Establishment and Transformation of Callus and Cell Suspension Cultures of the Prickly-Pear (*Opuntia ficus-indica)*

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ABSTRACT

Friable callus cultures were initiated from cotyledons and hypocotils of *Opuntia ficus-indica*. Propagation and growth of calli were observed on MS medium supplemented with 2,4-D, Pickloram and Casein. Cell transformation was achieved by particle bombardment. Kanamycin resistance, GUS expression, and PCR analyses indicated successful integration of foreign DNA.

Key words: Cell suspension, friable callus, *Opuntia ficus indica*, particle gun

Abbreviations: K: 6-Furfurylaminopurin; 2,4-D: 2,4-Dichlorophenoxyacetic acid; Pickloram: 4-Amino 3,5,6-Trichloropicolinic acid

INTRODUCTION

Over many years the prickly-pear (*Opuntia ficus-indica*) has shown its usefulness as a forage crop for small farmers in northeastern Brazil. This is especially true in the years of drought, where, on many occasions, its fleshy cladodes are the sole source of water and nutrients for farm animals. We are exploring the possibilities of using biotechnology methods to improve the quantity and quality of the protein profile in the cladodes of the prickly-pear (*Opuntia ficus-indica*). We are developing protocols for plant regeneration and transformation as well as studying the deposition pattern of proteins in seeds and cladodes. Here we present a method for the induction, establishment, and transformation of friable callus and cell suspension cultures

MATERIALS AND METHODS

Induction of Friable Callus

Seeds from mature fresh fruits of *Opuntia ficus-indica* were scarified to break the physical barrier of the hard lignified seed coat. Then seeds germinated in a mixture of river sand and compost (1:1) under 16 hours daily low-intensity illumination and constant 28°C. After germination (21 days) the seedlings were sterilized in 1.5% sodium hypochlorite and washed 3 times in distilled sterile water. Callus cultures were initiated from cotyledons and hypocotils. The explant's sections were transferred aseptically to petri dishes containing 30 ml of friable callus medium (FCM), consisting of full-strength MS salts and vitamins (Murashige and Skoog, 1962), 0.2 mg/l 6-furfurylaminopurine (K), 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l 4-amino 3,5,6-trichloropicolinic acid (Pickloram), 400 mg/l casein hydrolysate, 3% sucrose and 0.8% agar. All media were adjusted to pH 5.8 with 1M KOH and autoclaved at 121°C (2 kg/cm^2) for 15 minutes. The cultures were

incubated in the dark at 28°C. The calli were separated from cotyledons and hypocotils and subcultured at 3- to 4-week intervals on FCM medium.

Cell Suspension

Cell suspension cultures were initiated by shaking 35 to 40 g of friable callus at 150 rpm in the dark at 28°C in a 300 ml Erlenmeyer flask containing 150 ml of FCM without agar (FCMs). After 48 hours the cells and small cell aggregates were separated from the callus using a 1 mm stainless mesh, and the liquid medium was transferred to Falcon plastic tubes (50 ml) for sedimentation. Inocula of 7.5 to 10 ml of the cell suspension (sediment) were transferred to a 300 ml Erlenmeyer flask containing 30 ml of FCMs medium. After the first week of culture, 20 ml of fresh FCMs medium was added. The cells were separated every 15 days from the old medium using a 1 mm stainless mesh and transferred to fresh liquid medium. The cultures were placed on a gyratory shaker at 150 rpm in the dark at 28°C.

Transformation

For introduction of reporter genes we used the vector pPARGUSH (van der Hoeven *et al.*, 1994) which contains the *nptII* gene under control of the *nos* promoter, the *uidA* gene under control of the *par* promoter (Landsmann *et al.*, 1988), and the *tet* gene under control of a bacterial promoter. The DNA-coated particles for bombardment with the particle gun were prepared according to Sanford *et al.* (1993) and modified by Mahn *et al.* (1996). Friable callus sections were kept in solidified FCM (0.6 M) medium for a maximum of 4 hours after bombardment with a Biolistic PDS-1000He instrument from Bio-Rad Laboratories. The distance between the flying disk and the target tissue was 7.5 cm and the shooting pressure was adjusted to 1200 psi. The bombarded callus sections were transferred to petri dishes containing 30 ml of FCM medium supplemented with 100 mg/l kanamycin. Callus resistant to kanamycin was maintained under kanamycin selection and transferred to fresh medium at 3- to 4-week intervals.

GUS Expression

Friable callus was subjected to assays of GUS activity at 3- to 4-week intervals. For the histochemical GUS assay (Jefferson *et al*, 1987), tissue was immersed in a sterile solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and after 24 hours, was soaked in 70% ethanol. Assayed tissue was observed under a binocular microscope.

For the photometric GUS assay (Jefferson *et al.*, 1986), extracts of tissue (standardized to 35 µg protein) were transferred to a solution containing p-Nitrophenyl-ß-D-glucuronide substrate. The enzymatic reaction was stopped at 0, 30, and 60 minutes after incubation at 37°C. The reaction product, p-nitrophenol (pNP), was quantified photometrically at 415 nm. The amount of pNP was calculated in nMol pNP/mg protein/minute. One mg ß-glucuronidase enzyme produces 36 nMol pNP/minute (Van der Hoeven, 1992).

Polymerase Chain Reaction

The successful introduction of foreign genes into the plant genome was determined by PCR amplification with *gus* gene primers (5`-AAC GGC AAG AAA AAG CAG TC-3` and 5`-GAG CGT CGC AGA ACA TTA CA-3`), *npt II* gene primers (5`-GGT GCC CTG AAT GAA CTG-3` and 5`-TAG CCA ACG CTA TGT CCT-3`) and *tet* gene primers (5`-CGT GTT GCT AGG ATG GTT GT-3` and 5`-CTT TGG GTC ATT TTC GGC GA-3`). The expected size of the fragments is 895 bp, 700 bp, and 510 bp, respectively. PCR amplification was carried out in 50 µl containing 5 µl of DNA solution (0,005 µg/µl), 0.2 mM dNTPs, 3 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 10 mM KCl, 0.2 mM of each primer, and 2.5 units of Taq DNA polymerase. The DNA amplification was subjected to 35 cycles of

1 minute at 94°C and 1.5 minute at 53°C and 72°C. The PCR products were analyzed on a 1% agarose gel.

RESULTS

Induction of Friable Callus

Primary callus cultures were obtained from cotyledons and hypocotils of seedlings as described in Materials and Methods. Friable callus developed after 12 weeks of culture in the dark on FCM medium. The first callus formation was observed after 7 to 10 days. The percentage of successful callus development was 18.1%, a majority of the calli being nonfriable calli. For further propagation white-yellow calli were isolated from mother explants and subcultured in the same medium.

After 10 to 12 weeks of subculture in FCM medium, two types of calli (soft-friable and hard calli) were found. The percentage of friable calli was 11.4%; only the friable aggregates were propagated. Friable callus showed rapid growth compared to the nonfriable callus. The growth of friable callus was evaluated by diameter size. During the 28- to 30-day period, the diameter of the calli increased approximately 4.5 times from 0.32 to 1.43 cm. The best growth of friable callus tissue was obtained in the linear phase between 21 and 28 days of culture. However, after 30 to 40 days of culture, white and vigorous calli started to show brown discoloration. This suggests that the best time for transferring healthy friable callus to fresh medium is approximately 28 days after subculture.

Cell Suspension

Twenty-eight-day-old friable calli were used for starting cell suspension cultures in the FCMs medium. The friable calli rapidly disintegrated to single cells and small cell aggregates during vigorous shaking. After six passages of selective subculturing, the cell suspension looked homogeneous and contained spherical or elongated single cells and small aggregates. The growth rate of the cell suspension culture was determined by fresh weight. Fresh weight of the cell suspension after 10 days of culture increased from 8.9 to 26.8 g, and after 20 days the culture had increased by a factor of five. The nutrient composition and pH of the culture medium changed during cell suspension growth. The sucrose concentration decreased from initially 0.087 M to 0.052 M after 9 days. The pH of the medium decreased from 5.8 to 4.0. The decrease of the pH supports the notion that CAM plant cells contain high amounts of organic acid in their vacuole (Cook *et al.*, 1995).

Transformation

Transgenic friable calli were obtained on kanamycin containing medium after particle bombardment. The presence of the foreign genes was confirmed by histochemical and photometric assays for GUS activity and by PCR amplification of DNA with *gus*- gene primers, *nptII* gene primers and *tet* gene primers.

Histochemical analysis of the expression of the *gus* gene was done with individual cells, with groups of cells of friable callus and with cell suspension (Figure 1) which had been supplied with X-Gluc, the chromogenic substrate of the GUS enzyme.

Photometric GUS assays were conducted with the stable kanamycin resistant friable calli. GUS activity was determined with p-Nitrophenyl-ß-D-glucuronide as a substrate. The photometric GUS activity range was 47 ±18.6 nMol p-Nitrophenol pNP/mg protein/minute.

The incorporation of foreign genes into the plant cells was determined by agarose gel electrophoresis of PCR-amplified DNA from friable calli. The presence of the *gus*-fragment, the

nptII-fragment and the *tet*-fragment were detected in kanamycin-resistant friable calli. No amplification could be detected with DNA from unbombarded friable calli (Figure 2).

DISCUSSION

We have developed methods for the establishment and transformation of friable callus and cell suspension cultures of *O. ficus-indica*. A prerequisite in establishing friable calli was the supplementation with Pickloram and casein hydrolysate in MSK medium and selection of white-yellow soft calli. For establishment of cell suspensions, the characteristics of the inoculum (density, age, and growth phase) are important for growth improvements (Arias-Castro *et al.*, 1993). Subculturing every 15 days using an inoculum ratio of 1:3 (v/v) greatly enhanced the growth of the cell suspensions. The constitutive expression of the *gus* and *nptII* genes under the control of the *par* and *nos* promoters, respectively, indicated successful delivery of foreign DNA into cells of *O. ficus indica*.

Regeneration of whole transformed plants will be the next developmental step. To test every potential route towards this goal we are currently developing protocols for the integrative transformation of somatic embryos via particle bombardment.

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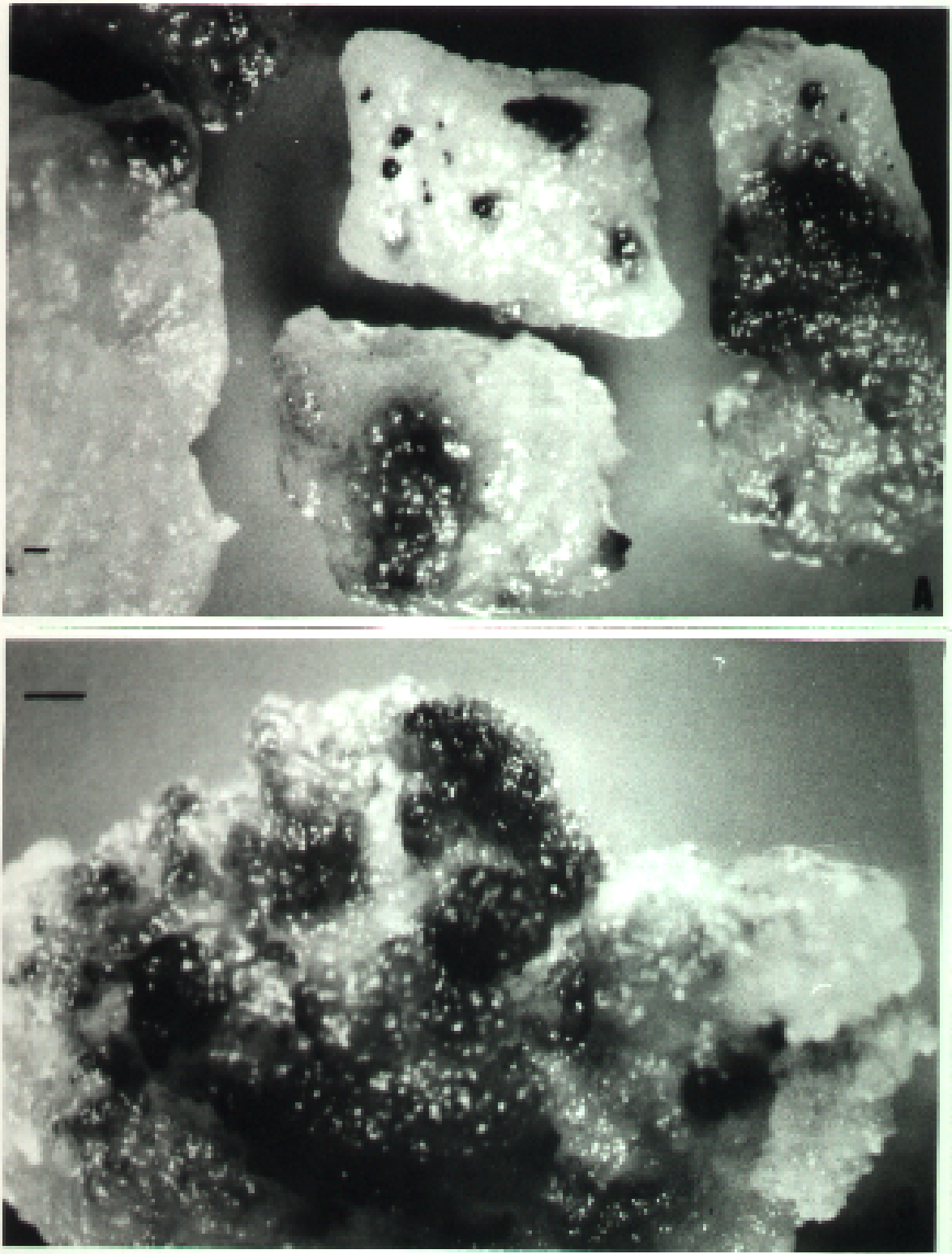

Figure 1. Histochemical assay of the glucuronidase (*GUS*) gene expression (dark spots) in friable callus directly after bombardment (A) and in kanamycin-resistant friable callus (B) (bars represent 1 mm)

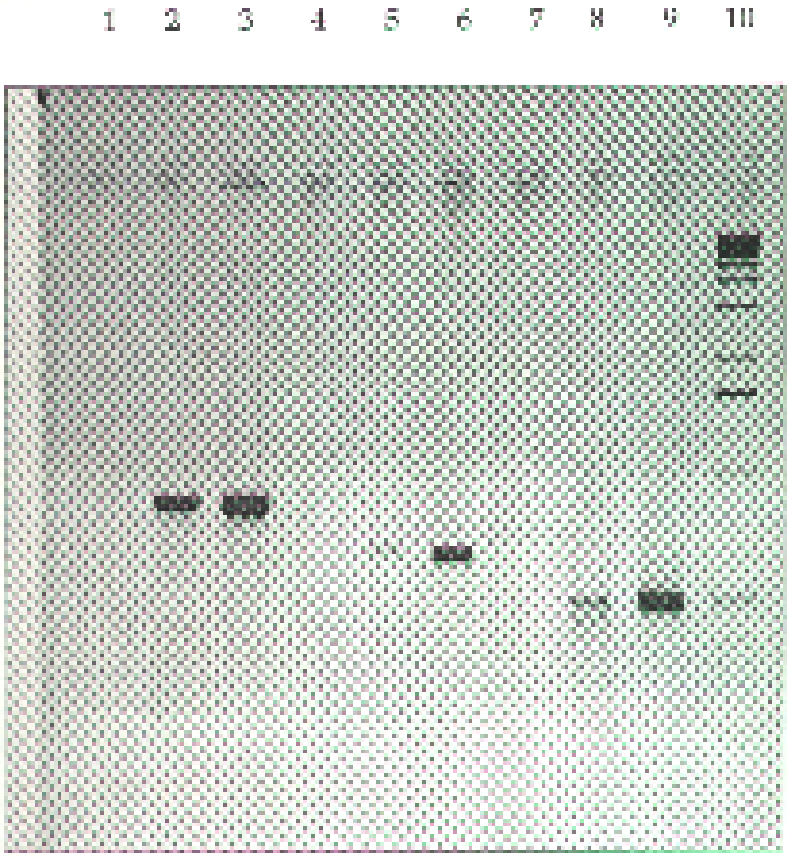

Figure 2. PCR amplification of DNA from friable callus the with *gus* primers (1, 2, 3), 895 bp fragment; *npt*II primers (4, 5, 6), 700 bp fragment; and *bar* primers (7, 8, 9), 510 bp fragment. lane 1, 4, 7: Negative control (untransformed); lane 2, 5, 8: transgenic friable callus; lane 3, 6, 9: positive control (pPARGUSH); and lane 10: 1 kb ladder marker (GIBCO BRL).