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Transient gene expression in transformed banana (*Musa* cv. Bluggoe) protoplasts and embryogenic cell suspensions

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Summary

In order to introduce currently-available genes with agronomical value into banana, two genetic transformation protocols have been optimized.

Firstly, regenerable protoplasts isolated from embryogenic cell suspensions of the cultivar Bluggoe have been used for the introduction of several chimaeric *uidA* gene constructs by electroporation. With the inclusion of polyethylene glycol and heat shock, the frequency of transiently expressing protoplasts reached 1.8% as shown by an *in situ* β-glucuronidase assay. A duplicated 35S promoter with an alfalfa mosaic virus leader sequence (pBI-426) induced the highest expression rate among the constructs tested.

Embryogenic cell suspensions of cv. Bluggoe have also been bombarded with accelerated particles coated with a high expression *uidA* gene construct (pEmuGN) using a biolistic gun. After a partial optimization of the procedure, transient GUS assays reproducibly demonstrated the presence of 400 blue foci in 30 μl of settled cell volume (approximately 25 mg cells). Selection and characterization of antibiotic-resistant transformed cultures is in progress.

Abbreviations: AMV – alfalfa mosaic virus, GUS – β-glucuronidase, TGE – transient GUS expression, *uidA* – gene for β-glucuronidase

Introduction

Banana (including plantain) is the world's largest fruit crop with an annual production of 74 million tons (FAO, 1993) and is the staple food for nearly 400 million people. The main factors threatening this level of production are fungal and viral diseases. The sigatoka disease complex (caused by *Mycophaerella* spp.) may cause yield losses of up to 30–50% (Mobambo et al., 1993), while plants infected with the banana bunchy top virus may become completely unproductive.

Since most cultivated bananas are triploid and highly sterile, application of classical breeding methods for disease resistance has resulted in rather limited success (Vuylsteke et al., 1993). By a one-step introduction of well-characterized genes conferring disease resistance to banana, genetic engineering may provide a powerful tool for breeding programmes. To exploit this potential, (i) highly performing *in vitro* regeneration systems and (ii) efficient genetic transformation procedures coupled with high gene expression are needed for banana.

Very recently, significant progress has been achieved in the *in vitro* manipulation of banana. Regenerable embryogenic cell suspensions have been established in the authors' laboratory from proliferating meristems (Dhed'a et al., 1991) and this technique has been successfully applied to several genetically distinct cultivars (Dhed'a, 1992). Protoplasts can be isolated from these embryogenic cell suspensions and plants have been regenerated therefrom through somatic embryogenesis at a high frequency (Panis et al.,

1993b). Furthermore, the established embryogenic cell suspensions can be stored by cryopreservation without loss of regenerating ability (Panis et al., 1992). Other laboratories have also succeeded in regenerating plants from cell suspensions (Novak et al., 1989) as well as from protoplast cultures (Megia et al., 1993).

Direct DNA transfer 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 transformation of a range of dicotyledonous (Riggs & Bates, 1986; Lindsey & Jones, 1989; Chupeau et al., 1989) and monocotyledonous species (Toriyama et al., 1988; Huang & Dennis, 1989). However, numerous factors affect the efficiency of gene transfer, including capacitance and field strength, duration and shape of electrical pulses, buffer composition and temperature, type of chimaeric gene constructs, and the concentration and form of DNA. In a recent report, we have demonstrated the introduction of foreign DNA into banana protoplasts by electroporation at a high frequency as assessed by a transient *in situ* GUS assay (Sagi et al., 1994).

Alternatively, banana cell suspensions may be directly transformed by particle bombardment obviating the need for protoplast isolation and culture techniques. An inexpensive but efficient biolistic gun model accelerates particles directly in a stream of helium without macrocarriers (Takeuchi et al., 1992; Finer et al., 1992). This technique has produced stable transformation of soybean and maize (Finer et al., 1992; Vain et al., 1993). According to our initial observations, this method resulted in high transient GUS expression in banana embryogenic cell suspensions (Panis et al., 1993a).

In the present report, data are provided on transient expression of the *uidA* gene of *E. coli* in banana protoplasts after transformation by electroporation. Transient GUS expression is also demonstrated after particle bombardment of suspension cells using a flowing helium gun.

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 & Skoog, 1962) supplemented with 5 μM 2,4-D and 1 μM zeatin (ZZ medium). The cell suspensions were cultured for at least 1 year prior to use in the experiments and consisted of small clusters of isodiametric, cytoplasm-rich cells.

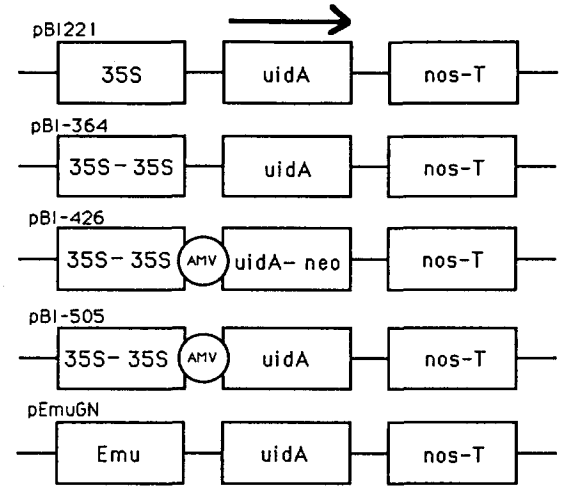

Fig. 1. Schematic representation of chimaeric GUS constructs used for transformation experiments. 35S = CaMV 35S promoter, 35S-35S = the tandem repeat CaMV 35S promoter, AMV = alfalfa mosaic virus leader sequence, uidA = GUS reporter gene, uidA-neo = GUS-NPTII fusion gene, nos-T = NOS terminator, Emu = Emu promoter (Last et al., 1991).

The plasmid pBI221 was obtained from Clontech Laboratories, Inc. This 5.7 kb vector consists of pUC19 and a 3.0 kb *Hind*III-*Eco*RI fragment carrying the CaMV 35S promoter, the GUS gene from *E. coli* and the NOS polyA site from *Agrobacterium tumefaciens*. The plasmids pBI-364 pBI-426 pBI-505 and pEmuGN were kindly provided by William Crosby, Plant Biotechnology Institute, Saskatoon, Sask., Canada and David Last, CSIRO, Canberra, Australia, respectively. The structure of chimaeric *uidA* gene constructs located on these plasmids is shown in Fig. 1. The plasmids were propagated in *E. coli* DH5α strain in the presence of 50 mg l^{-1} ampicillin. Plasmid DNA prepared by alkaline lysis was purified on Qiagen columns (Diagen GmbH). DNA concentrations were determined by absorption measurements at 260 nm and by gel electrophoresis.

Protoplast isolation. Isolation and purification of protoplasts from embryogenic cell suspensions was done according to Panis et al. (1993). The purified protoplasts were resuspended in electroporation buffer at a protoplast density of 1.25×10^6 ml^{-1}. The electroporation buffers are described in Table 1. Protoplasts

were counted using a modified Neubauer haemocytometer. Complete removal of the cell wall was confirmed by Calcofluor white staining while the viability of freshly-isolated or electroporated protoplasts was assessed by staining either with fluorescein diacetate or Evans' blue.

Transformation procedures. Protoplast transformation by electroporation was carried out as described by Sagi et al. (1994). Briefly, a 800 μl aliquot containing 10^6 protoplasts in electroporation buffer was placed into cuvettes with a 0.4 cm gap. After addition of plasmid DNA to a concentration of 60 μg ml^{-1}, cuvettes were stored on ice for 10 min, then electroporated with a 960 μF capacitor of a Bio-Rad Gene Pulser^{TM} transfection apparatus at a field strength of 800 V cm^{-1}. After electroporation, the cuvettes were placed on ice for 10 min and then for 10 min at room temperature. Protoplasts were diluted with ZZ medium supplemented with 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.

For particle bombardment of suspension cells, a flowing helium gun has been constructed based on the description of Takeuchi et al. (1992) and the procedure will be described in detail elsewhere (Sagi et al., in press). Briefly, for one bombardment 1 μg DNA was precipitated according to Sanford et al. (1993) onto 300 μg gold (Bio-Rad) or M-17 tungsten particles (Sylvania) and applied into a Swinney syringe filter unit. Particles were prepared and stored at 4° C as suspensions in 50% glycerol or 40% polyethylene glycol 6000 (PEG). Suspension cells were collected 4 to 6 days after subculture and 30 μl settled cell volume (approximately 25 mg cells) was used for bombardment. Particles were accelerated at a pressure of 3 to 6 bars. Cells were then cultured in ZZ medium for 1 or 2 days and assayed for transient GUS expression.

Transient β-glucuronidase expression assay. For the histochemical *in situ* assays, protoplasts were collected 48 hours after electroporation, resuspended in 50 mM sodium phosphate buffer, pH 7.0 and incubated for periods ranging from overnight up to 10 days at 37° C in the presence of 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) as described by Jefferson (1987). Protoplast transformation frequency was assessed by counting blue-stained protoplasts and relating this to the total number of protoplasts. Two to four internal repeats were counted and averaged for each treatment in each experiment. On average 10^5 protoplasts were observed for each repeat. The total number of treated protoplasts was determined independently for each repeat. For bombarded cells, transient GUS expression frequencies were expressed as number of blue foci per shot averaged over three to six replicates per treatment. Cultures of *E. coli* were used in parallel as positive controls for the GUS assay.

Statistical evaluation was carried out after *arcsin* transformation ($y' = 2$ *arcsin* $y^{1/2}$) of data from electroporation experiments. 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.

Results and discussion

Transient GUS expression (TGE) in electroporated protoplasts

Viability of freshly-isolated protoplasts was always over 90% with an average of 93%, as assessed by fluorescein diacetate staining or by the dye exclusion test with Evans' blue. After 24 hours, viability of the control cultures (no electroporation) in ASP-buffer (Tada et al., 1990) or in Cl-buffer (Fromm et al., 1985) was reduced from 93% to 75% and 67%, respectively.

Representative results in Table 1 illustrate that ASP buffer was superior to Cl-buffer, and that heat shock (45° C, 5 min) in ASP-buffer further increased TGE. Maximum TGE reached 1.8% of total electroporated protoplasts (Fig. 2a). Similar frequencies were observed by other groups in different species (Zhang & Wu, 1988; Dhir et al., 1991; Diaz & Carbonero, 1992).

Comparison of different plasmid constructs

In the absence of PEG, the rather low frequency of transformation was associated only with limited differences between the plasmid constructs tested (Table 2). However, when 5% PEG was added and transformation frequencies increased in accordance with our previous findings (Sagi et al., 1994), significant differ-

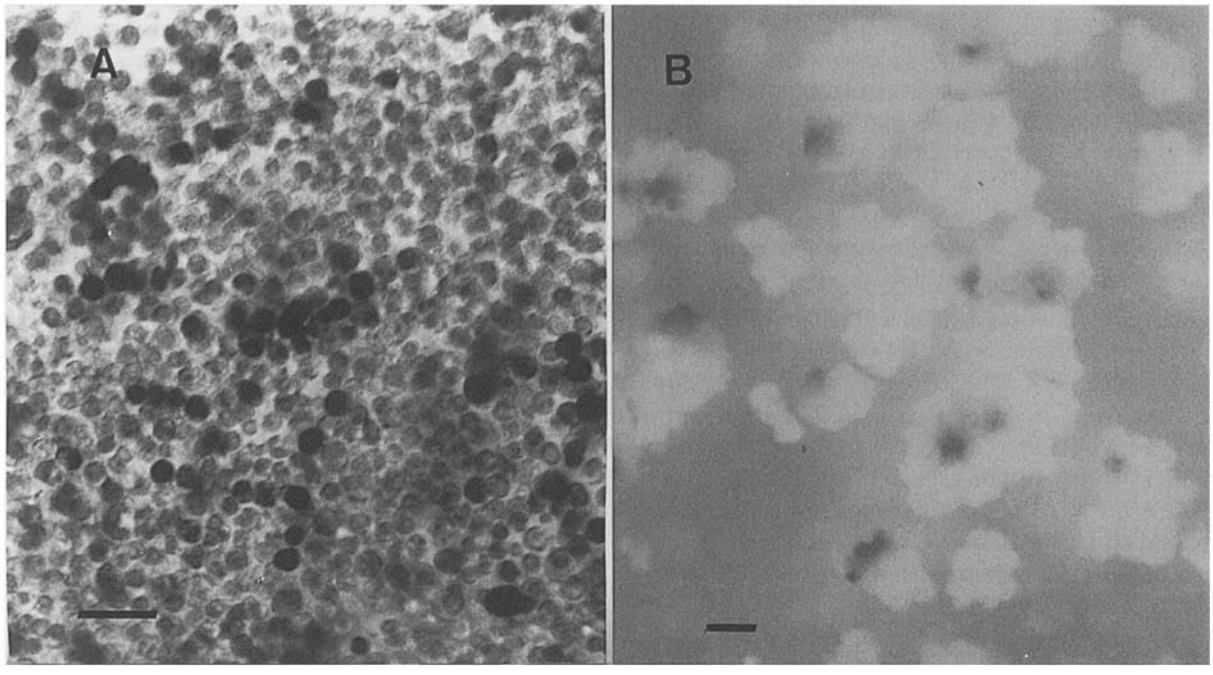

Fig. 2. Transient GUS expression in a) cv. Bluggoe banana protoplasts electroporated with plasmid pBI-426 in 5% PEG, b) cv. Bluggoe embryogenic cell suspensions after particle bombardment with plasmid pEmuGN coated gold particles. Bar = 100 μm.

Table 1. Effect of electroporation buffers and heat shock (45° C, 5 min) on transient GUS expression in banana protoplasts

Electroporation buffer	Treatment[a]		Mean[a]
	Control	Shocked	
ASP[1]	1.082 b	1.868 a	1.468 a
Cl[2]	0.734 b	0.758 b	0.746 b

TGE frequencies are expressed as percentages based on the number of blue protoplasts in two replicates of 10^5 protoplasts for each treatment electroporated with plasmid pBI-426 in 5% PEG and assessed 48 hours after electroporation.

[a] Entries within these headings followed by the same letter are not significntly ($P \leq 0.05$) different by Duncan's test after *arcsin* transformation. Standard deviation did not exceed ± 20% of the mean.

[1] 70 mM K-aspartate, 5 mM Ca-gluconate, 5 mM MES, 0.55 M mannitol, pH 5.8 (Tada et al., 1990).

[2] 150 mM NaCl, 4 mM $CaCl_2$, 10 mM HEPES, 0.55 M mannitol, pH 7.2 (Fromm et al., 1985).

Table 2. Effect of plasmid constructs on transient GUS expression in electroporated banana protoplasts

Plasmid	Construct	PEG concentration (%)	
		0	5
pBI221	35S	0.048 b	0.198 c
pBI-364	35S-35S	0.092 ab	0.205 c
pBI-426	**35S-35S-AMV**	0.073 ab	**0.434 a**
pBI-505	**35S-35S-AMV**	0.104 a	**0.329 b**

TGE frequencies are expressed as percentages based on the number of blue protoplasts in at least two replicates of 10^5 protoplasts for each treatment and assessed 48 hours after electroporation. Entries within columns followed by the same letter are not significantly ($P \leq 0.05$) different by Duncan's test after *arcsin* transformation. Standard deviation did not exceed ± 20% of the mean.

ences were found between the constructs in the following order: pBI-426 > pBI-505 > PBI-364 ≈ pBI221. Obviously, constructs with the AMV leader sequence gave significantly higher TGE than those without this sequence. The tandem repeat 35S promoter alone was not sufficient to increase transient gene expression significantly.

In accordance with the results obtained by Charest et al. (1993) in conifer species and Hobbs et al. (1990) in pea protoplasts, this observation supports the conclusion of Jobling & Gehrke (1987) that the AMV untrans-

Table 3. Effect of age suspension cells on transient GUS expression in electroporated banana protoplasts

PEG concentration (%)	Age of suspension cells (week) 1	2
0	0.053 e	0.057 e
3	0.485 c	0.607 bc
5	**0.765 ab**	0.276 d
8	0.815 a	0.007 f

TGE frequencies are expressed as percentages based on the number of blue protoplasts in two replicates of 10^5 protoplasts for each treatment electroporated with plasmid pBI-426 and assessed 48 hours after electroporation. Entries followed by the same letter are not significantly ($P \leq 0.05$) different by Duncan's test after *arcsin* transformation. Standard deviation did not exceed ± 10% of the mean.

Table 4. Transient GUS expression in banana suspension cells after particle bombardment

Particle Type	Bombarded in	Storage solution 50% glycerol	40% PEG
Tungsten	water	9.3 ± 7.1	49.0 ± 3.7
	ethanol	188.3 ± 56.1	166.3 ± 49.0
Gold	water	56.3 ± 31.4	74.3 ± 39.6
	ethanol	242.0 ± 14.4	324.3 ± 89.0

TGE frequencies are expressed as the number of blue foci per shot (± S.E.) with plasmid pEmuGN coated tungsten or gold particles and assayed 2 days after bombardment.

lated leader sequence increases translational efficiency of chimaeric mRNAs.

Effect of age of suspension cells

The reaction of protoplasts isolated from 1- or 2-week old suspension cells to various PEG levels was compared (Table 3). It was found that PEG exposure was not damaging to protoplasts isolated from 1-week old cell suspension. In contrast, protoplasts from 2-week old suspensions showed a declining TGE to increasing PEG concentration. Though 8% PEG treatment resulted in the highest TGE in 1-week old protoplasts, at this concentration results were highly unreproducible and aggregation of protoplasts was observed. One-week old cell suspensions were clearly superior to 2-week old cells for protoplast transformation. Moreover, in agreement with our previous results (Sagi et al., 1994), inclusion of 5% PEG during electroporation had the best influence on TGE.

Transient GUS expression in bombarded suspension cells

Effect of particles and precipitation factors

Table 4 illustrates TGE frequencies in bombarded banana suspension cells in relation to particle type, storage solution of particles and suspending agent prior to bombardment. When gold particles were used for coating, TGE frequencies were clearly higher for all treatments than with tungsten particles. It may be that gold particles bind DNA more efficiently and that they are more inert to plant cells than tungsten. Russell et al. (1992) have indeed observed that tungsten particles were toxic to tobacco suspension cells while gold particles were not toxic.

Using 40% PEG to prepare particle stock solutions usually resulted in higher TGE than 50% glycerol. This difference was more marked when particles were bombarded as an aqueous suspension, and was probably caused by more complete precipitation of DNA in the presence of PEG (Lis, 1980). Moreover, particles were more easily resuspended in PEG and could be preserved for coating for as long a period of time as in glycerol. Finally, in our laboratory, resuspension of coated particles in ethanol prior to bombardment was clearly more efficient than the removal of most of the aqueous solution from precipitation reactions and the use of the rest for bombardment. Double bombardment of cells further improved TGE frequency and resulted in an average of 494 ± 60.3 blue foci per sample.

Effect of age of suspension cells

There were only slight differences between suspensions of various ages when TGE was determined 2 days after transformation. Five- and six-day old suspensions produced more blue foci per shot than 4-day old suspension cells. However, 1 day after bombardment, 4-day old suspension cells showed higher TGE than cells at 5 days after subculture (Table 5). When comparing gold and tungsten particles, gold proved to be superior, as already shown in Table 4.

As the result of partial optimization of bombardment conditions, we have reproducibly observed at least 400 blue foci per shot (Fig. 2b) where for each shot approximately 25 mg cells are prepared. This frequency is comparable to what has been previously reported in other monocotyledons (Wang et al., 1988; Chibbar et al., 1993; Ritala et al., 1993) and it appears to be high enough to allow the production of stable transformants. Recently, we have been able to select regenerable cul-

Table 5. Effect of age of banana suspension cells on transient GUS expression after particle bombardment

Particle bombardment	GUS-assay days after	Age of suspension cells (days after subculture)		
		4	5	6
Gold	1 day	508.3 ± 178.3	387.3 ± 112.9	ND
	2 days	341.3 ± 57.8	468.3 ± 31.5	423.0 ± 48.2
Tungsten	2 days	ND	229.3 ± 15.2	300.3 ± 43.4

TGE frequencies are expressed as the number of blue foci per shot (± S.E.) with plasmid pEmuGN coated gold or tungsten particles and assessed 1 or 2 days after bombardment. ND – not determined.

tures after bombarding suspension cells with plasmids carrying the gene for hygromycin resistance. Molecular and histochemical analysis of these cultures is now in progress (Sagi et al., in press).

Based on these results, our goal is to introduce agronomically-important genes into banana. Our first targets are genes coding for new types of antifungal proteins (AFPs). These AFPs are stable, cysteine-rich small peptides which are isolated from seeds of different plant species (Broekaert et al., 1992; Cammue et al., 1992; Terras et al., 1992). More uniquely, they have a broad antifungal effect, and showed high antifungal activity *in vitro* to *Mycosphaerella fijiensis* and *Fusarium oxysporum*, the main fungal pathogens in banana, while they exert no toxicity for human and plant (including banana) cells (Cammue et al., 1993). In addition to fungal resistance, engineering virus resistance in banana should also be considered. Banana bunchy top virus has recently been isolated and the virus genome is now being sequenced (J. Dale, personal communication) with the aim of isolating the essential genes for replication of the virus.

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