Transgenic Plantain (cv. Harton) Plants Resistant to Herbicide Basta Obtained by Electroporation

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Abstract

Bananas and plantains (*Musa* spp.) are crops of vital importance to hundreds of millions of people in developing countries. In Venezuela, for instance, plantain represents one of the most socio-economically important crops. In order to improve the crop profitability, besides controlling nematodes, insects, fungi, bacteria and virus attack, it is also necessary to develop an efficient weed control method to decrease the incidence of pests and diseases. In this research we establish suitable parameters to obtain Basta (herbicide) resistant plants through electroporation of plantain shoot apices with plasmid pCAMBIA 3201 carrying the gene *bar*, which codifies for the enzyme phosphinothricin acetyl transferase, giving resistance to phosphinothricin, the active compound of the Basta herbicide. Initially, shoot apices were isolated and grown on Murashige and Skoog medium for 3 days under dark conditions, the tissue was pretreated with three hourly washes using buffer at pH 7.6, and then it was incubated along with plasmid pCAMBIA 3201, on the same buffer. Electroporation was performed applying 200 V/cm discharge from a 1000 µF capacitor. This protocol ensures high electroporation efficiency, less tissue recuperation time and greater percent of regenerated plants. Plasmid incorporation was traced using GUS as reporter gene, selection assays using Basta as selective agent, and PCR analysis. The minimal inhibitory concentration of Basta was 1 mg/ml.

INTRODUCTION

Biotechnology for agriculture has a great potential in stabilizing yields at high levels by alleviating biotic and abiotic stresses. The fundamental reason for plant breeding, using either conventional breeding or biotechnology, to be cost-effective is that the benefits of a one-time investment at a central research location can be multiplied over time across nations all over the world (Bouis and Sco, 2002). Because each developing country region presents particular challenges, each country has to develop a particular solution to its agronomical problems.

Among the major food crops in developing countries are banana and plantain, which are easy to grow with a relatively stable production; an estimated 20 million people eat banana or plantain as a major source of dietary input. The average world yield was 15 tons per hectare for banana and about 6 tons per hectare for plantain. Highest yields of both banana and plantain were found in the Caribbean region, and most of the 10% of the world's bananas and plantains that are grown for export come from Latin America and the Caribbean (IITA, 2000). In Venezuela plantain (*Musa* AAB 'Harton') is a very important crop because it is highly consumed locally and also is an important export crop to industrial countries.

Weeds are one of the harmful factors in bananas and plantain production, as they interfere with plant growth and production, due to the fact that weeds compete with crop plants for soil moisture and nutrients. Furthermore weeds increase the incidence of fungus and bacterial diseases, and also the attack of nematodes, pests and viruses. Basta is one of

the most commonly used nonselective herbicides for weed control, of which the active compound is ammonium glufosinate.

In the present investigation we establish a suitable protocol to obtain Basta tolerant plants through the electroporation of plantain ('Harton') shoot apices with plasmid pCAMBIA 3201 carrying the gene *bar*, which codifies for the enzyme phosphinothricin acetyl transferase, giving resistance to ammonium glufosinate.

MATERIAL AND METHODS

Plant Material

Shoot apices (0.2 to 0.2 cm) were isolated from in vitro produced plants of plantain (*Musa* AAB subgroup plantain 'Harton'), and inoculated in multiplication banana media (Gómez and García, 1994), based on Murashige and Skoog (1963) salts, supplemented with Morel vitamins, sucrose, agar, cysteine and 0.5 mg/L BA. Tissues were incubated under dark conditions for three days, and then pretreated with three hourly washes with buffer ASPm (a modification of ASP buffer, Tada et al., 1990) pH 7.6. Two shoot apices were incubated for one hour in a 0.4 cm micro-vessel, along with 20 µl pCambia 3201 plasmid and 500 µl ASPm buffer, at room temperature condition.

Electroporation

The electroporation was performed applying a 200 V/cm discharge from a 1000 µF capacitor (Gene Pulser® II, BIORAD). The plasmid used was the pCAMBIA 3201 with 11450 Kpb, which contains a multiple cloning site from the pUC18 plasmid, the resistant *cloramphenicol* gene, the reporter *gus* gene and the selectable *bar* gene, which confers resistance to the herbicide ammonium glufosinate (Basta). After electroporation shoot apices were placed for 10 minutes at 4°C, then 15 minutes at room temperature, and transferred to multiplication medium. They were incubated two days in dark condition and finally placed in a growth chamber at 25°C. After one month of growth, shoot apices were tested for *gus* assay, tolerance to Basta and PCR analysis of genes *gus* and *bar*.

Test of Tolerance of Basta

Sixty shoot apices of plantain meristems-derived plants produced in vitro were inoculated to multiplication media, with different concentrations of the herbicide Basta treatments: T_1 control, T_2 1 mg/L, T_3 2 mg/L and T_4 5 mg/L (Table 1).

Gus Assay

Sections of tissue were incubated in Eppendorf tubes along with 500 µl of the substrate 5Br-4Cl-3Indolil glutamic acid (X-Glu), for 24 to 48 hours at 37°C (Jefferson et al., 1987). Tissues were observed in a light microscope (Nikon II) to detect the blue coloration in transformed tissues due to the glucoronidase (*Gus*) activity. The results were registered with a photo camera Nikon FDX-35.

PCR Analysis of *Gus* Gene

DNA was isolated from transformed tissue (Doyle and Doyle, 1990). A *Gus* gene fragment of 1200 bp was amplified using the following primers: 5'-GGTGGGAAAGCG CGTTACAAG-3' (400 to 420 bp) and 5'-GTTTAGGCGTTGCTTCGGCCA-3' (1579 to 1599 bp). The reaction mixture (25 µl) contained 13.75 µl water, 2.5 µl buffer (25 Mm Tris-HCl, pH 8), 2 µl $MgCl_2$ (25 Mm), 0.5 µl of each primer (0.5 pmol/µl), 5 µl dNTP (0.2 mM), 0.25 µl *Taq* polymerase (1.25 U), 5 µl template DNA (10 ng/µl). PCR used one cycle of 95°C (5 min), 40 cycles of 94°C (30 sec), 65°C (30 sec), 72°C (1 min), and finally one cycle of 72°C (10 min). PCR products (5 µl) were electrophoresed on 1.5% agarose in 0.5x TBE and visualized under UV light on an ethidium-bromide-stained gel, washed and analyzed in a Gel Doc 2000 (BIORAD).

PCR Analysis of the *Bar* Gene

The PCR was also used to examine tissues for the presence of the *bar* gene. The procedure to extract DNA was similar to the one used in the previous experiment. A *bar* gene fragment of 400 bp was amplified using the following primers: 5'-CCA GAA ACC CAC GTG ATG CC, and 5'-CAG GAA CCG GCA GGA GTG GA. The reaction mixture (25 µl) contained 13.75 µl water, 2.5 µl buffer (25 Mm Tris-HCl, pH 8), 2 µl $MgCl_2$ (25 mM), 0.25 µl of each primer (25 pmol/µl), 0.5 µl dNTP (0.2 mM), 0.25 µl *Taq* polymerase (1.25 U), 5 µl template DNA (10 ng/µl). PCR used 30 cycles of 94°C (2 min), 60°C (2 min), 72°C (2 min), and one cycle of 72°C (1 min). PCR products (5 µl) were electrophoresed and analyzed as in the previous experiment.

RESULTS AND DISCUSSION

Test for Tolerance of Basta

A test was set up to screen for Basta tolerance to determine the minimal inhibitory concentration (MIC), defined as the lowest concentration of Basta that consistently induced necrosis in shoot apices (SA). It was found that after 21 days in culture, 0% of SA growing in T_1 (control), 50% of SA growing in T_2 (1 mg/L Basta), 85% of SA growing in T_3 (2 mg/L Basta), and 100% of SA growing in T_4 (5 mg/L Basta) were necrotic (Table 2). Based on these results, a 1 mg/L Basta concentration was used for selection of Basta tolerance in this system. This result is similar to the value found for other monocotyledons such as sugar cane (Chowdhury et al., 1992) and maize (Gordon-Kamm et al., 1990). Nevertheless there are other monocotyledon species that require higher concentrations of Basta for transgenic plant selection such as *Pennisetum glacum* (Goldman et al., 2003). In some cases, a similar test has been used to screen transgenic plants (Brukhin et al., 2000).

***Gus* Assay**

Positive *gus* activity (blue color) was observed in the vascular bundles, specifically in the tracheary elements. Figures 1 and 2 illustrate the comparison between a transgenic and a non-transgenic meristem.

PCR Analysis of *Gus*

The insertion of the *gus* gene in the DNA of the transformed plants can be found in Fig. 3, an electrophoretic band of 1200 bp can be observed in the DNA of *Cambia* plasmid (lane 3), as well in the DNA of transgenic plantain plant (lane 4). Meanwhile in the DNA of the negative control (non-transgenic plant), the 1200 bp band is not present.

PCR Analysis of the *Bar* Gene

PCR was used to examine the plants for the presence of the *bar* gene. It was demonstrated by the observation of a 400 bp fragment in transformed tissues (Fig. 4) observed in lanes 1 and 4 (DNA of plantain transformed plants), and lane 6 (DNA of pCambia containing the *bar* gene). These results are consistent with the findings in the PCR analysis of the *gus* gene in this investigation. These results demonstrate that, despite the fact that we used meristems as a target tissue to obtain transgenic plantain plants, we established a method that shows 50% efficiency in incorporating the *bar* gene in the DNA plant tissue as it was demonstrated by PCR analysis of the *gus* and *bar* genes. This result is similar to the values found by Zhong et al. (1996), working with transformation of maize meristems. Glucoronidase activity was detected in the mesophyll and bundle-sheath cells. We conclude that this system can be an alternative to laboratories that have not established the somatic embryogenesis in *Musa* spp. It is also a sensible system since the minimal concentration of Basta for screening of transgenic plants is 1 mg/L. This protocol ensures high electroporation efficiency, less tissue recuperation time and greater percent of regenerated plants.

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Tables

Table 1. Test of tolerance to Basta. Experimental design.

Treatment	Basta (mg/L)	N° dishes	N° meristems/dishes	N° meristems/treat
1	0	4	4	16
2	1	4	4	16
3	2	4	4	16
4	5	4	4	16

Sixty shoot apices of plantain in vitro plants were inoculated in multiplication media, with different concentrations of the herbicide Basta.

Table 2. Results of the test for Basta tolerance. Necrosis was evaluated in meristems growing with Basta, under light condition for 30 days.

Treatment	necrosis(%) (7 days)	necrosis(%) (14 days)	necrosis(%) (21 days)	necrosis(%) (30 days)
1	0	0	0	0
2	0	0	50	80
3	0	50	85	95
4	0	100	100	100

Figures

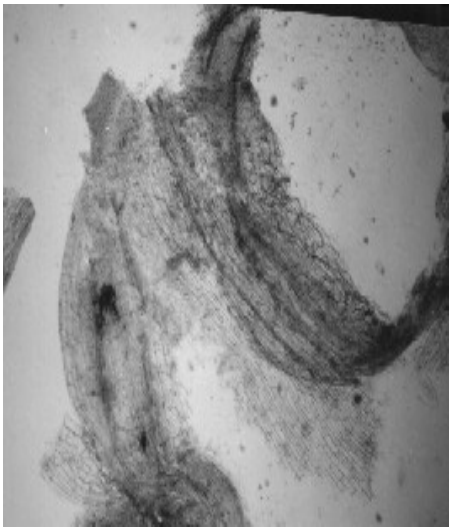

Fig. 1. Negative *Gus* reaction in a meristem electroporated without plasmid.

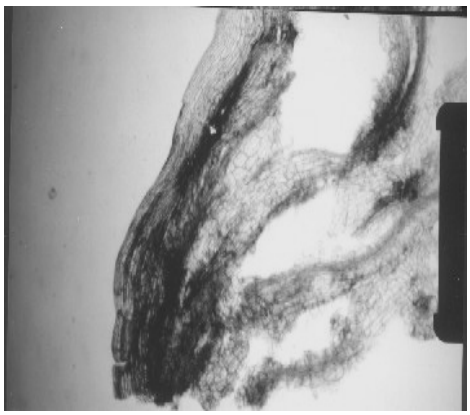

Fig. 2. Positive *Gus* reaction in a meristem electroporated with the plasmid described.

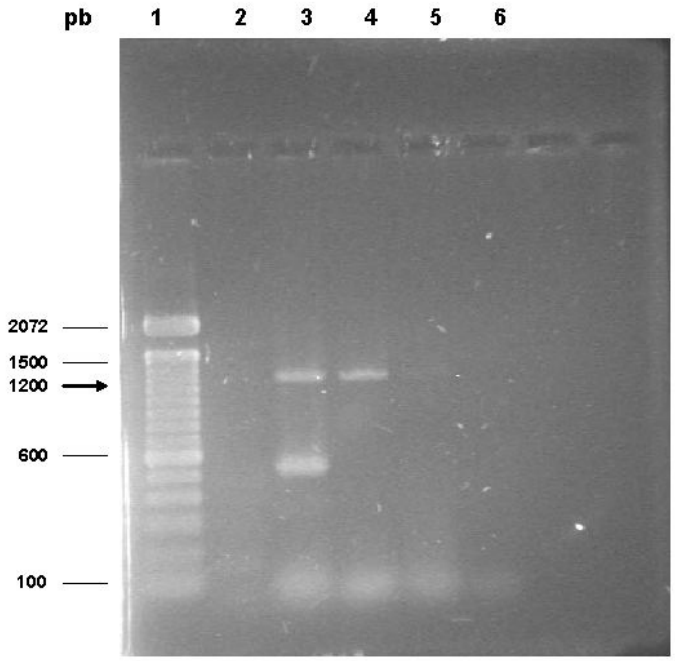

Fig. 3. A predicted fragment of 1200 bp in DNA of pCambia with the *gus* gene (lane 3), and in a transformed plantain plant (lane 4).

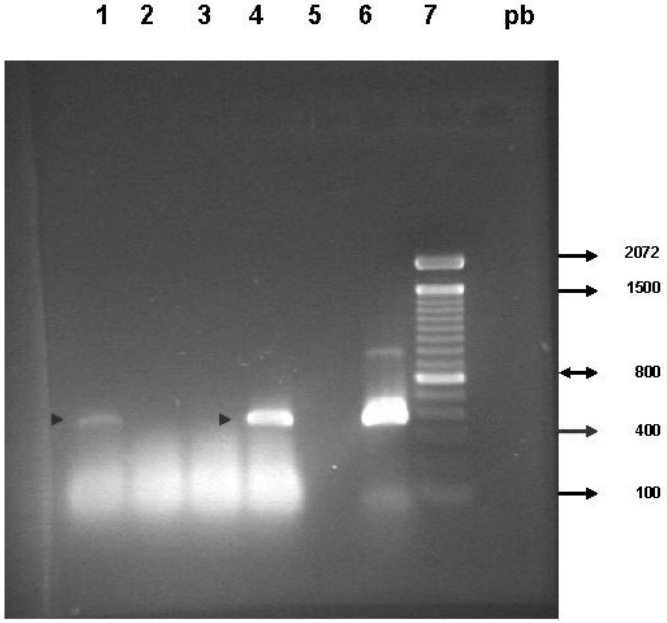

Fig. 4. A fragment of 400 bp is observed in lanes 1 and 4 (DNA of plantain transformed plants), and lane 6 (DNA of pCambia containing the *bar* gene). Lanes 2 (water) and 3 (plant electroporated without plasmid) do not show the same fragment.