# II.12 Transformation in Cassava (*Manihot esculenta* Crantz)[1]

C. Schöpke[2], C. Franche[3], D. Bogusz[3], P. Chavarriaga[2], C. Fauquet[2], and R.N. Beachy[2]



## 1 Introduction

### 1.1 Cassava and Biotechnology

Cassava is cultivated throughout the tropics for its starchy, tuberous roots. In Africa it plays an important role as a subsistence crop, while in Latin America cassava is also an industrial crop that is processed into more than 300 products. In Asia it is mainly used for the production of animal feed and for exportation. In terms of caloric production it ranks fourth after rice, wheat, and maize in developing countries (FAO 1987; cited in De Bruijn and Fresco 1989). Compared to other crops, cassava has played a minor role in agricultural research. However, during the last decade, the interest in cassava has grown (Cock 1982; Cooke and Cock 1989) and it was recognized that biotechnology might be a useful tool for cassava improvement (Roca 1984, 1989; CIAT 1989; Bertram 1990). With the aim to concentrate and coordinate efforts using biotechnology for the improvement of cassava, in August 1992 the First International Scientific Meeting of the Cassava Biotechnology Network (CBN) was held in Cartagena, Colombia.

One of the major constraints for cassava production is its susceptibility to viruses, especially African cassava mosaic virus (ACMV; Fauquet and Fargette 1990) and cassava common mosaic virus (CCMV; Costa and Kitajima 1972). Today, breeding of virus-resistant and -tolerant cultivars and the improvement of agricultural practices are used to control this problem. In spite of these efforts, cassava yield losses caused by these two viruses are still significant. In Africa an estimated average of 50% of the possible yield is lost due to ACMV infection (Fauquet and Fargette 1990).

Recent developments in genetic engineering showed that it is possible to protect agronomically important crops against viruses through the insertion of virus coat protein genes into the plant genome (Beachy et al. 1990). The International Cassava-Trans Project (ICTP), which was established to apply biotechnology to

[1]The studies described in this paper were completed at the Department of Biology at Washington University in St. Louis, MO 63130, USA
[2]Division of Plant Biology - MRC 7, ILTAB, The Scripps Research Institute, 10666 North Torrey Pines Rd., La Jolla, CA 92037, USA
[3]BSSFT (CTFT/ORSTOM), 45 bis Avenue de La Belle Gabrielle, 94736 Nogent-sur Marne Cédex, France

Biotechnology in Agriculture and Forestry, Vol. 23
Plant Protoplasts and Genetic Engineering IV (ed. by Y.P.S. Bajaj

cassava improvement, is investigating the possibility of using the coat protein-mediated resistance strategy to protect cassava against the above mentioned viruses (Fauquet et al. 1992).

Within this project, two approaches are being tested for their applicability in cassava transformation: the introduction of accelerated, DNA-coated microparticles into cells (Sanford 1990), and transformation using *Agrobacterium tumefaciens* (Klee and Rogers 1989).

## 1.2 The Choice of an In Vitro Regeneration System for Cassava Transformation

A basic requirement for any transformation program that proposes to develop improved plants is the availability of a reliable regeneration system. In cassava, nearly all plant parts have been used to establish in vitro cultures (Roca 1984). However, successful regeneration to plants is mainly restricted to young leaves (Fig. 4A, C), embryos (Fig. 4B), and shoot meristems. Using young cassava leaves, somatic embryogenesis can be induced and plants can be regenerated (Stamp and Henshaw 1982, 1986, 1987a, b; Stamp 1987). These results were confirmed and the method was applied to various cassava cultivars (Szabados et al. 1987a). Although it is now possible in principle to regenerate cassava plants from a limited number of cultivars, the efficiency achieved so far is relatively low. In their best experiment, Szabados et al. (1987a) obtained an average of 1.15 plantlets per primary explant. Stamp and Henshaw (1987a) used 2–8 mm long somatic embryos to study plant regeneration of somatic embryos derived from cotyledon explants. Fourteen percent of the embryos with which they started eventually regenerated into plants established in the greenhouse.

Meristems or shoot tips might provide an alternative source of explants for transformation experiments (Ulian et al. 1988; Schrammeijer et al. 1990). Meristem culture and subsequent regeneration of cassava plants have been reported (Kartha et al. 1974; Bajaj 1983). However, compared to the somatic embryogenesis system, it is difficult to obtain a number of meristems adequate for transformation experiments.

Cassava protoplast isolation and culture have been performed by various authors (Mabanza and Jonard 1983; Mabanza 1984; Szabados et al. 1987b; Villegas et al. 1988; Nzoghe 1989), but regeneration was observed only in one instance (Shahin and Shepard 1980) and has not been repeated.

If one compares the different options for in vitro regeneration in cassava, it becomes evident that somatic embryogenesis from young leaves is the only system at this moment that shows potential for transformation experiments with subsequent regeneration of plants. Somatic embryos can be obtained from a number of cultivars, although the regeneration procedure still needs to be optimized for each of them. Field tests with plants derived from somatic embryos showed that somaclonal variation does not seem to be a problem in cassava (Roca, pers. comm.).

Since the transformation/regeneration system in cassava is not yet developed to an extent that allows the production of transformed plants, we have used indirect

methods to test the expression of gene constructs in parallel with the transformation experiments with cassava. The two cassava viruses studied are both able to infect *Nicotiana benthamiana*, which is easy to transform and regenerate. This means that the chimeric genes which are to be expressed in cassava, can first be tested in *N. benthamiana* to evaluate their effect on resistance to viruses.

### 1.3 Cassava Transformation

Calderón (1988) was the first to describe transformed callus lines of cassava. He infected leaf pieces, stem pieces, and embryogenic callus with *Agrobacterium* containing plasmids with the coding sequences for neomycin phosphotransferase II (*nptII*), phosphinotricin acetyltransferase (*bar*), or β-glucuronidase(*uidA*). He isolated callus lines expressing the phenotype expected from transformed tissue, i.e., resistance to kanamycin and phosphinotricin, and containing β-glucuronidase activity. Southern blot analysis with one callus line demonstrated the stable integration of T-DNA into the cassava genome.

Transient expression of the GUS gene introduced into protoplasts by electroporation was demonstrated by Villegas (1988) and Cabral et al. (1991). Our results, some of which have been published (Franche et al. 1991) are detailed here.

## 2 Experiments with a Particle Gun: Transient Expression of the GUS Gene

### 2.1 Optimization of Delivery of Particles into Cassava Leaf Cells

The optimal way to study plant genes is through the analysis of transgenic plants. However, where efficient transformation and regeneration procedures do not yet exist, transient gene expression systems are valuable tools for the analysis of gene expression in plants. We used a particle gun to investigate transient gene expression in cassava leaves. Transient expression of genes transferred by high velocity microprojectiles has now been achieved with several economically important crops, including corn, tobacco, rice, wheat, soybean, and others (Klein et al. 1990). It appears that transient tissue-specific gene expression is similar to that in transgenic plants (Twell et al. 1989).

The particle gun used for the experiments was an air pressure device built at Washington University in St. Louis. It is composed of two steel cylinders connected by an electronically controlled valve. The upper cylinder contains compressed air (max. 5.5 MPa), the lower one represents the barrel of the gun. The bore of the barrel is 8 cm long and 5 mm wide. During bombardment, Teflon macroprojectiles carrying the DNA-coated microparticles are retained by a steel stopping plate that is screwed onto the barrel. The gun is positioned within a vacuum chamber, which is evacuated to −95 kPa during bombardment. The macroprojectiles are sized through a metallic cylinder that adjusts their diameter to 25 μm larger than the diameter of the bore.

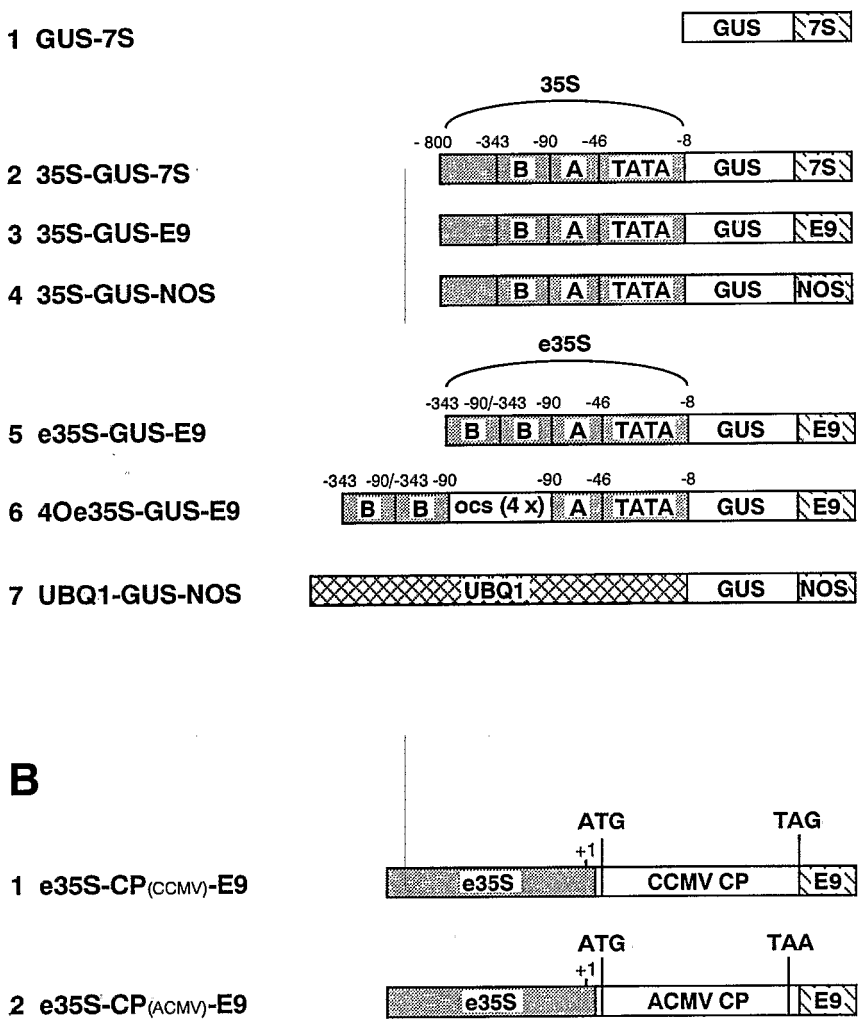


**Fig. 1.** **A** Schematic representation of the GUS gene constructs used in experiments for transient and stable gene expression (not drawn to scale). For details on transient expression in cassava leaves and for references, see Franche et al. (1991). *7S* 3′ end of the α-subunit gene of soybean β-conglycinin; *35S* cauliflower mosaic virus (CaMV) promoter; *A, B* Domains from the 35S promoter; *E9* 3′ end of the small subunit ribulose-1,5-bisphosphate carboxylase gene from *Pisum sativum*; *e35S* enhanced cauliflower mosaic virus promoter containing a duplication of the 35S region between nucleotides − 343 and − 90; *GUS* coding sequence for the β-glucuronidase gene (*E. coli uidA* gene); *NOS* 3′ end of the nopaline synthase gene of *Agrobacterium tumefaciens*; *ocs* activator region from the promoter of the octopine synthase gene of *Agrobacterium tumefaciens*; *UBQ1* promoter of the ubiquitin 1 gene from *Arabidopsis thaliana*. **B** Schematic representation of the coat protein gene constructs used in experiments for stable gene expression in cassava calli (not drawn to scale). The sites corresponding to transcription start ( + *1*), translation start (*ATG*), and translation stop (*TAA*) are indicated. *ACMV* African cassava mosaic virus; *CCMV* cassava common mosaic virus; *CP* coat protein; other abbreviations, see **A**

Conditions of bombardment were determined using leaves of greenhouse-grown plants of the cassava cultivar Señorita. The microparticles were coated with the plasmid p35S-GUS-7S (construct 2; Fig. 1A). Several factors which are known to affect the efficiency of the particle bombardment were studied, including the size of particles, the distance between the stopping plate and target, and the age of the cassava leaves. The efficiency of gene introduction was monitored by counting the number of foci (spots) on the leaves which exhibited transient expression of the GUS gene (Franche et al. 1991).

## 2.2 Transient Expression of the GUS Gene in Cassava Leaf Lobes After Bombardment with Microprojectiles

Tungsten particles with average diameters of 0.5, 1.2, and 2.0 μm (corresponding to sizes M5, M10, and M17, respectively; GTE Sylvania) were tested at shooting distances from 2 to 8 cm. A pressure of 5.5 MPa was used to accelerate the macroprojectiles. The leaf lobes were tested histologically for GUS expression 24 h after the bombardment (Jefferson 1987).

The best results were obtained with 1.2-μm particles shot at a distance of 4 cm (Fig. 2). The smaller particles probably did not have sufficient velocity to penetrate

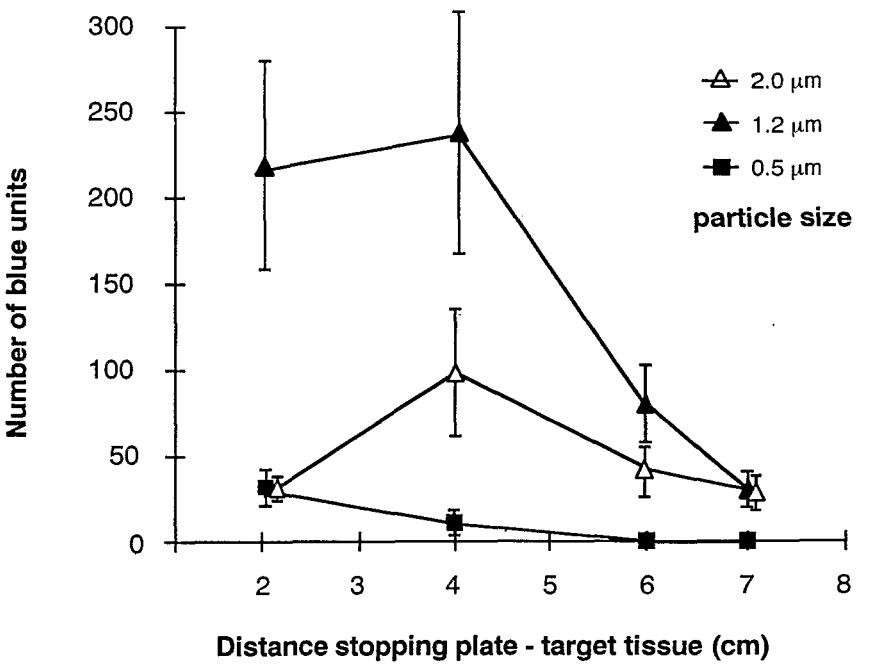


**Fig. 2.** Effect of the distance between stopping plate and target tissue on the number of GUS-expressing units in cassava leaves. Tungsten particles with diameters of 0.5, 1.2 and 2.0 μm were coated with the plasmid pUC18 (Messing 1983) containing the construct 35S-GUS-7S. Five leaf lobes were bombarded for each treatment. The lobes were assayed for GUS activity 24 h after the bombardment (Jefferson 1987). The average number of GUS-expressing units and the standard error of the mean are shown.

leaf cells efficiently, while the larger particles increased the damage caused by the bombardment.

Leaf age was also an important parameter in particle bombardment. Using the first fully expanded leaf from 1- to 2-month-old greenhouse plants, the average number of GUS-expressing units obtained was 268 ± 138 per leaf lobe (N = 5). This number decreased to 106 ± 77 with the next older leaf and to 7 ± 4 with the following leaf. Several factors might be responsible for this. As the leaves get older, the cuticle becomes thicker and the cell walls become more rigid, which likely affects the penetration of microprojectiles. In addition, cells from older leaves might be not as efficient as young ones in expressing the DNA introduced by particle bombardment.

The highest air pressure that we could use with our device was 5.5 MPa, which gave the highest number of GUS-expressing units (data not shown).

## 2.3 Quantitative Analysis of the Expression of the GUS Gene Under the Control of Different Promoter and Termination Sequences

Bombarded cassava leaves were used to perform the fluorometric GUS assay described by Jefferson (1987). Details of the procedure have been published elsewhere (Franche et al. 1991). The β-glucuronidase gene from *E. coli* was used as a reporter gene to investigate the effect of several promoter and termination sequences on transient expression (constructs 2–6; Fig. 1A). Based on the fluorometric assays (Table 1), we found that both the e35S promoter and the 4Oe35S promoter were three times more active in cassava leaves than was the unmodified

**Table 1.** Fluorometric GUS assay of cassava leaves bombarded with chimeric gene constructs[a] (After Franche et al. 1991)

| Construct | Specific activity | Relative expression (%) |
|---|---|---|
| 35S-GUS-7S | 70.5 ± 23 | 100 |
| 35S-GUS-E9 | 83 ± 34 | 118 |
| 35S-GUS-NOS | 63 ± 31 | 89.5 |
| e35S-GUS-E9 | 211 ± 78 | 299 |
| 4Oe35S-GUS-E9 | 202 ± 119 | 287 |

[a] Cassava leaf lobes were bombarded three times; 24 h later, quantitative GUS assays were performed. The data represent the average of ten experiments. Specific activity is expressed as production of nmol 4-MU (4-methyl umbelliferone) $min^{-1}$ $mg^{-1}$ protein less the background observed in leaves bombarded with the promoterless construct. The endogenous glucuronidase activity in fully expanded cassava leaves was 3.1 nmol 4-MU $min^{-1}$ $mg^{-1}$ protein. The relative GUS activities are expressed as a percentage of GUS activity induced by the construct 35S-GUS-7S (see Fig. 1A; table adapted from Franche et al. 1991, with permission).

35S promoter. The two 3′ ends used in our study did not lead to significantly different levels of GUS activity. The ubiquitin 1 promoter induced similar expression levels as the 35S promoter (data not shown).

## 3 Transformation of Leaf Disks with *Agrobacterium tumefaciens*

### 3.1 General Transformation Procedure

An *Agrobacterium* binary vector system was used for the transformation experiments. Leaf disks from 2-month-old greenhouse-grown plants (cv. MCol 22) were cocultivated for 3 days with overnight cultures of the avirulent *Agrobacterium* strain GV3111 carrying the vector pMON505 (Horsch and Klee 1986). This plasmid vector contains the NPT II gene as a selectable marker (Fraley et al. 1983). It confers resistance to the aminoglycoside antibiotics kanamycin and geneticin (G418). The basal culture medium was composed of MS salts (Murashige and Skoog 1962) and vitamins, 20 g/l sucrose, and 1.6 g/l Phytagel (Sigma). The pH was adjusted to 5.7 before autoclaving. After 3 days of cocultivation, the explants were transferred to a selection medium containing 1 mg/l BAP (benzylaminopurine), 1 mg/l NAA (α-naphthalene acetic acid), 100 mg/l kanamycin, and 500 mg/l carbenicillin. Calli emerging from the cut edges of the leaf disks were excised 30 days after inoculation and subsequently transferred onto selection medium every third week for 60 additional days of incubation.

### 3.2 β-Glucuronidase Gene Construct

Four weeks after the treatment of leaf disks with *Agrobacterium* containing the vector pMON505 that contains the 35S-GUS-7S gene (Fig. 1A, construct 2), 80 to 90% of the leaf disks developed an average of three calli on three of the four different selection media assessed. Stable integration of the T-DNA was demonstrated by Southern blot analysis (Fig. 3).

Interestingly, a medium containing only 2,4-D (2,4-dichlorophenoxyacetic acid) (4 mg/l) as growth regulator instead of 1 mg/l BAP and 1 mg/l NAA did not permit callus growth. This was an unexpected result, since controls that were not treated with *Agrobacterium* and that grew on medium without antibiotics did form callus with only 2,4-D as growth regulator. Addition of a cytokinin seems to be important for the development of transformed callus.

The level of expression of a newly introduced gene generally determines whether its transfer has the desired effect on a transformed plant. To find sequences that are suitable for the expression of foreign genes in cassava, we made several GUS chimeric gene constructs using known promoters and 3′ polyadenylation signals. The constructs 2, 3, 5, 6, 7 (Fig. 1A) were integrated into pMON505, transferred to *Agrobacterium* strain GV3111, and then used to transform cassava leaf disks. Infected disks were cultured on selection medium with 1 mg/l BAP and

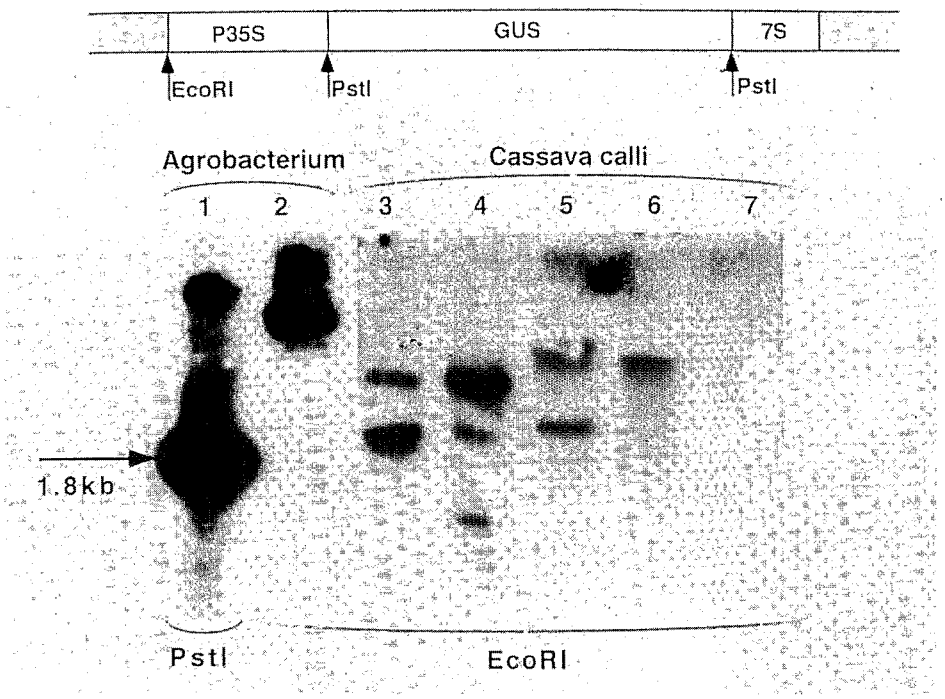


**Fig. 3.** Southern blot analysis of calli which had formed 3 months after transformation of cassava leaf lobes with *Agrobacterium tumefaciens* containing the plasmid pMON505:35S-GUS-7S. They were selected on medium with 100 mg/l kanamycin and 500 mg/l carbenicillin. DNA extracts were digested either with Eco R1 (*Agrobacterium* DNA) or with Pst 1 restriction nuclease (cassava DNA). The digested extracts were probed with the GUS-coding sequence. The *arrow* indicates the position which corresponds to the 1.8-kb Pst 1 fragment containing the GUS-coding sequence. *Lane 1* plasmid pMON505:35S-GUS-7S; *lane 2* linearized plasmid pMON505:35S-GUS-7S; *lane 3-6* DNA extract from transformed calli; *lane 7* DNA extract from a nontransformed callus

1 mg/l NAA. Ninety days after infection, kanamycin-resistant calli were checked for expression of the $\beta$-glucuronidase gene using the fluorometric GUS assay (Jefferson 1987). Constructs with the same promoter but with different 3′ end sequences showed similar GUS activity. The constructs containing either the e35S or the 4Oe35S promoter displayed higher GUS activity than those with either the nonmodified 35S promoter or the ubiquitin gene promoter of *Arabidopsis* (Franche et al., submitted).

### 3.3 Coat Protein Gene Constructs

Two chimeric genes containing the CCMV and the ACMV coat protein coding sequences inserted in the vector pMON505 (Fig. 1B) were introduced into cassava leaf disks using *Agrobacterium* strain GV3111 (Franche et al., submitted). Southern, Northern, and Western blot analyses were performed on kanamycin-resistant calli after 4 months on selection medium with 1 mg/l NAA, 1 mg/l BAP,

100 mg/l kanamycin, and 300 mg/l carbenicillin. These calli showed the presence of the corresponding coding sequence and the accumulation of the encoded mRNA. The CCMV coat protein construct was detected at a high level in transformed cassava tissue. Cassava calli expressing the ACMV coat protein were also identified, although Western blot analysis of the ACMV coat protein was more difficult.

## 4 Transformation of Leaf Lobes by Bombardment with an *Agrobacterium tumefaciens* Suspension

Preliminary transformation experiments were performed with clumps of young, small, somatic embryos. These experiments showed that either the wounding or the cocultivation with *Agrobacterium* or both damaged the embryos to such an extent that most did not survive the treatment.

This led us to use a particle gun to bombard embryo clumps with an *Agrobacterium* suspension. During the bombardment, a bacterial suspension is dispersed into many, very small droplets. When these droplets hit an embryo, small colonies should develop instead of the confluent growth observed during experiments with embryo clumps immersed in an *Agrobacterium* suspension. The overall damage caused by cocultivation with *Agrobacterium* should therefore be reduced. In addition, the bombardment with droplets itself should provide wounds necessary for efficient *Agrobacterium* infection. These wounds would cover only small areas of the embryos and so reduce the risk of too much damage. To get an idea on the usefulness of the method, we first applied it to in vitro leaf lobes (Schöpke et al. 1991).

### 4.1 General Procedures

The air pressure particle gun (see Sect. 2.1) was used for the experiments. In vitro leaf lobes from cassava cv. CMC 76 (syn. MCol 1505) were placed on a filter paper in a Petri dish with 1% agar medium containing only MS salts and bombarded with an overnight suspension of *Agrobacterium* strain GV3111 harboring the vector pMON505: 35S-GUS-7S. After 3 days of cocultivation on medium with MS salts and vitamins, 2 mg/l glycine, 20 g/l sucrose, 2 g/l Phytagel (Sigma), and 200 $\mu$M acetosyringone, the lobes were transferred to selection medium with 100 mg/l kanamycin, 300 mg/l carbenicillin, and either 2 mg/l 2,4-D or 1 mg/l NAA + 1 mg/l BAP.

### 4.2 Bacterial Growth; Expression of $\beta$-Glucuronidase

During the first 3 days of cocultivation, bacteria grew in small colonies (Fig. 4D) as opposed to the confluent growth observed after using the standard dipping method. Using the histological GUS assay (Jefferson 1987, modified) immediately after the cocultivation and 6 days later, the number of dark blue spots increased

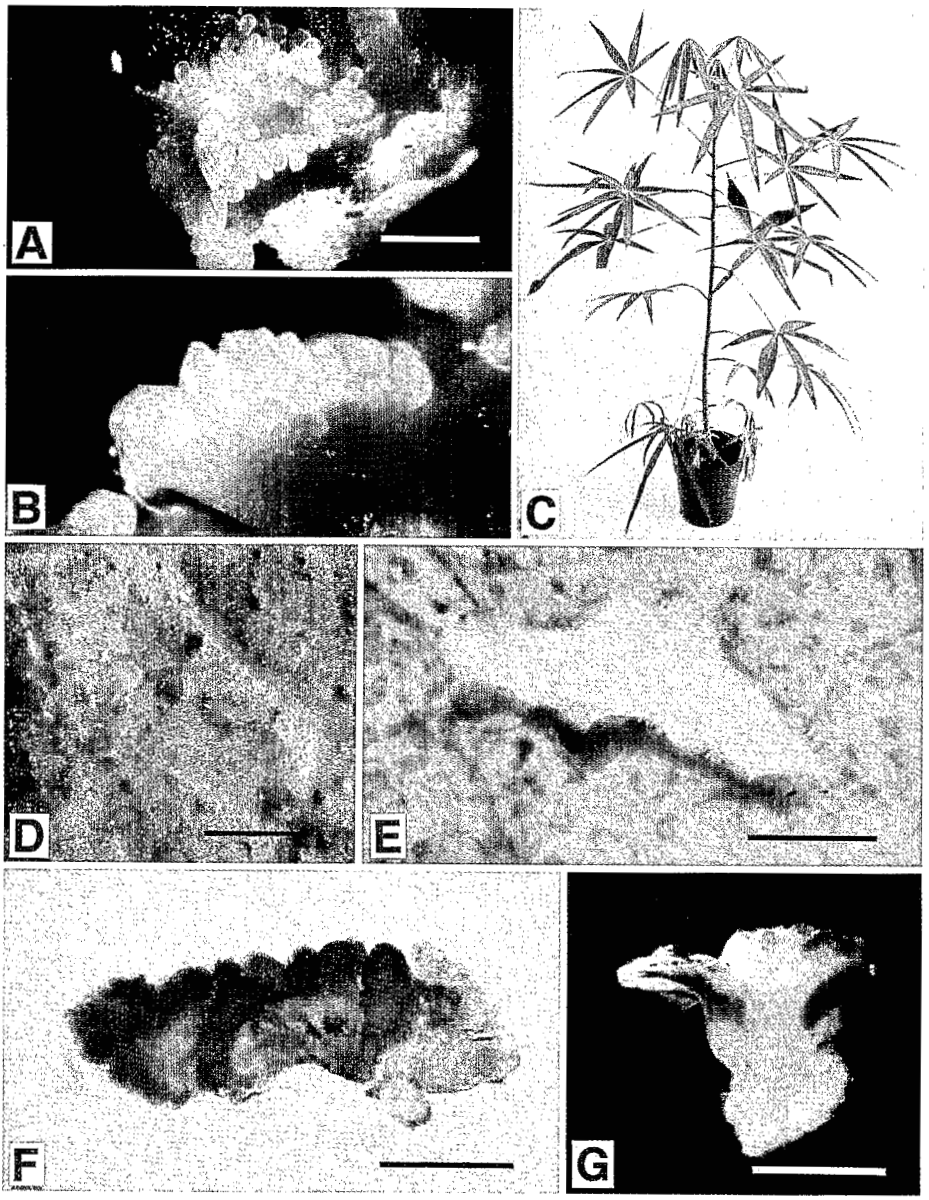


**Fig. 4.** **A** Somatic embryos that developed from a young cassava leaf lobe after 3 weeks on embryo induction medium. **B** Secondary somatic embryos emerging from the upper side of a cotyledon from a primary embryo, 2 weeks after transfer of primary embryos onto medium for the induction of secondary embryos. **C** Cassava plant regenerated from a somatic embryo, 6 months after transfer to the greenhouse (height approx. 80 cm). **D** Abaxial side of leaf lobe bombarded with *Agrobacterium* suspension, 3 days after the bombardment. Small impacts and bacterial colonies are visible. **E** A leaf lobe as shown in **D** was cultured on selection medium containing 100 mg/l kanamycin and 300 mg/l cabenicillin. Three weeks later the lobe was subjected to the histological GUS assay and cleared with 70% ethanol. The view of the abaxial side in the area of the main impact of the bombardment reveals many single GUS-expressing cells and one dark blue spot composed of several dark blue cells,

from very few to about 150 per bombarded lobe (Fig. 4E). Dark blue cells were found in the upper and lower epidermis, in spongy and palisade parenchyma, and in vascular tissues. After 4 weeks on selection medium, the explants on medium with NAA/BAP had formed calli with diameters from 1–3 mm. After 9 weeks, calli were divided into three classes according to size (Ø < 2, 2–5, and > 5 mm) and subjected to the histological GUS assay with X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide). All calli with a diameter larger than 5 mm expressed GUS. Of those with a size of 2–5 mm, 80% were GUS-positive, while only 16% of the calli smaller than 2 mm showed the blue stain. This means that the size of calli growing under selective conditions can give preliminary information concerning the success of a transformation experiment. All of six callus lines that were tested for GUS expression after 4 months on selection medium stained blue with the histological GUS assay (Fig. 4F).

Six days after cocultivation with *Agrobacterium*, the explants on selection medium with 2 mg/l 2,4-D showed an average of only about 30 dark blue cells per leaf lobe, most of them concentrated near the midvein. Later, they expanded slightly, with some swelling of the midvein. As in the case of leaf disk transformation, no callus was formed on this medium.

## 5 Transformation of Somatic Embryos with *Agrobacterium tumefaciens*

### 5.1 General Transformation Procedure

Somatic embryos were initiated from young in vitro leaves of cv. CMC 76 on a medium with 4 mg/l 2,4-D (Stamp and Henshaw 1986). A continuous production of embryos was achieved by monthly transfer to medium with 2 mg/l 2,4-D. Since clumps of very small embryos are easily damaged by cocultivation with *Agrobacterium*, in this experiment, embryos of a later developmental stage were used. If left for longer than 1 month on the same medium, the hypocotyl of embryos at the periphery of embryo clumps begins to grow and green cotyledons are formed. This type of embryo (2–3 mm) was used for transformation experiments with *Agrobacterium* strain GV3111, harboring the vector pMON505 carrying the gene 35S-GUS-7S. The embryos were cut into pieces with a scalpel and immersed for 30 min in an overnight culture of *Agrobacterium*. Subsequently, they were treated in a similar way as the leaf disks described in section 3. After 3 days of cocultivation, the pieces were transferred to selection medium that contained 100 mg/l kanamycin, 300 mg/l carbenicillin, 2 mg/l 2,4-D, and 0.05 mg/l BAP.

presumably derived from divisions of one transformed cell. **F** Section through a GUS-expressing callus derived from leaf lobes bombarded with *Agrobacterium* suspension, 4 months after the bombardment. **G** Longitudinal section of a somatic embryo infected with *Agrobacterium*. After 12 days on selection medium favoring secondary embryogenesis, many cells show GUS expression. Blue spots were detected on the surface of the swollen cotyledons where secondary embryos develop (bars always 2 mm)

### 5.2 Expression of β-Glucuronidase

The histological assay revealed a low level of GUS expression due to *Agrobacterium* on the surface of the explants during the first days on selection media, but very rarely later. On the other hand, there were very few dark blue plant cells during the first 2 days, but many more after 1 week. After 2 weeks on selection medium, out of 130 embryo pieces assayed, 53 (40%) had dark blue cells. Their number varied from 20 to 50 per piece. Approximately 5% of the explants also had large dark blue spots composed of more than one cell. Some of these probably derived from cell divisions of one transformed cell. Most of the blue cells were found on the hypocotyls and the lower side of the cotyledons. However, some were located on the upper side of the cotyledons near the leaf margin (Fig. 4G) where controls (not treated with *Agrobacterium*; on medium without antibiotics) produced secondary embryos (Fig. 4B).

Using the histological GUS assay with nontransformed somatic embryos, it was found that these have a weak endogenous GUS expression. After 8 h in the assay solution, few light blue cells can be observed, and after 24 h in some embryos nearly all the cells of the hypocotyl contain some blue crystals (about 1-5 per cell). Endogenous GUS expression has been reported for many species and for different types of tissue (Plegt and Bino 1989; Hu et al. 1990). Alwen et al. (1992) found that the endogenous β-glucuronidases of different plant families have their optimum at pH 5, while the glucuronidase from *E. coli* used for transformation studies has its optimum near pH 7. When GUS assay was performed with nontransformed somatic embryos at pH 5, the embryos were stained completely dark blue, indicating the presence of endogenous β-glucuronidase. Nontransformed in vitro leaves were stained light blue at pH 5, while at pH 7 no color was observed. These results underline the importance of using negative controls and using the correct pH during GUS assays with cassava tissues.

## 6 Antibiotics and Selection of Transformed Tissues

A problem that is still under investigation is how to select and grow transformed cells. Since we are using the NPT II gene as a selectable marker, we tested the susceptibility of nontransformed cassava tissues to various concentrations of kanamycin and geneticin. The induction of somatic embryogenesis from young leaf lobes was completely inhibited with 4 mg/l kanamycin and 16 mg/l geneticin. Callus formation was completely inhibited by 16 mg/l geneticin, and greatly reduced with 32 mg/l kanamycin. No root formation from in vitro shoot tip explants was observed after 10 days on shoot propagation medium containing 25 mg/l kanamycin (control: average of 1.8 roots/explant). Therefore we concluded that cassava is very sensitive to kanamycin and geneticin. However, in contrast to the results with primary explants, callus that had formed on young leaf lobes after 1 week on medium without kanamycin was able to survive and grow after transfer to medium with 100 mg/l kanamycin, although at a very slow rate. Similar results were obtained with embryo clumps: secondary embryogenesis was strongly suppressed, but the tissues were not killed and some callus growth was observed on medium with 400 mg/l kanamycin.

Fitch et al. (1990) described a comparable phenomenon in transformation experiments with papaya. Induction of embryogenesis from hypocotyls and zygotic embryos was suppressed by 75 mg/l kanamycin, while the growth of somatic embryos established on nonselective medium was inhibited only by 150 mg/l kanamycin.

In those experiments where leaf disks were treated for 3 days with *Agrobacterium* with a vector containing the NPT II gene and the GUS gene, the selection of transformed calli was readily achieved. Since the explants were placed onto selection medium with 100 mg/l kanamycin immediately after cocultivation, very little callus developed from nontransformed cells. When rapidly growing calli developed, 85–90% showed GUS activity. The callus lines showing GUS activity that were used for Southern blot assays all had the GUS gene stably integrated into their genome. In contrast, when embryo pieces were cocultivated for 3 days with *Agrobacterium* and were then transferred immediately to selection medium, a different picture emerged. Secondary embryogenesis was suppressed, but little cell proliferation on the surface of some embryos was observed on medium with 100 mg/l kanamycin. Those cells that were possibly transformed (as indicated by the histological GUS assay several weeks after *Agrobacterium* treatment) behaved similarly, i.e., they formed neither fast-growing callus nor secondary embryos. It seemed that nontransformed tissues as well as transformed cells were inhibited by kanamycin. The conclusion one can draw based upon these observations is that kanamycin apparently is not an efficient antibiotic for the selection of transformed somatic embryo cells of cassava.

An example for the effect of different antibiotics belonging to the group of aminoglycosides on plant regeneration is reflected in the work of Escandón and Hahne (1991) in sunflower. They found striking differences in the efficiency of kanamycin and paromomycin on the selection of transformed tissues.

A way to overcome the problems of selection of transformed cassava tissues with kanamycin might be either the use of alternative antibiotics or different selectable markers. Another strategy that would avoid problems resulting from selection with antibiotics in the early stages of regeneration would be to apply selection pressure either at a later stage after transformation, or not at all. However, in the latter case, one would have to screen hundreds or thousands of regenerated plants for the few expected transformants.

## 7 Summary and Conclusions

Cassava transformation is still in an early phase, i.e., many of the parameters involved in this process have either to be optimized or have not been investigated. In a first attempt to learn more about expression of foreign genes in cassava, we used an air pressure-driven particle gun to introduce DNA into leaf cells. With this system it was possible to obtain information about the expression efficiency of different chimeric gene constructs. Two promoters derived from the CaMV 35S promoter turned out to be good candidates to drive heterologous gene expression in cassava leaves.

Using *Agrobacterium*-mediated transformation of leaf disks, cassava calli were obtained that expressed the $\beta$-glucuronidase gene, the CCMV coat protein gene,

and the ACMV coat protein gene in a stable manner. Infection of somatic embryos with *Agrobacterium* led to the expression of the GUS gene (after 2 weeks on selection medium) in all parts of the embryos. These included the region where, in controls, secondary embryos usually begin to develop.

For the first time we report here on the use of particle gun to introduce *Agrobacterium* into plant tissues. For systems where the damage caused by *Agrobacterium* is detrimental to the plant tissue, this method might be an alternative to the conventional dipping method.

Both of the experiments involving leaf disk transformation by dipping explants in an *Agrobacterium* suspension as well as by bombardment of leaf lobes with *Agrobacterium* had an unexpected result. While controls not treated with *Agrobacterium* and grown on nonselective medium formed callus on media with either 2,4-D alone or with a combination of NAA and BAP, the initiation and growth of transformed callus was observed only on the latter medium. This means that not only are the parameters concerning the infection with *Agrobacterium* important for the recovery of transformed cells, but also the growth regulators present in the selection medium. Similar results were obtained by Escandón and Hahne (1991) in sunflower. In transformation experiments with *Helianthus* seedlings these workers observed a variation in the number of transformed cells depending on the growth regulator balance of the selection medium.

We also attempted to improve the regeneration of cassava in the absence of transformation, i.e., the number of leaf lobes producing embryos and the number of embryos regenerating into plantlets. One key to a solution of the problem of cassava regeneration in vitro might be the mineral composition of the culture medium used. Meyer and van Staden (1986) found that the MS salts used in cassava culture media are suboptimal for the growth of nodal explants taken from greenhouse-grown plants. They devised a modified mineral medium that improved shoot growth of nodal explants of four cassava cultivars by more than two fold, root growth by two to five fold. Part of this effect was due to an increase in the micronutrients, molybdenum and zinc.

We completed preliminary experiments investigating the effect of micronutrients on shoot growth in vitro and on somatic embryogenesis. The results show that an increase in the copper concentration from 0.1 $\mu$M (MS medium) to 2 $\mu$M increases both shoot and root growth and the number of leaf explants producing somatic embryos. This indicates that a further investigation of the effects of micronutrients might be useful to improve the overall response of cassava in vitro, especially the response of recalcitrant cultivars.

In summary, it may be said that some of the essential requirements for the transformation of cassava with viral coat protein genes are now met. We showed that both the CCMV coat protein gene as well as the ACMV coat protein gene can be transferred to cassava tissues and that they are expressed. Using *Agrobacterium*, we were able to produce embryos that express the GUS gene in cells where secondary embryos usually develop.

*Acknowledgments.* We would like to thank Dr. W. Roca for providing in vitro shoot cultures of cassava cv. MCol 22, Prof. G.G. Henshaw for in vitro shoot cultures and embryo cultures of cv. CMC 76, Dr. S.K. O'Hair for cuttings of cv. Señorita, Dr. J. Ellis for providing the ocs tetramer, Dr. J. Callis for the UBQ1 construct, Dr. M. Hinchee for advice concerning the coating of tungsten particles, the Monsanto

Company (St. Louis, MO) for providing several plasmids and the *Agrobacterium* strain GV3111, and Mrs. M. Dimmick and Mrs. B. Delanney for their excellent technical assistance. The work was supported by ORSTOM (Institut Français de Recherche pour le Développement en Coopération), by the Rockefeller Foundation, by IFAR/USAID, by GTZ (Gesellschaft für Technische Zusammenarbeit, Germany), and by the Center for Plant Science and Biotechnology at Washington University in St. Louis.

## References

Alwen A, Benito-Moreno RM, Vincente O, Heberle-Bors E (1992) Plant endogenous β-glucuronidase activity: how to avoid interference with the use of the *E. coli*: β-glucuronidase as a reporter gene in transgenic plants. Transgen Res 1:63–70

Bajaj YPS (1983) Cassava plants from meristem cultures freeze-preserved for three years. Field Crops Res 74:161–167

Beachy RN, Loesch-Fries S, Tumer NE (1990) Coat protein-mediated resistance against virus infection. Annu Rev Phytopathol 28:451–474

Bertram RB (1990) Cassava. In: Persley GJ (ed) Agricultural biotechnology: opportunities for international development. CAB Int, Wallingford, UK, pp 241–261

Cabral GB, Matsumoto K, Aragão F, Teixeria JB, Rech EL (1991) Expressão transiente do gene de β-glucoronidase em protoplastos de mandioca (*Manihot esculenta* Crantz) utilizando electroporacão. In: Resumos III. Congr Brasileiro de Fisiologia Vegetal. Sociedade Brasileria de Fisilogía Vegetal, Universidade Federal de Vicosa, Viçosa, Brazil, p 88

Calderón A (1988) Transformation of *Manihot esculenta* (cassava) using *Agrobacterium tumefaciens* and expression of the introduced foreign genes in transformed cell lines. MSc Thesis, Vrije Universiteit Brussel, Belgium, 37 pp

CIAT (1989) Report on the founding workshop of the advanced cassava research network. CIAT Document 52, Cali, Colombia

Cock JH (1982) Cassava: a basic energy source in the tropics. Science 218:755–762

Cooke R, Cock JH (1989) Cassava crops up again. New Sci 122:63–68

Costa AS, Kitajima EW (1972) Cassava common mosaic virus. CMI/AAB, Description of plant viruses, No 90, 4 pp (Issued jointly by the Common Wealth Mycological Inst, Ferry Lane, Kew, Surrey, UK and the Assoc Appl Biol, Culross & Son Ltd, Coupar Angus, Pertshire, Scotland)

De Bruijn GH, Fresco LO (1989) The importance of cassava in world food production. Neth J Agric Sci 37:21–34

Escandón AS, Hahne G (1991) Genotype and composition of culture medium are factors important in the selection for transformed sunflower (*Helianthus annueius*) callus. Physiol Plant 81:367–376

Fauquet C, Fargette D (1990) African cassava mosaic virus: etiology, epidemology, and control. Plant Dis 74:404–411

Fauquet C, Bogusz D, Chavarriaga P, Franche C, Schöpke C, Beachy RN (1992) Cassava viruses and genetic engineering. In: Thotthapilly G, Monti L, Mohan Raj DR, Moore AW (eds) Biotechnology: Enhancing research on tropical crops in Africa. CTA/IITA co-publication, Ibadan, Nigeria pp 287–296

Fitch MMM, Manshard RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep 9:189–194

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803–4807

Franche C, Bogusz D, Schöpke C, Fauquet C, Beachy RN (1991) Transient gene expression in cassava using high-velocity microprojectiles. Plant Mol Biol 17:493–498

Horsch RB, Klee HJ (1986) Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: role of the T-DNA in the transfer process. Proc Natl Acad Sci USA 83:4428–4432

Hu CY, Chee PP, Chesney RH, Zhou JH, Miller PD, O'Brien WT (1990) Intrinsic GUS like activities in seed plants. Plant Cell Rep 9:1–5

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Kartha KK, Gamborg OL, Constabel F, Shyluk JP (1974) Regeneration of cassava plants from apical meristems. Plant Sci Lett 2:107–113

Klee HJ, Rogers SG (1989) Plant gene vectors and genetic transformation: plant transformation systems based on the use of *Agrobacterium tumefaciens*. In: Vasil IK, Schell J (eds) Cell culture and somatic cell genetics of plants, vol 6. Molecular biology of plant nuclear genes. Academic Press, San Diego, pp 1–23

Klein TM, Goff SA, Roth BA, Fromm ME (1990) Applications of the particle gun in plant biology. In: Nijkamp HJJ, van der Plas LHW, and van Artrijk J (eds) Progress in plant cellular and molecular biology. Kluwer, Dordrecht, pp 56–66

Mabanza J (1984) La culture in vitro des protoplastes de manioc (*Manihot esculenta* Crantz). Etude des possibilités d'induction d'une résistance à la bactériose provoquée par le *Xanthomonas manihotis*. PhD Thesis, Université des Sciences et Techniques du Languedoc, Montpellier, 193 pp

Mabanza J, Jonard R (1983) L'isolement et le développement in vitro des protoplastes de manioc (*Manihot esculenta* Crantz). C R Soc Biol 177:638–645

Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:20–79

Meyer HJ, van Staden J (1986) Inorganic nutrient requirements of in vitro cultured *Manihot esculenta* plants. S Afr J Bot 52:472–480

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nzoghe D (1989) Recherche des conditions permettant l'obtention de néoformations chez différents génotypes de Manioc (*Manihot esculenta* Crantz). Extension à la culture de protoplastes. PhD Thesis, Université de Paris Sud, Centre d'Orsay, 150 pp

Plegt L, Bino RJ (1989) β-Glucuronidase activity during development of the male gametophyte from transgenic and non-transgenic plants. Mol Gen Genet 216:321–327

Roca WM (1984) Root and tuber crops: cassava. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol 2. MacMillan, New York, pp 269–301

Roca WM (1989) Cassava production and utilization problems and their biotechnological solutions. In: Sasson A, Costarini V (eds) Plant biotechnology for developing countries. Proc Int Symp organized by CTA and FAO, 26–30 June 1989, Luxembourg. The Trinity Press, UK pp 215–219

Sanford JC (1990) Biolistic plant transformation. Physiol Plant 79:206–209

Schöpke C, Franche C, Chavarriaga P, Bogusz D, Fauquet C, Beachy RN (1991) Stable transformation of cassava tissues using *Agrobacterium tumefaciens* and a particle gun. Abstr 1991 World Congr on Cell and Tissue culture, 16–20 June 1991, Anaheim, California, In Vitro Cell Dev Biol 27(3), pt II: 151A

Schrammeijer B, Sijmons PC, van den Elzen PJM, Hoekema A (1990) Meristem transformation of sunflower via *Agrobacterium*. Plant Cell Rep 9:55–60

Shahin EA, Shepard JF (1980) Cassava mesophyll protoplasts: isolation, proliferation, and shoot formation. Plant Sci Lett 17:459–465

Stamp JA (1987) Somatic embryogenesis in cassava: the anatomy and morphology of the regeneration process. Ann Bot 59:451–459

Stamp JA, Henshaw GG (1982) Somatic embryogenesis in cassava. Z Pflanzenphysiol 105:183–187

Stamp JA, Henshaw GG (1986) Adventitious regeneration in cassava. In: Withers LA, Alderson PG (eds) Plant tissue culture and its agricultural applications. Butterworths, London pp 149–157

Stamp JA, Henshaw GG (1987a) Secondary somatic embryogenesis and plant regeneration in cassava. Plant Cell Tissue Organ Cult 10:227–233

Stamp JA, Henshaw GG (1987b) Somatic embryogenesis from clonal leaf tissues of cassava. Ann Bot 59:445–450

Szabados L, Hoyos R, Roca WM (1987a) In vitro somatic embryogenesis and plant regeneration of cassava. Plant Cell Rep 6:248–251

Szabados L, Narváez J, Roca WM (1987b) Techniques for isolation and culture of cassava (*Manihot esculenta* Crantz) protoplasts. Working Document 23. Biotechnology Research Unit, CIAT, Cali, Colombia, 42 pp (Spanish/English)

Twell D, Klein TM, Fromm ME, McCormick S (1989) Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Physiol 91:1270–1274

Ulian EC, Smith RH, Gould JH, McKnight TD (1988) Transformation of plants via the shoot apex. In Vitro Cell Dev Biol 24:951–954

Villegas L (1988) Expresión transitoria en protoplastos de yuca. In: Villegas L (ed) Cultivo de tejidos vegetales aplicado a la producción agrícola. Instituto Internacional de Estudios Avanzados, Unidad de Biotechnología, Caracas, Venezuela, pp 69–80

Villegas L, Santana MA, Bravato M, Zapata C (1988) Hibridación somatica: electrofusión de protoplastos de yuca. In: Villegas L (ed) Cultivo de tejidos vegetales aplicado a la producción agrícola. Instituto Internacional de Estudios Avanzados, Unidad de Biotechnología, Caracas, Venezuela, pp 145–153

*Note Added in Proof.* Recently two papers have been published describing results that might be important for cassava transformation. Mathews et al. report on the improvement of the regeneration efficiency from somatic embryos, Raemakers et al. describe the use of liquid medium for somatic embryogenesis.

Mathews H, Schöpke C, Carcamo R, Chavarriaga P, Fauquet C, Beachy RN (1993) Improvement of somatic embryogenesis and plant recovery in cassava. Plant Cell Rep 12: 328–333

Raemakers CJJM, Schavemaker CM, Jacobsen E, Visser RGF (1993) Improvements of cyclic somatic embryogenesis of cassava (*Manihot esculenta* Crantz). Plant Cell Rep 12: 226–229

# II.13 Transformation of Tobacco (*Nicotiana clevelandii* and *N. benthamiana*)

R. Tavazza and G. Ancora[1]

## 1 Introduction

A range of procedures has been developed for transferring specific DNA into plant cells. These include the uptake of DNA by protoplasts (Krens et al. 1982; Potrykus et al. 1985), DNA transfer mediated by *Agrobacterium*, and more recently the microprojectile (Klein et al. 1987) or shoot gun method, which is of great interest for the transformation of plants whose tissues are recalcitrant to regeneration of whole plants from cells.

When possible, the *Agrobacterium*-mediated DNA transfer represents the method of choice for transferring genes into plant cells. The major techniques which utilize *A. tumefaciens* to transform plant cells are: wounding and inoculation of intact plants (Lippincott and Heberlein 1965; Murai et al. 1983), inoculation of explants in vitro (Garfinkel and Nester 1980, Barton et al. 1983), cocultivation of protoplast-derived cells with the bacteria (Wullems et al. 1981; Fraley et al. 1983) and leaf-disk transformation (Horsch et al. 1985). The leaf-disk transformation method rapidly became the method of choice due to both speed and efficiency.

From the initial attempts of plant cell transformation, *Nicotiana* species have been frequently utilized. This is largely due to the fact that *Nicotiana* species are more amenable to subsequent tissue culture procedures which allow regeneration of plants from transformed tissues.

## 2 Transformation Studies on *Nicotiana*

Early transformation experiments in *Nicotiana* were performed using wild-type strains of *Agrobacterium tumefaciens*. In these experiments the selection of transformants was based on hormone autotrophy acquired by the transformed cells (Marton et al. 1979; Wullems et al. 1981). The normal Mendelian transmission of the octopine synthase (OCS) marker gene introduced into a tobacco plant by the Ti plasmid of *A. tumefaciens* was reported by Otten et al. (1981) and confirmed in successive studies in *Nicotiana* species (De Block et al. 1984; Horsch et al. 1984, 1985).

[1]ENEA Dip. Ricerca e Sviluppo Agroindustriali, Div. Ingegneria Genetica, C.P. 2400, 00100 Roma A.D., Italy

Biotechnology in Agriculture and Forestry, Vol. 23
Plant Protoplasts and Genetic Engineering IV (ed. by Y.P.S. Bajaj)