Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium*-mediated transformation of embryogenic suspension cultures

**Abstract** A protocol was developed for *Agrobacterium*-mediated transformation of embryogenic suspension cultures of cassava. The bacterial strain ABI containing the binary vector pMON977 with the *nptII* gene as selectable marker and an intron-interrupted *uidA* gene (encoding β-glucuronidase) as visible marker was used for the experiments. Selection of transformed tissue with paromomycin resulted in the establishment of antibiotic-resistant, β-glucuronidase-expressing lines of friable embryogenic callus, from which embryos and subsequently plants were regenerated. Southern blot analysis demonstrated stable integration of the *uidA* gene into the cassava genome in five lines of transformed embryogenic suspension cultures and in two plant lines.

**Key words** Embryogenic suspension cultures - β-Glucuronidase - *Agrobacterium tumefaciens* - Cassava - Paromomycin

**Introduction** Cassava plays a significant role as an edible source of carbohydrates in many tropical countries. The world production in 1997 was estimated at 164 million tons of fresh root (FAO/GIEWS 1997). Because of its high yield potential under suboptimal conditions (Cooke and Cock 1989), it has received increasing interest in recent years. Traditional breeding programs in cassava were successful in introducing improved cultivars (Hershey and Jennings 1992), but a high degree of heterozygosity, irregular flowering in some cultivars, low seed set, and variable germination rates have impeded faster progress via classical plant breeding. Genetic engineering can complement traditional cassava breeding programs in several areas, including breeding for resistance to viral diseases (Fauquet et al. 1992), to alter starch quality (Salehuzzaman 1993), to reduce the content of cyanogenic glucosides (Koch et al. 1994), and to extend the shelf-life of the harvested tubers (Jhro et al. 1996). A prerequisite for genetic engineering of cassava is a reliable transformation system. Cassava was considered to be 'recalcitrant' in this regard, but after nearly a decade of unsuccessful attempts, four groups eventually produced transgenic cassava plants in 1996. The target tissues were either embryogenic suspensions (Schöpke et al. 1996; Raemakers et al. 1996) or cotyledons from somatic embryos, from which plants were regenerated through organogenesis (Li et al. 1996 or through embryogenesis (Sarria et al. 1996). The embryogenic suspensions were transformed via particle bombardment, while the cotyledons were transformed via infection with *Agrobacterium tumefaciens*. The use of embryogenic suspensions for genetic transformation is advantageous because the selection of transgenic cells in liquid medium is very efficient. Also, the risk of regenerating chimeric plants is low compared to protocols that involve organogenesis from organized tissues, such as the cotyledons. In addition, microparticle bombardment requires costly equipment and supplies, which limits its usefulness in developing countries. On the contrary, *Agrobacterium*-mediated transformation does not require specific equipment and thus is less expensive. We describe here a method that combines the advantages of embryogenic suspensions as target tissue with those of *Agrobacterium*-infection for the production of transgenic cassava plants.

**Materials and methods**

Plant material and culture conditions

Embryogenic suspensions derived from a 6-month-old culture of friable embryogenic callus of the cassava cultivar 'TMS 60444' were
initiated and maintained for 2 years as described by Taylor et al. (1996). The settled cell volume (SCV) of tissue originating from embryogenic suspension cultures was determined by leaving suspensions undisturbed for 30 min in a 15-ml graduated centrifuge tube. Suspensions that were to be used for transformation experiments were initiated by transferring 1 ml SCV of embryogenic tissue to 50 ml culture medium in 250-ml flasks. Every 3 days the medium was replaced with fresh medium, and after 15 days the suspension was sieved to obtain the fraction consisting of embryogenic units ranging in size from 250–500 μm to be used for inoculation with Agrobacterium. The following culture media were used: SH-1: SH salts (Schenk and Hildebrandt 1972), MS vitamins (Murashige and Skoog 1962), 50 μM picloram, and 60 g/l sucrose; MS: MS salts and vitamins (Murashige and Skoog 1962), no growth regulators. During the different regeneration steps the MS medium was modified by adding 5 μM 2-naphthalene acetic acid (NAA), 5 g/l activated charcoal (Sigma # C 3790), or 4.4 μM 6-benzylaminopurine (BAP), respectively. MS-based media were solidified by the addition of 2 g/l Phytagel (Sigma). All media were adjusted to pH 5.7 prior to autoclaving. The sequence of transfers to various media during the course of regeneration is described in Fig. 1. The suspensions were incubated on a shaker (150 rpm) at 25°C–28°C in a photoperiod of 16 h at 20–25 μmol s−1 m−2 PAR provided by fluorescent lamps (Sylvania Cool White). Embryogenic tissue, embryos, and plantlets were cultured at the same temperature and photoperiod but at 90–100 μmol s−1 m−2 PAR.

Plasmid and bacteria used for transformation

A GUS-expression cassette (E35 S promoter – uidA coding sequence – 35 S terminator) was cloned at the HindIII site of pMON977 (Monsanto Co, USA) to obtain the binary vector pILTAB 188. The uidA coding sequence in this construct is interrupted by an intron to prevent gene expression in Agrobacterium (Vancanneyt et al. 1990). The vector pILTAB 188 was introduced via triparental mating into A. mucedo strain ABI (Koncz and Schell 1986). Bacterial cultures were initiated by plating a frozen glycerol stock onto agar-solidified LB medium (Sambrook et al. 1989) containing the antibiotics spectinomycin (100 mg/l), kanamycin (50 mg/l), and chloramphenicol (25 mg/l). After 2 days growth, single colonies were inoculated into 2 ml liquid LB medium containing the above antibiotics and were grown for 24 h. Samples of 200 μl were taken and transferred to tubes with 1.8 ml LB medium without antibiotics and with an additional 100 μM acetosyringone. Bacterin were grown for 3–5 h to an OD600 of 0.04–0.06 and diluted to a density of 5×106 cells/ml with SH-1 medium. The Agrobacterium cultures were grown at a temperature of 30°C.

Transformation of embryogenic suspensions and selection of transgenic tissue

Aliquots of 0.5 ml SCV tissue from embryogenic suspensions in 2 ml SH-1 medium were transferred to 9-cm petri dishes and mixed with 5 ml of bacterial suspension. After 1 h, the bacterial suspension was removed with a pipette. The tissue was transferred to petri dishes with solidified SH-1 medium (7.5 g/l Difco Bacto agar) overlaid with sterile filter paper. Forty-eight hours later the tissue was transferred to 50 ml SH-1 liquid medium with 50 mg/l carbenicillin in 250-ml flasks. The suspensions were cultured on a shaker as described above for non-transformed embryogenic suspensions, and the culture medium replaced every 4 days by fresh medium. Ten days later 25 μM paromomycin was added to the medium. Five to six weeks after inoculation with Agrobacterium, units of yellow, viable tissue were selected under a stereomicroscope from among a background of white and brown-colored dead tissue and transferred individually onto selection medium (SH-1, 7.5 g/l Difco Bacto agar, 25 μM paromomycin, and 500 mg/l carbenicillin). For the subsequent steps leading to embryo formation and plant regeneration see Fig. 1. Some of the putative transgenic callus lines were subcultured for faster growth in liquid SH-1 medium as described above.

Histochemical localization of GUS was performed as described by Schopke et al. (1996). The assay buffer included 0.08 M sodium phosphate buffer at pH 7.0, 0.77 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic 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 use with 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 distilled water. The tissue then was transferred to 70% ethanol for clearing and long-term storage.

Fig. 1. Flow diagram showing the steps leading from embryogenic suspensions of cassava inoculated with Agrobacterium to transgenic plants. SH-1 Medium based on Schenk and Hildebrandt 1972, MS medium after Murashige and Skoog 1962, car. carb. carbenicillin, par. paromomycin.

Histological GUS assays.
Fig. 2A–D Regeneration of transgenic plants from embryogenic suspension cultures transformed with a plasmid containing the uidA gene. A Histological GUS assay with an embryogenic suspension culture, 10 days after inoculation with Agrobacterium. GUS-expressing cells are stained blue. B A growing yellowish embryogenic unit and dead whitish tissue after 5 weeks culture in liquid selection medium containing 25 μM paromomycin. C Several transgenic plants derived from a single selected piece of embryogenic callus, 31 weeks after transformation. D GUS-positive leaf lobe and roots of a transgenic plant shown in C. Bars: 500 μm in A, 1.5 mm in B, and 5 mm in D.

Southern blot analysis

DNA from five putative transgenic lines of embryogenic callus and two lines of regenerated plantlets was isolated according to Delporte et al. (1983). DNAs were digested either with HindIII or XhoI restriction endonucleases, and undigested and digested DNAs (10 μg per well) were separated on a 0.7% agarose gel. DNA was then transferred onto a Hybond-N+ nylon membrane (Amersham, Arlington Heights, Ill.) according to the instructions of the manufacturer. The uidA probe (α-32P)dCTP-labeled) was prepared by random priming, and hybridization was carried out at 65°C overnight in hybridization buffer (0.1xSSC, 0.5% Denhardt solution, 0.5% SDS, and 100 ng/ml denatured fragmented salmon sperm DNA) containing the uidA probe. The blot was then washed at high-stringency conditions (0.1xSSC and 0.5% SDS at 65°C) and exposed to X-ray film for 2 days.

Results and discussion

The protocol developed to regenerate cassava plants from embryogenic suspension cultures transformed through Agrobacterium is shown in Fig. 1. The sequence of subcultures, including the selection of paromomycin-resistant embryogenic tissue, the formation of somatic embryos, and regeneration from these embryos is essentially the same as that used for microbombarded embryogenic suspensions (Schöpke et al. 1996), but differs in the timing of subcultures due to different growth rates of the two systems. After inoculation and cocultivation with Agrobacterium, the tissue was allowed to recover for 8–10 days in medium without selective antibiotic. GUS assays performed at this time revealed blue spots on many of the embryogenic units (Fig. 2A). During this phase and the subsequent selection with 25μM paromomycin, numerous embryogenic units developed areas with brown-colored, dead cells. This browning is probably a reaction to Agrobacterium, since microbombarded embryogenic suspension-derived tissue that is subjected to selection with paromomycin does not show this response but turns white under the selection pressure (Schöpke et al. 1996). Sensitivity of cassava tissues to Agrobacterium has been reported earlier for young somatic embryos (Schöpke et al. 1993; Li et al. 1996). The growth rate of paromomycin-resistant cells was slower than the growth of microbombarded suspensions. In the latter case, yellowish embryogenic units with a diameter of 0.5–1.5 mm had developed after 3–4 weeks of selection in liquid medium, while it took 5–6 weeks to reach this stage in the case of embryogenic suspensions transformed with Agrobacterium. (Fig. 2B). This may be due to damage caused by the bacteria during cocultivation.

Between 20 and 180 yellow embryogenic units were obtained from 0.5 ml SCV of inoculated tissue in six experiments (Table 1). This is about half the number of transformed units per volume of tissue reported for microbombarded embryogenic tissue (20–100 yellow units from samples of 0.2 ml SCV; Schöpke et al. 1996). Individual subculture of these paromomycin-resistant units onto so-
Table 1  Efficiency of Agrobacterium-mediated transformation of embryogenic suspensions cultures of cassava. In each experiment, 0.5 ml SCV of embryogenic tissue was inoculated with *Agrobacterium tumefaciens* carrying a plasmid with the uidA and nptII genes. Plants were regenerated from two of the friable embryogenic callus lines (FEC) obtained in experiment 1.

<table>
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<tr>
<th>Experiment</th>
<th>Number of resistant units after 5 weeks of selection in liquid medium</th>
<th>Number of GUS-positive callus lines derived from resistant units after 4 weeks on solid selection medium</th>
<th>Number of FEC lines transferred to regeneration medium</th>
<th>Number of FEC lines that produced embryos</th>
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<td>Average</td>
<td>64</td>
<td>37</td>
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* Lost to fungal contamination

Lidified medium supplemented with 25 μM paromomycin resulted in the establishment of paromomycin-resistant callus lines. After 4 weeks on this selection medium samples were taken from the callus lines and subjected to histological GUS assays. On average 65% of the lines were GUS-positive (Table 1). Only the GUS-positive callus lines showing the characteristics of friable embryogenic callus were transferred to regeneration media. At this point they were considered paromomycin-resistant and were subsequently cultured on media without the antibiotic. Following the culture regime outlined in Fig. 1, between 43% and 75% of the friable embryogenic callus lines produced embryos. Out of these lines, two eventually regenerated shoots that rooted in MS medium without growth regulators (Table 1; Fig. 3C). An additional four lines are currently regenerating shoots. The pattern of GUS expression driven by the 35 S promoter in the regenerated plantlets was the same as that observed for transgenic plants derived from microbombarded embryogenic suspension cultures (Schöpke et al. 1996), i.e., all tissues stained blue in histological GUS assays, with staining the darkest in young leaves and in vascular tissues (Fig. 3D).

Transgenic cell lines and plants were assayed for the integration of T-DNA by Southern blot analysis (Fig. 3). In all analyzed suspension and plant lines the undigested genomic DNA hybridized to the probe, suggesting that T-DNA was integrated into the plant genome. DNA of all lines digested with HindIII produced a band at 3 kbp, the size expected for the intact uidA cassette, while the additional bands are presumably due to incomplete restriction enzyme digestion. The hybridization patterns resulting from genomic DNA digested with XhoI can provide an estimate of the number of integration sites, since the plasmid used for transformation contained a single XhoI restriction site. The second XhoI site, therefore, must reside in the plant genome, and each DNA fragment produced by XhoI that binds to the probe represents a specific integration event. The number of integration sites varied from one to four.

Fig. 3  Southern blot analysis of genomic DNA (10 μg per lane) of different lines of paromomycin-resistant and GUS-positive embryogenic suspensions and plants derived from Agrobacterium-mediated transformation of embryogenic suspensions of cassava. The DNA was either undigested (U) or digested with HindIII (H) or XhoI (X) and hybridized to a probe binding to the uidA coding region. For plant P2 the amount of available DNA was limited, therefore the hybridization of undigested DNA was omitted. A band at the expected size of 3 kbp for the intact uidA cassette was produced after DNA digestion with HindIII in all lines. CS Non-transformed control suspension, lines 16–111 transformed embryogenic suspensions, CP non-transformed control plant, P1 and P2 transformed plants.
Selection of transformed cells in our system took place in liquid medium and therefore raises the concern that tissue pieces might break apart. Visual selection of yellow tissue pieces after the selection in liquid medium then could lead to the establishment of sibling lines. Except for cell line No. 16 (Fig. 3), all cell and plant lines analyzed are derived from the same flask. The unique banding patterns for each of these lines indicates that no siblings were produced.

Our results show that embryogenic suspension-derived tissue can be used as a target for Agrobacterium-mediated transformation of cassava. With relatively little effort, hundreds of transgenic lines of friable embryogenic callus can be produced. The difficulties encountered during plant regeneration from transgenic callus are similar to those described in the first report on cassava transformation. Improved procedures for the production of friable embryogenic callus (Schöpke et al. 1996), i.e., a low rate of transformed calli producing somatic embryos and a low conversion rate of embryos into plants. However, optimization of the selection and regeneration protocol for microbombarded tissues has led both to an improvement in the regeneration efficiency and the time needed for regeneration (Taylor et al. 1997; Taylor and Schöpke, unpublished results), which shows that it will likely be possible to improve Agrobacterium-mediated transformation of embryogenic suspension cultures. If, after optimization, the efficiency of this system for the production of transgenic calli can be translated into an improved efficiency for plant regeneration, this system represents a useful, simple, and relatively inexpensive alternative to cassava transformation through microbombardment. Furthermore, the technology can be transferred easily to laboratories that do not have access to the microbombardment method of cassava transformation.

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References
