

IMPROVEMENT OF BANANAS FOR BLACK SIGATOKA AND PANAMA DISEASE RESISTANCE THROUGH GENETIC MANIPULATION

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ABSTRACT

Bananas are a staple food in Eastern Africa, with 25.3% of the total world production. The production is, however, threatened by the presence of several diseases, of which the fungal diseases black sigatoka and Panama disease are the most important. With the development of embryogenic cell suspension cultures, the isolation of protoplasts therefrom and their successful regeneration, an invaluable vegetative material for genetic manipulation of bananas became available. The discovery of new types of antifungal proteins (AFP's) and the cloning of their encoding genes provide a source of resistance to fungal diseases that can be introduced into plant cells by genetic engineering. Transformation techniques are currently under investigation. Electroporation of banana protoplasts have resulted in transient expression frequencies of more than 1% as visualised by the action of the β -glucuronidase (GUS) marker gene. Experiments with particle bombardment on cell suspensions have resulted in transient expression frequencies up to 3300 blue spots/0.5 ml cells, and stable transformants have also been selected. Optimisation of the cell suspension culture technique and transformation methodology is in progress.

Key Words: Banana, black sigatoka, genetic manipulation, *Musa spp.*, Panama disease

RÉSUMÉ

Les bananes constituent l'alimentation de base en Afrique de l'Est avec 25,3 % de la production totale mondiale. Les contraintes de production sont constituées par de nombreuses maladies parmi lesquelles la Cercosporiose noire et la Maladie de Panama sont les plus importantes. Le développement des techniques de culture de suspension de cellules embryogéniques, l'isolement des protoplastes et la réussite de leur régénération permettent d'obtenir un matériel végétal pour la manipulation génétique du bananier. La découverte de nouveaux types de protéines antifongiques (PAF) et le clonage des gènes correspondants offrent des sources de résistance aux maladies fongiques pouvant être incorporées dans les cellules par génie génétique. Les techniques de transformation sont en cours d'évaluation. L'électroporation des protoplastes a abouti à des fréquences d'expression transitoire de plus de 1% pouvant être visualisées par l'activité du gène marqueur B-glucoronidase (GUS). Des essais de bombardement de particules sur les suspensions de cellules ont abouti à des fréquences d'expression transitoire de plus de 3300 points bleus par 0.5 ml de cellules; des transformants stables ont aussi été sélectionnés. L'optimisation de la technique de culture de suspension cellulaire et de la méthodologie de transformation est en cours d'études.

Mots Clés: Bananier, cercosporiose noire, manipulation génétique, *Musa spp.*, maladie de Panama.

INTRODUCTION

Bananas (*Musa* spp.) are a staple food crop for nearly 400 million people in the tropical zones of the world. While only 10% of the world production is exported as dessert bananas (INIBAP, 1989), millions of farmers in the tropics cultivate plantains, dessert, cooking or beer bananas for local consumption according to local preferences. East-Africa with its distinct highland beer and cooking bananas accounts for a considerable share (25.3%) in the world production (FAO, 1991).

Two of the most important threats to the productivity of bananas are the fungal diseases black sigatoka and fusarial wilt (or Panama disease). The former is caused by the wind-spread fungus *Mycosphaerella fijiensis*. Originating from the Pacific it spread through Asia and much of the African and Latin-American humid lowlands in the early seventies (Raemakers, 1975; Stover, 1980). Yield losses may be as high as 30-50% (Stover, 1983). Although chemical control is possible for large plantations at high cost (between 600 and 1800 US\$ ha⁻¹ in Central America), it is economically and environmentally unsound for the subsistence farmer who grows his bananas and plantains in the backyard. Fusarial wilt which is caused by the soil-habiting fungus *Fusarium oxysporum* f.sp. *cubense*, destroyed more than 40,000 ha of bananas in Central and South America in the first 50 years of this century, until the cultivar 'Gros Michel' was replaced all over by the resistant 'Cavendish' types. With the recent appearance of race 4 in some subtropical regions, all important edible clones are susceptible to one or more races of this destructive pathogen (Novak, 1992).

Genetic improvement of *Musa* seems to be the most effective long-term solution to cope with those diseases. Conventional breeding, however, has several drawbacks such as the triploid nature and low seed fertility of most edible clones (Rowe, 1984; Swennen and Vuylsteke, 1993), the low seed germination rate (Vuylsteke *et al.*, 1993) and the long cropping cycle. Only recently, some very promising plantain hybrids with black sigatoka resistance have been produced at IITA (International Institute of Tropical Agriculture, Nigeria) (Swennen and Vuylsteke, 1993) through wide crosses of plantains with wild banana diploids as a male parent. However, the progeny of bananas and plantains in wide crosses always shows

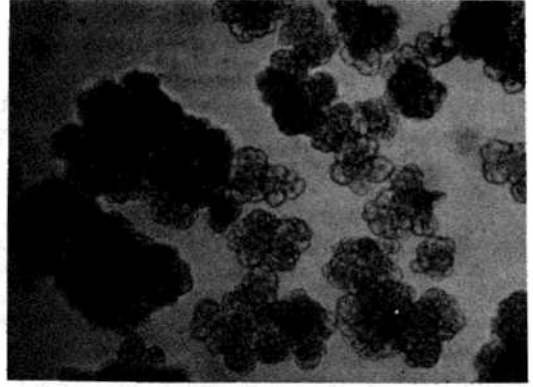


Figure 1. Cell suspension culture of *Musa* (ABB group) cv 'Bluggoe', consisting of embryogenic cell clusters with dense cytoplasm (x 131).

different characteristics from their female parents, and may produce seeds.

The most attractive strategy for disease control in *Musa* is probably the production of resistant transformants by introducing foreign genes. The requisites to exploit this potential are the disposal of an appropriate *in vitro* regeneration system and of genes coding for relevant characteristics, and the development of an efficient transformation technique.

VEGETATIVE MATERIAL

Embryogenic cell suspensions. The ideal *in vitro* system for transformation involves the regeneration of plants out of a single cell, such as cell suspension cultures or protoplasts. In this way chimaeric transformants (plants of which only a part of the somatic cells are transformed) are excluded. Embryogenic cell suspensions (ECS) have been produced from zygotic embryos from banana seed (Escalant and Teisson, 1989). However, as most edible bananas are practically seed-sterile, and as the exact genome constitution of a zygotic embryo is not known, vegetation tissue such as leaf bases, immature male flowers or meristems are preferred as a starting material by other research groups (Dhed'a *et al.*, 1991; Escalant *et al.*, 1993; Novak *et al.*, 1989). The

method described here has been developed at the Catholic University, Leuven (Dhed'a *et al.*, 1991). The starting materials are proliferating meristems ('scalps'), which makes the method theoretically applicable to all *Musa* genotypes. The ECS consist of round embryogenic cells that are continuously dividing (Fig. 1). The somatic embryogenesis closely resembles zygotic embryogenesis in the seed (Dhed'a *et al.*, 1991). No callus phase is involved in the regeneration. Such a callus phase is often considered undesirable as it increases the risk for somaclonal variation. Up to now ECS have been established for five different clones, i.e. 'Bluggoe', 'Saba' and 'Cardaba' (all of the ABB group), the plantain Three Hand Planty (AAB) and *Musa balbisiana*. Work is in progress to produce ECS of other cultivars in the genus *Musa*. Besides their use as a plant material for

(Catholic University, Leuven) (Panis *et al.*, 1993). After an enzymatic treatment of the ECS, protoplasts are purified by filtration. Regeneration is achieved by culturing the protoplasts on a feeder layer (Fig. 2) (an actively dividing cell suspension which "nurses" the protoplasts) or by plating at a sufficiently high density (10^6 protoplasts per ml). The main advantages of the method developed by Panis *et al.* (Panis *et al.*, 1993) over a previously described protocol for *Musa* protoplasts (Megia *et al.*, 1992) are the use of material of vegetative origin, the high protoplast yield (up to 6×10^7 protoplasts per ml packed cells, i.e. 100 times higher than in an earlier report (Megia *et al.*, 1992), the absence of a callus phase during regeneration and the high regeneration frequencies.

GENETIC MATERIAL

Recently researchers of the F.A. Janssens Laboratory of Genetics (Catholic University Leuven) have discovered new types of antifungal proteins (AFPs) from seeds of *Mirabilis jalapa* (Cammue *et al.*, 1992), *Amaranthus caudatus* (Broekaert *et al.*, 1992), and *Raphanus sativus* (Terras *et al.*, 1992). Because of some unique common properties these AFPs are excellent tools for transformation of banana for fungal disease resistance (Cammue and Broekaert, 1992):

- These AFPs have proven to induce a strong inhibitory effect on all phytopathogenic fungi tested so far *in vitro*. Hence, besides the ability to inhibit growth of *Mycosphaerella* and *Fusarium*, the causal agents of the two most important banana fungal diseases, they may have the potential to control any other fungal disease attacking bananas (other leaf spot diseases, post harvest rots, etc.) after introduction of their genes into the banana genome.
- The AFPs belong to the most potent antifungal plant proteins, as shown by *in vitro* tests.
- A major advantage is their non-toxicity for non-fungal plant cells (including banana cells) and different types of human cells. The latter is of course a first requirement for a protein of which the gene is to be introduced in an edible crop like banana.
- Microscopical studies have demonstrated at least two different modes of inhibition of

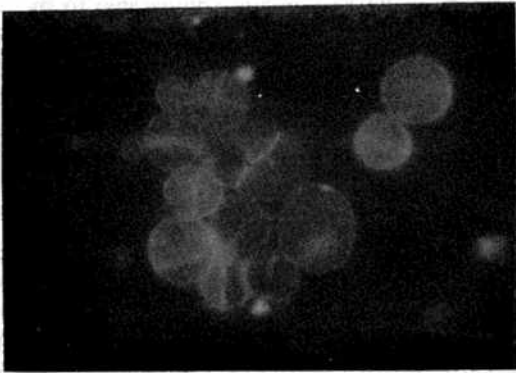


Figure 2. Cell wall regeneration and first and second division of *Musa* protoplasts, five days after inoculation on a feeder-layer culture (stained with calcofluor white and observed under ultraviolet illumination) (x 520).

transformations, ECS do have other important applications too, such as mass clonal propagation and cryopreservation (i.e. freeze-preservation of germplasm in liquid nitrogen) (Panis *et al.*, 1992; Panis *et al.*, 1993).

Protoplasts. The ECS are also a starting material for the isolation and regeneration of protoplasts following a protocol that has been optimized at the Laboratory of Tropical Crop Husbandry

fungus growth by the different AFPs. Hence, if two genes coding for AFPs with a different mode of action are introduced into the plant genome, the chance that the phytopathogenic fungus will develop resistance against both AFPs will be drastically reduced.

At this moment the corresponding genes of all three types of AFPs have been cloned and transformed to several crops (e.g. tobacco, oil seed rape, apple). The evaluation of the first transgenic tobacco plants demonstrated correct expression of the introduced AFPs resulting in an enhanced antifungal activity of the plant extracts.

TRANSFORMATION TECHNIQUES

The transfer of genes into plants has often been realized by the naturally occurring transformation system present in the common gall-inducing bacterium, *Agrobacterium tumefaciens*. The main drawback, however, is the fact that *Agrobacterium* does not affect monocots in nature. Although in some cases monocots have successfully been transformed using the *Agrobacterium* system (Mooney *et al.*, 1991), it seems appropriate to look for more rewarding techniques for *Musa*, like electroporation of protoplasts and particle bombardment.

Electroporation. Electroporation involves the formation of transient pores in biological membranes by the discharge of electric current, which allows DNA molecules to enter the protoplast or, eventually, the cell (Dandekar, 1992). Although the technique has already been proven to be applicable to walled cells (Dekeyser, *et al.*, 1990), protoplasts are preferred. Therefore, several experiments on the optimization of this technique on *Musa* protoplasts have been carried out at the Laboratories of Gene Technology and of Tropical Crop Husbandry of the Catholic University, Leuven (Sagi *et al.*, 1993). Transient expression was visualized by the action of the GUS (β -glucuronidase)-gene, a commonly used reporter gene in plants. Protoplasts expressing the gene stain blue in the presence of the substrate X-Gluc (Jefferson *et al.*, 1987). Parameters which were found to be highly influential were the composition of the electroporation buffer, a polyethylene glycol treatment and its duration before electroporation, the use of a heat shock, the

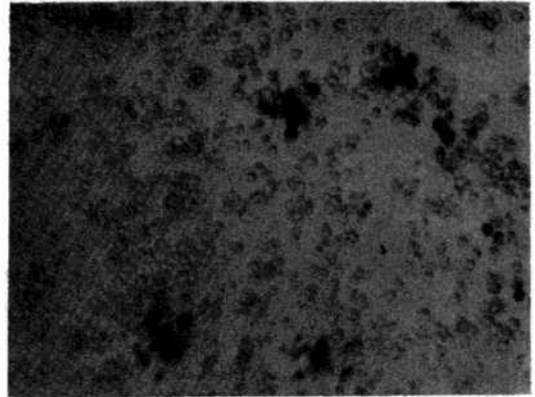


Figure 3. Transient expression of the β -glucuronidase gene in electroporated *Musa* protoplasts (x 20).

type of gene construct and the age of the suspension.

Transient expression frequencies amounted to 1.8% of the total number of protoplasts (Fig. 3). Those results are already comparable to those obtained by other research groups on other plants. Although further increase may still be expected by varying other parameters, the current frequencies already present a promising basis for the production of transgenic banana plants (Sagi *et al.*, 1993).

Particle bombardment (biolistics). The particle bombardment technique involves the acceleration of small tungsten or gold particles coated with plasmid DNA, that penetrate an intact cell. Several devices are used to accelerate particles, such as a "gun-like" explosion, a gas discharge or high voltage electric discharges. The device used in the Laboratory of Tropical Crop Husbandry in collaboration with the F.A.Janssens Laboratory of Genetics (both Catholic University, Leuven) is based on helium gas discharges. At this moment experiments are being carried out to optimize this system. The parameters to be optimized can be divided into device parameters, genetic parameters and target tissue parameters (Panis *et al.*, 1993):

- Device parameters, involving:
 - (i) the size and composition of the particles: usually tungsten particles of an average

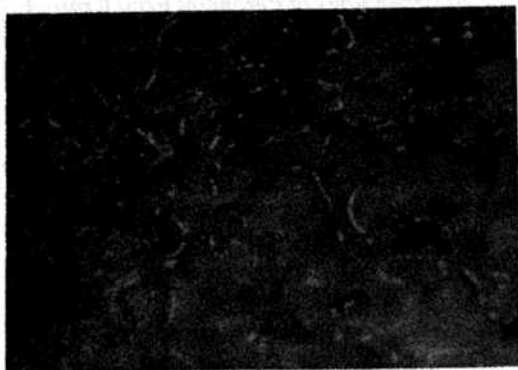


Figure 4. Transient expression of the β -glucuronidase gene in embryogenic *Musa* cells after helium-driven microprojectile bombardment (x 131).

- (ii) diameter of 1 μm are used;
- (ii) the DNA attachment to the particles: plasmid DNA is precipitated onto the particles with CaCl_2 and spermidine. The precipitation method and the quantities of the components have an influence on DNA attachment and aggregation of the micro-projectiles;
- (iii) the micro-projectile velocity: the optimal balance between transformation efficiency and tissue damage needs to be established.

- **Genetic parameters:** The plasmid DNA has to include an optimal promoter sequence for expression in *Musa*. A screenable marker like the GUS gene (see above) for assessment of the transient expression is required. Moreover, for the selection of stable transformants, a selectable marker should be present in the plasmid. Commonly used selectable markers are genes coding for antibiotic (e.g. hygromycin, kanamycin) or herbicide resistance. Only the transformed cells will survive on a medium containing the selective agent.

Target tissue parameters: They include both the age of the suspension and the amount of suspension to be used.

TABLE 1. Transient transformation frequencies in suspension cultures subjected to particle bombardment.

Crop	Transient transformation frequency (spots 0.5 ml^{-1})
Maize	200
Maize	2000
Banana	3333
Tobacco	3750
Cotton	4351

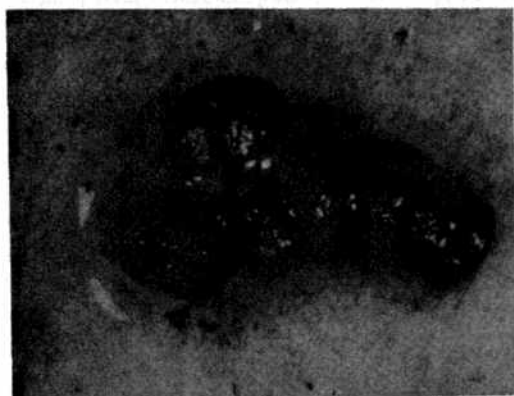


Figure 5. Antibiotic-resistant transformants, stained with X-Guc.

Before any optimization of the parameters, high transient expression frequencies have already been obtained with the *Musa* cultivar 'Bluggoe' (Fig. 4). The number of blue spots (i.e. cells expressing the GUS-gene) per 30 μl settled cell volume of cell suspension amounted up to 200, which corresponds to 3333 spots per 0.5 ml. Those values are already comparable to those obtained by other researchers on other crops (Table 1).

Estimates of rates of conversion from transient to stable incorporation of micro-projectile delivered DNA in plant cells range from 0.5 to 1% (Franks and Birch, 1991). This means that from each shot 1 to 2 stable transformants may be expected. Indeed, several antibiotic-resistant transformants also expressing the GUS-gene have been selected (Fig. 5) and are now cultured as callus or little plantlets.

CONCLUSION

With the establishment of embryogenic cell suspensions and the isolation of protoplasts therefrom, excellent tissue culture systems for genetic transformation in *Musa* have become available. A source of resistance against fungal diseases is provided by genes encoding antifungal proteins (AFPs). Successful introduction of these genes into the banana genome could induce resistance against most fungal diseases of banana, including black sigatoka and fusarial wilt. At the same time, all morphological, physiological, agronomic and post-harvest characteristics would remain unchanged. Experiments on both electroporation of protoplasts and transformation by particle bombardment have already resulted in promising rates of transient expression. The successful selection of antibiotic-resistant transformants issued from particle bombardment experiments further illustrates the feasibility of this transformation method. Soon, plasmids containing the genes for AFPs will be used for transformation. Hence, gene expression in a field-grown banana plant and its inheritance can be studied. It should be noted that the development of an efficient transformation system can also benefit the introduction of other interesting genes into *Musa* in the future. Several plant species have already acquired resistance to certain viruses through introduction of a gene encoding viral coat protein (Dandekar, 1992).

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