Plant Cell Reports (2000) 19:339–344

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Transgenic plants of cassava (*Manihot esculenta*) with resistance to Basta obtained by *Agrobacterium*-mediated transformation

Received: 9 January 1999 / Revision received: 10 May 1999 / Accepted: 15 June 1999

Abstract Transgenic plants of cassava (*Manihot esculenta*) resistant to the herbicide Basta were obtained through *Agrobacterium*-mediated transformation. The plants also expressed the *uid*A gene and two were positive for PCR- and/or Southern-based detection of the *npt*II gene. Somatic-embryo-derived cotyledons were used as source of explants. A non-disarmed *Agrobacterium* strain (CIAT 1182) was used to transfer the genes of interest into cassava cultivar MPer183. Greenhouse tests of resistance to Basta (Hoechst) showed three plant lines with different levels of tolerance to the herbicide. Based on Southern tests of transgenesis, the transformation efficiency was 1%. The results constitute the first report of the *bar* gene conferring herbicide resistance to cassava plants.

Key words *Manihot esculenta* · *bar* · ppt/Basta-resistance · useful transgenes

Abbreviations *BAP*: 6-Benzylaminopurine · *BM*: Basal medium · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *GUS*: β-Glucuronidase · *ppt*: Ammonium glufosinate

Communicated by H. Judelson

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Introduction

The importance of cassava as a global food source is reflected in the recent biotechnological accomplishments aimed to speed up non-conventional breeding in cassava. Transformation and regeneration of transgenic cassava plants expressing selectable and scorable markers has been reported by several laboratories (Sarria et al. 1993, 1995, 1997; Li et al. 1996; Raemakers et al. 1996; Schopke et al. 1996; Gonzalez et al. 1998). The establishment of *Agrobacterium*- and biolistic-mediated transformation methods now allows the introduction of agronomically useful genes into cassava (see Program and Abstracts CBN IV 1998). We report here the *Agrobacterium*-mediated tansformation of cassava cultivar MPer183 with the *bar* gene, resulting in the production of plants resistant to the herbicide Basta (Hoechst). The detection and/or expression of the inserted selectable and scorable transgenes *npt*II and *uid*A are also reported.

Materials and methods

Plant material and tissue culture

Somatic embryos were induced from apical meristems of cassava cultivar MPer183 on medium MS8 (CIAT 1997). MS8 medium was composed of MS basal salts [basal bedium (BM); Murashige and Skoog 1962] and supplemented with 8 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 50 mg/l casein enzymatic hydrolysate, 2 μm Cu_2SO_4 (Schopke et al. 1993), B5 vitamins (Gamborg et al. 1968), 2% sucrose, pH 5.7 and 5.8 g/l agar. Somatic embryos were induced in darkness for 30 days. Embryos proliferated on MS4 medium (same components as MS8 but with 2,4-D and casein halved) under a 12-h photoperiod (100 μE $m^{-2}s^{-1}$) for 30 days. Embryos matured on BM supplemented with 0.5 mg/l 6-benzylaminopurine (BAP), 2% sucrose, pH 5.7 and 5.8 g/l agar under a 12-h photoperiod at 300 μE $m^{-2}s^{-1}$. Embryo induction, proliferation and maturation were accomplished in a plant growth room at 25–28 °C. Green, cotyledonary leaves from 30 to 60-day-old, mature somatic embryos were used as explants for transformation (Sarria et al. 1993). Cotyledonary leaves were precultured on MS8 medium for 2–3 days in the light conditions (300 μE $m^{-2}s^{-1}$) at 25–28 °C before inoculation with *Agrobacterium*.

Bacterial strain and plasmids

A wild, agropine-mannopine, cassava-infecting *Agrobacterium tumefaciens* strain – CIAT 1182 – was selected according to early infectivity tests of several strains on different cassava cultivars (CIAT 1997). This strain was transformed with the binary vector pGV1040 (Fig. 1; PGS, Belgium; Calderon-Urrea 1988). The T-DNA of pGV1040 harbors the *npt*II, *bar* and *uid*A genes, which encode neomycin phosphotransferase, phosphinothricin acetyltransferase and β-glucuronidase (GUS), respectively. *Agrobacterium* cultures were maintained on solid AB-sucrose minimal medium (Chilton et al. 1974) and selected with 50 mg/l kanamycin and 100 mg/l each of rifampicin, spectinomycin and streptomycin.

Transformation, selection and plant regeneration

Bacterial cultures were shaken overnight at 250 rpm in bacterial induction medium (Arias and Sarria. 1995) at 27 °C until the OD_{560} was 1.2. Bacteria were then pelleted, supernatant discarded and they were resuspended in BM ($OD_{560}=0.8$) supplemented with 100 μm acetosyringone and 0.5% glucose for *vir* gene induction. Explants were inoculated by applying drops of bacterial suspension to them. Cocultivation was carried out on MS8 medium for 48 h in darkness at 28 °C. After cocultivation, explants were washed twice with sterile distilled water and once with liquid BM supplemented with 500 mg/l carbenicillin and 250 mg/l cefotaxime. Explants were dried on filter paper and cultured for 2–3 days on MS8 plus 500 mg/l carbenillin and 250 mg/l cefotaxim. The commercial herbicide Basta, which contains 200 g/l of ammonium glufosinate (ppt), was then used as selective agent. Selection was applied by inducing somatic embryos for 20 days on MS8 supplemented with 8–32 mg/l ppt. After selection, embryos were matured and elongated on solid BM containing 0.25 mg/l BAP, 0.25 mg/l gibberellic acid and 1.0 mg/l ppt. Plantlets were then micropropagated by nodal sections, rooted in vitro on 4E medium (Roca 1984) and transferred to the greenhouse.

Histological GUS test

Somatic embryos and shoot tips from greenhouse-grown plants, preselected on the basis of in vitro resistance to 8–32 mg/l ppt, were assayed for GUS activity as described by Stamp (1992), adding 20% methanol to the assay buffer to suppress endogenous GUS activity (Kosugui et al. 1990). Stained shoot tips and embryos were prepared for sectioning by standard histological procedures (Cann et al. 1965).

In vitro test of Basta resistance

Leaf discs from young leaves of in vitro plants were cultured on solid BM medium supplemented with 2, 4, 6, or 8 mg/l ppt. Susceptibility to Basta, scored as necrosis of leaf discs, was evaluated after 8, 15, and 24 days.

Greenhouse test of Basta resistance

Plants selected on the basis of in vitro resistance to ppt and GUS expression were sprayed (40 ml/m^2) with a 200 mg/l ppt solution from the commercial herbicide Basta. Two Basta applications were made, one every 15 days. Previous greenhouse tests (unpublished data) indicated that 100 mg/l ppt effectively kills cassava plants. Plants resistant to Basta were selected for PCR-based and Southern hybridization tests.

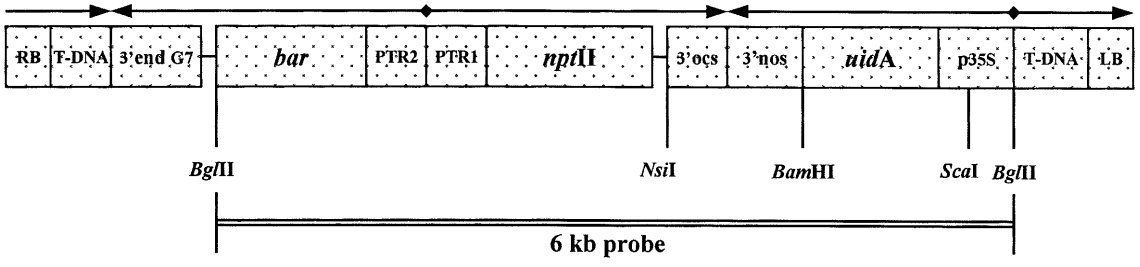
PCR-based tests and Southern hybridizations

Specific primers for the *npt*II gene were used to amplify 25 ng of genomic DNA from plants preselected on the basis of Basta resistance and positives for the GUS test. The final volume for PCR reactions was 25 μl. The expected PCR product was a 1.2-kb band for the amplified *npt*II gene. Amplifications were done in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.1 μM of each primer and 1 U of Taq polymerase (Perkin Elmer). Thermocycling was as follows: one cycle of 180 s at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 90 s at 72 °C. Thermocycling ended with a final extension cycle of 5 min at 72 °C. Amplification products were separated in 1.3% agarose gels and transferred to Hybond N^+ nylon membranes (Amersham) by Southern blotting. Genomic DNA was extracted from 3 g of mature, fresh leaves from greenhouse-grown plants according to the method of Dellaporta et al (1983). Genomic DNA (10 μg) was digested with either BglII or ScaI for 14 h at 37 °C, fragments separated on 0.8% agarose gels and Southern-blotted onto nylon membranes. Prehybridization of membranes containing PCR products or digested genomic DNA was carried out for 2 h at 65 °C using 5× SSPE (pH 7.0), 5× Denhardt's solution, 1% SDS and 150 μg/ml herring sperm DNA. For hybridization, a *Bgl*II 6-kb fragment from the T-DNA, containing the *npt*II, *bar* and *uid*A genes, was used as probe (Fig. 1). Two hundred nanograms of labeled probe (Amersham Multiprime Labeling System) was hybridized overnight to membranes. Filters were then washed once for 30 min. at 65 °C in 2×, 1× and then 0.5× SSPE containing 0.1% SDS, and exposed to X-ray film at –80 °C using intensifying screens.

Fig. 1 Map of T-DNA of plasmid pGV1040. Restriction sites are indicated. *RB*: Right border; *T-DNA*: sequence of the TL-DNA; *LB* Left border; *3'endG7*, *3'ocs* and *3'nos*: termination and polyadenylation signals from gene 7 of the octopine TL-DNA, the octopine synthase gene and the nopaline synthase gene, respectively; *PTR1* and *PTR2*: coupled promoters from the TR-region of the octopine Ti-plasmid; *p35S* promoter for the 35S transcript of the cauliflower mosaic virus; *uidA*: β-glucuronidase gene; *bar*: phosphinotricin resistance gene; *npt*II; kanamycin resistance gene

Results and discussion

A total of 100 cotyledonary leaves were inoculated with the *Agrobacterium* strain CIAT 1182-pGV1040. Seven



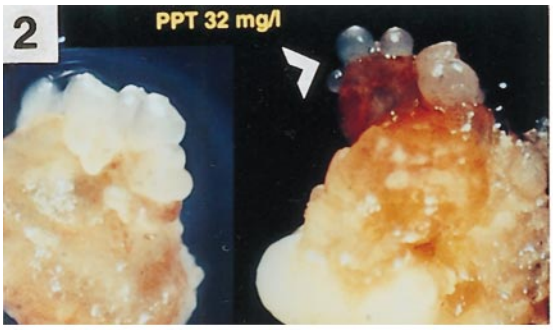


Fig. 2 Somatic embryos of cassava cultivar MPer183, transformed with *Agrobacterium* strain 1182-pGV1040 growing on MS8 selective medium (*arrowhead*). The approximate age of the embryos is 20 days. *PPT 32 mg/l* concentration of ppt in the medium

Fig. 3a Histological GUS assay for transformed (*right*) and non-transformed (*left*) shoot tips of plantlets of cassava cultivar MPer183 to show non-chimeric GUS expression. **b** Longitudinal section of a transformed, GUS-assayed shoot tip of cassava cultivar MPer183 demonstrating even (non-chimeric) distribution of GUS-expressing cells. The *arrow* indicates the evenly stained shoot apical meristem

explants produced somatic embryos after 20 days of selection on MS8 medium supplemented with 8–32 mg/l ppt (Fig.2). Initiation of embryo formation was observed on *Agrobacterium*-treated explants after 10 days on selection medium while control explants, not treated with *Agrobacterium*, produced embryos after 5 days on non-selective MS8 medium. Putatively transformed embryos matured and produced 42 plantlets on medium supplemented with 1 mg/l ppt. The low frequency of embryo induction (7%) under selective conditions was probably due to ppt itself. Leaf discs of in vitro cassava plants show complete necrosis after 5 days on BM supplemented with a ppt concentration as low as 2.0 mg/l (not shown). The ppt LD_{50} for cassava somatic embryos and green tissues is 16 and 1 mg/l, respectively (Sarria et al. 1993). Susceptibility of cassava embryogenic tissues to *Agrobacterium* infection (Schopke et al. 1993; Li et al. 1996; Gonzalez et al. 1998) may also be responsible for the observed low frequency of embryogenesis.

After germination and elongation of somatic embryos, histochemical localization of GUS activity was performed for 42 regenerated plantlets. Fifteen plantlets showed stronger blue staining in vascular tissues than in leaf tissue, indicating differential GUS expression. The number of explants expressing GUS reached 95% when vascular tissue was evaluated, while, with cotyledonary leaves, only 30% were positive. Whole shoot tips also displayed differential staining, i.e., intense blue color was observed in the apical part (Fig. 3a), but staining decreased in the differentiated and mature parts of shoot tips. However, histological sections of shoot apical meristems, assayed for GUS expression, demonstrated an even (non-chimeric) distribution of GUS-expressing cells (Fig. 3b). Differential expression of the *uid*A gene, driven by the CaMV35Spromoter, has been reported in cassava, e.g. young leaves and vascular tissues show darker-blue staining (Schopke et al. 1996; Gonzalez et al. 1998). In tobacco also, decreasing GUS expression in differentiating and mature tissues has been reported (Chinn and Luca 1996).

The 42 plantlets that regenerated from somatic embryos on selection medium were micropropagated through nodal explants to obtain 230 plants. Tests of resistance to 2–8 mg/l ppt for in-vitro-derived leaf discs were performed for all 230 plants. Simultaneously, PCR-based detection of the *npt*II and *uid*A genes was carried out for all 230 plants. The tests of resistance to ppt resulted in the selection of 15 plants that tolerated no less than 8 mg/l ppt after being on selective medium for a maximum of 24 days. All 15 plants were also positive for the PCR-based tests (not shown). Different levels of tolerance to Basta were observed. This observation could be due to different levels of expression of the *bar* gene, which can be affected by copy number, insertion site and rearrangement of the transgene into the genome (Cooley et al. 1995). Control leaf discs were completely necrotic at 2 mg/l ppt after 10 days on selection medium (Fig. 4).

The 15 plants mentioned above were transferred to the greenhouse. They were sprayed twice, one spraying every 15 days, with Basta (ppt at 200 mg/l, 40 ml/m^2). Three plant lines, 53–5.2, 137–3.1 and 207, showed different levels of resistance to Basta (Fig.5). Judging by the number and size of yellowish, necrotic areas on the leaves, plant line 53–5.2 showed the highest resistance of the three lines. The other 12 lines showed levels of resistance to Basta that were below those of plant lines 207 or 137–3.1, and some responded very similarly to control plants and were therefore considered non-resistant. Control plants showed complete necrosis 5 days after a single spraying, and after 5 days, leaves fell off the plant.

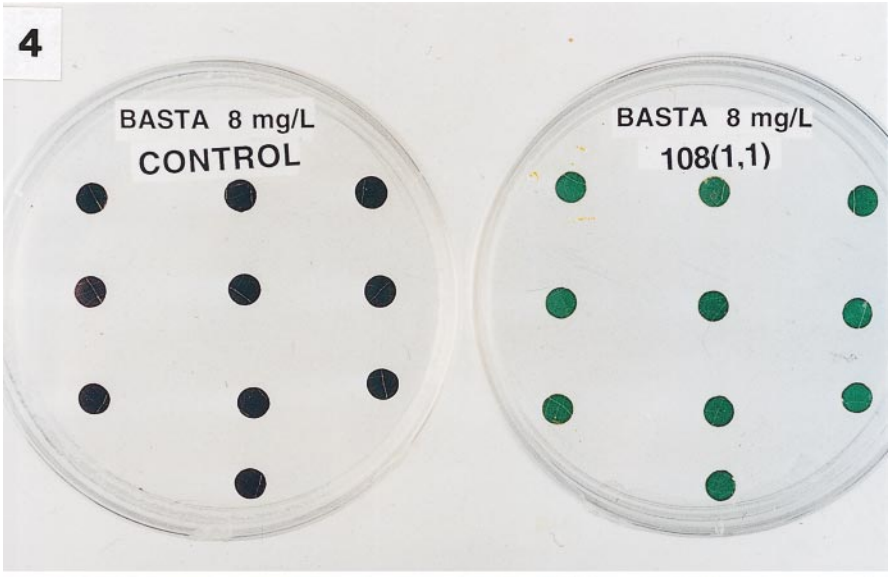


Fig. 4 In vitro test for resistance to Basta (8 mg/l ppt) for transformed (*right dish* plant line 108–1.1) and control, non-transformed leaf discs (*left dish*) of cassava cultivar MPer183

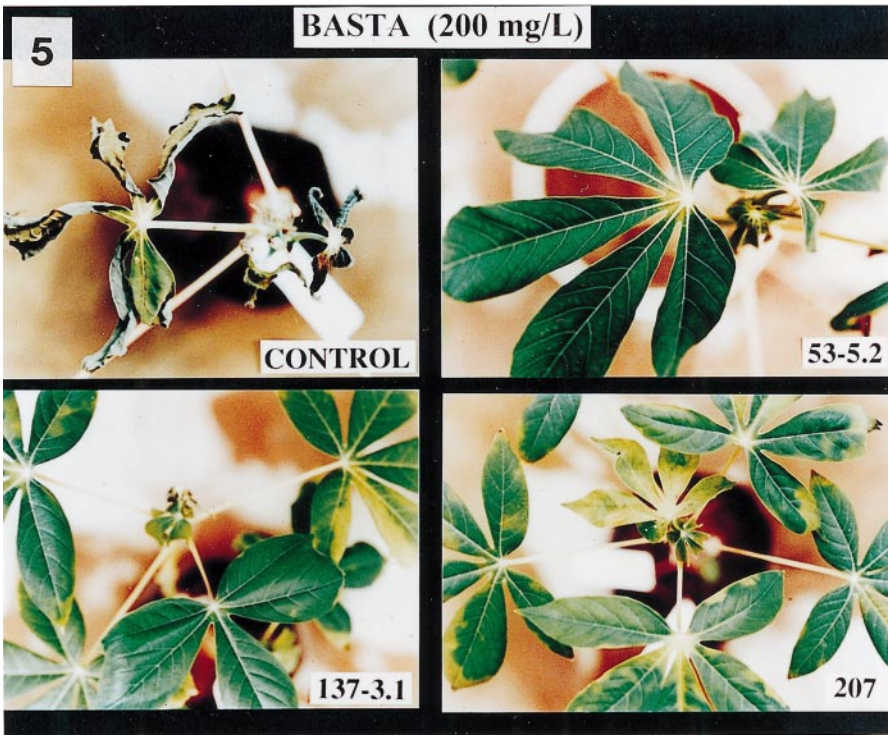


Fig. 5 Greenhouse test for resistance to Basta (200 mg/l) for three transgenic cassava plant lines (53–5.2, 137–3.1 and 207) and a non-transformed control (*upper left*) of cassava cultivar MPer183

DNA was extracted from the 15 plants mentioned above and Southern hybridizations were made for PCR-amplified products using the *npt*II probe (Fig. 1). Of the 15 plants analyzed, only plant 53–5.2 showed a strong signal. The detected band was 1.2 kb in size, corresponding to the expected size of the PCR-amplified fragment (Fig. 6). Total DNA was then extracted from all 15 plants, digested with *Bgl*II or *Sca*I and Southern-hybridized with the 6-kb probe containing the *bar*, *npt*II, and *uid*A genes. Plant 53–5.2 showed three strong signals (Fig. 7) indicating three possible T-DNA insertions into host DNA. Two of the three insertions in plant 53–5.2 may have lost *Bgl*II sites, generating bands larger than 6 kb. Integration of T-DNA into host DNA of plant 53–5.2 was confirmed by digesting host DNA with *Sca*I, which has a single site on the T-DNA (Fig. 1), and by hybridizing to the same 6-kb probe. Three bands were detected, confirming three different insertion events. The smallest band detected with *Sca*I was 9.3 kb, which indicated that the next *Sca*I site is at least 3.3 kb into host DNA from the *Bgl*II site in the right border flanking the T-DNA. As expected, no bands were observed in negative controls for cassava or tobacco (Fig. 7). DNA from the other 14 plant lines showed no detectable hybridization signals though these plants were positive for GUS expression, PCR-based tests and in vitro or greenhouse tests for resistance to Basta.

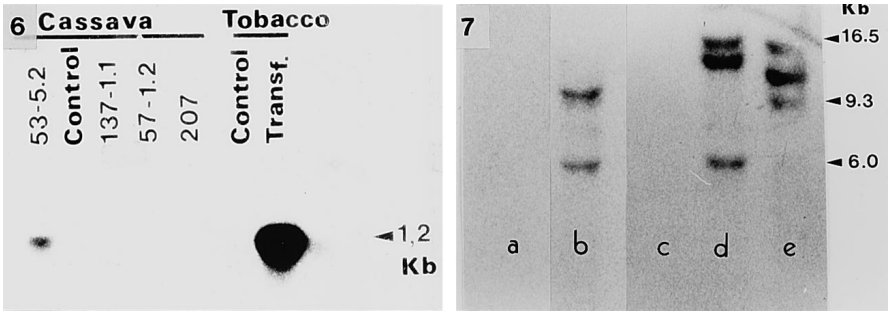


Fig. 6 Detection of *npt*II-specific, PCR-amplified products from transformed cassava plants of cultivar MPer183. From *left* to *right* under *Cassava*: plant line 53–5.2, non-transformed cassava plant (*Control*), and plant lines 137–1.1, 57–1.2 and 207; under *Tobacco*: non-transformed tobacco (*Control*) and tobacco transformed with *Agrobacterium* 1182-pGV1040 (*Transf.*). The *arrowhead* indicates DNA from cassava plant 53–5.2 and transformed tobacco showing the expected band at 1.2 kb

Fig. 7 Detection of the inserted T-DNA into the genome of cassava and a positive tobacco control. *Lane (a)* non-transformed tobacco control *lane (b)* tobacco transformed with *Agrobacterium* 1182-pGV1040 and digested with *Bgl*II showing two bands *lane (c)* non-transformed cassava control *lane (d)* cassava plant 53–5.2 digested with *Bgl*II showing three bands; *lane (e)* cassava plant 53–5.2 digested with *Sca*I showing three bands (see text for details). The probe used for Southern blots is depicted in Fig. 1

Our results show that a wild *Agrobacterium* strain – CIAT 1182-pGV1040 – was able to transfer and insert a T-DNA containing an agronomically useful gene (*bar* gene) into cassava cells from somatic-embryo-derived cotyledons. As a source of explants for cocultivation with *Agrobacterium*, somatic embryo-derived cotyledons have the advantage of being abundant and highly embryogenic, a finding first reported by Sarria et al. (1993) and validated by Li et al. (1996).

Selection of putatively transgenic tissues and plants, with up to 200 mg/l ppt by spraying in the greenhouse, produced three plants that tolerated the herbicide Basta, although only one plant was positive in Southern-based tests of transgenesis. The observed efficiency of transformation per initial number of inoculated explants was therefore 1%, a low but acceptable value. The improvement of cassava transformation efficiency is currently the focus of several laboratories that work with the crop (Program and Abstracts CBN IV 1998). Increasing the embryo-to-plant conversion rate, e.g. by reducing in vitro culture periods, may allow several transgenic lines to be obtained per transformation experiment, which will increase the chances of obtaining plants correctly expressing the genes of interest.

Acknowledgements The authors thank Dr. Alejandro Calderon-Urrea for his valuable guidance during the initial phases of this research. We also thank PGS (Belgium) for providing pGV1040.

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