium*-mediated transformation of Nigerian cultivars of tomato

PCR analysis of genomic DNA of transgenic plants

DNA was extracted from callus and different tomato tissues using established procedures (Dellaporta, 1983). Forward and reverse primers OLIV9 (5'-GGTGATCGGACGCGTCG-3') and OLIV13 (5'-CCGCTTCGCGTCGGCATC); 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 Sambrook *et al.*, (1989), 10 µl of PCR mixes contained 1.0µl 10X reaction buffer (100 mM Tris pH 9, 15 mM MgCl₂, 500 M KCl and 0.1% Gelatin), 0.8 µl dNTPs 200 mM, 1 µl of each forward and reverse primers 5 µM / 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 96°C for 3 min; 30 cycles at 95°C for 1 min (denaturing); 60°C for 1 min (annealing); 72°C for 2 min and one cycle (final extension) at 72°C for 5 min, kept at 4°C. The *nptII* gene amplification program consists of 1 cycle at 94°C for 4 minutes (initial denaturing), then 35 cycles at 94°C for 30 seconds (denaturing), 60°C for 30 seconds (annealing), then 72°C for 30 seconds (extension) and kept at 4°C. 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.

Agarose gel electrophoresis of DNA of transgenic plants

Using modified procedures of Rajput *et al.*, (2006), 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 (100 °C) until completely dissolved. The gel was allowed to cool slightly to about 40 °C 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; destained for 5-10 min in tap water, and then visualized at 302 nm on UV transilluminator and photographed with UVP bioimaging system (GDS-800).

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).

RESULTS AND DISCUSSIONS

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 2). 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 3). 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.

McCormick *et al.* (1991) and Rashid and Bal (2011) reported higher transformation rates with cotyledons than leaf; while in these experiments, higher transformation efficiencies were recorded in true leaves than in cotyledon explants. McCormick *et al.* (1991) also 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, calli were observed after about 6-8 wks on selective callus culture and whole transformed plants were regenerated from the developed calli in about 4-5 months. Majoul *et al.* (2007) reported that leaf explants showed higher organogenesis capacity (> 90%) than cotyledon explants. Higher regeneration rate was obtained on the media containing 1 mg L⁻¹ Zeatin. McCormick (1991) reported that although hypocotyls can be used for transformation, they are not as efficient in generating transgenic shoots and the shoots take longer to develop. Pino *et al.* (2010) reported regeneration frequencies of 37-38% from six independent transformation experiments.

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

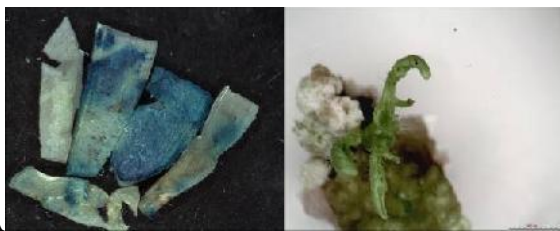


Plate A: +ve Gus assay in leaf explant after 4 d co-cultivation
Plate B: Shoots from transgenic callus (3 wks in XSRM4) – Ife
Plate C: Seedlings from transgenic plants (6 wks in XSRM4)
Plate D: *GUS* expression in independent transgenic plants (jbf)

Plate E: *GUS* expression in stems - IbL

Plate F: *GUS* expression in roots – Ife

Plate G: Flowering transgenic tomato plant

Key: b- Ibadan local; j- JM/94/46; f-ife

PCR analysis of transgenic plants

The presence of the *GUS* and *nptII* genes in the transgenic plants was further confirmed by the amplification of genomic DNA from thirteen transformed plants using *gus* and *nptII* primers. Plate 1 and Plate 2 shows the agarose gel electrophoresis of *GUS* and *nptII* genes respectively in amplified DNA product of 13 transformed leaves (lanes 1-13) that originated from seven independent calli with a negative (lane 14) and positive control (lane 15). Distinct bands of between 514 - 805 bp 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. The amplification of the *nptII* gene as shown in Plate 2 is also between 514-805 bp, although a little higher up than the *GUS* gene. There was however no amplification of the *nptII* positive control; but the transformed plants were amplified with the *nptII* primers. The amplification of the genes using PCR and their resolution on gel is an indication of the presence of the genes in the transgenic leaves.

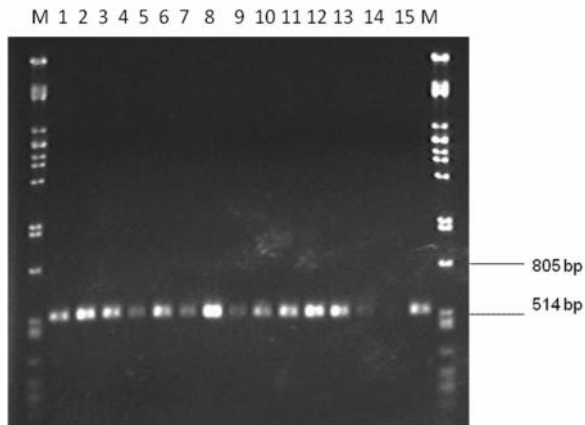


Plate 1: Amplified *GUS* gene in transformed plants

Lane M-*Pst* I Lambda marker; Lane 1-13-DNA of transgenic plants; Lane 14-ve control; Lane 15+ve control

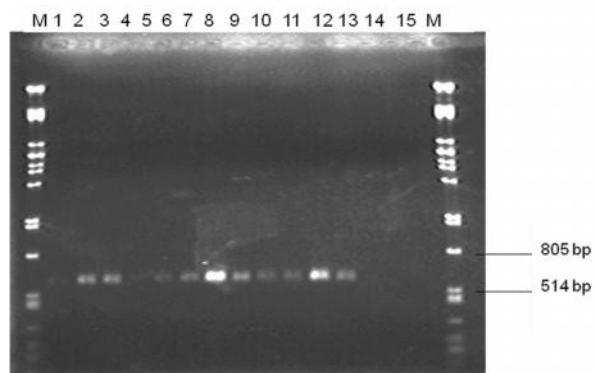


Plate 2: Amplified *nptII* gene in transformed plants

Lane M-*Pst* I Lambda marker; Lane 1-13-DNA of transgenic plants; Lane 14-ve control; Lane 15+ve control

Table 3: Frequency of GUS expression in transgenic tomato cultivars from cotyledon/pry explants

Cultivar	No. of individual plants	+ve	-ve	% +ve
IbL 1^o	15	5	10	33.33
2^o	15	9	6	60.0
Ife 1^o	15	8	7	53.33
2^o	15	9	6	60.0
JM 1^o	15	5	10	33.33
2^o	15	11	4	73.33

Table 4: Summary of frequency of GUS expression in transgenic tomato cotyledon/pry explants

Explant Type	Total No. of individual plants	+ve	-ve	% +ve
1^o	45	18	27	40.0
2^o	45	29	16	64.44

Results of contingency test (X^2) carried out in Table 3 showed that the responses of the various cultivars are dependent on the type of explant (cotyledon or leaf) used in transformation and regeneration. Since the calculated X^2 is larger than the tabulated value, it can be concluded that there are differences in the response of the three cultivars based on the explant type (age of explant).

$$X^2_{0.05 [2]} (\text{tabulated}) = 5.99$$

$$X^2_{0.05 [2]} (\text{calculated}) = 25.31$$

$$X^2_{[2]} (\text{calculated}) > X^2_{[2]} (\text{tabulated})$$

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