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

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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

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*

bombardment with genes. The *uidA* gene had an intron to prevent it from being

Particle bombardment

on solidified selection medium with 25 μM paromomycin

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

(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,

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

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

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