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Banana Protoplasts: Culture and its Applications

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ABSTRACT

Protoplast technology is an important adjunct to conventional plant breeding. The culture of banana protoplasts has advanced considerably in recent years through the use of embryogenic cell suspensions as a source of protoplasts, combined with nurse cultures. Protoplast transformation and somatic hybridization have also become feasible techniques to support the genetic improvement of banana. The present discussion reviews the advantages and disadvantages of the use of protoplasts compared with other methods of genetic transformation and conventional sexual hybridization. General protocols for protoplast culture, genetic transformation and somatic hybridization are presented.

Keywords: *Musa*, nurse culture, somatic hybridization, transformation

CONTENTS

INTRODUCTION

Bananas (*Musa* spp.), including plantain bananas, are one of the most important horticultural crops in tropical and subtropical countries. They also constitute the staple food of some 400 million persons living in developing countries. Banana is a perennial crop that provides more stability to daily living than the annual staple crops such as rice, wheat and corn (maize). However, as banana plants are more vulnerable to attack by pests and diseases, the best option to control these problems is by genetic manipulation through the generation of resistant varieties. The development of pest and disease resistant plants in *Musa* remains difficult by conventional breeding approaches because of the long generation time, sterility, and triploidy of most cultivated varieties. Non-conventional methods, such as genetic transformation, induced mutation and the selection of somaclonal variants, have been developed by several workers (Tripathi 2003; Arinaitwe *et al.* 2004; Chai *et al.* 2004; Roux 2004; Xu *et al.* 2006; Tripathi *et al*. 2008a; Roux *et al*. 2009), based on tissue culture approaches (Côte *et al.* 1996; Kosky *et al.* 2002; Matsumoto and Silva Neto 2003; Strosse *et al.* 2003, 2004). Indeed, since *in vitro* techniques have been established for banana, different source tissues such as corm slices, shoot tips, buds, callus, cultured cells and isolated protoplasts, have been exploited for genetic transformation (Hernández *et al.* 1999; Ságі *et al*. 2000; Tripathi 2003; Pei *et al*. 2005; Tripathi *et al.* 2005, 2008b; Sreeramanan *et al.* 2005, 2006; Arvanitoyannis *et al*. 2008; Ghosh *et al.* 2009).

Protoplasts are naked plant cells whose walls have been removed either mechanically or by enzymatic digestion. Isolated protoplasts are capable of incorporating foreign materials, such as DNA, or can be induced to fuse to transfer genetic material. This phenomenon has attracted scientists involved in gene transfer by transformation and somatic hybridization, and those plant physiologists investigating cell physiology and membrane behavior. During the 1980s and the first half of the 1990s, many scientific papers were published describing the use of isolated protoplasts to generate transgenic plants and somatic hybrids (Davey *et al*. 2005a, 2005b, 2005c). However, the focus on gene transfer by protoplast-based approaches declined since plant regeneration from isolated protoplasts was, and remains, extremely difficult in many species. Only relatively few species in which culture techniques were well established, such as tobacco, potato, citrus, *Brassica* and rice, were subjected to protoplast-based techniques (Davey *et al.* 2005a). In other species, protoplast technologies were superseded by other transformation technologies, and banana was no ex-

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Invited Mini-Review

Table 1 Some reports of banana protoplast isolation and culture.

Type of explants	Variety of *Musa*	Enzyme composition for isolation	Culture medium	Observation	References
Leaf and callus	Various (AA; BB; AAA; AAB; ABB)	2.5% Cellulase R10, 0.2% Hemicellulase (Sigma), 0.3% Pectolyase Y23, 0.6% Macerozyme	Not cultured	Protoplasts isolated only from callus	Bakry 1984
Youngest leaf	Cavendish (AAA); *acuminata* (AA)	0.5% Cellulysin, 0.5% Rhozyme HR-150, 0.125% Pectolyase Y23	½MS, 0.1 or 2 mg/L 2,4-D, 0.2 M glucose, 0.2 M mannitol, 0.17 M sucrose	Survived 6-15 days, no cell division	Chen and Ku 1985
Bracts	Maçã (AAB); Nanica (AAA); *balbisiana* (BB)	0.2% Cellulase R10, 0.2% Macerozyme R10, 0.2% Driselase	8p medium (Kao and Michayluk 1975)	Cell clusters in 20 days	Matsumoto *et al*. 1988
Suspension cells	Long Tavoy (AA)	5% Cellulase RS, 2% Pectolyase Y23	N6+8p organic acids, sugar alcohols, 0.35 M glucose, 0.12 M sucrose, 1.9 mM KH_2PO_4, 0.5 mM MES, with nurse cells	Callus	Megia *et al.* 1992
Suspension cells	Bluggoe (ABB)	1% Cellulase R10, 1% Macerozyme R10, 1% Pectinase 5S	½MS, 5 µM 2,4-D, 0.55M mannitol with nurse cells on reservoir medium of ½MS, 5 µM 2,4-D, 0.27 M mannitol	Plants regenerated	Panis *et al.* 1993
Suspension cells	Bluggoe (ABB)	1.5% Cellulase RS, 0.15% Pectolyase Y23, 0.2% Hemicellulase (Sigma)	N6+8p organic acids, sugar alcohols, 0.35 M glucose, 0.12M sucrose, 1.9 mM KH_2PO_4, 0.5 mM MES, with nurse cells	Plants regenerated	Megia *et al.* 1993
Suspension cells	Maçã (AAB)	1.5% Cellulase RS, 0.2% Pectolyase Y23	½MS, 5 µM 2,4-D, 0.55 M mannitol, 0.06 M sucrose with nurse cells on reservoir medium of ½MS, 5 µM 2,4-D, 0.27M mannitol	Plants regenerated	Matsumoto and Oka 1998
Suspension cells	Grande Naine (AAA)	2% Cellulase RS, 0.5% Macerozyme (Sigma), 0.2% Hemicellulase (Sigma), 0.25% Pectolyase Y23	N6+8p organic acids, sugar alcohols, 0.9 µM 2,4-D, 5.4 µM NAA, 2.3 µM zeatin, 0.4 M glucose, 0.12 M sucrose, 1.9 mM KH_2PO_4, 0.5 mM MES, with nurse cells	Plants regenerated	Assani *et al.* 2001
Suspension cells	Various (AAA); (AAB); (AA)	1.5% Cellulase RS, 0.15% Pectolyase Y23	N6+8p organic acids, sugar alcohols, 0.9 µM 2,4-D, 4.4 µM NAA, 2.3 µM zeatin, 0.4 M glucose, 0.12 M sucrose, 1.9 mM KH_2PO_4, 0.5 mM MES, with nurse cells	Plants regenerated	Assani *et al.* 2002
Suspension cells	Mas (AA)	3.5% Cellulase R10, 1% Macerozyme R10, 0.15% Pectolyase Y23	MS + Morel vitamins, 9 µM 2,4-D, 2.8 mM glucose, 0.12 M sucrose, 0.28 M maltose with nurse cells	Plants regenerated	Xiao *et al.* 2007

Abbreviations: **½MS,** half-strength Murashige and Skoog (1962) medium; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **MES**, 4-morpholineethanesulfonic acid; **Morel**, Morel and Wetmore (1951); **N6**, Chu *et al*. (1975) medium; **NAA**, α-naphthaleneacetic acid

ception. After demonstration of the possibility of transgenic plant production by biolistic and *Agrobacterium*-mediated transformation (May *et al*. 1995; Sági *et al*. 1995a), most procedures involving protoplast culture were abandoned. However, recent advances in protoplast culture, combined with marker gene technology, have stimulated a revival in protoplast-based techniques (Davey *et al*. 2005a, 2005b, 2005c). Haïcour *et al.* (2004) reviewed the isolation and culture of banana protoplasts. The present paper provides a brief history of the culture of banana protoplasts, and discusses its possibility in contributing to the genetic improvement of banana with focus on genetic transformation and somatic hybridization.

ISOLATION AND CULTURE OF BANANA PROTOPLASTS

Biotechnological approaches have been exploited for banana since the early 1970s through *in vitro* propagation and embryo rescue. The initial attempt at banana protoplast isolation was reported by Bakry (1984), but almost 10 years elapsed before the establishment of a protoplast-to-plant system (**Table 1**). The culture of banana protoplasts was successful only when suspension cells were used for protoplast isolation. Some of the authors listed in **Table 1** had already attempted to culture protoplasts from leaves or other tissues, but were unsuccessful in producing calli or regenerating shoots. Another important observation was the use of nurse (feeder) cells for protoplast culture. Panis *et al.* (1993) and Megia *et al*. (1993) reported simultaneously nurse culture techniques for protoplasts of banana with an ABB genome. They also showed that without nurse cells, protoplast densities exceeding 1.0×10^6 and 5.0×10^5/mL, respectively, were essential to sustain cell division. These critical densities might, however, be under estimated due to the excellent conditions of their embryogenic cells and the response to culture of the banana variety. Based on experiments by the present authors on cultivars of banana with the AAB and AAA genomes, a protoplast density of 2.0×10^6/mL was required in the absence of nurse cells, while a density of 1.0×10^5/mL was sufficient to culture protoplasts and to regenerate shoots from protoplast-derived tissues when the nurse culture technique was exploited (**Fig. 1**).

Protoplast isolation and culture in banana is now feasible. Using actively-dividing and fine embryogenic cell suspensions, protoplasts can be isolated in a enzyme solution consisting of 1.5–2% Cellulase RS and 0.15–0.2% Pectolyase Y23. When culture is associated with nurse cells it possible to maintain isolated protoplasts at a low density of 1 to 5×10^5/mL and to regenerate intact plants. Any treatment associated with genetic transformation and somatic hybridization generally causes death of some protoplasts which reduces the number of viable protoplasts. Consequently, the use of the nurse culture technique, combined with high quality banana suspension cells, is essential to generate genetically transformed plants and/or somatic hybrids (Matsumoto *et al.* 2002; Assani *et al.* 2005).

DIRECT GENE TRANSFER FOR PROTOPLAST TRANSFORMATION

Protoplast transformation mediated by treatment with polyethylene glycol (PEG), electroporation, or a combination of these two treatments, has been used to induce DNA uptake and to generate transiently and stably transformed cell lines. Protoplast transformation has an advantage over both *Agrobacterium*- and biolistic-mediated gene transfer to tissue explants in that protoplast regeneration is a single cell event. Thus, plants regenerated from transformed protoplasts are not chimeric. The major disadvantage of using protoplasts for genetic transformation is that shoot regeneration from protoplasts of many plants remains extremely difficult and

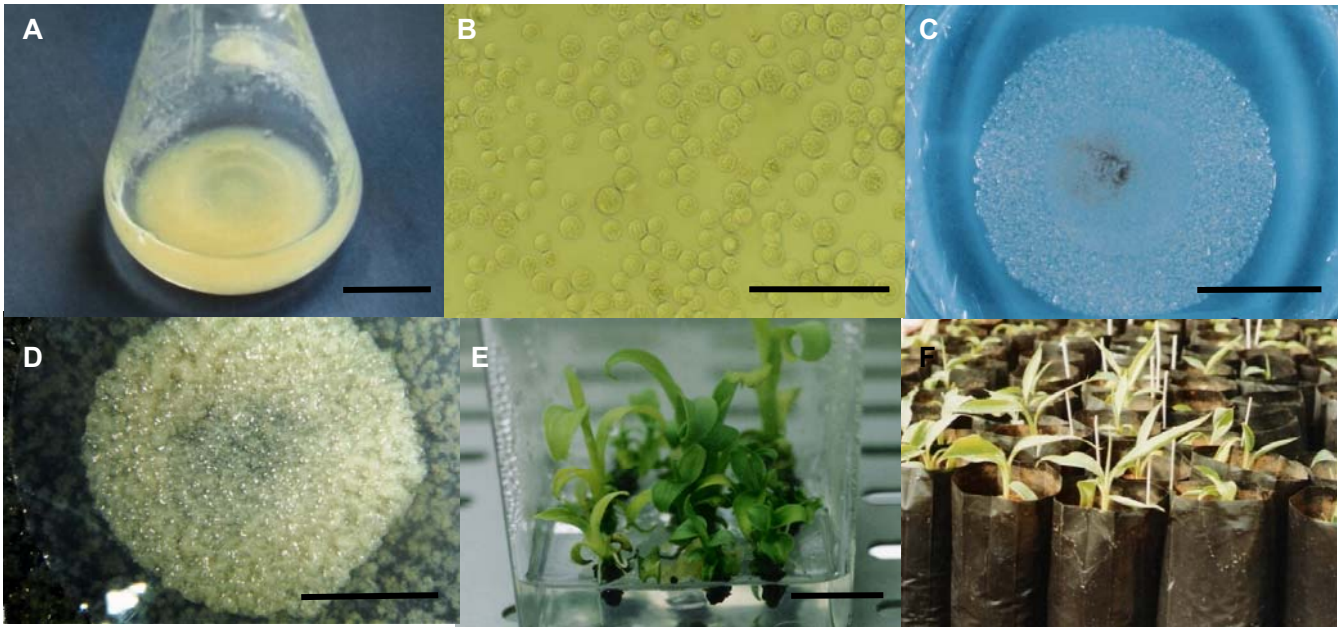

Fig. 1 Banana protoplast isolation and culture. (**A**) Cell suspension used as a source of protoplasts; scale bar = 20 mm. (**B**) Isolated protoplasts; scale bar = 100 µm. (**C**) Protoplasts on an Isopore membrane overlaying nurse cells; scale bar = 20 mm. (**D**) Protoplast-derived calli after 1-2 months of culture; scale bar = 10 mm. (**E**) Shoot regeneration; scale bar = 20 mm. (**F**) Acclimatization of regenerated plants in compost in 2-L plastic bags.

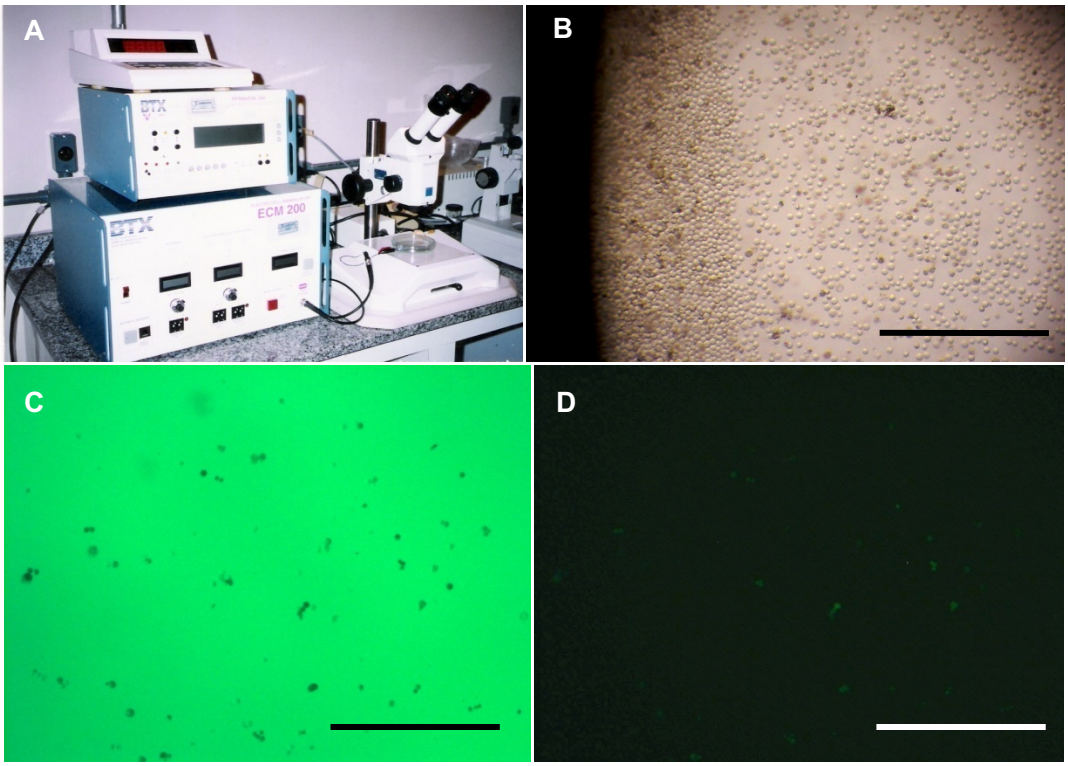

Fig. 2 Protoplast transformation by electroporation. (**A**) Electroporation system consisting of a DC square pulse generator (BTX; Electro cell manipulator; ECM 200), Petri dish-type electrode chamber and stereo-microscope. (**B**) Protoplasts in the electrode chamber immediately after electroporation. (**C**) Transient GFP expression in transformed protoplasts observed by bright field, and (**D**) under UV illumination 3 days after electroporation; scale bars = 300 µm.

generally involves an extended period of culture. For this reason, although protoplast transformation has been exploited for stable transformation, it has been directed primarily to investigations of transient gene expression (Davey *et al.* 2005a). As summarized in **Table 1**, a protoplast-to-plant system is now established in several banana cultivars. In these cultivars, protoplast-based transformation could become a feasible technique to generate transgenic plants.

Ságí *et al.* (1994) first reported transient gene expression in electroporated banana protoplasts. Under their experimental conditions, approximately 2% of the protoplast population expressed the β-glucuronidase (*gusA* or *uidA*) gene. After investigating various parameters, these authors concluded that optimum efficiency was when using a 960 µF capacitor, an electric field strength of 800 V/cm, ASP electroporation buffer (Tada *et al*. 1990), a PEG concentration of 5% (w/v), a heat shock of 45°C for 5 min before the addition of PEG, and protoplasts from 1-week-old embryogenic cell suspensions. Optimization of gene constructs was also essential (Ságí *et al*. 1994, 1995b, 1998, 2000).

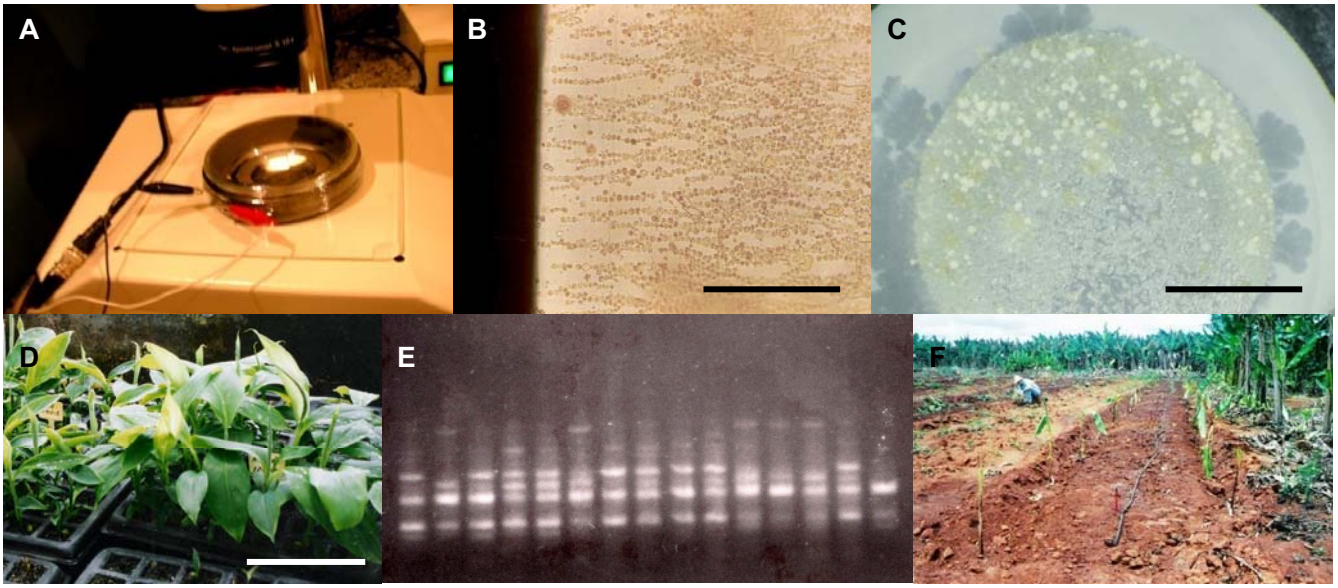

Fig. 3 Electrofusion of protoplasts and generation of somatic hybrids. (**A**) Petri dish-type electrode chamber for electrofusion (SHIMADZU; concentric chamber FTC-03), with 0.8 mL effective volume and 2 mm electrode gap distance. (**B**) Formation of protoplast chains ("pearl chains") in an AC electric field prior to fusion of adjacent protoplasts induced by DC electric pulses; scale bar = 300 µm. (**C**) Calli and somatic embryos regenerated after 2 months of culture; scale bar = 10 mm. (**D**) Regenerated somatic hybrid plants; scale bar = 20 cm. (**E**) Hybrid nature of regenerated plants evaluated by PCR/RAPD analysis using a primer OPAC5 (Operon Technology): From left to right, lanes 1and 2 - parental varieties; lanes 3 - 11 and 14 - somatic hybrids. Lanes 12, 13 and 15 may not be somatic hybrids or should be analyzed with other primers (reprinted from **Matsumoto K, Vilarinhos AD, Oka S** (2002) Somatic hybridization by electrofusion of banana protoplasts. *Euphytica* **125**, 317-324, ©2002, with kind permission of Springer Science + Business Media, Dordrecht, The Netherlands). (**F**) Somatic hybrid plants in the field.

Unfortunately, these investigators did not report further development of their methods. In laboratory of present authors, transient gene expression assays used a green fluorescent protein (*gfp*) gene (**Fig. 2**). More than 25% of the protoplasts that survived the DNA uptake treatment, expressed the *gfp* gene when diploid AA banana protoplasts were electroporated with 40 µg/mL of plasmid DNA, an electric field strength of 1250 V/cm for 50 µs, with 3 pulses in modified ASP electroporation buffer supplemented with 3% (w/v) PEG. However, this procedure was not considered to be 10-fold more efficient than the former work, because the gene expression frequency was assessed in protoplasts that survived the treatment rather than the total number of protoplasts inoculated, as in the former work. Additionally, the frequency of transformation was often greater when monitored by *gfp* expression than by expression of the *gus* gene (Sreeramanan *et al.* 2005, 2006). However, it is like that the transformation efficiency can be increased by further intensive studies. For example, in tobacco, *gus* gene expression frequency in nearly 90% of the protoplasts was obtained routinely by protoplast electroporation (Fisk and Dandekar 2005).

SOMATIC HYBRIDIZATION BY ELECTROFUSION AND PEG-INDUCED FUSION OF PROTOPLASTS

Until the present time, banana hybrid cultivars have not been generated by somatic hybridization. Hybrid cultivars have been obtained only by conventional cross-breeding, supported by embryo rescue and micropropagation (Tomekpe *et al.* 2004; Pedraza *et al.* 2005; Morán 2006). However, somatic hybridization involving protoplast fusion is currently the only way to obtain hybrids between highly sterile banana varieties, particularly in the triploid Cavendish group. Additionally, it is possible to manipulate some polygenic characters without DNA-level knowledge, by effecting chromosome and/or organelle transfer through asymmetric protoplast fusion. The first tentative attempts at protoplast fusion were reported by Chen and Ku (1985) and, subsequently, by Matsumoto *et al.* (1992). The former investigators used leaf protoplasts, while the latter employed bract protoplasts. Both research groups demonstrated the fusion of isolated protoplasts, but were unable to culture fusion-treated material. Somatic hybrids were obtained only after the protoplast culture protocol was established, using embryogenic suspension cells as source material (**Table 1**). Protoplast electrofusion was achieved using an AC electric field of 1 MHz at 200-230 V/cm for a period of 10-30 s, followed by a DC electric field of 1250-2300 V/cm for 30-100 µs with 3-20 pulses (**Fig. 3**; Matsumoto *et al.* 2002; Assani *et al.* 2005). Instead of electrofusion, a treatment with 50% (w/v) PEG solution could also induce reproducible protoplast fusion, although subsequent plant regeneration was not as efficient (Assani *et al.* 2005).

PROSPECTS FOR GENETIC IMPROVEMENT OF BANANA

Although protoplast culture is routine for some cultivars of banana, fine embryogenic cell suspensions are not available in many cultivars. Once embryogenic cell suspensions are established in a range of cultivated varieties, protoplast technology will be widely applicable to the genetic improvement of banana through induced mutation, symmetric and asymmetric somatic hybridization. The latter may include chromosome and organelle transfer which will be relevant to the breeding of heritable polygenic characteristics.

For the last 10 years, consumer acceptance of genetic transformation has become a key issue. Reduction of the possible environmental impact of transgenes has been discussed, with the eventual requirement to eliminate marker genes from transgenic plants (Day *et al.* 2005; Ebinuma *et al.* 2005). Cisgenesis, involving genetic modification of plants by inserting genes of the plant species itself or from sexually compatible relatives, has been suggested as a replacement for transgenesis (Joshi *et al.* 2008; Schouten 2008). The marker genes for selection, such as the neomycin phosphotransferase (*npt*II) gene that confers kanamycin resistance on transformed plant cells, is indispensable in the transformation of explants such as those of leaves, embryos or shoot tips, since foreign DNA becomes integrated and expressed in relatively few of the target cells. Consequently, millions of non-transformed cells must be eliminated during culture prior to shoot regeneration from transformed cells. This can be effected by expression of a selectable marker

gene in transformed cells. The situation is similar with embryogenic cell suspensions, since the cells mostly grow as multi-cell clusters. As all protoplasts have direct contact with the foreign DNA during electroporation or PEG transformation procedures, it should be feasible to generate transgenic bananas without the need for selection, provided the efficiency of protoplast transformation is increased to be comparable to that of tobacco. In this way, protoplasts will have true value in the genetic improvement of banana.

PROTOCOL FOR ISOLATION, ELECTROPORATION, ELECTROFUSION AND CULTURE OF BANANA PROTOPLASTS

Solutions and culture media

a) MCP solution for protoplast isolation and electrofusion: 0.6 M mannitol, 0.1 mM $CaCl_2$, 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 3.5 mM MES and 8 mg/L bromocresol purple, pH 5.7 (Sterilize by autoclaving at 121°C for 20 min and store at 4°C).
b) Enzyme solution for protoplast isolation: 1.5% (w/v) Cellulase Onozuka RS and 0.2% (w/v) Pectolyase Y23 in MCP solution (Filter-sterilize, 0.2 µm pore size and store at -20°C).
c) KA-MCP solution for electroporation: MCP solution plus 70 mM potassium aspartate, pH 5.6 (Sterilize by autoclaving and store at 4°C).
d) PA3 liquid medium for protoplast culture: ½ strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.06 M sucrose, 0.55 M mannitol, pH 5.8 (Sterilize by autoclaving and store at room temperature).
e) PA3 semi-solid medium for immobilization of nurse cells: PA3 liquid medium plus 1.6% (w/v) agarose of low gelling temperature (Agarose Type VII, Sigma) (Sterilize by autoclaving and store at 4°C).
f) PA5 semi-solid medium for protoplast culture: ½-strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 µM 2,4-D, 0.06 M sucrose, 0.27 M mannitol, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).
g) Cell multiplication medium: ½-strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 µM 2,4-D, 1 µM zeatin, 0.09 M sucrose, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).
h) Germination medium: MS salts and vitamins, 2 µM indole-3-acetic acid (IAA), 2 µM benzylaminopurine (BA), 0.06 M sucrose, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).

Protoplast isolation

1) Prepare 5 mL of the enzyme solution and decant into a 5-cm Petri dish.
2) Harvest embryogenic suspension cells (**Fig. 1A**) at 3-5 days after the last subculture and transfer approximately 0.2 mL settled-cell-volume (SCV) to the enzyme solution in the Petri dish.
3) Incubate at 28 ± 2°C in the dark at 50 rpm on a horizontal platform shaker for 15 hours (overnight).
4) Pass the suspension through a nylon mesh of 25 µm pore size.
5) Centrifuge the suspension at 900 rpm for 4 min.
6) Remove the supernatant and re-suspend the pelleted protoplasts in MCP solution.
7) Repeat twice steps (5) and (6).
8) For electroporation, add 1 mL of KA-MCP solution to gently resuspend the protoplasts. For electrofusion, add 1 mL of MCP solution. When only protoplast culture is carried out, add 1 mL of PA3 liquid medium, count the protoplasts using a hemocytometer, adjust the density to 3.0×10^5 protoplasts/mL with PA3 liquid medium and proceed to step 24 (**Fig. 1B**; See Note 1).

Electroporation

9) Count the protoplasts using a hemocytometer and adjust the protoplast density to 1.0×10^6 protoplasts/mL by the addition of KA-MCP solution.
10) Add 40-80 µg/mL of plasmid DNA and mix thoroughly but gently.
11) Add PEG to a final concentration of 3% (w/v) [75 µL/mL of 40% (w/v) PEG stock solution].
12) Transfer the protoplast/DNA/PEG suspension to the electrode chamber of the electroporation apparatus and maintain the chamber on ice for 10 min.
13) Apply an electric field of DC 1250 V/cm, with 3 pulses, each of 50 µs duration (**Fig. 2A, B**).
14) Keep the chamber with the electroporated protoplasts on ice for 10 min, followed by room temperature for 1 h in the dark.
15) Proceed to the protocol for protoplast culture.

Electrofusion

16) From step (8), count the protoplasts using a hemocytometer and adjust the protoplast density to 1.0×10^6 protoplasts/mL by addition of MCP solution. Prepare protoplasts of the 2 banana varieties to be fused, using the same protocol.
17) Mix, in equal numbers, the protoplasts of the 2 varieties.
18) Transfer the protoplast mixture to the electrode chamber of the electrofusion apparatus (**Fig. 3A**).
19) Apply an AC electric field of 1 MHz, 200 V/cm for 10 s to align the protoplasts (formation of "pearl chains"; **Fig. 3B**).
20) Apply a DC electric field of 1250 V/cm with 3 pulses each of 100 µs.
21) Maintain the chamber containing the protoplast suspension at room temperature, in the dark for 1 h.
22) Proceed to the protocol for protoplast culture.

Protoplast culture

23) During the 1-hour incubation of electroporated or fusion-treated protoplasts in the electrode chamber, prepare the nurse cells as detailed below.
24) Liquefy the semi-solid PA3 medium using a microwave oven and maintain the molten medium at 35-40°C.
25) Take 1.5 mL settled cell volume (SCV) of nurse cells (rapidly growing banana, rice or *Lolium perenne* suspension cells) and suspend in 13.5 mL of PA3 liquid medium.
26) Mix with an equal volume of molten PA3 medium.
27) Wait approximately 2 min. until the medium becomes slightly viscous.
28) Distribute 2-mL of the cell-containing medium over 25-mL of PA5 semi-solid medium in a 9-cm Petri dish and allow the medium containing the nurse cells to become semi-solid.
29) Place an Isopore membrane (25-mm diameter, 5-µm pore size) over the surface of the medium containing the nurse cells.
30) Harvest the protoplasts from the electroporation/electrofusion chamber adding more 2 volumes of PA3 liquid medium. (If the chamber volume is 0.8 mL, add 1.6 mL of the PA3 liquid medium, giving a final protoplast density of 3.0×10^5 per mL.)
31) Dispense 0.2 ml of the protoplast suspension on the surface of the Isopore membrane (**Fig. 1C**); seal the Petri dish (e.g. with PVC film or Parafilm); incubate the cultures at 28 ± 2°C in the dark. Set up all of the treated protoplasts in this way (See Note 2). [If the plasmid DNA contains the *gfp* gene and transient expression assay is intended, dispense approximately 4 mL of the

suspension of electroporated protoplasts into a 5-cm diameter Petri dish and culture at 28 ± 2°C in the dark at 50 rpm on a horizontal platform shaker for 3 days. Observe transient gene expression with a microscope and UV-blue light at 395 nm (**Fig. 2C, 2D**)].

32) After 20-30 days of culture, transfer the protoplast-derived cells attached to the Isopore membrane to the cell multiplication medium (**Fig. 1D**). [In the case of electroporation, plasmid DNA containing the hygromycin phosphotransferase (*hpt)* gene may be used, and transgenic protoplast-derived tissues may be recovered on multiplication medium supplemented with 20-30 mg/L hygromycin B. In the case of electrofusion for somatic hybridization, a selection agent is not applied and somatic hybridity is evaluated by PCR/RAPD analyses (**Fig. 3E**), and/or ploidy analysis by flow cytometry, after shoot regeneration (See Note 3)].

33) Transfer somatic embryos when they are each approximately 1 mm in size (**Fig. 3C**) to germination medium for shoot development (**Figs. 1E, 1F**).

NOTES

1. In our laboratory, 1.0×10^7 protoplasts are obtained routinely from 0.2 mL SCV of embryogenic suspensions of AAA, AAB and AA group bananas. The enzyme solution of 1.5% Cellulase RS + 0.2% Pectolyase Y23 may be suitable for several different banana cultivars, since enzyme mixtures of similar composition have been used by other workers (**Table 1**). The quality of cell suspensions should be assessed if protoplast isolation is not successful. Cell suspensions should be rich in embryogenic cells, small cell clusters (each < 200 µm in size) being generally preferable as source material for protoplast isolation.
2. In the case of the banana var. Embrapa/CNPMF 2803-01 (AA group), 200 – 500 proembryos are regenerated on a membrane when 0.6×10^5 protoplasts (0.2 mL/membrane of a suspension of 3.0×10^5 protoplasts / mL) are cultured without electroporation or fusion treatments. When electroporation or fusion procedures are employed, the regeneration efficiency is reduced to less than 50 proembryos/membrane. Consequently, the density of protoplast suspension should be adjusted following preliminary experiments, to facilitate the selection of target cells or proembryos.
3. The protoplast fusion efficiency is 30–40% when observed by light microscopy. Following 3 fusion experiments between AAB and AA genome cultivars, more than 200 plants were regenerated from electrofused protoplasts, and 16 of the 24 plants evaluated showed hybrid characteristics as assessed by PCR/RAPD and flow cytometry analyses.

REFERENCES

Arinaitwe G, Remy S, Strosse H, Swennen R, Sági L (2004) *Agrobacterium*- and particle bombardment-mediated transformation of a wide range of banana cultivars. In: Mohan Jain S, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science Publishers, Enfield, NH, USA, pp 351-357

Arvanitoyannis IS, Mavromatis AG, Grammatikaki-Avgeli G, Sakellariou M (2008) Banana: cultivars, biotechnological approaches and genetic transformation. *International Journal of Food Science and Technology* **43**, 1871-1879

Assani A, Chabane D, Haïcour R, Bakry F, Wenzel G, Foroughi-Wehr B (2005) Protoplast fusion in banana (*Musa* spp.): Comparison of chemical (PEG: polyethylene glycol) and electrical procedure. *Plant Cell, Tissue and Organ Culture* **83**, 145-151

Assani A, Haïcour R, Wenzel G, Côte F, Bakry F, Foroughi-Wehr B, Ducreux G, Aguillar ME, Grapin A (2001) Plant regeneration from protoplasts of dessert banana cv. Grande Naine (*Musa* spp., Cavendish sub-group AAA) via somatic embryogenesis. *Plant Cell Reports* **20**, 482-488

Assani A, Haïcour R, Wenzel G, Foroughi-Wehr B, Bakry F, Côte F, Ducreux G, Ambroise A, Grapin A (2002) Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (*Musa* spp.). *Plant Science* **162**, 355-362

Bakry F (1984) Choix du materiel à utiliser pour l'isolement de protoplastes de bananier (*Musa* spp.), Musacées. *Fruits* **39**, 449-452

Chai M, Ho YW, Liew KW, Asif JM (2004) Biotechnology and *in vitro* mutagenesis for banana improvement. In: Mohan Jain S, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science Publishers, Enfield, NH, USA, pp 59-77

Chen WH, Ku ZC (1985) Isolation of mesophyll cells and protoplasts, and protoplast fusion and culture in banana. *Journal of the Agriculture Association of China, New Series* **129**, 56-67 (in Chinese)

Côte FX, Domergue R, Monmarson S, Schwendiman J, Teisson C, Escalant JV (1996) Embryogenic cell suspensions from the male flower of *Musa* AAA cv. Grand naine. *Physiologia Plantarum* **97**, 285-290

Davey MR, Anthony P, Power JB, Lowe KC (2005a) 2004 SIVB congress symposium proceedings "Thinking outside the cell": Plant protoplast technology: status and applications. *In Vitro Cellular and Developmental Biology – Plant* **41**, 202-212

Davey MR, Anthony P, Power JB, Lowe KC (2005b) Plant protoplast technology: Current status. *Acta Physiologiae Plantarum* **27**, 117-129

Davey MR, Anthony P, Power JB, Lowe KC (2005c) Plant protoplasts: Status and biotechnological perspectives. *Biotechnology Advances* **23**, 131-171

Day A, Kode V, Madesis P, Lamtham S (2005) Simple and efficient removal of marker genes from plastids by homologous recombination. In: Peña L (Ed) *Transgenic Plants: Methods and Protocols, Methods in Molecular Biology* 286, Humana Press, Totowa, NJ, USA, pp 255-269

Ebinuma H, Sugita K, Endo S, Matsunaga E, Yamada K (2005) Elimination of marker genes from transgenic plants using MAT vector systems. In: Peña L (Ed) *Transgenic Plants: Methods and Protocols, Methods in Molecular Biology* 286, Humana Press, Totowa, NJ, USA, pp 237-252

Fisk HJ, Dandekar AM (2005) Electroporation: introduction and expression of transgenes in plant protoplasts. In: Peña L (Ed) *Transgenic Plants: Methods and Protocols, Methods in Molecular Biology* 286, Humana Press, Totowa, NJ, USA, pp 79-90

Ghosh A, Ganapathi TA, Nath P, Bapat VA (2009) Establishment of embryogenic cell suspension cultures and *Agrobacterium*-mediated transformation in an important Cavendish banana cv. 'Robusta' (AAA). *Plant Cell, Tissue and Organ Culture* **97**, 131-139

Haïcour R, Assani A, Matsumoto K, Guedira A (2004) Banana protoplasts. In: Mohan Jain S, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science Publishers, Enfield, NH, USA, pp 111-125

Hernández JBP, Remy S, Galán-Saúco V, Swennen R, Sági L (1999) Chemotactic movement and attachment of *Agrobacterium tumefaciens* to banana cells and tissues. *Journal of Plant Physiology* **155**, 245-250

Joshi S, Soriano M, Kortstee A, Schaart JG, Krens FA, Jacobsen E, Schouten HJ (2008) Development of cisgenic apples having durable resistance to apple scab. In: *1st International Symposium on Biotechnology of Fruit Species*, Dresden, Germany, JKI, 2008, Program and Abstract Book, p 85

Kosky RG, Silva MF, Pérez LP, Gilliard T, Martínez FB, Vega MR, Milian MC, Mendoza EQ (2002) Somatic embryogenesis of the banana hybrid cultivar FHIA-18 (AAAB) in liquid medium and scaled-up in a bioreactor. *Plant Cell, Tissue and Organ Culture* **68**, 21-26

Matsumoto K, Crepy L, Teixeira JB, Ferreira FR (1988) Isolation and culture of bract protoplasts in banana plants. In: International Rice Research Institute (IRRI) and Academia Sinica (Eds) *Genetic Manipulation in Crops*, Cassell Tycooly, Philadelphia, USA, pp 414-415

Matsumoto K, Kobayashi S, Barbosa ML, Teixeira JB (1992) Isolamento e fusão elétrica dos protoplastos de bananeira. *Revista Brasileira de Fruticultura, Cruz das Almas* **14.1**, 27-33

Matsumoto K, Oka S (1998) Plant regeneration from protoplasts of a Brazilian dessert banana (*Musa* spp., AAB group). *Acta Horticulturae* **490**, 455-462

Matsumoto K, Silva Neto SP (2003) Micropropagation of bananas. In: Mohan Jain S, Ishii K (Eds) *Micropropagation of Woody Trees and Fruits*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 353-380

Matsumoto K, Vilarinhos AD, Oka S (2002) Somatic hybridization by electrofusion of banana protoplasts. *Euphytica* **125**, 317-324

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Bio/Technology* **13**, 486-492

Megia R, Haïcour R, Rossignol L, Sihachakr D (1992) Callus formation from cultured protoplasts of banana (*Musa* sp.). *Plant Science* **85**, 91-98

Megia R, Haïcour R, Tizroutine S, Bui Trang V, Rossignol L, Sihachakr D, Schwendiman J (1993) Plant regeneration from cultured protoplasts of the cooking banana cv. Bluggoe (*Musa* spp., ABB group). *Plant Cell Reports* **13**, 41-44

Morán JFA (2006) Híbridos de banana desenvolvidos pela FHIA. In: Soprano E, Tcacenco FA, Lichtemberg LA, Silva MC (Eds) *Reunião Internacional ACORBAT, 17., Joinvile, SC, Brasil. Bananicultura: Um Negócio Sustentável – Anais*. ACORBAT/ACAFRUTA, 1, pp 173-177

Panis B, Van Wauwe A, Swennen R (1993) Plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa* spp.). *Plant Cell Reports* **12**, 403-407

Pedraza TR, Díaz LG, Martín JCV, Morales SR, Guerra JRG (2005) Production of banana and plantain hybrids in Cuba. *InfoMusa* **14.1**, 11-13

Pei XW, Chen SK, Wen RM, Ye S, Huang JQ, Zhang YQ, Wang BS, Wang ZX, Jia SR (2005) Creation of transgenic bananas expressing human lysozyme gene for Panama wilt resistance. *Journal of Integrative Plant Biology* **47**, 971-977

Roux NS (2004) Mutation induction in *Musa* – review. In: Mohan Jain S, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science Publishers, Enfield, NH, USA, pp 23-32

Roux NS, Toloza A, Strosse H, Busogoro JP, Doležel (2009) Induction and selection of potentially useful mutants in banana. *Acta Horticulturae* **828**, 315-322

Sági L, May GD, Remy S, Swennen R (1998) Recent developments in biotechnological research on bananas (*Musa* spp.). *Biotechnology and Genetic Engineering Reviews* **15**, 312-327

Sági L, Panis B, Remy S, Schoofs H, De Smet K, Swennen R, Cammue BPA (1995a) Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. *Bio/Technology* **13**, 481-485

Sági L, Remy S, Cammue BPA, Maes K, Raemaekers T, Panis B, Schoofs H, Swennen R (2000) Production of transgenic banana and plantain. *Acta Horticulturae* **540**, 203-206

Sági L, Remy S, Panis B, Swennen R, Volckaert G (1994) Transient gene expression in electroporated banana (*Musa* spp., cv. 'Bluggoe', ABB group) protoplasts isolated from regenerable embryogenetic cell suspensions. *Plant Cell Reports* **13**, 262-266

Sági L, Remy S, Verelst B, Panis B, Cammue BPA, Volckaert G, Swennen R (1995b) Transient gene expression in transformed banana (*Musa* cv. Bluggoe) protoplast and embryogenic cell suspensions. *Euphytica* **85**, 89-95

Schouten HJ (2008) Cisgenesis in fruit trees. In: *1st International Symposium on Biotechnology of Fruit Species*, Dresden, Germany, JKI, 2008, p 29 (Abstract)

Sreeramanan S, Maziah M, Abdullah MP, Sariah M, Xavier R, Nor'Aini MF (2005) Physical and biological parameters affecting transient GUS and GFP expression in banana via particle bombardment. *Asia Pacific Journal of Molecular Biology and Biotechnology* **13**, 35-57

Sreeramanan S, Maziah M, Abdullah MP, Sariah M, Xavier R (2006) Transient expression of *gus* gene in *Agrobacterium*-mediated banana transformation using single tiny meristematic bud. *Asian Journal of Plant Sciences* **5**, 468-480

Strosse H, Domergue R, Panis B, Escalant JV, Côte F (2003) Banana and plantain embryogenic cell suspensions. *INIBAP Technical Guidelines 8*, The International Network for the Improvement of Banana and Plantain, Montpellier, France, 31 pp

Strosse H, Van Den Houwe I, Panis B (2004) Banana cell and tissue culture – review. In: Mohan Jain S, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science Publishers, Enfield, NH, USA, pp 1-12

TadaY, Sakamoto M, Fujimura T (1990) Efficient gene introduction into rice by electroporation and analysis of transgenic plants: use of electroporation buffer lacking chloride ions. *Theoretical and Applied Genetics* **80**, 475-480

Tomekpe K, Jenny C, Escalant JV (2004) A review of conventional improvement strategies for *Musa*. *InfoMusa* **13.2**, 2-6

Tripathi L (2003) Genetic engineering for improvement of *Musa* production in Africa. *African Journal of Biotechnology* **2**, 503-508

Tripathi L, Odipio J, Tripathi JN, Tusiime G (2008a) A rapid technique for screening banana cultivars for resistance to *Xanthomonas* wilt. *European Journal of Plant Pathology* **121**, 9-19

Tripathi L, Tripathi JN, Tushemereirwe WK (2008b) Rapid and efficient production of transgenic East African Highland banana (*Musa* spp.) using intercalary meristematic tissues. *African Journal of Biotechnology* **7**, 1438-1445

Tripathi L, Tripathi JN, Hughes Jd'A (2005) *Agrobacterium*-mediated transformation of plantain (*Musa* spp.) cultivar Agbagba. *African Journal of Biotechnology* **4**, 1378-1383

Xiao W, Huang XL, Huang X, Chen YP, Dai XM, Zhao JT (2007) Plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **90**, 191-200

Xu L, Li ZY, Du ZJ (2006) A pilose fruit mutant in banana (*Musa* spp. 'Williams'). *Scientia Horticulturae* **107**, 315-318