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Scrutinising the Most Efficient Explant for *Agrobacterium* Mediated Transformation in Indian Cassava Variety, H226

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Abstract

The most reliable and competent explant for producing transgenic lines were identified in farmer preferred Indian cassava variety, H226, utilizing four different tissue types *viz.,* apical meristem (AM) young cotyledon (YC), compact embryogenic structures (CES) and friable embryogenic callus (FEC) through transformation with *Agrobacterium tumefaciens* strain, AGL0 harbouring the plant transformation vector pOYE153 having *uidA* gene conferring *GUS* activity and *Npt* II marker (AGL0/ pOYE153). The transgenic lines were confirmed for the presence and expression of transferred selectable marker gene *Npt* II as well as visual marker gene *GUS* by molecular analysis (PCR and Nucleic Acid Spot Hybridisation (NASH)) and GUS histochemical assay. This study reported maximum frequency of *GUS* gene expression (64%) in cotyledon explants, producing six putative transgenic lines and it was followed by CES explants, producing two lines in selection medium. Although FEC *GUS* assay proved positive for 43% of the tested samples, only one transgenic line was regenerated and the recovery was quite difficult. In the present study, young cotyledon was identified as the most suitable target tissue for *Agrobacterium* mediated genetic transformation of cassava variety, H226.

Key words: Cassava, *GUS* assay, friable embryogenic callus, *Agrobacterium* mediated transformation

Introduction

Cassava (*Manihot esculenta* Crantz), considered as one of the future crop, is cultivated both under irrigated, as well as rainfed marginal lands in many countries including Africa, Latin America, the Pacific Islands and Asia. There will be an increase in demand for the tuber crops over the other cash crops like wheat and rice, mainly due to their decline in global production in addition to the substantial increase in global population. In India, cassava is cultivated in a total area of 0.23 million ha producing 35.6 t ha^{-1} (FAOSTAT, 2017) especially in southern Kerala, Tamil Nadu and Andhra Pradesh, mainly for domestic and starch production. A major threat for cassava cultivation is disease and pests, of which cassava mosaic disease (CMD) is of high importance and all the cultivars used by farmers are highly susceptible to CMD. Any attempt to introduce resistance in these cultivars requires an efficient genetic transformation protocol that is not yet available for Indian cassava cultivars.

Attempts have been made since many years by different research groups all over the world, for a guaranteed and successful gene transfer system for cassava coupled with proper regeneration of transgenic plant with desirable trait. The major constraints encountered for cassava transformation includes its low transformation efficiency when compared to other crops, difficulty in analysis of transformants through southern blot because of larger cassava genome, difficulty in identifying independently transformed lines, loss of transgene with time (Sarria et al., 2000) due to deletion. Furthermore, not all varieties

are acquiescent to both transformation and regeneration (Hankoua et al., 2006).

Understanding all these constraints, researchers have managed to optimise genetic transformation in some of the farmer- and industry-preferred cassava varieties of Africa (Li et al., 1996; Vanderschuren et al., 2012; Nyaboga et al. 2013, 2015); South America (Siritunga and Sayre, 2003); Indonesia (Koehorst-van Putten et al., 2012) and Asia (Ntui et al., 2015, Anuradha et al., 2016). Compared to other continental cassava varieties, reports on Indian cassava genetic transformation were limited or greatly delayed as different criteria has to be optimised at each stage of transformation, right from the choice of explants for establishing an efficient transformation system.

The different *in vitro* cassava regeneration system previously used for cassava transformation include somatic embryos (Raemakers et al., 1997; Ntui et al., 2015), somatic cotyledon (Jorgensen et al., 2005, Prakash et al., 2011), axillary bud (Rossin, 2008, Msikita et al., 2006), embryogenic suspension (Chellappan et al., 2004, Vanderschuren et al., 2009), FEC (Raemakers et al., 2001, Liu et al., 2011, Bull et al., 2009, Chetty et al., 2013, Chauhan et al., 2015).

Among the different gene transfer techniques, *Agrobacterium* mediated transformation had been most widely preferred for cassava and has been utilised in the present study considering the advantage of this system over the other gene transfer techniques, which includes greater transformation efficiency, requirement for only simple equipment, reduced risk of generating chimeras, greater stability of transformants, usually transfers only one or few copies of transgene (Gonzalez et al., 1998; Schreuder et al., 2001; Nyaboga et al., 2013).

H226, a hybrid cultivar (Magoon et al., 1970), predominantly cultivated in Kerala and Tamil Nadu, are among the first three high yielding varieties of cassava released from ICAR-CTCRI in 1971. Due to non-availability of resistant gene in this farmer preferred cultivar, the present study had been focussed on identification of the most efficient regeneration system by targeting tissue/explants ie., apical meristem (AM), young cotyledon (YC), compact embryogenic structures (CES) and friable embryogenic callus (FEC)) using *Agrobacterium* mediated transformation system with *GUS* gene construct. The expression of *GUS* gene and molecular analysis of putative transformants have been used to detect the incorporated *GUS* transgene.

Materials and Methods

Explant source for cassava transformation

Virus free *in vitro* mother plants of cassava variety, H226 produced through meristem culture were multiplied on MS basal medium (Murashige and Skoog, 1962) supplemented with sucrose (20 g l^{-1}), agar (6.8 g l^{-1}), pH 5.8-5.9, and was used as the source material for the production of explants for *Agrobacterium* mediated transformation.

Four different tissue types were cultured to determine the best explant for optimum transformation frequencies *viz.*, (i) apical meristem (AM) cultured on cassava meristem media, [CMM, MS medium supplemented with 20g l^{-1} sucrose (w/v), benzyl adenine (BA, 0.1 mg l^{-1}), naphthalene acetic acid (NAA, 0.2 mg l^{-1}), gibberellic acid, (GA_3, 0.04 mg l^{-1}) along with $CuSO_4$ (0.3 mg l^{-1})] for 2-4 days, 28º C, dark (ii) young cotyledon (YC) developed from primary somatic embryos cultured on MSNB medium [MS medium supplemented with 20g l^{-1} sucrose (w/v), benzyl adenine (BA, 1 mg l^{-1}), naphthalene acetic acid (NAA, 2 mg l^{-1})] for 15-18 days (iii) compact embryogenic structures (CES) of 14 weeks old cultured on GD (Gresshoff and Doy, 1974) medium containing 12 mg l^{-1} Picloram (GDP) (iv) friable embryogenic callus (FEC) of 3 month old were cultured on GDP (Fig. 1). All the explants were transferred to fresh media 3-4 days prior to transformation.

Cassava transformation

Agrobacterium strain and preparation of culture for co cultivation

The culture of *Agrobacterium tumefaciens* strain, AGL0 harbouring the plant transformation plasmid, pOYE153 having *uid A* gene, conferring *GUS* activity and *Npt II* marker gene, encoding resistance to aminoglycoside antibiotics like geneticin was used to study the most suitable explant for transformation in cassava variety, H226. *Agrobacterium* carrying the constructs was allowed to grow in YEB medium containing acetosyringone (200 µM). When the culture reached 0.8-1.0 at OD_{600}, the cells were pelleted at 5000 rpm for 10 min., the pellet was washed twice in liquid medium (MS liquid for apical meristem and young cotyledon; liquid GD for CES and

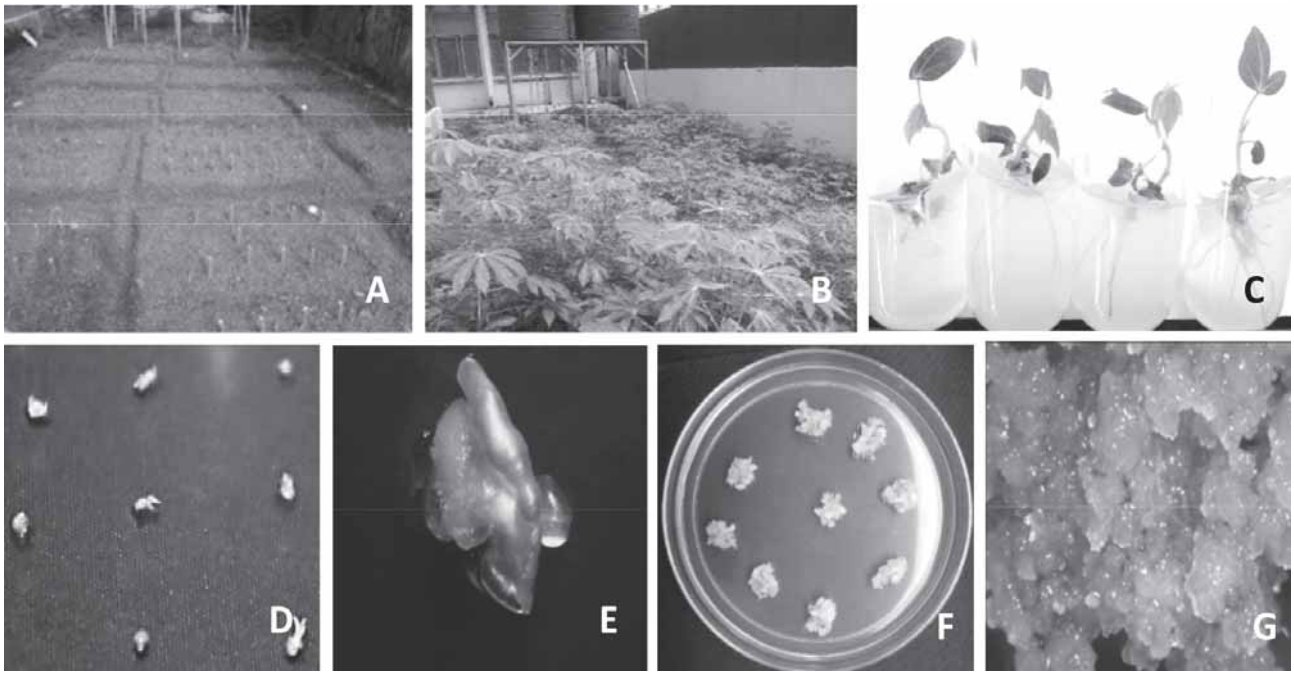

Fig. 1. Production of explant for cassava transformation : (A-B) Establishment of cassava varieties in experimental field by planting stem cuttings (15-20cm) (C) Meristem derived virus free plants, (D-G) Different types of cassava explants used for agrobacterium mediated transformation: (D) apical meristem (E) young cotyledon (F) compact embryogenic structures and (G) friable embryogenic callus

FEC), followed by re-suspending in liquid medium with acetosyringone (200 µM); diluted to final OD_{600} = 0.8-1.0, and was further used for co-cultivation.

Co cultivation and selection of transformants

Four different explants have been scrutinized in the present work to evaluate the best explant giving maximum transformation efficiency in one of the most farmer preferred variety in south India, H226. The various media used for cassava transformation using different explant is summarized in Table 1.

The apical meristem (including 1 or 2 leaf primordia) were pricked using sterile needle without causing any injury to the meristematic dome before incubating in bacterial suspension for 30 min. Direct shoot regeneration from apical bud meristem were considered as an advantage of apical meristem based transformation system since this could avoid the intermediate callus phase, preventing the

Table 1. Culture media used for transformation using different explants

Medium	Explants			
	Apical meristem (AM)	Young cotyledon (YC)	Compact embryogenic structure (CES)	Friable embryogenic callus (FEC)
Pre-culture medium	CMM	MSNB	GDP	GDP
Co-cultivation medium	AM-CC	YC-CC	CES-CC	FEC-CC
Wash solution	AM-WS	YC-WS	CES-WS	FEC-WS
Post-cultivation medium	AM-PC	YC-PC	CES-PC	FEC-PC
Transformant selection medium	AM-TS1	YC-TS1	CES-TS1	FEC-TS1
	AM-TS2	YC-TS2	CES-TS2	FEC-TS2
	AM-TS3	YC-TS3	CES-TS3	FEC-TS3
Embryo maturation medium	AM-MM	YC-MM	CES-MM	FEC-MM
Shooting medium	AM-SM	YC-SM	CES-SM	FEC-SM
Cassava propagation medium	CPM	CPM	CPM	CPM

chances of somaclonal variation, thus maintaining the transgenic nature of regenerated plant, and also reduce the time between transformation and regeneration phase which helps in early recovery of transgenic lines. While using cotyledon as explant, the margin of these young cotyledon (15-18 days old) were chopped off, pricked with needle, and were immersed in culture for 30-45 min at 28º C with agitation. The CES are densely aggregated, hard, compact embryogenic structures. The CES (10 clumps/ plate) were taken in a 50 ml oakridge tube and were washed in liquid GD medium before co-cultivation. CES were then incubated in culture for 20-30 min, with constant shaking inside the sterile hood. While using FEC, the *Agrobacterium* culture was added on the top of the FEC clumps placed over the nylon mesh and incubated for 15 min.

The total number of explant used for co-cultivation in three different experiments are as follows: 500 apical meristem (AM); 700 young cotyledon (YC), 50 mg of CES (0.5 cm dia. clumps) and 50 mg of FEC (0.5 cm dia. clumps). After the short *Agrobacterium*-infection period, the explants were briefly blotted on sterile filter paper to remove excess bacteria. All the treated explants and the control tissues (without treatment) were placed on respective co-cultivation media: AM-CC, CMM supplemented with acetosyringone (200 µM) for AM; YC-CC, MSCP supplemented with acetosyringone (200 µM) for YC; CES-CC and FEC-CC, GDP medium supplemented with acetosyringone (200 µM) for CES and FEC, respectively. Co-cultivation was carried out at 22ºC for 4 days (16 h light and 8 h dark). After co-cultivation, the infected tissues were washed thoroughly using sterile distilled water, followed by a wash in washing solution. AM and YC were washed in AM-WS and YC-WS (liquid MS medium supplemented with ticarcillin (500 mg l^{-1})), respectively. CES and FEC were washed in CES-WS and FEC-WS (GD liquid medium supplemented with ticarcillin (500 mg l^{-1}), respectively, for 5-8 min to kill the *Agrobacterium*.

The washed explants were transferred to post cultivation medium: AM-PC (CMM containing 500 mg l^{-1} ticarcillin) for AM, YC-PC (MSPC medium containing 500 mg l^{-1} ticarcillin) for YC, CES-PC and FEC-PC (GDP medium containing 500 mg l^{-1} ticarcillin) for CES and FEC, respectively, for 3 days. Then transferred to somatic embryo selection medium: YC-TS1 (MS medium supplemented with 12mg l^{-1} picloram, 500 mg l^{-1} ticarcillin and 15 mg l^{-1} geneticin) for YC, CES-TS1 and FEC-TS1 (GD medium supplemented with 12mg l^{-1} picloram, 500 mg l^{-1} ticarcillin and 15 mg l^{-1} geneticin) for CES and FEC, respectively. While the AM was placed in AM-TS1 (CMM supplemented with 500 mg l^{-1} ticarcillin and 15 mg l^{-1} geneticin). They were sub-cultured every week onto same selection medium with stepwise increase in geneticin concentration (5 mg l^{-1} every subculture) maximum upto 25 mg l^{-1} geneticin (AM-TS3), in order to maintain appropriate selective pressure and to avoid over growth of *A. tumefaciens* cells. For the development and regeneration of transformed apical meristem, AM-MM (MSC medium supplemented with 250 mg l^{-1} ticarcillin and 25 mg l^{-1}geneticin) was used. The transformed embryos from other transformation system were transferred to embryo maturation medium (MS medium with 1mg l^{-1} NAA, 250 mg l^{-1} ticarcillin and 25 mg l^{-1} geneticin): YC-MM for YC explant, CES-MM for CES explant, FEC-MM for FEC, for cotyledon development and scored each time during subculture. The young cotyledonary stage embryos obtained during 4-5 subcultures were transferred to shooting/ germination medium (MSC medium supplemented with 0.4 mg l^{-1} BA, 250 mg l^{-1} ticarcillin and 25 mg l^{-1} geneticin): AM-SM for AM, YC-SM for YC, CES-SM for CES explant, FEC-MM for FEC explants and finally to cassava propagation medium, CPM containing antibiotic selection (MSC containing 25 mg l^{-1} geneticin).The untransformed cotyledonary stage embryos (control) were also subcultured on to same medium as well as to plain CPM medium. The newly emerging leaflets from putative transformants were further used for *GUS* assay and molecular analysis for the detection of transgene.

Confirmation of transformants through GUS assay and molecular analysis

GUS histochemical analysis

The transformed tissues were analysed for histochemical β-glucoronidase *GUS* activity by incubating the different putatively transformed tissues (callus and leaf tissues) as well as control tissues in *GUS* buffer (Jefferson et al., 1987) with the substrate 1 mM X-Gluc (5-bromo-4-chloro- 3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium, Sigma) at 37º C overnight. After incubation, the stained tissues were washed several times in 70% ethanol to remove the chlorophyll content. After washing

they were observed visually and microscopically to detect the *GUS* expression. The percentage of cell clusters / transformed tissues showing *GUS* expression was assessed. The transient *GUS* transformation frequency was calculated based on the number of *GUS* positive transformed tissue or calli as a proportion (%) of the total number of tissues/ calli used for *GUS* assay.

Statistical analysis

The number of transformed embryo responded on selection medium, percentage of embryo germinated and the percentage of transformed tissues cell clusters showing *GUS* expression were scored and statistically analyzed through one way analysis of variance (ANOVA) ($p < 0.05$) using SAS system version 9 (SAS, 2010).

PCR analysis

Total DNA was isolated from newly emerged leaf samples of putative regenerated plantlets derived from different transformation system using various explants of cassava variety, H226 except the apical meristem (AM) since none of the explants survived after transformation , as well as from non-transgenic plants using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle., 1987). 100 mg of leaf tissue was powdered using liquid nitrogen and pre-warmed (65ºC) extraction buffer [2% CTAB, 2% PVP, 100 mM Tris- HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 2 % β-mercaptoethanol (v/ v)] was immediately added to it. The grinded tissue was incubated at 60ºC in water bath for 30 min, with intermediate inversion every 10 min and centrifuged at 10,000 rpm for 10 min to collect the supernatant. 10 µl of RNase (stock 10 mgl^{-1}) was added to the supernatant. Prior to addition, the RNase stock was activated by incubating at 95ºC for 5 min followed by sudden quenching in ice for 5 min. The mixture was incubated at 37ºC for 1 hour; equal volume of Chloroform: Isoamyl alcohol (24:1) was added to it and mixed well by repeated inversion. Then centrifuged at 10,000 rpm for 10 min and 0.8 volume of ice-cold iso-propanol was added to the transferred aqueous layer. It was then incubated at -20 ºC for 1 hr and centrifuged at 15000 rpm at 15 min. The pellet was washed with 0.5ml of 70% ethanol and centrifuged at 12,000 rpm for 5 min. The pellet was then air dried and re-suspended in 50 µl of 1 X TE buffer and stored at -20 ºC.

The total DNA from putative transgenic plants of other three transformation system namely, cotyledon, CES and FEC were subjected to PCR analysis with *Npt II* and *GUS* gene specific primers (*Npt II*-F: 5'-TATTCGGCTAT GACTTGG-3'; *Npt II*-R: 5'GCCAACGCTATGTCC TGATA-3' and for *GUS* gene specific primer is, *GUS*-F: 5'- GGGCATTCAGTCTGGATC-3' and *GUS*-R: 5'-GTGCGGATTCACCACTTG-3') to identify the stably transformed progeny that survived in geneticin (25 mgl^{-1}) medium The PCR was carried out using 5 ng μl^{-1} DNA, 1X PCR buffer, 0.5 mM dNTP mix, 0.5 pmol μl^{-1} each of forward and reverse primer, 0.05 Units μl^{-1} Taq DNA Polymerase. The PCR condition for *Npt II* specific primers were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 1 min., 56°C for 1 min and 72°C for 1 min and a final extension of 72°C for 5 min. The DNA was further amplified with *GUS* specific primers and incubated at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min and a final extension of 72°C for 15 min. The vector plasmid as well as the DNA isolated from untransformed tissue was used as positive control and negative control, respectively.

Nucleic Acid Spot Hybridisation (NASH) for transgene detection

For the preparation of DNA probe, the plasmid DNA was isolated from the pOYE 153 construct having *uidA* gene and *Npt II* gene using GenElute™ Plasmid isolation Kit (Sigma Aldrich). A non-radioactive, gene specific, biotin labelled DNA probe was generated from the eluted 660 bp amplicon of *GUS* gene using North2South™ Biotin Random Prime Labelling Kit (Thermo Scientific) as per manufacture's instruction, and was used for hybridisation.

Sample Spotting and Hybridization was done as follows: The total DNA isolated from the transgenic samples (CES, FEC and newly emerged leaves from cotyledon explants), non-transgenic samples (negative control) and pOYE 153 plasmid DNA (positive control) were spotted on squares of 1 x 1 cm^2 on Hybond N+ positively charged nylon membrane (Sigma Aldrich) and UV cross linked using UV transilluminator equipped with 254 nm bulbs for 66s. The blot was pre hybridized at 55°C for 30 min, with gentle rotation, then hybridization done at 55°C, overnight using (10^{-3} diluted) denatured biotinylated DNA probe, followed by stringency washes with 1X North2South® Hybridization Stringency Wash buffer (2X SSC/0.1%

SDS). Post hybridisation washes and detection was done using Streptavidin-Horseradish Peroxidase Conjugate in the presence of substrate (Peroxide Solution -Luminol/ Enhancer) according to manufacture's instruction of North2South Chemiluminescent Nucleic Acid Detection module (Thermo Scientific). The blot was placed in the X-ray cassette after blotting the excess buffer on a paper towel for 2-5 s. The X-ray film was developed and observed for black spots and were photographed.

Results and Discussion

Cassava, which was once considered recalcitrant to transformation, can now be transformed, certainly for some of the farmer preferred and industrially important varieties from many continents and very few Asian varieties *via* different gene transfer techniques (Chavarriaga, 2016). But most of the transformation protocols are variety / cultivar / genotype specific. The hindrance in technology transfer to other varieties may be due to lack of competence of certain cell or targeted tissues to transform. In certain varieties, cells that are competent to transformation may not be competent for organogenesis. The success rate of cassava transformation greatly depends on varied factors including A*grobacterium* strain, co-cultivation time, media composition, variety, explant used and many other unknown factors which are yet to be studied intensively at molecular level. Moreover, the major limitation observed in different transformation systems is mainly due to intrinsic variation in explant type, explant age, tissue quality and low regeneration capacity of transgenic lines from somatic embryos (Baba et al., 2008). It is also important to optimise each step to overcome these limitations (Zhang et al., 2005) and the present study is focussed on optimising the best explant for cassava transformation in the most popular Indian cassava cultivar, H226.

Effect of different explant on cassava transformation

Cassava apical meristem transformation

When apical meristem was used as explants, it was observed that none of the explants survived after two subculture in regeneration medium supplemented with geneticin 20 mg l^{-1}. The transformed explant became soft, lost their regeneration capacity became dark and died. Seventy percent of non-transformed AM explants on non-antibiotic selection medium regenerated into healthy plants, while all the non-transformed tissue in antibiotic selection medium turned white and died. The apical meristem was also not competent to transformation, and may be due to failure of agrobacterium from entering the meristematic zone which was covered by leaf-primordia. This proves the difficulty of using meristem as explant for the transformation of recalcitrant crop like cassava. Rossin et al., 2008 revealed that axillary bud transformation and direct regeneration are not feasible in African cassava local variety, T200 and TMS 60444. While a success story of cassava axillary bud transformation giving a transformation efficiency between 1 and 5% have been reported by Msikita et al., 2006. These findings from different reports supports the fact that cassava transformation is highly dependent on explant type and genotype.

Cotyledon mediated transformation

In this study, young cotyledon of 18-21 days produced through somatic embryogenesis have been utilized as another explant type. The initiation of callus formation was observed within 10-12 days and the first set of embryos was transferred after 18 days on callus induction medium with antibiotic selection. After three to four weeks, a total of 127 putative transformed embryos that survived on embryo induction medium under selection pressure (YC-TS3) were transferred to YC-MM, for the maturation and production of cotyledons. The embryos were sub-cultured every week in order to prevent growth of non-transformants and 35% of the transformed embryo germinated to produce cotyledonary staged embryos. After two cycles on embryo maturation medium, 15 putative transgenic lines regenerated into healthy plantlet and took 8-9 weeks to reach 1-2 leaf stage plantlets (Table 2). Seventy five percentage of the non-transformed young cotyledonary explants induced embryos, and regenerated into healthy plantlets in non-selection medium, while all the untransformed tissue in antibiotic selection medium died. The different stages of putative transgenic plant developed using cotyledon as explant is shown in Fig. 2 (a-d). Puonti- Kaerlas (1998) reported that using cotyledons from somatic embryos cultured for 15 days on embryo maturation medium showed higher regeneration and transformation frequencies while very young cotyledon from newly germinated embryos resulted in poor transformation efficiency since the latter are very sensitive to *Agrobacterium* and poorly survived the co-cultivation procedure.

Table 2. Selection, regeneration of transgenic plants expressing *GUS* gene of cassava variety, H226 derived from different explants

Explant type	No. of putative transformed embryo surviving on selection medium	*No. of embryos germinated to cotyledonary stage (%)	No. of regenerated plants (1-2 leaflet stage)
Cotyledon	127	45 (35.43%)	15
CES	74	22 (29.72%)	07
FEC	85	17 (20.00%)	05

*Percentage with respect to number of surviving somatic embryos on selection medium

Embryogenic callus mediated transformation

In cassava, induction of different type of callus have been reported earlier (Taylor et al., 1996) and either callus or various explant type derived from these tissues at different stages of development/ embryogenesis have been utilised in transformation studies. It includes somatic embryo (Siritunga and Sayre, 2004; Ntui et al., 2015), embryogenic suspension (Schopke et al., 1996, Schrender et al., 2001), FEC (Raemakers et al., 2001, Taylor et al., 2012, Nyaboga et al, 2015).

In CES based transformation system, pre-washing of CES in liquid GD medium before co-cultivation helped to discard the translucent matrix surrounding the embryogenic structures, and thus increased the chance of contact of agrobacterium with target tissues, which enhanced the transformation frequency. The different stages for the development of transgenic cassava using compact embryogenic callus as initial explant is shown in Fig. 2 (e-h). These clumps were monitored weekly under stereo microscope for any signs of embryo development. From 50 mg of CES, a total of 74 putative transformed embryos were produced, respectively, after 8 cycles of 14 days each on MSN medium with geneticin selection. The germination potential of transformed embryos were 30% for H226 CES, scored after 3-4 weeks of incubation on embryo maturation and germination medium under geneticin selection pressure and seven putative transgenic lines regenerated into healthy plantlet (Table 2).

In FEC mediated transformation system, the transformed FECs were monitored weekly and scored for embryo development, germination and regeneration (Fig. 2 (i-l)), as done for CES transformation system. FEC clusters produced 85 putative transformed embryos, after 8 cycles of 7 days each on MSN medium with geneticin selection. It was observed that H226 FEC showed a lower germination frequency of 20% than other explants. Only five cotyledonary staged embryo regenerated into 1-2 leaflet staged plants (Table 2).

GUS assay of transformants in different transformation system

Many researchers have reported the production of transgenic cassava varieties harbouring selectable marker gene or genes expressing traits of interest for cassava improvement (Liu et al., 2011, Chavarriaga et al., 2016). Visual markers like *GUS*, luciferase and *GFP*, have been

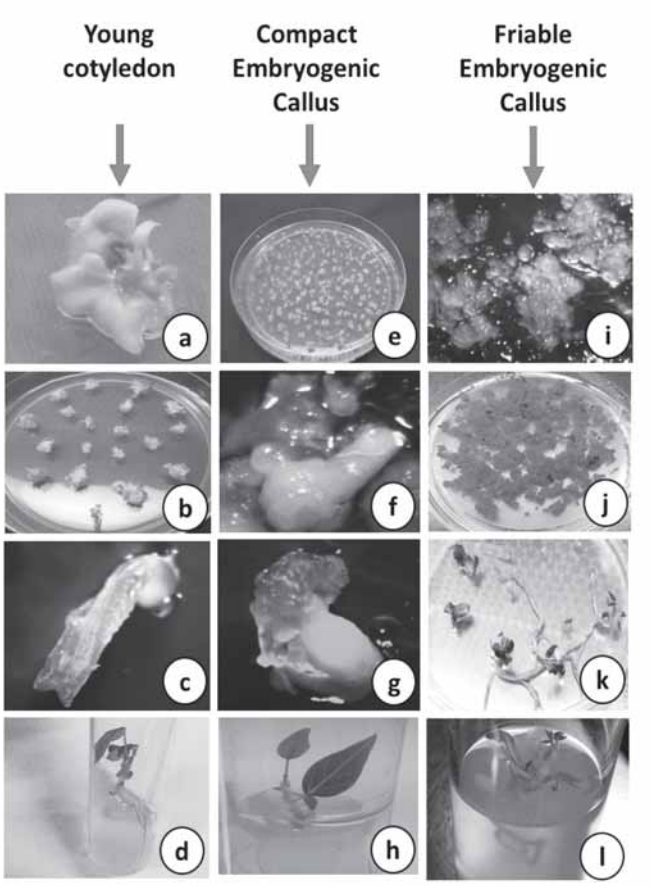

Fig. 2. Different stages of transgenic cassava development using different explants of cassava variety, H226: cotyledon mediated transformation (a-d), compact embryogenic callus mediated transformation (e-h), friable embryogenic callus mediated transformation (i-l)

extensively exploited in many research laboratories for studying the transgene expression or for gene constructs validation (Sarria et al., 2000, Nyaboga et al., 2013). *GUS (uid A*) gene had been routinely used as a tag for the identification of gene of interest through *GUS* assay and for validation of gene construct for the development of efficient transformation system (Jefferson et al., 1987). The transformation frequency was calculated in different ways by different research team by scoring the number of PCR positive regenerated putative transgenic plants on selection medium or by scoring the dot blot or southern positive samples from the regenerated transgenic lines or by scoring the number of positive calli / tissues expressing visual markers like *GUS/ GFP* over the total number of explants inoculated / co-cultivated (in percentage).

In the current study, the frequencies of *GUS* gene expression were evaluated in four different transformation system of H226, confirming the presence and expression of *GUS A* gene in co-cultivated explants as well as leaf samples isolated from regenerated transgenic plants. It was observed that the apical meristem explants were not a suitable target tissue for *Agrobacterium* transformation for the selected cassava variety at existing laboratory conditions and parameters, since none of the transformed explants survived after second subculture in the antibiotics selection medium and hence could not use for *GUS* assay.

The transient *GUS* transformation frequency for the cotyledon transformation system was determined based on the percentage of newly emerged leaf explants showing *GUS* expression over the total number of leaf explants used for *GUS* assay. The newly regenerated shoot primordia from putative transformants on selection media developed through cotyledon mediated transformation system showed transient to stable *GUS* expression in older and younger leaves on *GUS* assay (Fig. 3 D). The result showed a maximum *GUS* expression frequency of 64 % while using cotyledon as explant (Table 3).

The transformation frequency of two different types of callus *ie* Compact embryogenic structures (CES) and Friable embryogenic callus (FEC) were also determined based on the percentage number of callus lines showing transient *GUS* expression over the total number of calli used for *GUS* assay. In this study, the FEC lines and the CES were analysed for *GUS* assay only after three subculture on selection medium, in order to avoid *Agrobacterium* contamination (Fig. 3A and 3B). 57% of transient *GUS* expression were observed in geneticin resistant embryos / cell lines generated from H226 CES (Table 3). The embryos developed from CES explants, germinated and regenerated very gradually and showed a stunted growth after repeated subculture on regeneration medium. This result showed that the CES that were competent to transformation, were not competent to regeneration. Hankoua et al., 2006, suggest the same possibility for inefficient regeneration of transgenic plant expressing gene of interest may be due to methylation of antibiotic resistant gene that eventually prevents growth of these transformed cells or tissues on selection medium.

The FEC clumps of H226 after three subcultures on embryo maturation under antibiotic selection pressure, showed a lower *GUS* transformation frequency of 43% (Table 3). While the non-transformed FEC (negative control) die off or either failed to develop embryos or germinate at the second round of antibiotic selection and did not show any signs of blue colouration, confirming no *GUS* expression (Fig. 3 C). There was significant difference in *GUS* expression between the three explants within the variety (Table 3).

Table 3. Validation of putative transgenic plants of cassava variety, H226 using various explant

Explant type	Percentage of explants/cell clusters showing transient *GUS* expression	No. of randomly analysed putative transformants		Percentage of PCR/NASH positive samples	
		PCR	NASH	PCR (*GUS* gene)	NASH (*GUS* probe)
Cotyledon	64.20 ± 3.96[a]	6	8	100	75
CES	56.75 ± 3.71[b]	3	6	67	50
FEC	43.05 ± 1.46[c]	5	8	20	25

Values in a column followed by different letters are significantly different from each other at $p < 0.05$

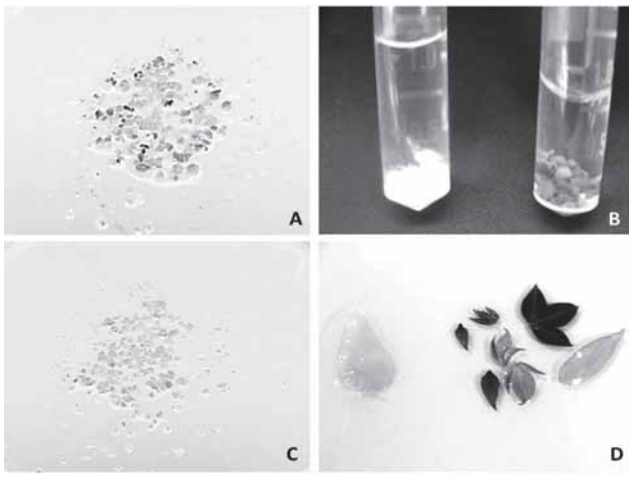

Fig. 3. Histochemical *GUS* expression of transformed tissues of cassava variety, H226: (A) Transformed FEC showing *GUS* expression, (B) Non-transformed CES control (left) and CES showing *GUS* expression (right), (C) Non-transformed FEC (D) Non-transformed leaf sample (left) and leaf sample from transformed cotyledon showing *GUS* expression (right).

Molecular detection of putative transformants from different transformation system

The DNA isolated from randomly selected putative transformants from three different transformation system namely, cotyledon, CES and FEC were subjected to PCR analysis using *Npt II* and *GUS* gene specific primers; produced an amplified product of 280 bp product for *Npt II* gene and 660bp for *GUS* gene which confirmed the presence of *GUS A* transgene in stably transformed progenies (Fig. 4) that survived in geneticin selection. Six, two and one putative transgenic lines derived from transformed young cotyledon, CES and FEC explants gave amplified product of 660 bp for *GUS* gene, respectively. While the non-transformants failed to give any amplified product for *Npt* II gene and *GUS* gene. The non-transformants can be easily identified in the preliminary phase and removed through routine PCR based detection technique.

The presence of transgene was also confirmed through nucleic acid spot detection using biotin labelled *GUS* probe, in which black spot was observed in randomly selected PCR confirmed transformants generated through different transformation system, corresponding to the black spot obtained for positive control plasmid DNA (pOYE 153) (Fig. 5).

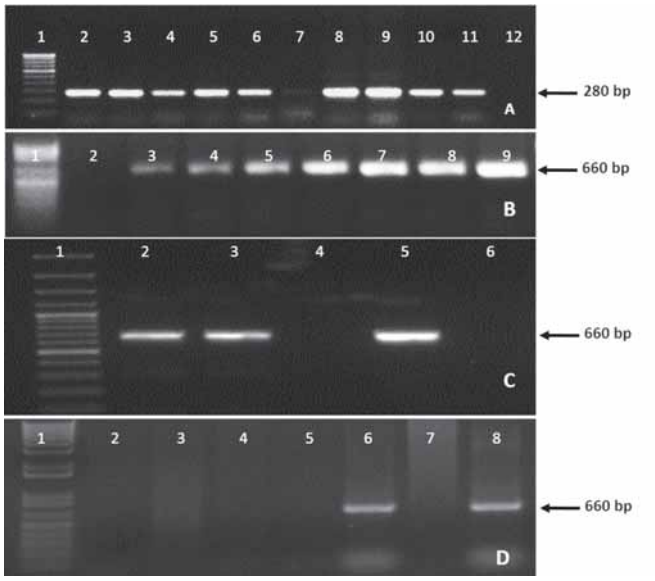

Fig. 4. Molecular analysis of *Npt* II gene and *GUS* gene in transgenic lines derived from YC, CES, FEC explant of cassava variety, H226 (A) Analysis of *Npt* II gene: Lane 1: 100 bp marker, lanes 2-4: YC samples, lanes 5-7: CES samples, lanes 8-10: FEC samples, lanes 11: pOYE 153 plasmid DNA (positive control), lane 12: non-template (negative control). (B) *GUS* gene confirmation in cotyledon derived transgenic samples: Lane 1: 100 bp marker, lanes 2: Non-template (negative control), lanes 3-8: Cotyledon derived samples, lane 9: pOYE 153 plasmid DNA (positive control). (C) *GUS* gene confirmation in CES derived transgenic samples: Lane 1: 100 bp marker, lanes 2-4: CES derived samples, lane 5: pOYE 153 plasmid DNA (positive control), lanes 6: non-template (negative control). (D) *GUS* gene confirmation in FEC derived transgenic samples: Lane 1: 1 kb plus ladder, lanes 2-6: FEC derived samples; lane 7: non-template (negative control); lanes 8: pOYE 153 plasmid DNA (positive control)

The blot analysis of transgenic lines produced through *Agrobacterium* transformation in cotyledon explants showed positive signal in 75% of tested samples, while 50% of the spotted DNA samples of CES derived transgenic plants gave positive signal. Two transgenic samples derived from FEC of H226 gave very weak signal when hybridised with *GUS* probe. (Table 3). None of the non-transformants produced black spot on the nylon membrane which confirmed that the positive signal for the integration of *GUS* gene in transgenic lines.

Anuradha et al., 2016 obtained forty transgenic lines and six shoots (integrated with SLCMV Rep RNAi constructs) from 3000 young leaf-lobe and 500 green somatic

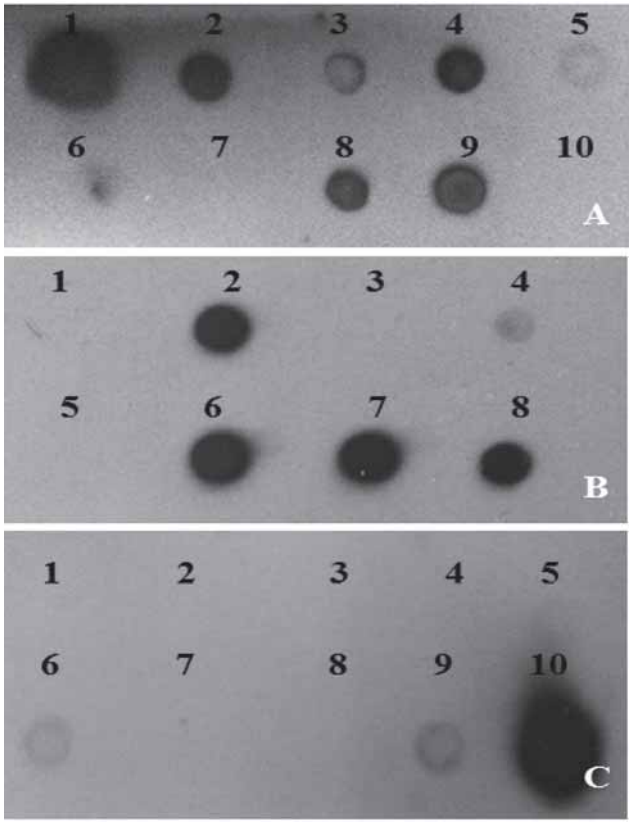

Fig. 5. NASH blots of transgenic lines derived from YC (A), CES (B), FEC (C) explant of cassava varieties, H226 (A) Spot 1: pOYE 153 plasmid DNA (positive control), Spot 2-9: YC derived transgenic samples, Spot 10: Untransformed sample (negative control) (B) Spot 1: Untransformed sample (negative control), Spot 2: pOYE 153 plasmid DNA (positive control), Spot 3-8:CES derived transgenic samples (C) Spot 1: Untransformed sample (negative control), Spot 2-9 FEC derived transgenic samples, Spot 10: pOYE 153 plasmid DNA (positive control)

cotyledon of cultivar, H226, respectively; but the recovery of transgenic plants from cotyledon was found to be poor. Prakash et al., 2011 optimised *Agrobacterium* mediated transformation of cotyledon in cassava variety, H226 using *Agrobacterium* strain, AGL0 (OD_{600} 1.0), and obtained a maximum transformation efficiency of 65.66% (Percentage transformation efficiency scored in terms of blue color development on transformed tissues from different trials). Ntui et al., 2015 successfully produced transgenic plants resistant to SLCMV, in Asian cultivar, KU50, using somatic embryo as initial explant. Only few genetic transformation studies have been reported in Indian cassava cultivar and it was found that the transformation frequency of African cassava cultivars was higher than Indian cultivars. Nyaboga et al., 2015 reported regeneration of about 70-80 independent transgenic lines per ml settled cell volume (SCV) of FEC of cassava cultivar, TME 14. Nyaboga et al., 2013 reported 22, 17, 14 and 28 transgenic lines from 100 mg FEC of Serere, Ebwanatereka, Kibandameno, and 60444, respectively. However, Chetty et al., 2013 obtained 45 transgenic lines from 140 FEC clusters. Thus there always exist a variability in transformation frequency in cassava and it cannot be compared due to the heterozygous nature of the crop, difference in transformation method or capacity for somatic embryo development / variation in germination and the plant regeneration efficiency between different varieties.

In the present study, six putative transgenic plants were produced through cotyledon mediated transformation, and two putative transgenic plants were generated while using compact embryogenic structures as explant. Only one putative transgenic lines were derived from FEC explants, which showed faint band on molecular analysis indicating *GUS* gene integration but the recovery was found to be very poor. AM was also found to be non-target tissues for *Agrobacterium* mediated transformation. This supports the fact that success of transformation is highly dependent on explant type as well as on genotype. The presence of *GUS* transgene was confirmed through molecular analysis. It can be concluded that for H226, cotyledon remain as the best explant type for transformation, as it showed a maximum *GUS* expression and a significantly ($p \leq 0.05$) high efficiency for germination and regeneration of transformed embryo compared to other explants.

Conclusion

Young cotyledon of cassava cv. H 226 was identified as the most suitable target for Agrobacterium mediated genetic transformation based on *GUS* and molecular analysis. This finding will pave way for creating disease resistance in this cultivar which is highly susceptible to *Sri Lankan cassava mosaic virus* infection.

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