J. Plant Biotechnology(2002) *Vol. 4(2). pp. 71~76*

Journal of Plant Biotechnology

Repetitive Somatic Embryogenesis in Cacao and Optimisation of Gene Expression by Particle Bombardment

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Key words: Biolitic, cacao transformation, osmotic treatment, *Theobroma cacao*

Abstract

In order to achieve repetitive somatic embryogenesis in cacao (*Theobroma cacao* L.), callus derived from floral tissues were continuously cultured in a medium containing 2,4-D. In 5% of the explants, repetitive somatic embryogenesis was observed after 8 weeks and maintained in a globular stage for several weeks. This is the first report showing repetitive somatic embryogenesis in cacao. The calli were bombarded with a plasmid containing β-glucuronidase (*gus*) as reporter gene. Two week-old calli showed the high average number of cells expressing the *gus* gene. The effect of osmotic agents (mannitol, sorbitol and sucrose) on gene expression was evaluated. Pre-treatment during 16 h with 0.25 M mannitol revealed an improvement in gene expression. The potential utilization of the repetitive embryogenesis, combined with osmotic treatment, is discussed as an alternative to achieve stable transgenic cacao plants.

Introduction

Cacao (*Theobroma cacao* L.) is one of the most important crops for the food, pharmaceutical and cosmetic industries. The main cacao producers are tropical countries such as Ivory Coast, Indonesia, Ghana, Nigeria and Brazil. Consequently, there is considerable interest in introducing useful traits, such as biotic and abiotic stress tolerance in this important crop. The use of genetic engineering techniques for the introduction of genes responsible for agronomic characteristics, associated with sexual breeding methods, may accelerate the production of new genotypes.

The development of transgenic plants depends on several factors, such as the establishment of an efficient regeneration and transformation systems. During the last years, zygotic and somatic cells have been able to regenerate plants from cacao tissues (Pence et al., 1980; Figueira and Janick, 1993; Li et al., 1998). Early efforts to transform cacao (*T. cacao*) demonstrated its susceptibility to *Agrobacterium tumefaciens* and some transgenic tissues from leaf discs, have been achieved (Purdy and Dickstein, 1989; Sain et al., 1994). Guiltinan et al. (1997) were able to achieve transgenic cacao plants by the co-cultivation of somatic embryos with *Agrobacterium*. However, this system is time consuming and the frequency of transgenic plants obtained was much lower than required for obtaining elite events for the breeding programs. Perry et al. (2000) have demonstrated the applicability of particle bombardment to introduce and express the gus gene in cacao tissues. Despite the development of several systems for cacao *de novo* regeneration, their combination with the biolistic process for transformation has not been successfully achieved.

Repetitive somatic embryogenesis is the ability to develop secondary somatic embryos from a single cell line after additional cycles of culture. In combination with a gene transfer system, it allows an appropriated selection, preventing the development of chimerical transgenic plants. The development of repetitive embryogenesis from

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Received Apr. 20, 2002; accepted Mary. 11, 2002.

established somatic embryogenesis systems, in association with the biolistic process, has been successfully used in plant transformation (Santarém and Finer 1999; Magbanua et al., 2000) and would be an alternative to achieve stable transgenic cacao plants. In the present work we developed a repetitive somatic embryogenesis system for cacao. In addition, this system was associated with the biolistic process in order to access its potential for cacao transformation.

Materials and Methods

Plant material and repetitive somatic embryogenesis

Floral buds (8-9 cm long), from the genotype 'Cenargen 2', were taken prior to opening, surface sterilized in 70% (v/v) ethanol for 1 min followed by 2% (v/w) sodium hypochlorite for 20 min, and rinsed 6 times in sterile distilled water. Staminodes (vestigial stamens) were aseptically excised from buds and cultivated for 2 weeks on PCG (primary callus growth) medium [DKW salts (Driver and Kuniyiki, 1994) supplemented with 20g/L glucose, 250 mg/L glutamine, 100 mg/L myo-inositol, 2.5 mg/mL TDZ (Thidiazuron), 2 mg/L 2,4-D and 2g/L phytagel] (Li et al., 1998). Obtained calli were then submitted to 2 treatments: (A) subcultured on the PCG medium every 2 weeks for 8 weeks, transferred to SCG (secondary callus growth) medium [WPM salts (Lloyd and McCown, 1981) containing 20 g/L glucose, 2 g/L phytagel, 50 ml/L coconut water, 2 mg/L 2,4-D, 100 mg/mL kinetin] for additional 2 weeks (Li et al., 1998), and cultivated on ED (embryos development) medium (DKW salts supplemented with 30 g/L sucrose and 1 g/L glucose) (Li et al., 1998) until the development of embryos; (B) subcultured to SCG medium every 2 weeks for 8 weeks, transferred to ED medium until development of embryos. Cultures were maintained in Petri dishes in the dark at 26±2°C. The treatments were repeated 3 times with 300 explants each. Explants were evaluated for the development of somatic embryos, after 4 weeks on ED medium.

Plasmid vector and particle bombardment

The plasmid pBI426, used for the particle bombardment assay, containing the *gus* gene fused with *neo* (neomycin phosphotransferase II) gene (Datla et al., 1991) driven by a double 35S cauliflower mosaic virus (CaMV) promoter plus a leader sequence from alfalfa mosaic virus (AMV), was provided by Dr. William Crosby (Plant Biotechnology Institute, Saskatoon, Canada). Plasmid DNA was precipitated onto WP-100 tungsten particles (Atlantic Equip. Eng., New Jersey, USA) as previously described by Aragão et al. (1996). Calli at different culture stages were bombarded in the conditions previously established in our laboratory (Aragão et al., 1996). Staminodes were cultured for 0, 1 or 2 weeks on PCG medium, stages I, II and III respectively. Staminodes cultured for 2 weeks on PCG followed by 1 or 2 weeks on SCG medium, stages IV and V, respectively. Staminodes were cultured for 2 weeks on PCG, followed by 2 weeks on SCG and 1 week on ED medium, stage VI. Tissues were placed at a distance of 12 cm from the stop screen and a helium pressure of 1,200 psi was applied. Bombarded tissues were maintained in each respective bombardment medium (supplemented with 0.8 g/L phytagel) for 24 h, and transferred to PCG (stages I and II), SCG (stages III and IV) or ED (stages V and VI) media. Treatments were repeated 2 times with 12 explants each.

Effects of osmotic treatments

In order to test the effect of an osmotic agent on transformation efficiency, 2-week-old calli cultivated on PCG medium were placed for 16 h prior to bombardment on the PCG medium containing 0, 0.25, 0.5 and 1.0 M mannitol, sorbitol, sucrose or a mixture of mannitol and sorbitol. After bombardment, the calli were maintained in the same medium for 24 h and analysed for the gus gene expression 48 h after bombardment. The treatment with mannitol was utilised in an additional experiment to evaluate the effect of the period of osmotic treatment prior bombardment (4 and 16 h) 48 h after the bombardment. The treatments were repeated 3 times with 12 explants each.

Bombardment of repetitive somatic embryos

Clusters of repetitive somatic embryos were positioned on a plate containing PCG medium supplemented with 0.25 M mannitol (16 h of pre-treatment) and bombarded as described above. The explants were transferred to PCG liquid medium 24 hours after bombardment and cultivated during 2 weeks in the dark at 26± 2°C , in a shaker at 100 rpm. The explants were subcultured every 2 weeks in the PCG liquid medium containing 400 mg/L kanamycin and analysed for *gus* expression after a total of 8 weeks of cultivation.

GUS histochemical assay

Tissues were analysed for *in situ* localization of GUS activity 48 h and 2 weeks after bombardment as previously

described (Jefferson et al., 1987). Transformation efficiency was expressed as the number of blue spots per bombarded explant.

Statistical analysis

The experiments were carried out in a completely randomised design. Data were shown as the mean±standard deviation (S.D.). Treatments were compared by analysis of variance (ANOVA) and Tukey's test.

Results and Discussion

Repetitive somatic embryogenesis

All cultivated staminodes showed callus formation after 2 weeks of culture on PCG medium. These calli were then subcultured either on PCG (treatment A) or SCG medium (treatment B) for 8 weeks in order to achieve repetitive embryogenesis. Part of the calli (5%) continuously subcultured on PCG (treatment A) has shown repetitive somatic embryogenesis 8 weeks after culture initiation. During further subcultures on PCG medium, secondary embryogenesis was maintained at the globular stage of differentiation, for at least 10 weeks (Figure 1). These repetitive somatic embryos were also able to differentiate in a bipolar structure when transferred to SCG medium and subcultured on ED medium (Figure 1). Sixty percent of these embryos were converted into phenotipically normal plants (Figure 1).

The induction of repetitive somatic embryogenesis has been achieved in several species, such as peanut (Baker and Wetzstein 1995; Little et al., 2000), *Medicago trunculata* (Neves et al., 1999), tea (Akula et al., 2000) and *Helianthus maximiliani* (Vasic et al., 2001) by continual cultivation in media containing any kind of auxins, particularly 2,4-D. The use of others auxins (dicamba, picloran, NAA and p-chlorophenoxyacetic acid) have also being proposed (Little et al., 2000) to induce repetitive somatic embryogenesis.

Particle bombardment

In order to access the efficiency of gene introduction into callus cells, an experiment was carried out bombarding calli at different cultured stages. The best transformation efficiency was achieved in 2-week-old calli (an average of 13 blue spots per explant), which was significantly different from that observed in calli from the other stages (0, 1, 3, 4 and 5 weeks in culture) (Figure 2). The 2-week-old calli were cultured on PCG medium, which contains TDZ and 2,4-D. TDZ is a potent cytokinin-like regulator that acts synergistically with 2,4-D to induce somatic embryogenesis (Hutchison et al., 1996). Consequently, at this stage, 2-week-old calli are stimulated to cell division and faster growth, affecting cell transformation efficiency. In general, healthy and actively dividing cells are the best targets for particle bombardment because they can easily overcome the stresses promoted by microparticles penetration (Brettschneider et al., 1997; Vain et al., 1993). The expression has dramatically decreased 2 weeks after bombardment (Figure 2). This observation suggested that most blue spots observed in the GUS assay represent non-integrative events. In addition, cytoplasmatic contents release and lack of an effective selection agent must be involved.

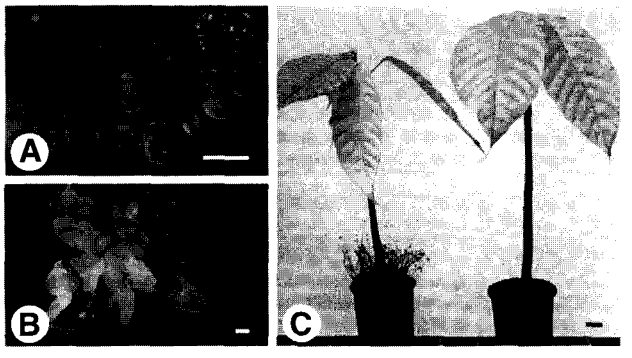

Figure 1. Repetitive somatic embryogenesis in cacao. A) Secondary embryos after 8 weeks subcultured on PCG medium, B) Differentiated embryo in a bipolar structure and C) Acclimated plants generated from somatic embryo. Bars represent 5 mm (A and B) and 20 mm (C).

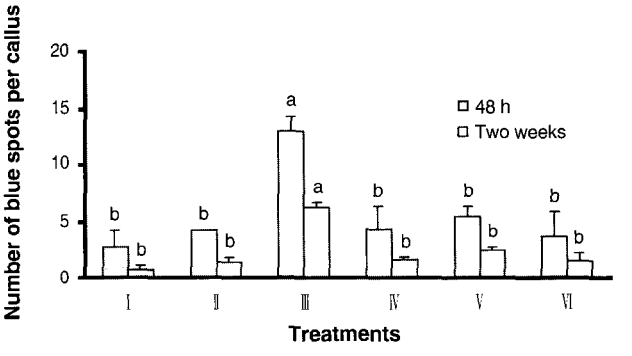

Figure 2. Effect of calli culture stage on gene introduction and expression. Treatments I, II and III (0, 1 and 2 weeks of cultivation on PCG medium); treatments IV and V (1 and 2 weeks of cultivation on SCG medium); treatment VI (1 week of cultivation on ED medium). Calli were assayed for *gus* expression 48 h and 2 weeks after bombardment (bars mean standard deviation). Treatments bearing the same letter are not significantly different according to Tukey's test at 5% probability.

The number of blue spots produced on 2-week-old calli were higher even 2 weeks after bombardment, indicating that it would be the best age for bombardment.

Effects of osmotic treatments on gene expression

Effects of osmotic treatments on gene introduction and expression on 2-week-old calli were tested. There were no significant differences observed among the osmotic agents tested (Figure 3). Nevertheless, the *gus* gene expression was higher when the calli were pre-treated for 16 h with a concentration of 0.25 M of osmotic agents (Figure 3). When the period of pre-treatment was reduced to 4 h, it was observed a reduction in the *gus* gene expression (Figure 4). Osmotic agents, such as mannitol and sorbitol, have been used to increase genetic transformation of plant cells by particle bombardment. It has been proposed that osmotic treatment enhances transformation efficiency rate by the reduction of cell turgor, which prevents damages on cell membrane and loss of cytoplasm, provoked by the shock wave generated during the particle acceleration (Sanford et al., 1993; Vain et al., 1993). In addition, the osmotic treatment reduces the volume of the vacuoles, increasing the possibility to reach the nucleus, resulting in a larger number of cells successfully expressing the introduced genes (Yamashita et al., 1991). Increasing the concentration of the osmotic agents resulted in a decrease of the number of blue spots per callus (Figure 3). It may have resulted from osmotic adjustment, which may have allowed the cells to return to hypertonic state (Santarém and Finer, 1999).

Bombardment of repetitive somatic embryogenic clusters

Bombardment of cacao repetitive somatic embryos showed intense GUS activity at high frequency of blue spots compared to control, mainly in the embryogenic sector (Figure 5). It was found ca. 1,300 blue spots per 100 mg of bombarded tissues pre-treated for 16 h with 0.25 M

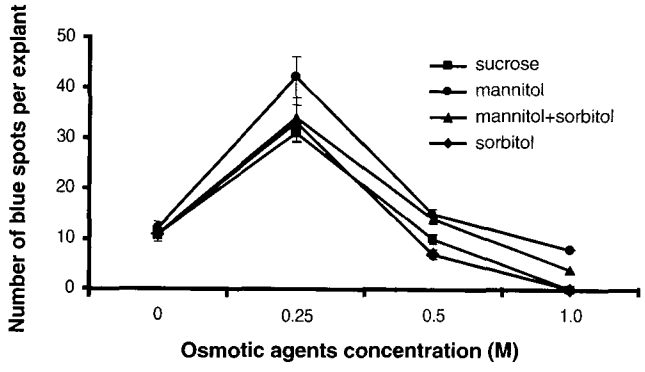

Figure 3. Effect of osmotic pre-treatment on gene expression in cacao embryogenic calli. Bars mean standard deviation.

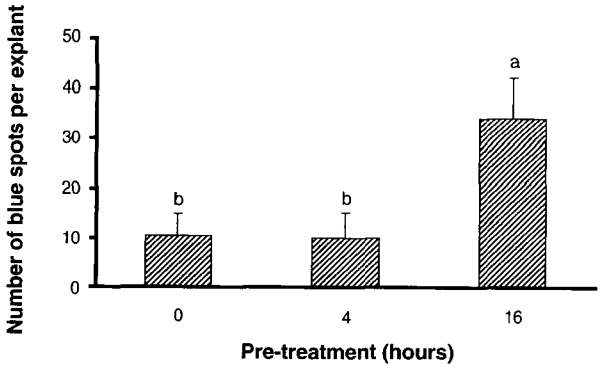

Figure 4. Effect of the period of pre-treatment of cacao embryogenic calli with 0.25 M mannitol. Treatments bearing the same letter are not significantly different according to Tukey's test at 5% of probability.

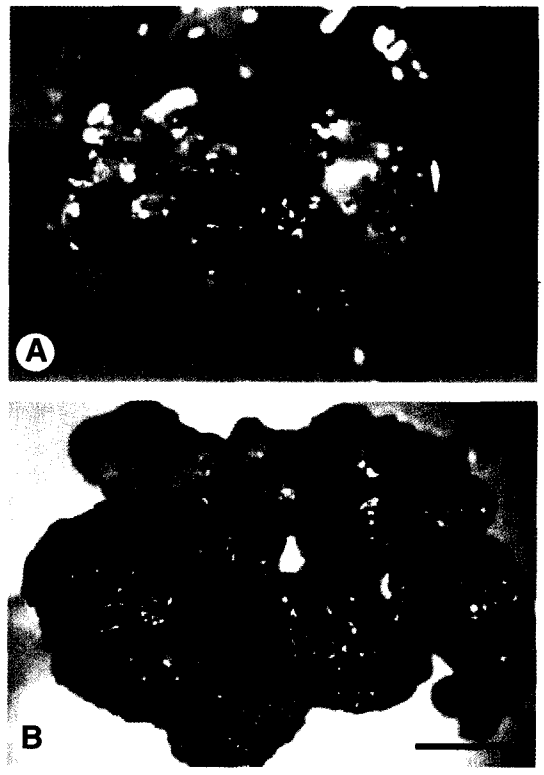

Figure 5. Particle bombardment of repetitive somatic embryogenic cluster. A) Two clusters (arrows) of repetitive somatic embryos expressing the *gus* gene, 48 h after bombardment. B) Transgenic calli derived from a bombarded cluster cultured for 8 weeks, showing intense *gus* gene expression. Bars represent 5 mm.

mannitol. The bombarded cluster of repetitive embryos cultivated for 8 weeks showed extensive mass of transgenic calli (Figure 5).

This is the first report showing repetitive somatic embryogenesis in cacao. Our results indicated that the repetitive embryogenic callus, treated with an osmotic agent, could serve as a convenient source for cacao transformation. One important constraint would be the achievement of an appropriate selection system for transformed cells in combination with an efficient regeneration and transformation protocol as described in this report. Recently, we have developed a novel system to select transgenic cells based on the use of imazapyr, an herbicidal molecule capable of systemically translocating and concentrating in the apical meristematic region (Aragão et al., 2000). We are now extending these findings in order to regenerate transgenic cacao plants. These results are of particularly importance to the understanding of the basic process of integration of foreign genes in cacao, which, in turn, may form the foundation to the effective and practical utility of genetic engineering to introduce useful traits in this important crop.

Acknowledgments

This work was supported by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)-Brazil, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)-Brazil and Coordenação de Aperfeioamento de Pessoal de Nível Superior (CAPES)-Brazil. This work is part of PhD thesis in Molecular Biology of M.O.S. at Universidade de Brasília, Brazil.

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