Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) from microbombarded embryogenic suspension cultures

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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 was detected histochemically. Molecular analysis of paromomycin-resistant embryogenic calli and of plants regenerated from these calli, confirmed the stable integration of bombarded DNA into the cassava genome.

Keywords: *Manihot esculenta*, embryogenic suspensions, microbombardment, transformation

Cassava (*Manihot esculenta* Crantz) is an important source of dietary carbohydrates in many tropical countries, especially in Africa. Overall world production in 1994 was estimated to be as much as 158 million tons[1]. Despite the importance of cassava for the livelihood of millions of people it was, until recently, considered a neglected crop[2]. To promote the genetic improvement of cassava it has been recommended that genetic transformation of cassava be considered a research priority[3]. Areas in which transformation might complement, or even circumvent, traditional cassava breeding programs include increased resistance to pests and diseases[4], improved starch quality[5], and reduction of cyanogenic glucosides in the edible tubers[6]. Improvement of cassava by genetic engineering, however, has been limited by 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[7]. However, the use of the embryogenic structures generated by this culture system as target tissues for genetic transformation via *Agrobacterium*[8,9], particle bombardment[10], and electroporation[11] has yielded, at best, only chimeric embryos. Recently, an alternative regeneration system was developed[12] 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. We describe the use of such cultures for the genetic transformation and regeneration of the first confirmed transgenic plants of cassava.

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. The results for three antibiotics against which *nptII* confers resistance are summarized in Figure 1. After 1 week of growth in culture medium with antibiotic, kanamycin (25 µM to 300 µM) had no effect on the viability of the embryogenic suspensions, 25 µM geneticin killed all cells, while 25 µM paromomycin reduced the viability to 4.5% 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 300 µM phosphinotricin, respectively. Therefore, we decided to use the *nptII* gene in transformation studies and to use geneticin and paromomycin as selective agents.

Establishment of paromomycin-resistant cell lines. Geneticin and paromomycin were compared for their ability to select transformed tissue. Tissue from embryogenic suspensions was bombarded with pILTAB313 (Fig. 4A), a plasmid that contains the *nptII* gene as selectable marker and the *uidA* gene (also referred to as *gus* gene) as a reporter. One month after bombard-

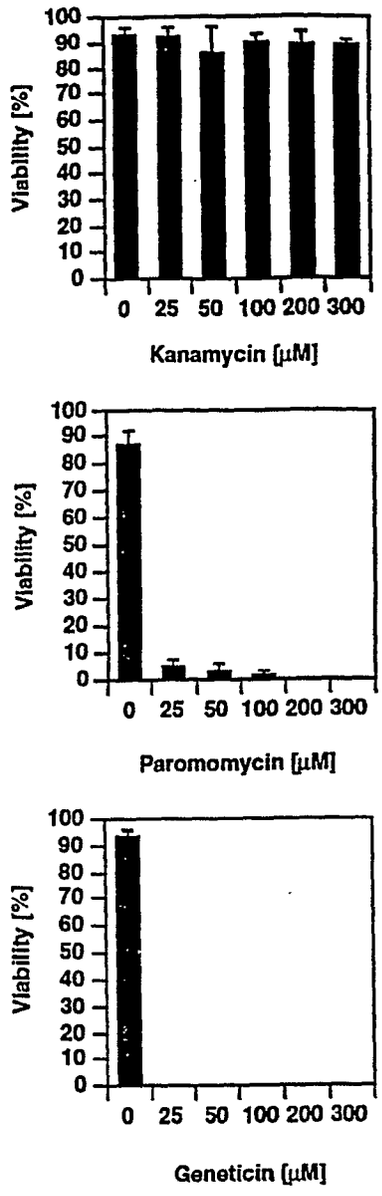

Figure 1. The effect of kanamycin, paromomycin, and geneticin on the viability of embryogenic suspensions of cassava. Embryogenic suspensions were cultured in SH-1 medium with different concentrations of kanamycin, paromomycin, and geneticin. After 1 week, viability was assessed with the fluorescein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

ment (the tissue had been treated with antibiotic for 3 weeks and then moved to medium without antibiotic) histological β-glucuronidase (GUS) assays revealed single GUS-positive, dark blue cells in all treatments, but there was a higher proportion of blue cell clusters in the suspension cultures treated with paromomycin versus those treated with geneticin (about 100 per bombarded sample of 0.2 ml settled cell volume (SCV) after selection with paromomycin versus about 10 after selection with geneticin). Furthermore, few tissue pieces per bombarded sample were recovered several weeks after selection in medium with geneticin (0 at 25 μM, four pieces at 50 μM). Selection with paromomycin resulted in a sixfold greater success (24 pieces at 12.5 μM, 28 pieces at 25 μM).

For all of the following experiments, 25 μM of paromomycin were chosen to select resistant cells following bombardment of embryogenic tissues with the *nptII* gene. The protocol for the steps from microbombardment of embryogenic suspensions to plant regeneration is summarized in Figure 2. An initial growth phase in the absence of antibiotic is followed by 4 to 5 weeks' selection with paromomycin, after which most of the tissue is killed. However, under a stereomicroscope, small (0.5 to 1.5 mm), yellowish embryogenic units can be distinguished among white, dead units (Fig. 3B). Their numbers range 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 (Fig. 2, step 3) and produce friable, embryogenic callus.

In previous attempts to transform cassava cells, the formation of transgenic callus was achieved using the *nptII* gene as selectable marker[9]. However, plant regeneration from kanamycin-selected tissue was not reported. Our results with nontransformed embryogenic suspensions support the conclusion that kanamycin is not a suitable selective agent for cassava tissues. In addition, we found that only paromomycin was efficient in killing nontransformed embryogenic cells while permitting transformed cells to develop into embryogenic callus. Paromomycin has not been used extensively in tissue culture, although it has been reported to be beneficial for the selection of transformed sunflower tissues[13] and for the generation of transgenic oat[14].

Plant regeneration from paromomycin-resistant embryogenic tissue. It has been shown that plants can be regenerated from embryogenic suspensions of cassava[12]. 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 (Fig. 2, step 4). In liquid medium the volume of tissue doubles within 2 to 4 days if the culture medium is renewed every 2 days. On solidified medium the growth rate is much slower: The volume of tissue doubles within 3 to 4 weeks.

Using the protocol outlined in Figure 2, 18 independently transformed lines were established from embryogenic callus or suspensions, 16 of which were GUS-positive in histological assays. Attempts were made to regenerate plants from seven of the lines. From all lines, embryos with green cotyledons were recovered (Fig. 2, steps 5 to 7). Transfer of these embryos to medium with 4.4 μM benzylaminopurine (BAP) induced single or multiple shoot formation (Fig. 3H). Subsequent culture of 1- to 2-cm-long shoots in medium without growth regulators led to rooting and plantlet development (Fig. 3G). To date, shoot formation has been induced in six lines coming from three independent bombardments. Plantlets with a shoot length >5 cm from two of these lines were grown in vitro on SP medium without growth regulators, and plants from another line were transferred to soil for growth in a greenhouse (Fig. 3I; line 44.3). 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 nontransformed 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 >30).

GUS expression in transgenic cassava tissues. During the course of culture of tissues subsequent to bombardment with pIITAB313, the following general pattern of GUS expression was observed: 3 days after bombardment many single, dark blue cells were visible (Fig. 3A). Two to 4 weeks after bombardment, multicellular, dark blue units were observed (Fig. 3C). When the GUS-positive units had a size larger than about 200 μm (4 to 8 weeks after bombardment), the pattern of GUS activity became less uniform. The units had a light blue center with sections of

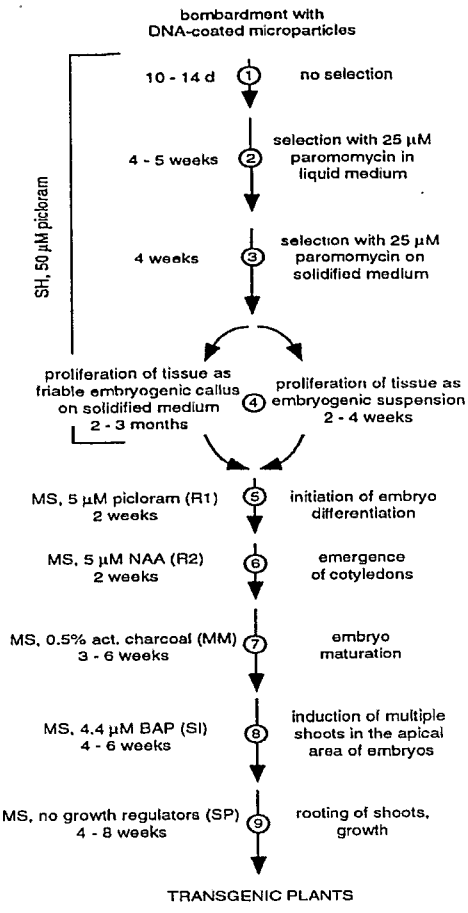

Figure 2. Flow diagram of the steps leading from microbombarded embryogenic suspensions of cassava to transgenic plants.

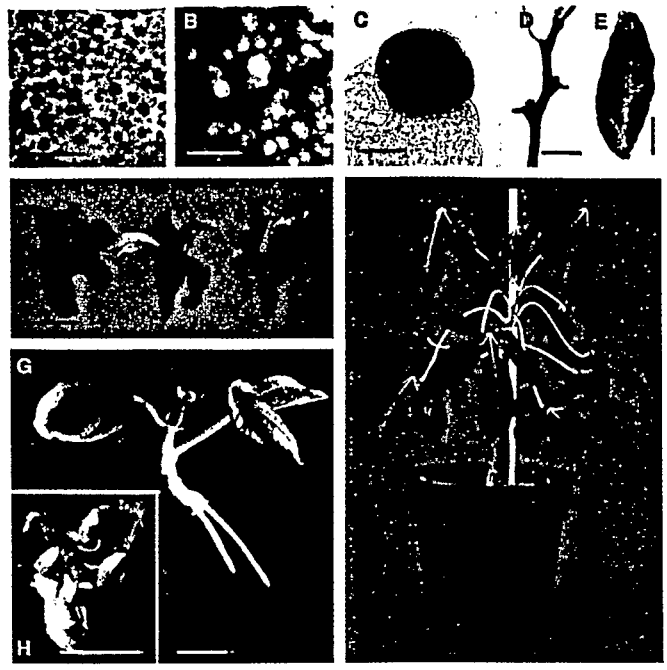

Figure 3. Regeneration of transgenic plants from microbombardment embryogenic suspensions of cassava. (A) Embryogenic units with GUS-positive cells, 3 days after bombardment (a.b.). (B) Sample of an embryogenic suspension that was cultured without antibiotic for 10 days a.b. followed by 4 weeks in medium 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 1 year a.b. (F) GUS-positive embryos, 10 months a.b. (G), (H) Plantlet and organogenic tissue from which it developed, 6 and 5½ months a.b. (I) Transgenic plant of line 44.3, 14 months a.b. Bars in (A) and (B) are 2 mm; in (C) 50 µm; in (D) 5 mm; in (E) and (F) 2.5 mm; in (G) and (H) 0.5 cm.

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 (Fig. 2, step 6) stained completely blue (Fig. 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 (Figs. 3D and 3E). Preferential staining of vascular tissue of plants transformed with *uidA* in histological GUS assays has been reported previously[15]. It is highly probable that the differential staining patterns in these shoots are due to differential gene expression rather than chimerism, since these patterns were very similar in independently recovered lines and were stable over time. 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.

Southern blot analysis. The fact that paromomycin-resistant embryogenic tissue could be 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 4B 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 (Fig. 4B; lanes 3, 5, 7, 9). The undigested DNA of the nontransgenic 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 would be expected if integration of the introduced DNA occurred at random sites (Fig. 4B; 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 (Fig. 4B; lanes 3, 4, 9, 10), show that the individual tissue pieces collected at the end of the initial selection phase (see Fig. 2, 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.

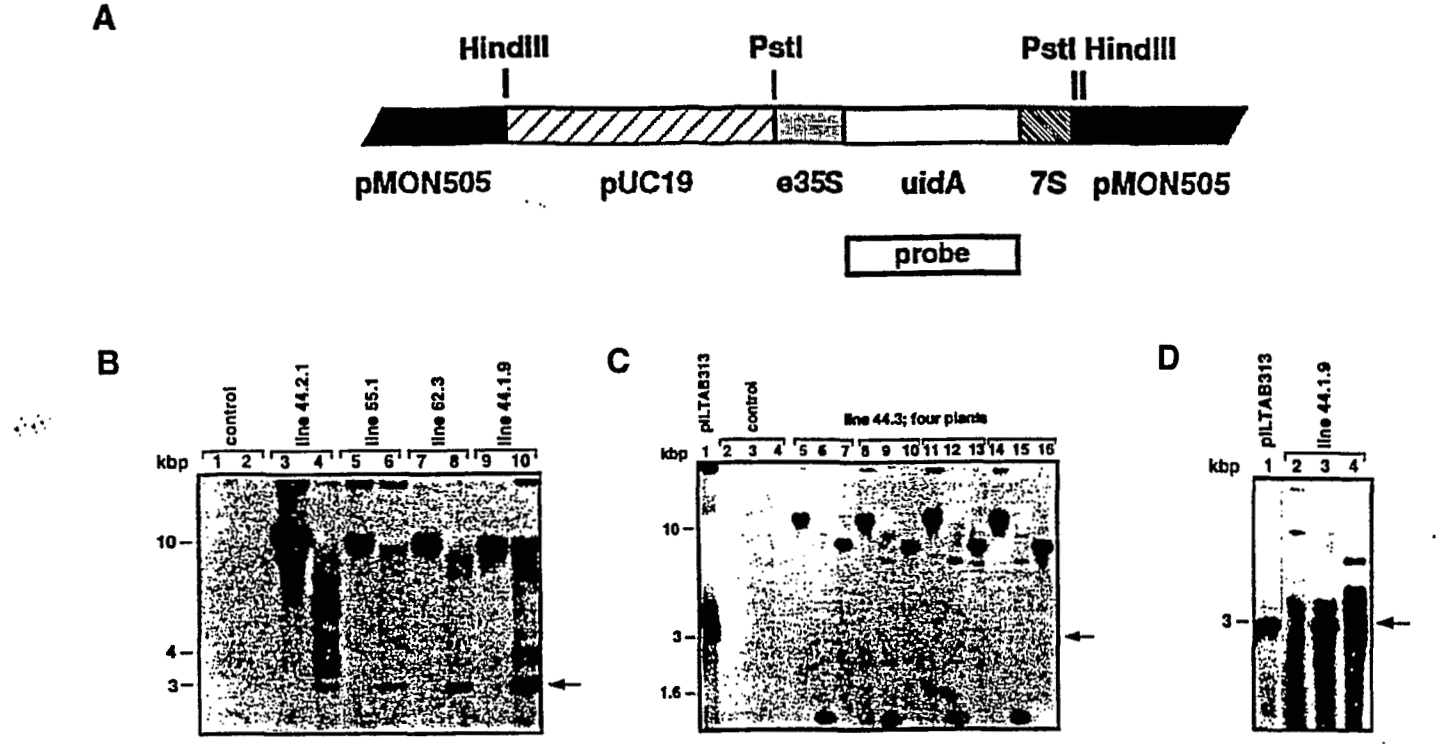

Figure 4. Southern blot analysis of total genomic DNA from putative transgenic embryogenic suspensions and plants. (A) Schematic representation of pILTAB313, showing the restriction sites for PstI and HindIII and the relative size of the *uidA* probe (not drawn to scale). (B) DNA from embryogenic suspension cultures (5 μg DNA pr lane). Lanes 1, 2: DNA from nonbombarded control suspension; lanes 3, 4, 9, 10: DNA of independently established suspensions derived from the same bombardments (lines 44.2.1 and 44.1.9); lanes 5 to 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. (C) 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 to 4: DNA of an untransformed control plant; lanes 4 to 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. (D) 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.

In one experiment, more than 30 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 (Fig. 4C; lanes 5 to 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 fragment of about 1.4 kb in size (Fig. 4C; 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 (Fig. 4D). The identity of the hybridization patterns of DNA from this plantlet and of the embryogenic suspension from which it had been regenerated (Fig. 4B, lane 10) demonstrates the stability of the integrated gene during the course of development from embryogenic cell to plantlet.

In conclusion, we have developed a method for the recovery of genetically transformed cassava plants via microparticle bombardment. The use of embryogenic suspensions as target tissue and the use of paromomycin for the selection of transformed tissues were crucial to 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. 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.

Experimental protocol

Plant material and culture media. Embryogenic suspensions were initiated from a 6-month-old line of embryogenic callus of cassava cultivar TMS 60444 and maintained as described by Taylor et al.[12]. Suspension cultures were used for particle bombardment 18 to 22 months after their initiation. The following culture media were used: SH-1 (SH salts[16], MS vitamins[17], 50 μM picloram, and 60 g/L sucrose); SH-2 (SH-1 with 7.5 g/L Difco-Bacto agar); R-1 (MS salts 1/2 concentrated, MS vitamins, 20 g/L sucrose, 7.5 g/L Difco-Bacto agar, 5 μM picloram); R-2 (as R-1, but picloram replaced by 5 μM α-naphthalene acetic acid); 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); and SP (MS salts and vitamins, 20 g/L sucrose, 2 g/L phytagel [Sigma Chemical Company, St. Louis, MO], no growth regulators). All media were adjusted to pH 5.7 prior to autoclaving.

Effect of antibiotics on nontransformed embryogenic suspensions. Embryogenic suspensions were sieved to isolate cell clusters 250 to 350 μm in diameter. Aliquots of 5000 units were transferred to 15-ml graduated centrifuge tubes containing 5 ml of SH-1 medium with antibiotic. The medium was buffered with 0.5 g/L 2-[N-morpholino]ethanesulfonic acid. 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, Edison, NJ) and rotated at 10 rpm. After 7 days, samples contain-

ing between 300 and 400 embryogenic cell clusters were removed from each tube, and their viability was assessed with the vital dye fluorescein diacetate (FDA)[18]. Samples were stained for 10 min in SH-1 medium with 0.05% (w/v) FDA. They were viewed under an Olympus IMT2 inverted microscope (10x 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.

Plasmid used for microbombardment. The enhanced 35S promoter from cauliflower mosaic virus[19] was linked to the *uidA* gene coding for GUS coupled to the 7S polyadenylation signal isolated from a β-conglycinin gene[20] and cloned into pUC19[21] at the PstI site. The resulting plasmid was cloned into the polylinker site of the binary vector pMON505[22], which contains the *npt*II gene. This plasmid was designated pILTAB313 (Fig. 4A).

Microprojectile 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 μm to 500 μm in size from 12- to 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 in diameter (BioRad, Hercules, CA) were coated with plasmid DNA according to Sivamani et al.[23]. Five μl of the particle-DNA suspension were distributed onto each macrocarrier (BioRad) and kept in a desiccator until used. Bombardment took place with the use 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 1100 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 petri 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 2. After bombardment, the tissue was cultured for 10 to 14 days in SH-1 medium without antibiotic followed by a selection phase (4 to 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 nontransformed 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 2. In the experiment that resulted in the first transgenic plants, the initial growth phase without antibiotics was 3 days long.

Histochemical assay for GUS. A modification of the assay by Jefferson[24] 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-β-D-glucuronide cyclohexylammonium salt), 20% (v/v) methanol and 0.16% Triton X-100. Potassium ferricyanide and potassium ferrocyanide were added to the buffer at 6.4 mM for embryogenic suspensions and 0.64 mM for roots, stems, and leaves. Tissues were covered with assay buffer and kept for 2 h (embryogenic suspensions) or 16 h (stems, leaves, roots) at 37°C in darkness. Assays were stopped by washing the tissue several times with water. For clearing and long-term storage the tissue then was transferred to 70% ethanol.

DNA isolation and Southern blot analysis of putative transgenic tissues. Genomic DNA was isolated according to Dellaporta et al.[25]. 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 h. 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 approximately 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 [α-32P]dCTP. Hybridization was carried out at 65°C in hybridization buffer (3x SSC, 2x Denhardt solution, 0.1% sodium dodecylsulfate [SDS], 6% PEG) overnight. The final wash was done at 65°C with (0.1x SSC; 0.1% SDS). The membrane was then exposed to x-ray film overnight.

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