



Production of embryogenic tissues and regeneration of transgenic plants in cassava (*Manihot esculenta* Crantz)

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Summary

Disorganised embryogenic tissues have been utilised as target tissues for transgene insertion and transgenic plant regeneration in cassava (*Manihot esculenta*). The production of friable embryogenic callus in fourteen geographically diverse cassava cultivars, from which eleven were established as embryogenic suspension cultures, is reported. Embryogenic tissues were similar in nature in all cultivars tested although there was variation in the time required to generate friable callus and the growth rates of suspension cultures. Regeneration of plants has been achieved from eight cultivars but varied significantly in efficiency, with cv. TMS 60444 and Line 2 from Zimbabwe being the most responsive. Tissues from the remaining eight cultivars became arrested at globular and torpedo stages of regeneration indicating that they most likely possess an inherent ability to produce plants but require further research to allow this to be realised. Significant numbers of transgenic plants containing transgenes for putative resistance to important viral diseases of cassava in addition to visual marker genes have been regenerated. Transgenic plants from three of the cultivars TMS 60444, Bonoua Rouge and M.Col 1505 were recovered after particle bombardment of embryogenic suspension cultures. Correlations have been made between abnormal leaf morphology and plant vigour with the use of embryogenic suspension cultures for transgene insertion. As a result friable embryogenic callus is now being successfully utilised as the target tissue for genetic transformation and plant regeneration at ILTAB.

Abbreviations: ACMV – African cassava mosaic virus; BAP – benzylaminopurine; CsVMV – Cassava vein mosaic virus; FEC – friable embryogenic callus; GD – Gresshoff and Doy basal medium; ILTAB – International Laboratory for Tropical Agricultural Biotechnology; MS – Murashige and Skoog basal medium; NAA – naphthaleneacetic acid; SH – Schenk and Hildebrandt basal medium; 2,4-D – 2,4-dichlorophenoxy acetic acid; SCV – settled cell volume

Introduction

Development of robust protocols for the genetic transformation of cassava (*Manihot esculenta* Crantz) is essential if biotechnology is to be applied to addressing yield constraints and product enhancement in this important tropical root crop (Taylor et al., 1999; Thro et al., 1999). The heterozygous nature of cassava means that zygotic embryos of this species have unknown

and unpredictable phenotypes, and are therefore unsuitable as starting material for genetic transformation programmes. Instead, organised somatic embryos generated from leaf lobe explants are utilised for the production of friable embryogenic callus which in turn is used as the target tissue for gene insertion. Friable embryogenic callus and embryogenic suspension cultures are favoured target tissues for the production of genetically transformed plants in many crop

species (Christou, 1996; Birch, 1997). Their ability to regenerate whole plants from single cells makes these cultures amenable to both direct gene transfer and genetic transformation by *Agrobacterium*.

We previously reported the development and use of embryogenic suspension cultures as target tissues for the recovery of transgenic plants of the African cassava cultivar TMS 60444 (Taylor et al., 1996; Schöpke et al., 1996). In this procedure, organised somatic embryos were induced from immature leaf lobe explants cultured on Murashige & Skoog (1962) basal medium supplemented with a potent auxin such as 2,4-D or picloram. These structures are highly organised and are formed directly from the explant without an intervening callus phase and as such they are unsuitable as a target for transgene insertion and recovery of transgenic plants. To overcome this problem friable embryogenic callus was generated from the organised somatic embryos by transferring them to a medium containing Gresshoff & Doy (1974) basal salts and 50 μM picloram. After 3–4 subculture cycles on this medium homogeneous friable embryogenic callus (FEC) was generated which consisted of thousands of sub-millimeter sized, pro-embryogenic units (Taylor et al., 1996) per gram of tissue. Embryogenic suspension cultures were established from the FEC by transferring the callus to liquid Schenk & Hildebrandt (1972) basal medium supplemented with 50 μM picloram. Plants were recovered from the embryogenic suspensions and callus tissues by sequential differentiation, maturation and germination of somatic embryos on a series of MS based media.

Embryogenic suspension cultures of cv. TMS 60444 have been utilised as target tissues for transgene insertion. Genetically transformed cassava plants expressing the *uidA* (GUS) and luciferase markers genes have been recovered after particle bombardment (Schöpke et al., 1996; Raemakers et al., 1996), and more recently *Agrobacterium tumefaciens* transformation (Gonzalez de-Schöpke et al., 1998), of the disorganised embryogenic tissues. Since these initial reports, efforts have been directed towards optimisation of the genetic transformation process and its extension into further cassava cultivars.

Here we report progress in the production of embryogenic tissues and recovery of plants from a range of geographically diverse cassava cultivars. In addition, we review the status of genetic transformation and the insertion of marker genes and genes of putative agronomic interest into cv. TMS 60444.

Materials and methods

Culture conditions

Basal media were obtained from Sigma Chemical Company. Sucrose, growth regulators and other supplements were added before adjusting the pH to 5.8, addition of 7.8 g l^{-1} Noble Agar and autoclaving. Media were dispensed at 25 ml per 9 cm Petri dish and cultured in a growthroom at 27 ± 1 °C with 16 hours illumination at 30 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Plant tissue

Cassava cultivars were maintained as shoot cuttings cultured on Murashige & Skoog basal medium supplemented with 2% w/v sucrose (MS2). Mother plantlets for the production of embryogenic tissues were prepared by transferring ten nodal cuttings to 9 cm Petri dishes containing MS2 medium and culturing for 4 weeks prior to explant excision.

Induction and determination of embryogenic tissues

Unfolded leaf lobe explants 2–6 mm in length were excised from *in vitro* mother plantlets and placed on MS2 medium supplemented with 50 μM picloram. Twenty eight days later, embryogenic structures were removed and the associated callus cut away. Friable embryogenic callus was induced by transferring these somatic embryo structures to Gresshoff & Doy basal medium supplemented with 2% w/v sucrose and 50 μM picloram (GD2 50P) (Taylor et al., 1996). Once established the FEC was maintained by four-weekly subcultures on GD medium containing 6% sucrose and 50 μM picloram.

Embryogenic suspension cultures were initiated by transferring 0.5 g of FEC to 50 ml of liquid Schenk & Hildebrandt basal medium supplemented with 50 μM picloram and 6% w/v sucrose (SH6 50P) in 250 ml Erlenmeyer flasks, and maintained as described by Taylor et al. (1996). Growth of the suspension cultures was determined by measuring the settled cell volume (SCV), fresh and dry weights. SCV was assessed by pipetting embryogenic suspensions into 15 ml graduated test tubes and allowing the tissues to settle for 30 minutes.

Distribution of size fractions within the suspension cultures was determined by pipetting embryogenic tissues into a 50 ml test tube and vortexing for three minutes to encourage disaggregation. Suspension tissues were then passed sequentially through 1000, 500,

350, 250 and 100 μm sieves and the retained embryogenic units collected at each stage. The number of units in each fraction was recorded using a Sedgewick Rafter Cell counter under the compound microscope.

Plant regeneration

Plant regeneration was achieved via a multiple-stage embryo and germination process. FEC was transferred to MS2 medium containing 10 μM 2,4-dichlorophenoxy acetic acid (2,4-D) for 3 weeks, followed by subculture onto MS2 medium with the addition of 5 μM naphthalene acetic acid (NAA). After a further 2–3 weeks, cotyledon-stage embryos were removed and placed on MS2 medium containing 5% w/v activated charcoal. Seven days later the matured embryos were transferred to MS2 medium supplemented with 5 μM benzylaminopurine (BAP). Germinated plantlets were maintained on MS2 medium. Plants were transferred to soil by washing the agar from the roots of *in vitro* regenerants and placing in tap water in an open Magenta box for three to four days. Hardened plantlets were planted in 4 inch pots containing MetroMix 360 potting compost and maintained at 90–100% relative humidity in a growth chamber at 26–28 °C. Seven to ten days later the plants were transferred to the greenhouse.

Genetic transformation

Embryogenic suspension culture tissues were utilised as the target tissue for gene transfer by particle bombardment. Tissue was removed from liquid culture and passed through 500 μm and 100 μm sieves. Embryogenic units retained on the 100 μm sieve were transferred to a graduated tube and allowed to settle for 30 minutes. Aliquots containing 0.2 ml SCV of tissue were placed onto a 100 μm plastic mesh, spread to form a monolayer approximately 2 cm in diameter and bombarded using the Biolistic[®] particle bombardment system at 1100 psi and 27 inHg, as described by Schöpke et al. (1996).

After gene transfer, tissues were returned to liquid SH6 50P medium. Three days later 25 μM of the antibiotic paromomycin was added to this medium. Seven days after gene insertion the tissues were spread evenly over semisolid GD2 50P medium containing 25 μM paromomycin. Plated tissues were examined for growing, yellow coloured embryogenic units ten days after plating, and weekly thereafter, for a total of six weeks following bombardment. Growing

units were removed from the first selection medium and transferred to fresh semi-solid medium of the same type. Embryogenic units which grew and established colonies of FEC after 21 days culture on the second round of semi-solid selection medium were considered as putatively transgenic and underwent testing for presence of the transgenes by PCR and/or histochemical staining.

PCR and Southern blot analysis

For PCR, genomic DNA from callus and leaf tissues were extracted according to Dellaporta et al. (1983). PCR conditions were those described by the *Taq* polymerase manufacturer (Life Technologies, Gaithersburg, MD). Reaction mixtures were incubated for 4 minutes at 94 °C followed by 30 cycles at: 1 minute at 94 °C, 50 seconds at 60 °C (for the CsVMV coat protein gene) or 56 °C (for the AC1 replicase gene) and 1 minute at 72 °C. Primer sequences used to amplify the transgenes are shown in Table 3.

To perform Southern blot analysis genomic DNA was isolated from young leaves of *in vitro* transgenic cassava plants using the Qiagen plant DNA miniprep kit (Qiagen, Valencia, California) according to manufacturer's recommendations. Ten micrograms of DNA was digested with Bgl II and loaded onto each lane of a 0.8% agarose gel and run for 16 hrs at 25V. DNA was blotted onto a Boehringer Mannheim (Germany) hybond N⁺ membrane according to standard methods. PCR was used to amplify the AC1 coding region from plasmid pILTAB 382 using conditions mentioned above. The PCR product was TA cloned into the pCRII TOPO TA cloning vector (Invitrogen, Carlsbad, California, USA) to produce pCRII AC1. An antisense RNA probe complementary to the AC1 coding region was generated using the Boehringer Mannheim Digoxigenin RNA labeling kit according to the manufacturer's recommendations. Southern hybridisation was performed using the DIG high prime starter kit II (Boehringer Mannheim) according to the manufacturer's specifications. Chemiluminescence from the hybridised membrane was detected using Lumi-film Chemiluminescence detection film (Boehringer Mannheim).

GUS staining

Transgenic tissues were examined for GUS expression according to Jefferson (1987).

Table 1. Production of friable embryogenic callus, embryogenic suspensions and subsequent plant regeneration in a range of cassava cultivars

Cultivar	Country of origin	Time to establish FEC (months)	Embryogenic suspension established	Regeneration achieved to the stage of
TMS 60444	Nigeria	3	+	plants
TMS 60142	Nigeria	9	+	cotyledon embryos
TMS 83350	Nigeria	9	–	FEC
TMS 90853	Nigeria	4	+	cotyledon embryos
Bonoua Rouge	Ivory Coast	6	+	plants
Kataoli	Togo	6	+	cotyledon embryos
Okouta	Gabon	6	+	globular embryos
Line 2	Zimbabwe	4	+	plants
Line 11	Zimbabwe	4	+	cotyledon embryos
M.Aus 7	Zimbabwe	4	na	plants
M.Col 22	Colombia	6	na	plants
M.Col 1505	Colombia	4	+	plants
M.Col 2215	Colombia	6	na	plants
M.Tai 5	Thailand	4	+	plants
M.Tai 25	Thailand	4	+	torpedo embryos

FEC: friable embryogenic callus., na: not attempted.

FEC production was attempted but was unsuccessful in cvs. TMS 30572, TMS 90059, TMS 90257, TMS 30337, TMS 30040 and Minis.

Results

Production of friable embryogenic tissues

Organised embryogenic structures were induced from immature leaf lobe explants in a range of cassava cultivars on an MS based medium (Raemakers et al., 1993; Taylor et al., 1997). Transfer of somatic embryogenic structures to GD2 50P medium and subsequent selection and cyclic subculture of FEC on fresh medium of the same type, facilitated production of homogenous friable embryogenic callus from a range of cassava cultivars. From a total of twenty cultivars attempted to date, fourteen; nine African, three South American and two from Asia, have proved amenable to the formation of FEC (Table 1). The time required to generate homogenous lines of FEC from the organised embryogenic structures varied between the cultivars and reflected the ease with which this could be achieved. TMS 60444 was the most responsive cultivar, producing FEC within 2 months (or 3×21 day subculture cycles). Formation of FEC from cultivars Bonoua Rouge and L2 was also relatively rapid, but in the case of TMS60142 and TMS 83350 took as long as 9 months. Once established FEC callus lines were easily maintained in all the cultivars listed in Table 1.

Embryogenic suspension cultures were successfully established from FEC of all the cultivars attempted except for TMS 83350 (Table 1). The growth of TMS 60444 tissues in liquid culture was studied in detail by measuring increases in SCV, fresh weight and dry weight over a 21 day period. All three growth parameters displayed the same pattern, with embryogenic tissues increasing approximately 15 times over the first 15 days and by a total of 22 times over the complete three-week culture period (results not shown).

Having established that SCV is an accurate method for measuring the growth of suspension cultures in cassava, this simple and non-destructive technique was used to determine the performance of embryogenic suspensions from five different cassava cultivars (Figure 1). Growth rates varied between the cultivars screened. TMS 60444 and TMS 90853 grew most rapidly with the inoculum increasing by a factor of 22–24 times over the culture period while Bonoua Rouge and TMS 60142 increased approximately 15 times over the same time. Although remaining healthy throughout the culture cycle, Kataoli was significantly the poorest of the five cultivars tested. Tissues from this cultivar increased by only 8 times, three times less than TMS 90853 over the same 21 day period.

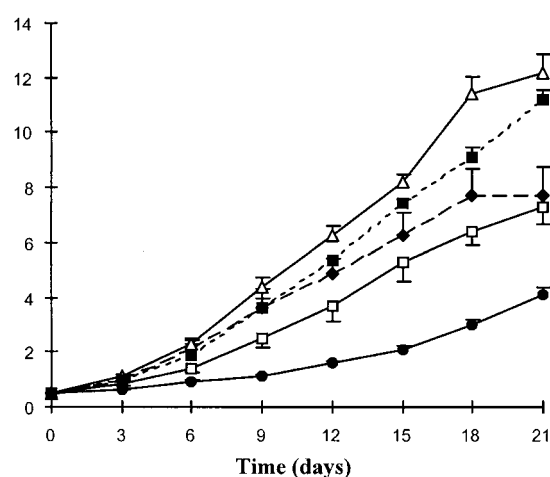


Figure 1. Growth of embryogenic suspension cultures in various cassava cultivars.

Embryogenic cultures of cassava cultivars TMS 90853 (Δ), TMS 60444 (\blacksquare), TMS 60142 (\blacklozenge), Bonoua Rouge (\square) and Kataoli (\bullet), were cultured in 50 ml SH6 50P medium and refreshed every three days. Growth was determined by measurement of the settled cell volume (SCV). Values shown are the mean of six flasks plus/minus the standard deviation.

Composition of embryogenic suspension tissues was determined for 15 day old cultures. Suspension cultures were fractionated sequentially through 1000, 500, 250 and 100 μm sieves and the number of embryogenic units retained on each mesh was counted. Figure 2 shows the percentage distribution of embryogenic units by size for four cassava cultivars. Some vacuolated, single cells were seen in the fraction which passed through the 100 μm mesh, but non-embryogenic tissues constituted an insignificant proportion of these suspension cultures. Although there were differences in their growth rates (Figure 1), all four cultivars showed a very similar pattern of size distributions within the embryogenic tissues of their respective suspension cultures. The fine, dispersed nature of the suspensions generated by the four cultivars is illustrated by the fact that less than three percent of the total embryogenic units were greater than 1 mm in diameter, and that approximately 90% of all units were between 500 and 100 μm in diameter. Fifty percent or more of the embryogenic units were less than 350 μm in diameter in all the cultivars except Kataoli. The latter genotype had a higher proportion of its embryogenic units between 500 and 350 μm in diameter. Embryogenic units were seen in the fraction which passed through the 100 μm sieve. However, these were not numerous and being

Table 2. Regeneration of mature somatic embryos and plants from friable embryogenic callus of cassava

Cultivar	# cotyledon-stage embryos regenerated/g FEC \pm SD	% cotyledon-stage embryos germinating into plants
TMS 60444	1520 \pm 90	72
Line 2	2480 \pm 245	74
M.Col 1505	910 \pm 305	24
M.Col 22	300 \pm 51	36
TMS 60142	230 \pm 75	0
TMS 90853	55 \pm 10	0
Kataoli	70 \pm 20	0
Line 11	90 \pm 45	0

Regenerated embryos are shown as the mean number of cotyledon-stage embryos, \pm standard deviation (SD), regenerated from ten 0.1 g samples of FEC after culture on MS2 medium supplemented with 10 μM 2,4-D followed by three weeks culture on MS2 medium containing 5 μM NAA. Percentage of embryo germinating was determined by transferring 25 cotyledon-stage embryos to MS2 medium supplemented with 0.5% w/v activated charcoal for seven days followed by culture on MS2 medium containing 5 μM BAP.

composed of less than 100–200 cells each, they constituted an insignificant proportion of the suspension's biomass.

Regeneration of mature embryos and plants from FEC

In order to assess the regeneration potential of the embryogenic tissues generated, 0.1 g samples of FEC from embryogenic callus of various cultivars were subcultured through the embryo regeneration, maturation and germination sequence described in Materials and methods. Cotyledon-stage embryos were recovered in all the cultivars tested, but the regeneration efficiency varied significantly (Table 2). The cultivars could be split into three groups depending on their regeneration potential. TMS 60444, Line 2 and M.Col 1505 were highly regenerative, producing on average 1000 or more cotyledon-stage embryos per gram FEC. M.Col 22 and TMS 60142 were capable of regenerating several hundred cotyledon-stage embryos from the same amount of tissue, while TMS 90853, Kataoli and Line 11 produced less than 100. In the latter three, the majority of the FEC developed to the globular and especially the torpedo stages but failed to mature further (Table 1). Those which did form foliose structures were generally of poor quality, possessing malformed cotyledons or badly formed shoot and root poles.

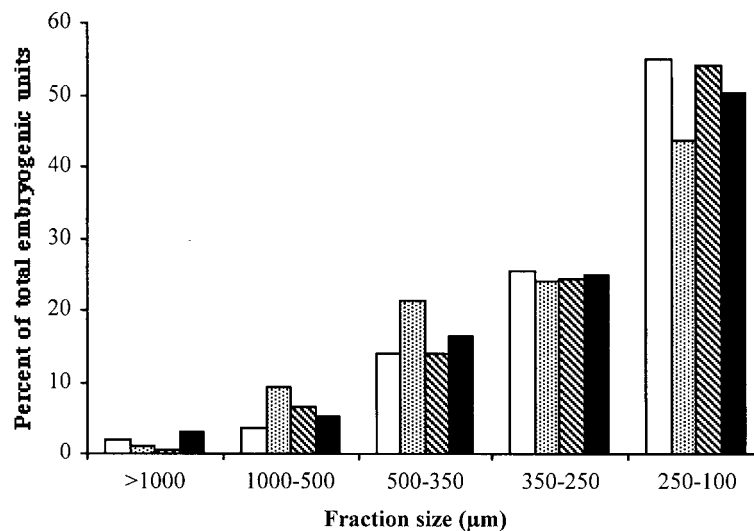


Figure 2. Size distribution of embryogenic units in suspension cultures of cassava. Embryogenic suspension tissues from cassava cultivars TMS 60142 (empty bar), Kataoli (stippled bar), M.Col 1505 (diagonal bar) and TMS 60444 (solid bar) were fractionated and the number of units retained on each sieve counted. Values shown are the mean from four separate flasks of each cultivar.

Twenty five cotyledon-stage embryos from each cultivar were tested for their ability to germinate and produce plants by subculture onto MS2 medium containing activated charcoal for seven days followed by transfer to MS2 supplemented with 5 μ M BAP. The number of germinating embryos was recorded 28 days later. The percentage of embryos forming shoots was also found to be cultivar dependent and followed the same pattern as that for embryo formation (Table 2). Thus, embryos of cvs. TMS 60444 and Line 2 germinated at high frequency, approximately 70%, while the two South American cultivars, M.Col 1505 and M.Col 22 produced plants at much lower rates of 24 and 38% respectively. Embryos from cvs. TMS 60142, TMS 90383, Kataoli and Line 11 grew and developed large green cotyledon-like structures but failed to germinate under these culture conditions.

In addition to the above, plants have also been recovered from FEC of cultivars Bonoua Rouge, M.Aus 7 and M.Tai 5 (Table 1) bringing to a total of eight the number of cultivars from which plants have been recovered from disorganised embryogenic callus of cassava.

Robust plants from TMS 60444, Line 2 and M.Col 22 have been transferred to soil and established in the greenhouse. Phenotypic variation was observed from plants regenerated from FEC which had been maintained as disorganised callus on GD medium for longer than six months. These plants grew more slowly than the controls and were characterised by

epinastic curling of their leaves and in some cases reduced apical dominance. In order to determine whether a reduction in exposure to the auxin containing medium had an effect on the quality of regenerated plants, FEC from cv. TMS 60444 which had been established as a homogenous embryogenic callus line for only eight weeks, was taken through the regeneration stages described above. Cotyledon-stage embryos were recovered and 100 of these placed on MS2 medium supplemented with 5 μ M BAP in order to induce germination. Within six weeks, 69% percent of the embryos produced a shoot and could be established and maintained as *in vitro* plantlets on MS2 medium. Transfer to soil and establishment in the greenhouse was attempted from 50 of these plantlets. In all, 29 (58%) transferred successfully and grew to produce robust plants. Strong *in vitro* plantlets which possessed stems of 3 or more millimeters in diameter and an actively growing root system, were considerably easier to transfer to soil. Of 34 such plants, 25 (74%) were successfully establish as plants in the greenhouse. Examination of the 29 independent plant lines growing in soil revealed no morphological differences within the regenerants or in comparison with control plants regenerated from micropropagated shoot cuttings (Figure 3).

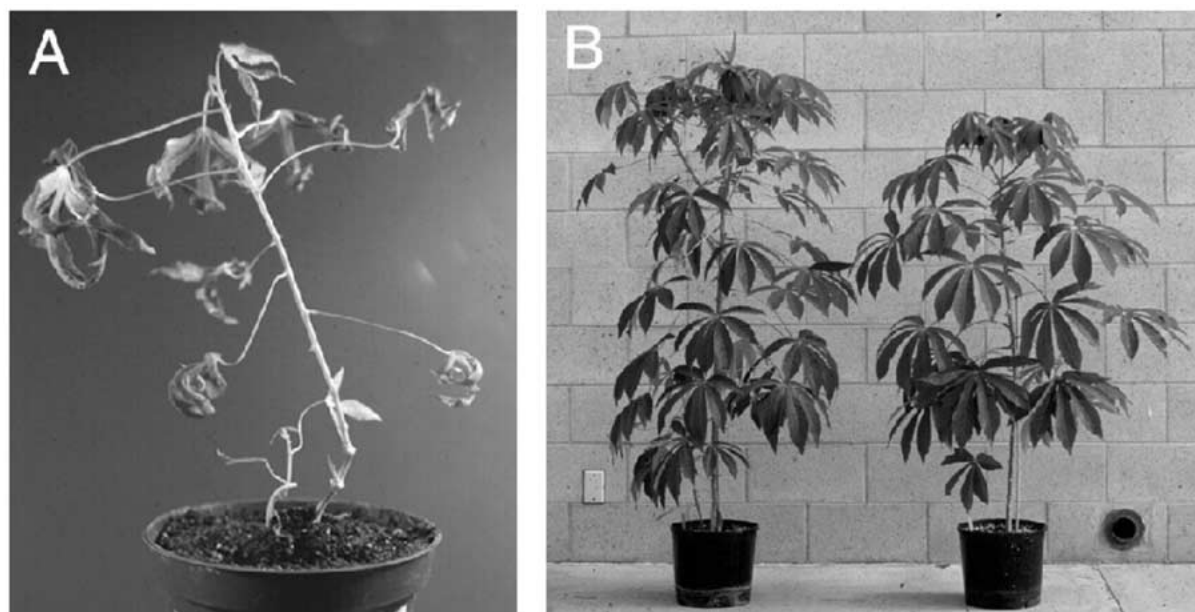


Figure 3. Cassava plants regenerated from somatic embryos of cassava cultivar TMS 60444. A: malformed plantlet derived from embryogenic suspension culture, note severe curling of the leaves, B: 12 month old cassava plants regenerated from friable embryogenic callus, these plants are 1.5 m in height and indistinguishable from controls.

Table 3. Numbers of transgenic cassava plants regenerated from microparticle bombardment of embryogenic suspension cultures

Cultivar	Transgene of interest				Total
	CsCMV CP	AC1	Δ AC1	<i>uidA</i>	
TMS 60444	29	12	29	14	84
Bonoua Rouge	–	–	10	–	10
M.Col 1505	14	–	–	–	14

Presence of the transgene was confirmed by PCR and in some cases Southern blot analysis from leaf tissues (see also Figure 4). All transgenes were contained within the pMon 977 vector and driven by the CsVMV promoter (Verdaguer et al., 1998). CsCMV CP: cassava common mosaic virus coat protein, AC1: replicase gene from African cassava mosaic virus (Hong and Stanley 1996), Δ AC1: replicase gene from African cassava mosaic virus with truncated CsVMV promoter, *uidA*: GUS visual marker gene. Primer sequences used to amplify the transgenes were as follows:

CsVMV CP	forward 5'-GAAGATCTAGGGCCACCCCTACTTCAAC-3' reverse 5'-GGGGTACTACTCATCCACTCCTGTGA-3'
AC1	forward 5'-ATGACAACTCCTCGTCCCCAGA-3' reverse 5'-CACTCTCCTACTACGGCCGGATATA-3'

Genetic transformation

Table 3 summarises the transgenic plants produced by particle bombardment of embryogenic suspension cultures at International Laboratory for Tropical Agricultural Biotechnology (ILTAB). More than 100 independent plant lines from the three cultivars TMS

60444, Bonoua Rouge and M.Col 1505 have been confirmed by PCR to contain marker genes or constructs containing transgenes for putative resistance to Cassava common mosaic virus (CsCMV) and African cassava mosaic virus (ACMV). Southern blot analysis performed on 12 of these plant lines has confirmed the integration of between 1 and 4 transgene copies. Figure 4 displays the results from four such plant lines, showing integration of the transgene into the genome of cassava. Expression of the coat protein of CsCMV has been confirmed by western analysis in nine out of 29 transgenic TMS 60444 plants which were PCR positive for the presence of this transgene (Schöpke et al., 2000).

Callus lines positive for the presence of the Δ AC1 transgene (Hong & Stanley, 1996) a promoter-less, non-transcribed version of the replicase gene from ACMV, were investigated for their regeneration potential. No significant difference was seen between the transgenic and non-transgenic control tissues, in either the number of mature cotyledon-stage embryos produced per gram of tissue, or the percentage of these embryos which were capable of germinating to produce plantlets. The frequency of plant regeneration was similar to that observed from FEC of TMS 60444 shown in Table 2. The starting material for gene insertion with the AC1 and Δ AC1 genes had

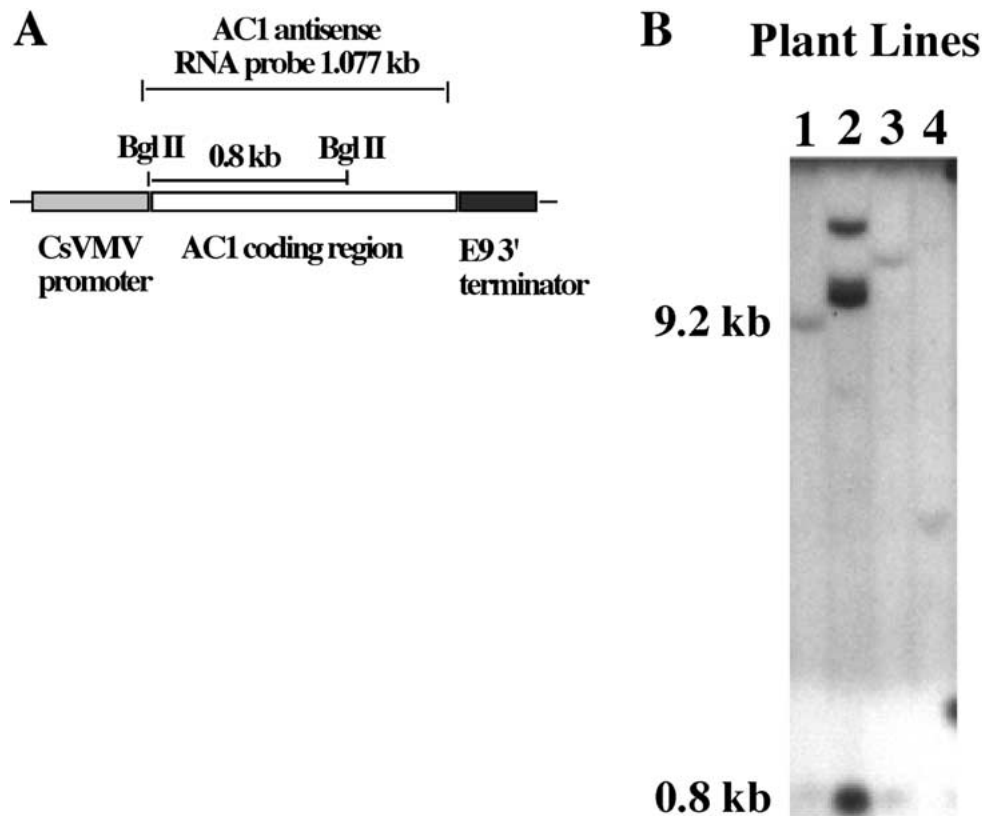


Figure 4. Southern blot analysis of four cassava plants of cv. TMS 60444 genetically transformed with the AC1 gene coding for replication associated protein (*Rep*) from African cassava mosaic virus (ACMV-Kenya). A: schematic linear representation of the probed region of the transgene showing positions of the Bgl II restriction sites. B. Southern blot of four independent transgenic plant lines showing presence of the 0.8 kb Bgl II fragment and variable sized fragments corresponding to hybridization of the probe with the 3' end of the AC1 coding sequence which indicate integration of the introduced cassette.

been maintained in suspension culture for more than six months prior to particle bombardment. The regenerated embryos were strong and germinated at high frequency (45–55%) but the resulting plants showed reduced vigour and leaf curling. Although most could be rooted in soil, these characteristics proved to be persistent and the plants did not revert to a normal phenotype in the greenhouse (Figure 3). This was also the case for the transgenic lines recovered from cv. M.Col 1505 and to a lesser extent Bonoua Rouge.

Tissues bombarded with the *uidA* marker gene under control of the CsVMV promoter (Verdageur et al., 1998) had spent only six weeks in liquid culture prior to gene insertion. From five bombarded samples 53 lines of FEC were recovered which were resistant to the antibiotic medium containing 25 μ M paromomycin. Of these, 32 callus lines were confirmed to express GUS at various levels. Fourteen independent transgenic plant lines were regenerated and are undergoing

analysis for their phenotype and GUS expression patterns. To date of the 14 plant lines ten are vigorously growing and have been established in the greenhouse.

Discussion

Much progress has been made since the initial reports of transgenic plant recovery in cassava (Schöpke et al., 1996; Raemakers et al., 1996; Li et al., 1996). By particle bombardment of embryogenic suspension tissues it is now possible to regenerate large numbers of cassava plants expressing both marker genes and genes of putative agronomic interest (Table 3).

A prerequisite for the recovery of transgenic plants for any given cultivar by the methods described in this report, is the capacity to produce embryogenic callus from that germplasm. Application of the techniques first described by Taylor et al. (1996) has enabled these elite tissues to be generated in 14 different cultivars

from diverse geographic backgrounds. Demonstration of the widespread capability to generate totipotent target tissues for use in transgenic improvement programmes is an important indication that transgenic technologies can be applied to address the agronomic improvement of cassava across diverse regional situations. Nevertheless, much future research is required. Although induction of FEC and establishment of embryogenic suspension cultures has been successful, and has shown that these tissues have significant similarities across different cultivars (Figures 1 & 2), plant regeneration remains poor or impossible from a significant proportion of the cultivars screened (Tables 1 & 2). To date, the culture systems described for the induction of, and regeneration from, FEC and embryogenic suspensions of cassava have been developed for the cultivar TMS 60444. Future efforts should be directed at adapting the culture conditions to the specific needs of other genotypes. The fact that several cultivars, for example TMS 90853 and Kataoli were able to regenerate to the torpedo or cotyledon-stage (Table 1), would indicate that significant regeneration potential exists within these, and most likely, other cultivars. The challenge is to discover what specific culture conditions are required to release this arrested state and allow full plant regeneration to take place. Focused research on this crucial area will surely result in the required breakthroughs and allow the genetic transformation of a greater range of cassava cultivars.

PCR positive, transgenic plants have been recovered from the cvs. Bonoua Rouge and M.Col 1505 in addition to TMS 60444. To date all PCR positive plants for which Southern blots have been carried out have also proved to be positive for transgene integration by the latter method. To date more than 100 independent transgenic plant lines have been recovered showing that this transformation system has significant potential for use in genetic transformation programmes.

A factor of significant importance has arisen concerning the relationship between the age of the embryogenic tissues, especially when maintained as embryogenic suspension cultures, and the quality of the subsequently regenerated plants. Narrow leaves with epinastic curling are reportedly a characteristic of herbicide drift damage from applications of 2,4-D and picloram in field-grown plants (Lozano et al., 1981). Very possibly transgenic plants and others regenerated from older embryogenic tissues which display a similar phenotype (Figure 4), are suffering from some imbalance of their endogenous auxin metabolism caused

by extended exposure to picloram in the tissue culture medium. It is now considered to be most important to minimise the number of subcultures the target tissues are exposed to, prior to transgene insertion and regeneration. To this end, friable embryogenic callus is a preferable target tissue to embryogenic suspension cultures as the latter requires longer to establish and the tissues are totally immersed in the auxin containing medium. Establishment of almost 30 plants derived from young FEC, less than two months old, in the greenhouse and which have shown no indications of somaclonal variation, indicate that this tissue will be preferable to suspension cultures for the generation of transgenic plants. Indeed, all genetic transformations at ILTAB now utilise this tissue as the target for transgene insertion.

Whilst work is still required to optimise the genetic transformation protocols, the ability to recover significant numbers of transgenic cassava plant in a routine manner indicates that the technology has advanced considerably over the last 2–3 years. The major focus for cassava biotechnology is now turning towards the study of transgene stability and expression in cassava tissues. Expression of the CsCMV coat protein gene at various levels has been shown in cassava plants genetically transformed with this gene (Schöpke et al., 2000). These and newly generated plants transgenic for the CsCMV coat protein gene are now undergoing challenge with the virus at ILTAB in order to determine whether the transgene imparts elevated resistance to the pathogen. In a similar manner, large number of plants transgenic for the AC1 gene and the defective interfering (DI) particle (Frischmuth & Stanley, 1991) from ACMV, are undergoing analysis and challenge with the respective viruses. It is hoped to transfer some of these plant lines to Africa for field testing before the end of the year 2001.

Considering the low levels of funding available for cassava biotechnology research and the small number of research groups dedicated to this activity, progress over the last ten years have been remarkable good. However, many challenges remain. Bringing more cultivars into the transformation system is essential, as is expanding the experience base for cassava genetic engineering. Only in this way can the various different constraints facing cassava in different regions be address and improved products delivered to farmers.

Acknowledgements

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