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Stable transformation of cassava (*Manihot esculenta* Crantz) by particle bombardment and by *Agrobacterium*†

C. Schöpke[1]*, N. Taylor[1], R. Cárcamo[1], A.E. González de Schöpke[1], N.K. Konan[1], P. Marmey[1], G.G. Henshaw[2], R.N. Beachy[1] & C. Fauquet[1]

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[1]International Laboratory for Tropical Agricultural Biotechnology (ILTAB/ORSTOM-TSRI), Division of Plant Biology-BCC206, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA [2]School of Biological Sciences, University of Bath, Claverton Down, Bath, BA2 7AY, UK. * corresponding author (e-mail: chriss@scripps.edu)

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Abstract/Résumé

A protocol was established for the introduction of DNA into embryogenic suspension-derived tissues of cassava via microparticle bombardment, for the selection of genetically transformed cells, and for the regeneration of fully transgenic plants from these cells. The plasmid DNA used for bombardment contained a gene encoding neomycin phosphotransferase (nptII) and a gene encoding ß-glucuronidase (*uidA*). Selection of bombarded tissue with paromomycin resulted in the establishment of putative transgenic embryogenic calli. In most of these calli ß-glucuronidase (GUS) was detected histochemically. Molecular analysis of paromomycin-resistant embryogenic calli and of plants regenerated thereof confirmed the stable integration of bombarded DNA into the cassava genome. After the establishment of a selection and regeneration protocol for microbombarded tissue, we modified this protocol to use it for *Agrobacterium*-mediated transformation of embryogenic suspensions. Embryogenic tissue was infected with two *Agrobacterium* strains containing a plasmid with the *nptII* and the *uidA* genes. Many paromomycin-resistant and GUS positive lines of embryogenic callus were recovered from the infected tissue. Some of these lines are now in the process of shoot regeneration.

Key words/Mots clés: cassava, *Manihot esculenta*, embryogenic suspensions, microbombardment, transformation

Transformation stable du manioc *(Manihot esculenta* Crantz) via le bombardement de particules et via *Agrobacterium*

La dernière décennie a vu un intérêt croissant pour la transformation geénétique du manioc. Mais la plupart des efforts ont abouti soit à la production de cals transgéniques non regénérables ou soit à la production d'embryons chimériques, a partir desquels des plants complètement transgéniques n'ont pu être obtenus. Les raisons en sont probablement le manque d'un système de sélection efficace pour les tissus transformés et l'emploi d'embryons somatiques organisés pour des expériences de transformation. Les auteurs décrivent ici l'utilisation d'un système different de régéneration mis au point récemment, c'est-à-dire des suspensions cellulaires embryogènes, en conjonction avec le microbombardement de particules enrobées d'ADN ou avec la transformation via *Agrobacterium*. Pour le microbombardement, des particules d'or (1.0 ,um) ont été enrobées d'un plasmide contenant a la fois le gène *gus* (marqueur visible) et le gène *npt II (marqueur de selection)*. Le bombardement a été fait au moyen du Particle Delivery System 1000/He (BioRad). Les tissus bombardes ont été mis en culture pendant 10-14 jours dans un milieu liquide contenant 50 µM de picloram. Des tissus résistants a un antibiotique ont été ensuite selectionnés avec la paromomycine (25 µM). Le développement des embryons matures a été induit en transférant les tissus transformés sur un milieu ayant une concentration réduite en picloram (5 µM), suivi d'un traitement avec du NAA (5 µM) et une période de croissance sur un milieu contenant 0.5% de charbon actif. Un traitement avec du BAP (4.4 µM) a induit le développément de parties aériennes à partir de ces embryons. Eventuellement, des plantes ont été régénérées à partir de plusieurs lignées transformées. La plupart des tissus résistants à la paromomycine et les plantes qui en sont dérivées ont donné des résultats positifs lors des essais histologiques pour repérer l'expression du gène *gus*. L'intégration de l'ADN introduit dans le génome du manioc a été confirmée par l'analyse de Southern des tissus embryogènes et des plantes. La transformation via *Agrobacterium* des suspensions cellulaires embryogènes a été effectuée en utilisant des procédes standards. La souche ABI *d'Agrobacterium* contenant le plasmide pMON977 avec un gène *gus* contenant un intron et le gène *npt II* a été utilisée. La procédure de sélection et de régénération ont été similaires a celles utilisées pour les tissus microbombardés. A ce jour, des embryons verts capables d'exprimer le gène *gus* ont été obtenus. Le repiquage ultérieur sur des milieux d'induction de parties aériennes devrait aboutir à la régénération de plantes transgeniques.

Introduction

To promote the genetic improvement of cassava it has been recommended that genetic transformation of cassava be considered a research priority (Roca & Thro 1993). Areas in which transformation might complement, or even circumvent, traditional cassava breeding programs include increased resistance to pests and diseases (Fauquet et al. 1992), improved starch quality (Salehuzzaman et al. 1993), and reduction of cyanogenic glucosides in the edible tubers (Koch et al. 1994). The limiting factor for the improvement of cassava by genetic engineering, however, has been the lack of a reproducible transformation system to facilitate the introduction of foreign genes into this crop.

A method for regeneration of cassava plants through somatic embryogenesis has been available since 1982 (Stamp & Henshaw). However, the use of the embryogenic structures generated by this culture system as target tissues for genetic transformation via *Agrobacterium* (Calderón 1988, Schöpke et al. 1993a), particle bombardment (Schöpke et al. 1993b)

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and electroporation (Luong et al. 1995) has yielded at best only chimeric embryos. Recently an alternative regeneration system was developed (Taylor et al. 1996), in which clusters of embryogenic cells are suspended in liquid medium (hereafter referred to as embryogenic suspensions). With regard to accessibility of regenerable cells and selection procedures these suspensions are far more suitable for genetic transformation protocols. Here we describe the use of such cultures for the genetic transformation and regeneration of the first confirmed transgenic plants of cassava (Schöpke et al. 1996). In addition, results on the use of embryogenic suspensions for *Agrobacterium*-mediated transformation are reported.

Materials and Methods

Plant material and culture media. Embryogenic suspensions were initiated from a six months old line of embryogenic callus of cassava cultivar TMS 60444 and maintained as described by Taylor et al. (1996). Suspension cultures were used 18 to 22 months (particle bombardment) or 36 to 40 months (*Agrobacterium*-mediated transformation) after their initiation. The following culture media were used: SH-1, SH salts (Schenk & Hildebrandt 1972), MS vitamins (Murashige & Skoog 1962), 50 µM picloram, and 60 g/l sucrose; SH-2, SH-1 with 7.5 g/l Difco-Bacto agar; R-1, MS-salts (Murashige & Skoog 1962), MS vitamins, 20 g/l sucrose, 7.5 g/l Difco-Bacto agar, picloram 5 µM; R-2, as R-1, but picloram replaced by 5 µM α-naphthalene acetic acid (NAA); MM, MS salts, no growth regulators, 5 g/l activated charcoal, 30 g/l sucrose; SD, as R-1, but 4.44 µM BAP instead of picloram; SP, MS-salts and vitamins, 20 g/l sucrose, 2 g/l phytagel (Sigma), no growth regulators. All media were adjusted to pH 5.7 prior to autoclaving.

Effect of antibiotics on non-transformed embryogenic suspensions. Embryogenic suspensions were sieved to isolate cell clusters 250–350 µm in diameter. Aliquots of 5,000 units were transferred to 15 ml graduated centrifuge tubes containing 5 ml SH-1 medium with antibiotic. The medium was buffered with 0.5 g/l 2-[N-morpholino]ethanesulfonic acid (MES). Stock solutions of the antibiotics kanamycin, geneticin, paromomycin, hygromycin, and the herbicide phosphinotricin were filter-sterilized and aliquots added to the autoclaved culture medium to obtain concentrations of 25, 50, 100, 200, and 300 µM. Controls were cultured without antibiotics. Four replicate tubes per treatment were placed in a tissue culture rotator (New Brunswick Scientific, USA) and rotated at 10 rpm. After seven days samples containing between 300 and 400 embryogenic cell clusters were removed from each tube and their viability was assessed with the vital dye fluorescein diacetate (FDA; Widholm 1972). Samples were stained for ten minutes in SH-1 medium with 0.05% (w/v) FDA. They were viewed under an Olympus IMT2 inverted microscope (10 x objective) with epifluorescence attachment and with a FITC filter set and scored for their viability. An embryogenic cell cluster was assessed as alive if it gave a strong yellow fluorescent signal

Particle bombardment. Embryogenic suspensions were initiated with 1 ml SCV in 50 ml SH-1 medium. The medium was renewed every 2 days. Cell clusters 250–500 µm in size from 12–14 day old embryogenic suspensions were used for bombardments. Aliquots of 200 µl SCV in a volume of 1 ml culture medium were pipetted onto polypropylene grids (Spectra/Mesh #146428, opening 210 µm) in such a way that the liquid formed a drop kept in place by surface tension. The grids were placed in petri dishes on top of a dry filter paper. Immediately before bombardment, the liquid was absorbed from the droplet, leaving a monolayer of embryogenic cell clusters on the grid. Gold particles of 1.0 µm diameter (BioRad; USA) were coated with plasmid DNA (pILTAB313, containing the *nptII* gene with the nos promoter and the *uidA* gene with the enhanced 35S promoter; Schöpke et al. 1996) according to Sivamani et al. (1996). Five µl of the particle-DNA suspension were distributed onto each macrocarrier (BioRad) and kept in a desiccator until used. Bombardment took place with of the Particle Delivery System PDS1000/He (BioRad) following the manufacturer's recommendations. The dishes with tissue were placed at a distance of 9 cm from the rupture disk retaining cap and the coated microparticles were accelerated using a pressure of 1,100 psi in a partial vacuum (9.1 kPa abs. pressure). Each sample was bombarded twice. To prevent desiccation of the tissue between bombardments the mesh was placed in a dish containing sufficient SH-1 liquid medium to just cover the tissue.

Selection of transformed tissue and regeneration of plants. The general protocol for the selection and regeneration is shown in Figure 1. After bombardment, the tissue was cultured for 10-14 days in SH-1 medium without antibiotic followed by a selection phase (4-5 weeks in SH-1 + 25 µM paromomycin). Surviving pieces of yellowish tissue were subcultured individually on solidified SH-2 medium with 25 µM paromomycin. Under these conditions, antibiotic-resistant tissue developed into yellowish, friable embryogenic callus indistinguishable from non-transformed embryogenic callus. The times for the following steps leading to embryo development, shoot induction and finally plant regeneration varied between experiments, as indicated in Figure 1. In the experiment that resulted in the first transgenic plants the initial growth phase without antibiotics was three days.

DNA isolation and Southern analysis of putative transgenic tissue. Genomic DNA was isolated according to Dellaporta et al. (1983). DNAs were digested either with PstI or HindIII restriction endonucleases (Gibco-BRL, Gaithersburg, MD). Undigested and digested DNAs (5 µg per well) were loaded on a 0.8% agarose gel and electrophoresis was carried out at 5 volt/cm for 6 hours. DNAs were then transferred onto a Hybond-N+nylon membrane (Amersham, Arlington Heights, IL) according to the instructions of the manufacturer. The *uidA* probe was a purified PCR product (size approx. 1.8 kb) that was synthesized with primers specific for sequences in the *uidA* coding region and with pILTAB313 as template. Labeling was done by random 9-mer priming with Exo-klenow (Stratagene, La Jolla, CA) and [a-32P]dCTP. Hybridization was carried out at 65°C in hybridization buffer (3 x SSC, 2 x Denhardt solution, 0.1% SDS, 6% PEG) overnight. The final wash was done at 65°C with (0.1 x SSC; 0.1% SDS). The membrane was then exposed to X-ray film overnight.

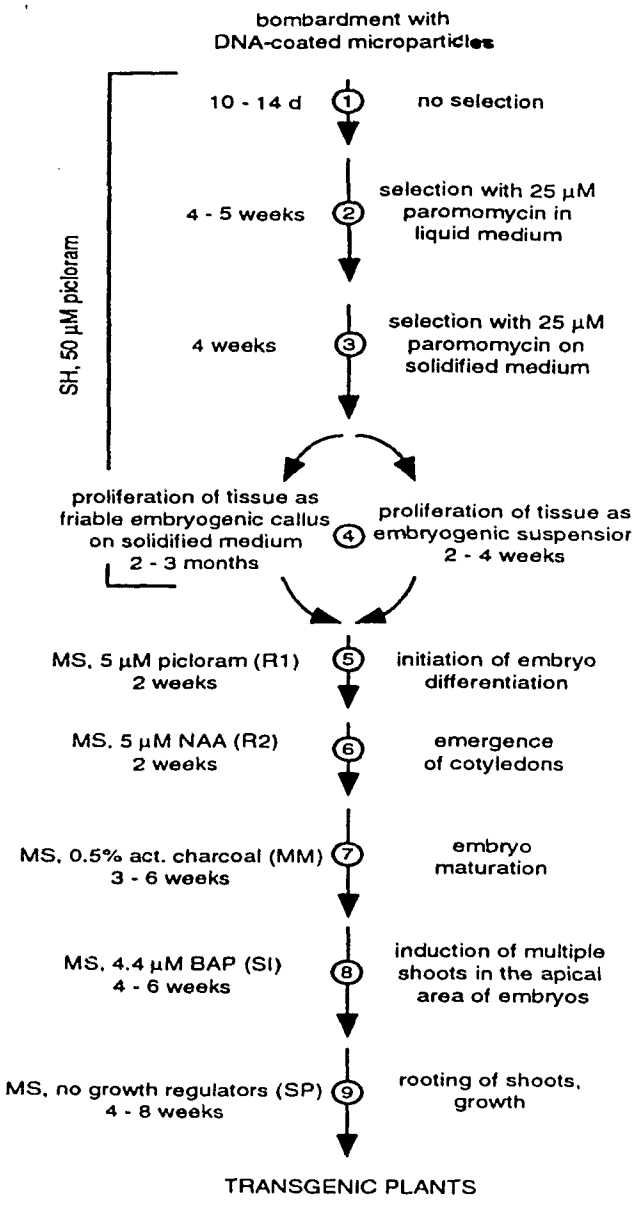

Figure 1. Flow diagram of the steps leading from microbombarded embryogenic suspensions of cassava to transgenic plants.

***Agrobacterium*-mediated transformation.** The embryogenic suspensions used for *Agrobacterium*-mediated transformation were cultured as described for particle bombardment. Embryogenic tissue was inoculated and cocultured with *Agrobacteria* containing a plasmid with the *nptII* and the *uidA* genes. The *uidA* gene had an intron to prevent it from being expressed in *Agrobacterium* (González de Schöpke, in preparation). Except for the addition of antibiotics to kill *Agrobacterium* remaining in the tissue after the transformation, the tissue was subcultured essentially as described for microbombardment-derived tissue (Figure 2).

Histochemical assay for ß-glucuronidase (GUS). A modification of the assay by Jefferson (1987) was used in which the assay buffer included 0.08 M sodium phosphate buffer at pH 7.0, 7.7 mM X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide cyclohexylammonium salt), 20% (v/v) methanol and 0.16% Triton X 100. The oxidation catalysts potassium ferricyanide and potassium ferrocyanide were added to the buffer at 6.4 mM for embryogenic suspensions and 0.64 mM for roots, tubers, stems and leaves. Tissues were covered with assay buffer and kept for 2 h (embryogenic suspensions) or 16 h (stems, leaves, roots, tubers) at 37°C in darkness. Assays were stopped by washing the tissue several times with water. For clearing and long-time storage the tissue then was transferred to 70% ethanol.

Results and Discussion

The choice of a selectable marker. Genes that are frequently used to select transformed plant tissues include *nptII*, *hph* and *bar*, encoding neomycin phosphotransferase, hygromycin phosphotransferase, and phosphinotricin acetyl transferase, respectively. These genes confer resistance to kanamycin and related aminoglycosides, to hygromycin, and to phosphinotricin. In order to determine the usefulness of each of these genes for the selection of transformed embryogenic tissue of cassava, killing curves were established for embryogenic suspensions with a variety of antibiotics. In Figure 2 the results for three antibiotics against which *nptII* confers resistance are summarized. After one week of growth in culture medium with antibiotic, kanamycin (25–300 µM) had no effect on the viability of the embryogenic suspensions, geneticin killed all cells at 25 µM, while paromomycin reduced the viability to 4.5% at 25 µM and to zero at 200 µM. Both hygromycin and phosphinotricin were inefficient in the concentration range tested: 7.9% and 7.3% of the embryogenic cell clusters survived treatments with 300 µM hygromycin and phosphinotricin, respectively. Therefore, we decided to use the *nptII* gene in transformation studies and to use geneticin and paromomycin as selective agents.

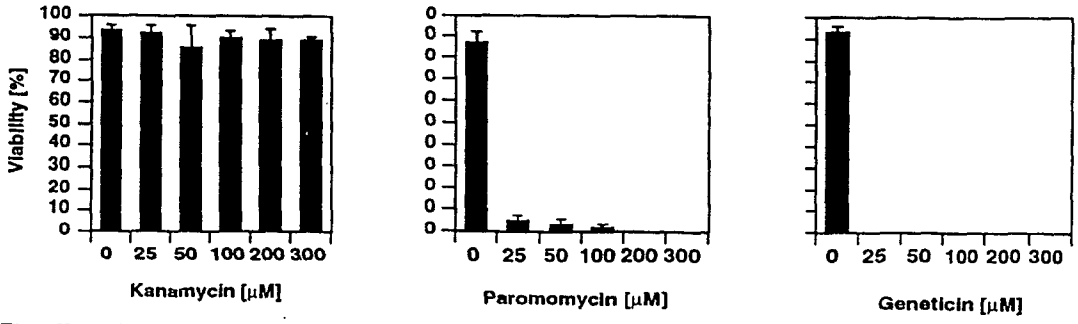

Figure 2. The effect of kanamycin, paromomycin and geneticin on the viability of embryogenic suspensions of cassava. Embryogenic suspensions were cultured in SH-1 medium with different concentrations of kanamycin, paromomycin and geneticin. After one week viability was assessed with the fluorescein diacetate test. Four replicates per treatment with 300-400 embryogenic units per replicate were scored. Viability is expressed as the average percentage (± SD) of embryogenic units that show bright yellow fluorescence under UV light.

Particle bombardment

Establishment of paromomycin-resistant cell lines and plant regeneration. The protocol for the steps from microbombardment of embryogenic suspensions to plant regeneration is summarized in Figure 1. An initial growth phase in the absence of antibiotic is followed by 4-5 weeks selection with paromomycin, after which most of the tissue is killed. However, under a stereomicroscope, small (0.5-1.5 mm), yellowish embryogenic units can be distinguished among white, dead units (Figure 3B). Their number ranges from 20 to 100 per bombarded tissue sample. About one third of these units continue to grow when cultured individually on solidified selection medium with 25 µM paromomycin (Figure 1, step 3) and produce friable, embryogenic callus.

To increase the chance of plant regeneration, paromomycin-resistant embryogenic tissue was amplified either by culture in liquid SH-1 medium or on solidified SH-2 (Figure 1, step 4). In liquid medium the volume of tissue doubles within two to four days if the culture medium is renewed every two days. On solidified medium the growth rate is much slower, i.e., the volume of tissue doubles within three to four weeks.

Using the protocol outlined in Figure 1, 18 independently transformed lines were established from embryogenic callus or suspensions, 16 of which were GUS-positive in histological

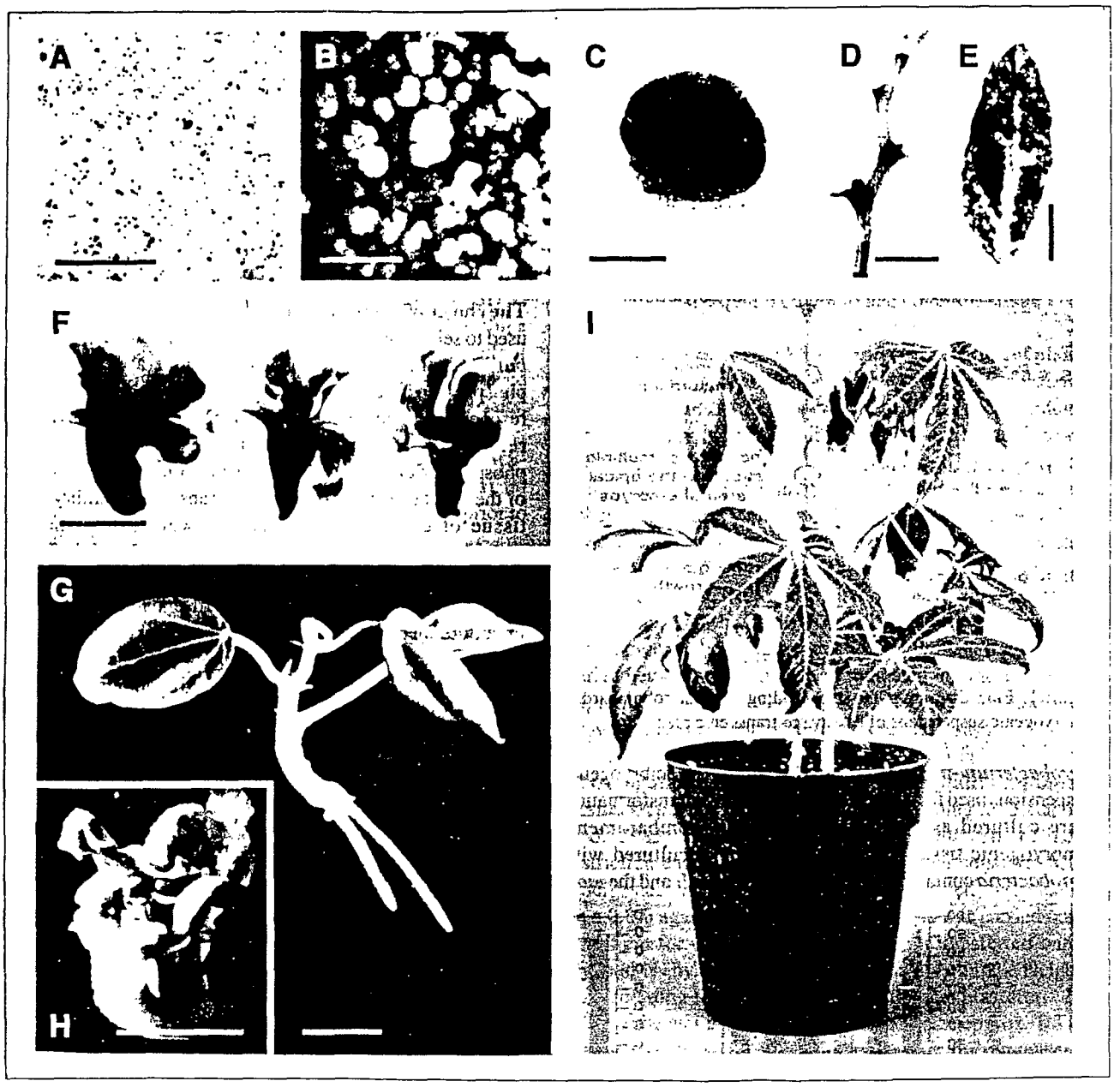

Figure 3. Regeneration of transgenic plants from microbombarded embryogenic suspensions of cassava. (A) Embryogenic units with GUS-positive cells, 3 days after bombardment (a.b.). (B) Sample of an embryogenic suspension that was cultured without antibiotic for ten days a.b. followed by 4 weeks in medium with 25 µM paromomycin. Note the difference between yellowish, growing pieces of tissue and the white tissue that did not survive selection. (C) GUS-positive embryoid, 4 weeks a.b. (D), (E) GUS expression in a stem and a leaf, respectively, of plant line 44.1.9, one year a.b. (F) GUS-positive embryos, 10 months a.b. (G), (H) Plantlet and organogenic tissue from which it developed, 6 and 5 1/2 months a.b. (I) Transgenic plant of line 44.3, 14 months a.b. Bars in (A) and (B) 2 mm; in (C) 50 µM; in (D) 5 mm; in (E) and (F) 2.5 mm; in (G) and (H) 0.5 cm.

assays. Attempts were made to regenerate plants from seven of the lines. From all lines embryos with green cotyledons were recovered (Figure 1, steps 5-7). Transfer of these embryos to medium with 4.4 µM benzylaminopurine (BAP) induced single or multiple shoot formation (Figure 3H). Subsequent culture of 1-2 cm long shoots in medium without growth regulators led to rooting and plantlet development (Figure 3G). Shoot formation has been induced in six lines coming from three independent bombardments. Plantlets with a shoot length greater than 5 cm from two of these lines were grown in vitro on SP medium without growth regulators, and plants from two other lines were transferred to soil for growth in a greenhouse (line 44.3, Figure 3I, and line 62.12). A limiting step in the regeneration process is the development of small shoots into vigorously growing plantlets. Once plantlets have formed a root and shoot system comparable to non-transformed controls, the success rate of transfer to soil is close to 100%. However, we observed considerable variation in the number of transferable plantlets derived from different lines of embryogenic tissue (from zero to over thirty).

Southern blot analysis. The fact that paromomycin-resistant embryogenic tissue was recovered and that most of the lines derived from resistant tissue were GUS-positive in histological assays strongly suggested the stable integration of at least the *nptII* gene, and in most cases of both the *nptII* and the *uidA* gene. To verify this conclusion, Southern blot hybridization analyses were performed both with putative transgenic embryogenic suspensions and with plants regenerated from such suspensions. Figure 4A shows the result of a hybridization reaction of genomic DNA from four GUS-positive lines of embryogenic suspensions derived from three different bombardment experiments. In all lines the radiolabeled *uidA* probe hybridized with undigested high molecular weight DNA (Figure 4A; lanes 3, 5, 7, 9). The undigested DNA of the non-transgenic control showed no hybridization with the probe. This confirms the integration of the transforming DNA into the chromosomal DNA. The digestion of genomic DNA with PstI (to release the complete 35S-uidA-7S cassette) produced different banding patterns for each of the suspension cultures, as expected if integration of the introduced DNA occurred at random sites (Figure 4A; lanes 4, 6, 8, 10). Furthermore, the digested DNA from all suspensions contained a fragment corresponding to the expected size (3 kb) of the intact *uidA* gene cassette. It is presumed that other bands of hybridization represent integration of fragments of the *uidA* gene. The banding patterns of DNA coming from two randomly selected embryogenic suspensions that are derived from the same bombardment experiment, but from different selected tissue pieces (Figure 4A; lanes 3, 4 and 9, 10) show that the individual tissue pieces collected at the end of the initial selection phase (see Figure 1, step 2) are the result of different transformation events. This is supported by a Southern blot analysis of DNA derived from five independently established embryogenic suspensions from another bombardment experiment (data not shown), which resulted in five different DNA hybridization patterns. Therefore, the probability of obtaining siblings from different antibiotic-selected tissue pieces coming from the same suspension culture is negligible.

In one experiment more than thirty plantlets were regenerated from a GUS-negative embryogenic callus derived from a single selected tissue piece (line 44.3). Southern blot analysis was performed using leaf DNA of four of these plantlets (Figure 4B, lanes 5-16). All DNAs showed an identical hybridization pattern when digested with PstI or HindIII, proving that the plantlets were siblings. As expected, DNA taken from GUS-negative plantlets did not contain the intact *uidA* gene cassette. Instead, the *uidA* probe bound to a

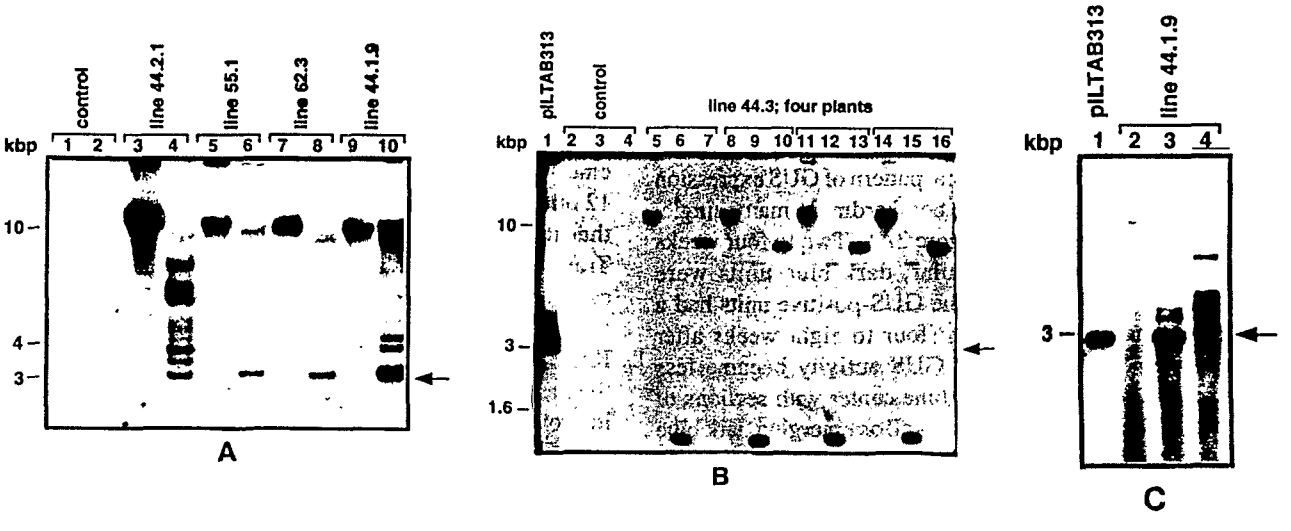

Figure 4. Southern blot analysis of total genomic DNA from putative transgenic embryogenic suspensions and plants. (A) DNA from embryogenic suspension (5 µg DNA per lane). Lanes 1, 2: DNA from non-bombarded control suspension; lanes 3, 4 and 9, 10: DNA of independently established suspensions derived from the same bombardment (lines 44.2.1 and 44.1.9); lanes 5-8: suspensions derived from different bombardments (lines 55.1 and 62.3). Lanes with odd numbers: undigested DNA; lanes with even numbers: DNA digested with PstI. (B) DNA from GUS-negative plants derived from a single selected putative transformed piece of tissue (line 44.3). Lane 1: pILTAB313 digested with PstI; lanes 2-4: DNA of an untransformed control plant; lanes 4-16: DNA of four transformed plants, 3 lanes per plant. Lanes 2, 5, 8, 11, 14: undigested DNA; lanes 3, 6, 9, 12, 15: DNA digested with PstI; lanes 4, 7, 10, 13, 16: DNA digested with HindIII. (C) DNA from a GUS-positive plantlet (line 44.1.9). Lane 1: pILTAB313 digested with PstI; lane 2: undigested; lane 3: DNA digested with PstI; lane 4: DNA digested with HindIII. Arrows indicate the size of the expected hybridization product.

fragment of about 1.4 kb in size (Figure 4B, lanes 6, 9, 12, 15), indicating that a portion of the *uidA* gene was deleted during the transformation event or during regeneration.

Southern blot analysis of leaf DNA from a plantlet derived from a GUS-positive embryogenic suspension revealed integration of the intact uidA gene cassette (Figure 4C). The identity of the hybridization patterns of DNA from this plantlet and of the embryogenic suspension from which it had been regenerated (Figure 4A, lane 10) demonstrates the stability of the integrated gene during the course of development from embryogenic cell to plantlet.

***Agrobacterium*-mediated transformation.** With the exception of the antibiotic treatment necessary to eliminate *Agrobacterium*, transformed tissue was subjected to essentially the same regeneration protocol that was used for tissue derived from particle bombardment. In most experiments, the number of paromomycin-resistant lines of embryogenic callus was in the range of 10 to 20 per 0.2 ml (SCV) tissue inoculated with *Agrobacterium* (González de Schöpke et al. in preparation). The transformation efficiency measured as the number of paromomycin-resistant lines of embryogenic callus per volume of tissue used for the a transformation experiment therefore is comparable to the efficiency achieved through particle bombardment. Several paromomycin-resistant, GUS positive lines of *Agrobacterium*-transformed embryogenic callus formed embryos and are now in the stage of shoot regeneration.

Genetic transformation of cassava through *Agrobacterium* has been demonstrated recently by Li et al. (1996). These authors used an organogenic system involving cotyledons from somatic embryos. At this moment it is difficult to say whether this system or the use of embryogenic suspensions in conjunction with particle bombardment or *Agrobacterium*-mediated transformation will eventually be more practical. However, because a method that works well in one cassava cultivar might not be applicable to another, the availability of different transformation methods is advantageous.

GUS expression in transgenic cassava tissues. During the course of culture of tissues subsequent to bombardment with pILTAB313, the following general pattern of GUS expression was observed: three days after bombardment many single, dark blue cells were visible (Figure 3A). Two to four weeks after bombardment multicellular, dark blue units were observed (Figure 3C). When the GUS-positive units had a size larger than about 200 µm (four to eight weeks after bombardment), the pattern of GUS activity became less uniform. The units had a light blue center with sections of dark blue cells on their surface, as well as emerging dark blue secondary units.

Up to this point all of the GUS-positive embryogenic lines showed similar staining patterns. However, during the subsequent series of steps differences between lines became apparent. In some lines emerging cotyledons (Figure 2, step 6) stained completely blue (Figure 3F), while in other lines the stain was restricted to veins and stomata. At this stage the root pole usually stained blue. In six lines shoot formation could be induced. Four of these were GUS positive, with blue stain restricted mainly to vascular tissues and to leaves (Figure 3D, 3E). Blue stain was always detected in all leaves of a plantlet, as well as in vascular tissue from the shoot base up to the tip. In two lines no GUS expression was detected with the histological assay.

Histological GUS assays performed with tissues derived from a 50 cm tall transgenic cassava plant growing in soil (line 62.12) revealed GUS expression in leaves, stems, roots, and tubers. The staining patterns depend on the composition of the assay buffer. Inclusion of 6.4 mM ferri- and ferrocyanide leads to the formation of dark blue stain in xylem parenchyma and lactifers, and to a lighter blue stain in the epidermis. All other cell types (depending on the age of the sample) stain only light to very light blue. Most of the development of the blue stain is finished after 2 h, since prolonged exposure of tissue to the assay buffer does not lead to great changes. In buffer with 0.64 mM ferri- and ferrocyanide, most cells stain with a blue of more or less the same intensity, which depends on the age of the tissue (e.g., younger tissues produce a darker blue than older ones) and on the incubation time. The stain also appears as precipitate in the staining buffer.

The reason for the differences in staining patterns lie in the catalytic properties of ferri- and ferrocyanide. When the substrate X-Gluc is added to a sample that contains ß-glucuronidase, in a first reaction X-Gluc is cleaved into glucuronic acid and X (= 5-bromo-4-chloroindoxyl), whose subsequent oxidative dimerization leads to the blue indigo stain. The monomer X is water soluble and therefore can diffuse, while the blue stain is insoluble and remains at the site where it was formed. Oxidation catalysts such as ferri- and ferrocyanide enhance the speed of dimerization and so reduce the diffusion of X, which leads to a clearer distinction between cells with a high level of GUS expression and cells with a low expression.

Conclusions

We have developed a method for the recovery of genetically transformed cassava plants of cv. TMS 60444 via microparticle bombardment. The use of embryogenic suspensions as target tissue and the use of paromomycin for the selection of transformed tissues were crucial for this success. Since embryogenic calli and embryogenic suspensions of at least 12 other cultivars are available (data not shown), we expect that the method should be applicable to other cultivars. Transient expression studies have shown that the *uidA* gene can be expressed with comparable efficiency in tissue derived from embryogenic suspensions of the cultivars Bonoua Rouge, Kataoli, Okouta, and TMS 60142. Although the efficiency of the transformation and regeneration procedures can likely be improved, we think that these methods can be applied to transform cassava with genes that are expected to be beneficial to cassava production. Experiments with the aim to transform embryogenic suspensions through *Agrobacterium* thus far have resulted in GUS positive shoots, and we expect to obtain fully transgenic plants from these shoots in the near future.

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