Transient gene expression in electroporated banana (Musa spp., cv. 'Bluggoe', ABB group) protoplasts isolated from regenerable embryogenic cell suspensions

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Summary. Electroporation conditions were established for transient expression of introduced DNA in banana (Musa spp., cv. 'Bluggoe') protoplasts isolated from regenerable embryogenic cell suspensions. The following parameters were found to be highly influential: electroporation buffer, polyethylene glycol treatment and its duration before electroporation, use of a heat shock, and chimaeric gene constructs. The maximum frequency of DNA introduction as detected by an in situ assay for transient expression of the uidA gene, amounted to 1.8% of total protoplasts. Since plants have recently been regenerated from banana protoplasts at a high frequency, the present results may contribute to the production of transgenic banana.

Key words: Direct gene transfer - Electroporation - β-Glucuronidase - Banana - Protoplast

Abbreviations. AMV = alfalfa mosaic virus, CaMV = cauliflower mosaic virus, 2,4-D = 2,4-dichlorophenoxyacetic acid, EODTA = ethylene glycol-O,C'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, GUS = β-glucuronidase, HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, MES = 2-morpholinoethanesulfonic acid, MS = Murashige-Skoog, NOS = nopaline synthase, NPTII = neomycin phosphotransferase, PEG = polyethylene glycol, TGE = transient GUS expression, X-Gluc = 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid

Introduction

Banana and plantain (Musa spp.) are the world's second largest fruit crop with an annual production of 74 million tons (FAO 1991) and the main yield reducing factors are fungal and viral diseases. The annual cost of chemical control of black sigatoka - the major fungal disease of banana caused by Mycosphaerella fijiensis - averages between 0.3 and 1.0 US $ per plant in banana plantations in order to avoid yield losses of 30-50%. Furthermore, plants infected with the banana bunchy top virus may become completely unproductive.

In the past 70 years the application of classical methods to breeding for disease resistance resulted in a limited success only, due to long generation times, high sterility and triploidy of most cultivated bananas. The integration of genetic engineering into breeding programmes may provide a powerful tool to overcome these limitations by inducing specific genetic changes within a short period of time.

In vitro culture in banana has been extensively used to quickly propagate vegetative clones of many genotypes (Vuylsteke and De Langhe 1985). Recently, a general method has been described for the establishment of regenerable embryogenic cell suspensions from proliferating meristems (Dheda et al. 1991) and has been successfully applied to several genetically distant cultivars (Dheda 1992). This method has a significant advantage over alternatives using immature zygotic embryos (Escalant and Teisson 1989), since most edible bananas rarely set seed. Protoplasts have also been isolated from an embryogenic cell suspension and plants have been regenerated through somatic embryogenesis at a high frequency (Panis et al. 1993). Furthermore, the established embryogenic cell suspensions can be stored by cryopreservation without loss of regenerating ability (Panis et al. 1991).

Direct DNA introduction by electroporation (Fromm et al. 1985) into viable and highly regenerative protoplasts provides an opportunity for efficient genetic transformation of banana. The technique is effective for genetic transformation of a range of dicot (Riggs and Dennis 1989) and monocot species (Toriyama et al. 1988; Huang and Dennis 1989). It has been utilized not only for the study of expression of chimaeric gene constructs at the transient level (Fromm et al. 1985; Bates et al. 1988) but also to produce stable transformants (Shillito et al. 1985; Fromm et al. 1986; Rhodes et al. 1988; Shimamoto et al. 1989). However, there are many variables affecting the efficiency of gene transfer, including capacitance and field strength; duration, shape, number and spacing of electrical pulses; buffer composition; temperature; concentration and form of DNA, etc.

In the present work we investigated a number of parameters influencing transient expression of the E. coli uidA gene, a general reporter gene in plants coding for the β-glucuronidase enzyme (Jefferson et al. 1986), after transformation of banana protoplasts by electroporation. The initial observations of this work have been recently described in a short account (Sagi et al. 1992). This is the first detailed report describing transformation with transient gene expression in banana.

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Materials and methods

Cell suspensions and plasmids. Embryogenic cell suspension lines of cv. 'Bluggoe' (Musa spp. ABB group) described by Dhed'a et al. (1991) were maintained and subcultured weekly in MS medium (Murashige and Skoog 1962) supplemented with 5 μM 2,4-D and 1 μM zeatin. The cell suspensions were cultured for more than 1 year prior to use in the experiments and consisted of small clusters of isodiametric, cytoplasm-rich cells (Fig. 2a).

The structure of the plasmids pBl221 (Clontech Laboratories, Inc.), pBl-426, and pBl-505 (kindly provided by William Crosby, Plant Biotechnology Institute, Saskatoon, Sask., Canada) used in this work is shown in Fig. 1. Plasmid DNA was purified using Qiagen columns.

![Diagram of plasmid structures](image)

**Fig. 1.** Schematic representation of the chimeric gene constructs used for electroporation experiments. 35S = CaMV 35S promoter, 35S-35S = tandem repeat CaMV 35S promoter, AMV = Alfalfa Mosaic Virus leader sequence, uidA = GUS reporter gene, uidA-neo = GUS-NPTII fusion gene, nos-T = NOS terminator

Protoplast isolation. Ten or 20 ml of 1-week-old suspension cells were transferred to an equal volume of enzyme solution containing 1% cellulase, 1% macerozyme, 1% pectinase, 0.55 M mannitol and 3 mM MES, pH 5.8, for 20-24 hours at 25°C in the dark. The protoplasts were then filtered through 80 μm and 25 μm mesh stainless steel screens and collected by centrifugation at 90g. The pellet was washed in 30 ml of the protoplast isolation solution but without enzymes followed by a second wash in 20 ml of electroporation buffer. Finally, the washed pellet was resuspended in electroporation buffer at a protoplast density of 10^6 800 μl^-1. The electroporation buffers are described in Table 1. This protocol usually resulted in a protoplast yield of 2 x 10^6 ml^-1 of cell suspension. Protoplasts were counted using a modified Neubauer haemocytometer.

Complete removal of the cell wall was confirmed by Calcofluor white staining while viability of freshly isolated or electroporated protoplasts was controlled by staining either with fluorescein diacetate or Evans' blue.

Electroporation. A 800 μl aliquot containing 10^6 protoplasts in electroporation buffer was placed into cuvettes of 0.4 cm gap and with aluminium electrodes glued onto opposite faces (Bio-Rad Laboratories). After addition of plasmid DNA to a concentration of 60 μg ml^-1, cuvettes were stored on ice for 10 min. Just before delivering the pulse, 40% PEG was added to a final concentration of 3-12% for 1 or 3-4 min and cuvettes were carefully vortexed to prevent sedimentation of protoplasts. Duration of PEG treatment means the time elapsed between addition of PEG and pulse delivery. The samples were then electroporated with a 960 μF capacitor of a Bio-Rad Gene Pulser™ transfection apparatus which generates an exponential decay pulse. The time constant of the pulse was monitored after each pulse delivery. After electroporation, the cuvettes were placed on ice for 10 min and then for 10 min at room temperature. Protoplasts were diluted with the MS medium supplemented with 5 μM 2,4-D, 1 μM zeatin and 0.55 M mannitol to a density of 10^5 ml^-1 and incubated in the dark at 24°C.

The following controls were used: (1) samples electroporated with pUC19 DNA, (2) samples electroporated without plasmid DNA, (3) non-electroporated samples incubated with plasmid DNA. Transient β-glucuronidase expression assay. For the histochemical in situ assays, protoplasts were collected at 24 or 48 hours after electroporation, resuspended in 50 mM sodium phosphate buffer, pH 7.0 and incubated from overnight up to 10 days at 37°C in the presence of 1 mM X-Gluc as described by Jefferson (1987). Transformation efficiency was assessed by counting blue stained protoplasts and relating to the total number of protoplasts. At least two internal repeats were counted and averaged for each treatment in each experiment. The number of blue-stained cells was counted on average in 10^5 protoplasts for each repeat. Total number of treated protoplasts was determined independently for each repeat. Since the number of blue protoplasts was significantly increasing after 10 days of incubation in comparison to 1 day incubation (Fig. 2b, c), for statistical evaluation data were obtained after a 10 day incubation in GUS-assay buffer. To avoid overestimation of GUS-expressing protoplasts due to the possible diffusion of the indigo product (Diaz and Carbonero 1992), each blue cluster of protoplasts was scored as a single transformant. Cultures of E. coli were used in parallel as positive controls for the GUS assay.

Statistical evaluation was carried out after the arcsin transformation (y = 2 arcsin y/2) which is necessary to stabilize the variances of the error terms for processing of data which are proportions. The ANOVA and Duncan's multiple range tests were performed using the statistical software package SAS (SAS Institute, Inc. Cary, North Carolina, USA). Where significant differences in the treatment means were found at the 5% probability level of an F-test, means were compared using Duncan's multiple range test at 5% level of significance.

![Images of banana cell suspensions](image)

**Fig. 2.** a cv. 'Bluggoe' banana cell suspension showing a cell aggregate after 3 days of subculture. b Transient GUS expression in banana protoplasts assayed 24 hours after electroporation in 5% PEG showing single blue protoplasts after 1 day incubation. c Transient GUS expression in banana protoplasts treated as in b but after 10 days of incubation. d Transient GUS expression in banana protoplasts treated as in b but electroporated without PEG. Bar = 100 μm.