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Genetic Modification of Cavendish Bananas (*Musa* spp.) in South Africa

by

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DECLARATION

I, the undersigned, thereby declare that this thesis submitted herewith for the degree Magister Scientia to the University of Pretoria, contain my own independent work and has not been submitted for any degree at any other University.

René Sutherland

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PREFACE

Bananas and plantains (*Musa* spp.) are considered the fourth most important staple food crop in the world after rice, maize and wheat (Ammar-Khodja, 2000). The crop is the most nutritious of all food crops, and provides food and an income to millions of people around the world (Stover and Simmonds, 1987). Worldwide, 400 million people rely on the banana plant as a basic food source (Ammar-Khodja, 2000). The South African banana industry has an annual turnover of approximately R600 million and provides jobs to approximately 18 000 people (Viljoen, personal communication). Like the rest of Africa, and indeed the world, diseases and pests are threatening continued production. With the limited genetic background of the seedless Cavendish banana, the only type cultivated in South Africa, these diseases and pests can easily overcome plant resistance responses and cause severe damage to commercial plantations. For many banana diseases and pests no sustainable control strategy is available, while small-scale farmers often do not have the financial resources to sustain production by means of the application of chemical fungicides and pesticides (Viljoen *et al*., 2004). The introduction of diseases and pests into banana fields, therefore, often spells the end of further production.

The parthenocarpic nature of cultivated bananas makes it difficult to breed for resistance to diseases and pests (Jones, 2000). Disease and pest resistant hybrids have been developed by means of classical breeding, and although good resistance has been obtained, most of these hybrids are not acceptable to local markets. Researchers have also tried to generate disease resistance in Cavendish plants by using tissue culture-based techniques such as *in vitro* mutagenesis. However, none of these techniques have produced a high yielding banana plant with sufficient resistance to Fusarium wilt in South Africa. Genetic modification now appears to be an excellent option whereby local banana cultivars can be improved for sustainable cultivation. The purpose of this thesis, therefore, was to establish the technology of genetic transformation of Cavendish bananas in South Africa at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Chapter 1 of the thesis presents a literature review on the improvement of bananas for fungal disease resistance. A brief overview is given on the banana plant and its

most economically important diseases and pests. These sections are followed by a discussion on the conventional and unconventional methods used for the improvement of bananas. The focus of this particular section is on plant improvement through *Agrobacterium*-mediated transformation and particle bombardment. Useful genes for possible resistance to fungal pathogens and common selectable and screenable markers used in plant transformation are highlighted.

Somatic embryogenesis is one of the most powerful tools that can be used for plant improvement and the micropropagation of disease-free plants. Embryogenic cell suspensions that are obtained by means of somatic embryogenesis can be used for genetic engineering of disease and pest resistant plants, *in vitro* mutagenesis, germplasm conservation and protoplast culture. The development of embryogenic cell suspensions from Cavendish banana cultivars is presented in **Chapter 2**. The use of embryogenic cell suspensions is preferred over other transformation techniques because of the higher transformation efficiency and the avoidance of chimeric plants.

The transformation of Cavendish bananas is described in **Chapter 3**. Two plasmids were incorporated into the plant genome using two different *Agrobacterium* strains. These plasmids contain genes with kanamycin resistance as well as the β-glucuronidase gene. A gene providing resistance to the banana weevil and the burrowing nematode (*OcI*) was also introduced into Cavendish bananas.

Genetic modification of banana is useful only when the transformed plants are genetically stable and similar to the original mother plant, except for the introduced property. Molecular fingerprinting provides a rapid means to test for such stability. In **Chapter 4**, the use of amplified fragment length polymorphisms (AFLPs) is investigated as a fingerprinting technique to detect minor differences in closely related cultivars, hybrids and varieties. Our results suggest that AFLPs could be considered as a molecular fingerprinting technique to detect small variations and off-types in genetically modified bananas.

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CHAPTER 1

Genetic modification of banana for fungal resistance

ABSTRACT

Bananas and plantains (*Musa* spp.) are cultivated commercially as a dessert fruit and by small-scale farmers as staple food crop in many countries of the world. Sustainable production, however, is threatened by a number of diseases and pests such as Fusarium wilt (*Fusarium oxysporum* f.sp. *cubense*), black Sigatoka (*Mycosphaerella fijiensis*), the banana weevil (*Cosmopolitus sordidus*) and the burrowing nematode (*Radopholus similis*). Many diseases and pests of banana cannot be managed by conventional control methods, and replacement cultivars are often not acceptable to local industries. Resistance can be introduced into banana by means of conventional and unconventional improvement methods. Conventional breeding programs have many limitations, due to sterility of cultivated bananas, long growth cycles, low seed set and hybrids that are often not accepted by consumers. Unconventional improvement for enhanced resistance involves methods such as *in vitro* mutagenesis, protoplast culture, and genetic modification. *In vitro* mutagenesis is time consuming and expensive, and protoplast cell culture is limited by the low frequency of plant regeneration. Genetic modification involves the introduction of foreign genes into banana by means of *Agrobacterium*-mediated transformation or by particle bombardment. Different banana plant tissue can be transformed, such as thinly sliced rhizome tissue, scalps or embryogenic cell suspensions. Of these, the use of embryogenic cell suspensions is preferred because of the higher transformation efficiency and avoidance of chimeric plants. Genes that can be incorporated into plants include those coding for phytoalexins, pathogen-related proteins, reactive oxygen species, ribosomal inactivating proteins, polygalacturonase-inhibiting proteins, resistance genes, lysozyme and non-plant antimicrobial genes. Successful transformation can be increased and monitored by the use of selectable and screenable markers. Several biosafety issues need to be considered before genetically modified plants can be released.

INTRODUCTION

Bananas and plantains (*Musa* spp.) are considered as the world's most important fruit and the fourth most important staple food crop (Swennen and Vuylsteke, 2001). The fruit is nutritional and contains high levels of potassium, vitamin C and vitamin B_6 (Samson, 1986; Robinson, 1996). There are two types of bananas: the sweet dessert and the cooking banana (including plantains) (Jones, 2000c). The dessert banana is left to ripen and then eaten raw, while the cooking banana is peeled and cooked into a dish (Robinson, 1996). It can further be used to brew alcoholic drinks and can be sliced up and dried to make 'chips' (Jones, 2000c). Plantains are either cooked or fried. The male flowers of both bananas and plantains can be eaten as a vegetable, and banana leaves can be used as umbrellas, for wrapping of food, or to make rope (Robinson, 1996).

Approximately 72 million tonnes of bananas are produced in the world, of which almost 16 million tonnes are traded internationally (FAO, 2006). The rest of cultivated bananas are consumed in their country of production, where it often makes up the main form of carbohydrate consumed by locals (Robinson, 1996). In Uganda, approximately 300 kg of banana fruit is consumed per capita per year (Onguso *et al*., 2004). The banana crop is a vital staple food crop for 400 million people in the tropics, and with 700 million people suffering of malnutrition (Ammar-Khodja, 2000), the importance of the crop is highlighted.

A number of diseases and pests threaten the international banana industry (Robinson, 1996). Among the diseases, Fusarium wilt (caused by *Fusarium oxysporum* Schlecht f.sp. *cubense* (Smith) Snyd. and Hans [*Foc*]) and black Sigatoka (caused by *Mycosphaerella fijiensis* Morelet) are considered most important (Carlier *et al*., 2000; Ploetz and Pegg, 2000), while bacterial wilt (caused by *Xanthomonas campestris* pv. *musacearum* [*Xcm*]) has recently caused severe damage to plantations in Uganda (Ndungo *et al.*, 2004). Of the known banana pests, the burrowing nematode (*Radopholus similis* Cobb) and the banana weevil (*Cosmopolitus sordidus* Germar) are most destructive. Many of the fungal and bacterial diseases and pests cannot be managed by conventional control methods (Ploetz and Pegg, 2000), and no commercially acceptable resistant cultivar has been identified (Tripathi *et al.*, 2004).

The use of fungicides and nematicides to manage *M. fijiensis* and nematode pests such as *R. similis* are considered to be environmentally damaging, and can not be afforded by small-scale farmers. In addition, no biological control agent has been found (Ploetz, 2004).

Resistance can be introduced into banana by means of conventional and unconventional improvement methods (Crouch *et al*., 1998). Some improvement methods, such as a long-term breeding program, have many limitations due to sterility of cultivated bananas, long growth cycles, low seed set and hybrids that are often not accepted by local consumers (Crouch *et al*., 1998; Sági, 2000). Improving disease resistance is vital for the future survival of bananas (Pearce, 2003). The annual losses in the bananas industry due to fungal diseases can be severe, with Fusarium wilt causing losses of approximately $400,000,000 up to 1960 (Ploetz, 2005). Further, losses of $350,000,000 were recorded between 1972-1985 for chemical application to control black Sigatoka (Carlier *et al*., 2000).

This review will give a brief background to banana, the most important diseases associated with it, followed by a description of the improvement of banana by conventional and unconventional methods. Furthermore, plant transformation through *Agrobacterium* and particle bombardment will be discussed, as will some useful genes to obtain possible resistance to fungal pathogens. Finally, it will briefly focus on the most common selectable and screenable markers in plant transformation.

THE BANANA PLANT

The genus *Musa* L. consists of four sections: Callimusa, Australimusa, Rhodochlamys and Eumusa (Daniells *et al*., 2001). Cultivated bananas belong to the section Eumusa and were formed by interspecific hybridization between *M. acuminata* Colla and *M. balbisiana* Colla (Stover and Simmonds, 1987), which contributed the A and B genomes to hybrid progeny, respectively. Somatic mutations gave rise to several clonal forms of important cultivars with morphologically differentiated characteristics (Robinson, 1996). Characterization of banana cultivars is based on 15 morphological characteristics that were proposed by Simmonds and Shepherd (1955).

The banana plant is a giant perennial herb that consists of a rhizome and a pseudostem (Robinson, 1996) (Fig. 1). The rhizome is found underground with a shallow root system and several vegetative buds, from which the suckers develop (Cronauer and Krikorian, 1986). The erect pseudostem is composed of several tightly packed leaf bases that are the initial outgrowths of a centrally located apical meristem. At the latter stages, the apical growing point differentiates into an inflorescence meristem (Cronauer and Krikorian, 1986). The inflorescence consists of two parts: the male bud and female flowers. The banana fruit develops from the female flowers (Stover and Simmonds, 1987).

Most of the commercially grown banana cultivars are sterile and parthenocarpic (Robinson, 1996). The banana plant reproduces by forming suckers from a vegetative bud on the rhizome (Cronauer and Krikorian, 1986). A banana mother plant can produce five to ten suckers per year. The suckers are removed from the mother plant and used as starting material in a new field (Cronauer and Krikorian, 1986). Planting material can also be generated in the laboratory by means of *in vitro* propagation (Crouch *et al*., 1998). For *in vitro* propagation, the shoot tips are isolated from the banana suckers under sterile conditions. Unlimited numbers of shoot tips can be obtained by subdividing the shoot clusters (Krikorian and Cronauer, 1984). Shoot tip culture of banana is an easy technique to accomplish a 10-fold increase in multiplication rate after every subsequent culture (Swamy and Sahijram, 1989). In addition, *in vitro* plants are an essential source of disease-free plant material apart from viruses (Crouch *et al*., 1998).

BANANA DISEASES

Bananas are susceptible to a wide range of pathogens and pests (Robinson, 1996). The annual losses in the bananas industry due to diseases and pests are not only severe but on the increase. In Central America, almost 40,000 ha of Gros Michel plantations were lost because of Fusarium wilt up to the 1960's (Ploetz and Pegg, 2000). Furthermore, susceptible cultivars cannot be planted in infested *Foc* soil for up to 30 years (De Vries and Toenniessen, 2001). Black Sigatoka was first discovered in Fiji in 1963, and within 30 years spread throughout the world. Currently, black Sigatoka costs producers in Central America $49,000,000 per year in the application of

fungicidal sprays (Carlier *et al*., 2000). In addition, banana yields has declined from 1970-1997 from 11 to 5.5 tonnes per hectare in Rwanda (De Vries and Toenniessen, 2001). The greatest damage in lost production is not suffered by the export industry, but by subsistence farmers in third world countries that rely on the crop as basic source of food and income.

The most important diseases of banana are caused by fungi such as *Foc* and *M. fijiensis*, bacteria such as *Xcm*, *Ralstonia solanacearum* (Moko disease) and *Pseudomonas celebensis* (Blood disease) (Thwaites *et al*., 2000), and viruses such as the banana streak virus (BSV) and the banana bunchy top virus (BBTV) (Thomas and Isra-Caruana, 2000). The damage caused by Fusarium wilt (also known as Panama disease) during the first half of the 20th century established the disease as one of the greatest epidemics in agricultural history (Ploetz and Pegg, 2000). Like most other banana pathogens and pests, the origin of *Foc* is believed to be Southeast Asia (Stover, 1962). Three races of the pathogen are recognized, with race 1 causing disease to Gros Michel and the AAB genotypes 'Silk' and 'Pome' (Ploetz and Pegg, 2000), race 2 affecting Bluggoe (*Musa* spp., ABB) and other cooking bananas (Ploetz and Pegg, 2000) and race 4 causing disease to race 1 and race 2 susceptible cultivars and Cavendish banana cultivars (Ploetz and Pegg, 2000). Black Sigatoka is a severe foliar disease that has replaced the less damaging yellow Sigatoka (caused by *Mycosphaerella musicola* Leach) wherever it was introduced in the tropics (Carlier *et al*., 2000). The only effective means to control the disease is by fungicidal applications (Carlier *et al*., 2000). However, small-scale farmers are unable to afford expensive chemicals, and the fungus has the ability to rapidly develop resistance (Marin *et al*., 2003). The symptoms caused by the three species of bacteria pathogenic to bananas and plantains are almost the same, with wilting from the youngest to the older leaves (Twaites *et al*., 2000). *Xcm* has been responsible for severe damage to banana plantations in central Africa recently (Tushemereirwe *et al*., 2003), and appears to be transmitted by insects from one bunch stalk to another (Addis *et al*., 2004).

Diseases caused by the BSV and BBTV can result in yield reduction of up to 90% (Swennen and Vuylsteke, 2001). The problem is further enhanced by the incorporation of BSV activable sequences in the B genome of *Musa* (Harper *et al*.,

1999), and consequently the episomal sequence is a major limitation to the banana breeding industry (Ndowora *et al.*, 1999). Both the banana weevil borer and nematode species such as *R. similis* can cause significant damage to bananas and plantains. All banana plants in east Africa are believed to be infected with the banana weevil (Swennen and Vuylsteke, 2001). The weevil lays eggs in the rhizome of a banana plant, and once the eggs have hatched the larvae will bore tunnels through the rhizome, thereby damaging it (Gold and Messiaen, 2000). The water and nutrient uptake is severely reduced and the roots are also damaged which causes toppling (Gold *et al.*, 2001). Apart from *R. similis*, nematode species such as *Pratylenchus coffeae* Goodey, *P. goodeyi* Sher and Allen, *Helicotylenchus multicinctus* Cobb and *Meloidogyne* spp. Göldi can cause substantial damage to banana roots (Gowen, 2000), resulting in yield losses of up to 80% (Swennen and Vuylsteke, 2001).

The control of diseases and pests of banana is difficult (Ploetz and Pegg, 2000). Diseases such as black Sigatoka and pests such as nematodes can be controlled by chemicals, but such control is only economically feasible in highly intensive commercial production (Carlier *et al*., 2000). In addition, fungi such as *M. fijiensis* and pests such as *C. sordidus* can develop resistance to chemicals (Marin *et al*., 2003). In resource poor areas, farmers have to make use of cultural control practices such as leaf trimming and the parring of suckers to reduce disease spread in plantations. These farmers can also not afford tissue culture plants that are produced free of all diseases and pests, apart from viruses. Controlling viral diseases is difficult, but it can be done by meristem culture in combination with thermotherapy or chemotherapy (Dahal *et al*., 1998). The most efficient way of controlling diseases and pests of bananas is by means of introducing disease resistance into cultivated varieties (Sági, 2000). However, no sources of resistance have been identified for diseases such as bacterial wilt (Tripathi *et al.*, 2004) and the BBTV (Thomas *et al*., 1994), and pests such *R. similis*.

Diseases and pests have spread throughout the world in infected planting material (Ploetz and Pegg, 2000). While such spread can be reduced by the use of *in vitro* propagated planting material, suckers and bits are still used as primary planting stock by most producers in the poorer countries of the world. The continuous culture of bananas in the same field further enhances disease incidence and severity, while the

lack of genetic diversity in cultivated bananas substantially enhances losses to diseases and pests (Pearce, 2003).

IMPROVEMENT OF BANANA FOR DISEASE AND PEST RESISTANCE

Developing cultivated bananas with resistance to the major diseases and pests is one of the greatest challenges in sustainable banana production (Becker *et al*., 2000). Management strategies such as chemical control and the use of tissue culture plants are often not feasible to small-scale farmers, and the most affordable and environmentally friendly way of disease and pest control would be the planting of disease and pest resistant plants. The development of such bananas can be achieved by means of conventional and unconventional improvement (Crouch *et al*., 1998).

Conventional Breeding

The threat that diseases and pests hold to bananas in monoculture has led to the establishment of conventional banana breeding programmes. The initial objective was to breed for resistance to diseases such as Fusarium wilt and black Sigatoka (Stover and Buddenhagen, 1986). The first breeding program was initiated at the Imperial College of Tropical Agriculture in Trinidad in 1922 (Stover and Buddenhagen, 1986). Two years later, a second breeding program was started in Jamaica (Stover and Buddenhagen, 1986). Today there are five major banana-breeding programmes in the world (Escalant *et al*., 2002). These include the Empresa Brasileira de Pesquisa Agropecuária – Mandioca e Fruticultura Tropical (EMBRAPA-CNPMF) in Brazil, the Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Département des productions fruitières et horticoles (CIRAD-FLHOR) in Guadeloupe, the Fundación Hondurereña de Investigacón Agrícola (FHIA) in Honduras, Centre Africain de Recherches sur Bananiers et Plantains (CARBAP) in Cameroon, and the International Institute for Tropical Agriculture (IITA) in Nigeria (Escalant *et al*., 2002).

There are two strategies employed in banana breeding programs. The first strategy is aimed at improving diploids as male parents (Escalant *et al*., 2002), before they are crossed with triploid females. During meiosis, the triploid female parent produces

unreduced triploid gametes that result in offspring that is tetraploid (3n+1n) (Jones, 2000b). Originally, the most commonly employed female parent was Gros Michel (*M. acuminata*, AAA). In some instances, however, Gros Michel has now been replaced with Highgate (*M. acuminata*, AAA) and later with Lowgate (*M. acuminata*, AAA) to produce shorter hybrids that are less susceptible to wind damage (Stover and Buddenhagen, 1986). The second strategy on banana breeding relies on the production of a tetraploid from a diploid with the aid of colchicine before the tetraploid is crossed with a diploid (Escalant *et al*., 2002). With this strategy the chance of obtaining a hybrid resembling a Cavendish type is greatly increased (Escalant *et al*., 2002).

Several biotechnological techniques are available to facilitate the improvement of banana breeding. Embryo rescue has significantly increased the germination rate of banana embryos (Rowe and Richardson, 1975; Crouch *et al*., 1998). The multiplication of banana plantlets through shoot tip culture has also dramatically increased the number of plants in contrast to vegetative multiplication by formation of suckers (Crouch *et al*., 1998). Tissue culture plants, however, do have some disadvantages, such as a greater susceptibility to Fusarium wilt (Smith *et al*., 1998). Another tissue culture technique that enhances the breeding programs is the production of haploids that can be treated with colchicine to form diploids (Assani *et al*., 2003). The advantage of using this type of diploid in breeding program is that it is homozygous. Molecular markers such as microsatellite markers can be linked to important characteristics such as resistance and yield (Crouch *et al*., 1998). This ensures that plants can be screened at a very young age for characteristics rather than during long and expensive field trials. Segregating populations can also be studied for the generation of genetic maps of the banana genome. CIRAD is currently assessing five segregating banana populations, one for black Sigatoka, two for nematodes and two for parthenocarpy (Escalant *et al*., 2002).

Banana breeding programs have several difficulties to overcome. The time between pollination and hybrid development is a minimum of 3 years, making the breeding effort an extremely slow process (Rowe and Richardson, 1975). The lack of knowledge on resistance to some pathogens such as *Xcm* is another drawback (Ndungo *et al*., 2004). BSV activable sequences were recently found incorporated

into the B genome of *Musa*, which threaten and hamper the efforts of banana breeders (Harper *et al*., 1999). Several hybrids have now been released with resistance to pathogens, but due to changes in the ripening process they are not acceptable to many markets. The small number of breeding programs in the world is also a problem (Escalant and Jain, 2004), as is the lack of an extensive germplasm collection. Despite all these shortcomings, banana breeding can still play an imortant role in the production of disease resistant bananas (Crouch *et al*., 1998).

Unconventional breeding

Unconventional breeding involves the use of biotechnology for the improvement of bananas for disease and pest resistance, improved agronomic traits, and enhanced secondary metabolites production (Novak *et al*., 1990; Kumar *et al*., 2005; Smith *et al*., 2006). Since most cultivated bananas are sterile, unconventional breeding can play a considerable role in improvement (Pei *et al*., 2005). The biotechnological techniques used in unconventional breeding include *in vitro* mutagenesis, protoplast culture and genetic transformation (Crouch *et al*., 1998).

In vitro mutagenesis

In vitro mutagenesis can lead to variability in banana clones that are generated from a single mother plant. This process, called somaclonal variation, can be the result of nuclear chromosomal re-arrangement, gene amplification, non-reciprocal mitotic recombination, transposable element activation, point mutations and reactivation of silent genes (Jain, 2001). Somaclones in banana are induced as the number of multiplication cycles is increased (Sahijram *et al*., 2003). Once the number of multiplication frequency in Cavendish bananas by means of shoot tip culture exceeds 12 cycles, the number of somaclonal variants increase substantially (Ko *et al*., 1991).

The Taiwan Banana Research Institute started a program on mutation breeding for resistance to Fusarium wilt of Cavendish bananas in 1984 (Hwang and Ko, 2004). Over 30 000 *in vitro* plants were screened for resistance in *Foc*-infested greenhouse soils, of which only six plants remained healthy after a further 2 years' of field testing (Hwang and Ko, 1992). Despite their tolerance to the Fusarium wilt pathogen, the

resistant plants had inferior agronomic traits. One of the plants, GCTCV-215-1, showed enhanced tolerances against *Foc* and had good agronomic traits. This cultivar was registered as Tai Chiao No. 1 in 1992 (Hwang and Ko, 2004). Due to its height and difficulty to adapt across environments this clone was not widely accepted (Hwang, 2001). GCTCV-216, GCTCV-219 and GCTCV-217 had even better tolerance against *Foc*, but were also not accepted because of undesirable agronomic characteristics and uneven ripening (Hwang, 2001). A Cavendish banana plant (GCTCV-218) has since been selected from a plantation in Taiwan that is highly tolerant to Fusarium wilt and with a substantial increase in yield compared to the mother plant. This plant has been registered as Formosana (Hwang and Ko, 2004). In similar studies, de Beer *et al*. (2001) found 15 tolerant Cavendish banana plants with poor agronomic traits in South Africa, while three somaclones tolerant to *Foc* race 1 (IBP 5-61, IBP 5-B and IBP 12) were obtained from Gros Michel in Cuba (Bermúdez *et al*., 2002).

Mutations for plant improvement can further be induced by gamma irradiation and by *in vitro* chemical treatment (Bhagwat and Duncan, 1998; Smith *et al*., 2006). During gamma irradiation the mutation rate is based upon the time the cells are subjected to the gamma irradiation, as well as the dosage. The survival rate of the banana plants, however, decreases inversely (Bhagwat and Duncan, 1998). There are three different techniques to apply gamma irradiation to banana plants. The first technique is to irradiate the sucker before the meristem tip is isolated. In the second technique, *in vitro* banana plantlets from shoot tip culture are irradiated (Novak *et al*., 1990). Thirdly, embryogenic cell suspensions can be exposed to gamma irradiation. By using embryogenic cell suspensions, chimeric plants can be avoided (Xu *et al*., 2005).

Mutation breeding by gamma irradiation has led to the production of several valuable plants. A mutant of a Cavendish banana cultivar, Dwarf Parfitt (*M. acuminata*, AAA) showed enhanced tolerance against *Foc* race 4 after *in vitro* plants were treated with gamma irradiation of 20 Gy (Smith *et al*., 2006). This mutant further had agronomical traits similar to Williams (*M. acuminata*, Cavendish subgroup, AAA). In a study by Novak *et al*. (1990), multiple clones of Grande Naine (*M. acuminata*, Cavendish subgroup, AAA), Highgate, SH-3142 (*M. acuminata*, AA), SH-3436 (*M. acuminata*, AAAA), AVP-67 (*Musa* spp., AAB), Saba (*Musa* spp., ABB) and Pelipita

(*Musa* spp., ABB) were treated with gamma irradiation. In this study, a mutant (GN-60 Gy/A) was found among the Grande Naine clones that flowered 6 months earlier than the control Grande Naine plants. Novak *et al*. (1990) reported that the early flowering mutant had a unique esterase isozyme profile, and it was later registered as Novaria (Mak *et al*., 1996). Minor research has been done on irradiation of cell suspension. However, Kulkarni *et al*. (2004) reported that embryogenic cells suspensions of Grande Naine were subject up to 100 Gy gamma rays. No disease or pest resistance were reported.

A number of chemicals are able to induce mutations in banana plants, such as ethylmethanesulphonate, sodium azide and diethyl sulphate (Omar *et al*., 1989; Bhagwat and Duncan, 1998). Bhagwat and Duncan (1998) reported that a number of Highgate plants showed improved tolerance to *Foc* after treatement with mutagenic chemicals.

There are several disadvantages to mutation breeding against pests and diseases (Crouch *et al*., 1998). Since most induced variants are of no commercial use, a large number of plants need to be screened for improved properties, a process that is both time consuming and expensive (Crouch *et al*., 1998). Mutations in banana plants cannot be controlled (Sahijram *et al*., 2003), and can be lost in the second or third generation (Hwang and Tang, 2000). Finally, variations acquired by induced mutations can differ between different genotypes (Roux, 2004).

Protoplast culture

Protoplasts are cells from which the cell wall has been removed mechanically and/or enzymatically (with pectinase, hemicellulase and cellulase) (Haïcour *et al*., 2004). Protoplasts are totipotent, meaning that total plant regeneration can occur from a single cell (Davey *et al*., 2005). Under suitable conditions, the protoplasts will re-synthesize the removed cell wall and continue to divide. Such cells then form clusters of cells and develop into callus that can be used to generate complete *in vitro* plants.

Protoplasts can be manipulated by protoplast fusion or transformation (Ságí *et al*., 1994; Matsumoto *et al*., 2002). Protoplast fusion allows for the genetic gene pool to

be widened and, therefore, can overcome the hurdle of sterile cultivars in conventional breeding (Davey *et al.*, 2005). Fusion between two different protoplasts permits the transfer of useful characteristics, even if molecular knowledge of the genes is absent (Haïcour *et al.*, 2004). There is, however, a major disadvantage to protoplast culture. Protoplast cell culture is limited by the low frequency of plant regeneration from the protoplasts culture (Smith and Drew, 1990). Still, protoplasts can compliment other techniques to improve banana against pests and diseases.

Banana protoplasts for unconventional plant improvement can be obtained from different sources, but those obtained from the inflorescence give the best results (Bakry, 1984). It is now accepted that cell suspensions provide the best source for protoplast isolation (Assani *et al.*, 2001). Megia *et al.* (1992) found that the presence of a feeder layer is a prerequisite for banana protoplast to multiply, since the feeder layer is important during cell division (Haïcour *et al.*, 2004). Megia *et al.* (1992) also proved that *Lolium mutiflorum* Lam. was the best type of feeder cell to use. Fusion between the protoplasts of Maçã (*Musa* spp., AAB) and Lidi (*M. acuminata*, AA) was reported by Matsumoto *et al.* (2002). Hybridization was confirmed by random amplified polymorphic DNA (RAPD) analysis, and the ploidy of the hybrids determined with flow cytometric ploidy analyses. Electroporation of Bluggoe protoplasts was developed by Sági *et al.* (1994). The *UidA* gene was expressed in 1.8% of the total protoplasts (Sági *et al.*, 1994).

GENETIC MODIFICATION OF BANANA

Genetic modification can be used to increase nutritional value of foods, minimize abiotic and biotic stresses of plants, produce secondary metabolites, and to gain more knowledge in plant-pathogen interaction (Chakraborty *et al.*, 2000; Melchers and Stuiver, 2000; Balint-Kurti *et al.*, 2001; Wang *et al.*, 2004; Kumar *et al.*, 2005). Bananas were genetically modified by Sági *et al.* (1994). To enhance transformation efficiency and avoid the production of chimeric plants, the modification of bananas has shifted from the use of thinly sliced wounded rhizome tissue to the use of embryogenic cell suspensions as starting material. Embryogenic cell suspensions were first transformed by Sági *et al.* (1995) by particle bombardment.

Agrobacterium-mediated transformation of embryogenic cell suspension technique was patented by Engler *et al*. (2000) and described by Ganapathi *et al*. (2001).

Techniques used for genetic modification of banana

Bananas and plantains can be genetically modified in a number of ways. The techniques that promise to have the greatest relevance to banana include *Agrobacterium*-mediated transformation, particle bombardment and plastid transformation of banana cell suspensions.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens was first used for the introduction of foreign genes into plants when tobacco was genetically modified in 1983 (Fraley *et al*., 1983). Today, *Agrobacterium*-mediated transformation is used as a routine technique to modify different plant species in many laboratories around the world.

The transfer of genetic material from *A. tumefaciens* into a plant is a complex process and involves many stages (Fig. 2). The gene of interest can be inserted into Ti-plasmid by two ways. Firstly, the gene of interest is inserted into a plasmid and introduced into *Agrobacterium* cells by conjugation or transformation. The gene of interest with a resistance marker is then incorporated into the T-region by double recombination (Gelvin, 2003). Secondly, the gene of interest can be incorporated with the aid of ColE1 replicons (Gelvin, 2003). For the plant transformation process to commence, *A. tumefaciens* has to attach and colonise the plant surface (De la Riva *et al*., 1998) (Fig. 2). This is followed by the recognition of sugars released by the wounded plant cells, and results in the expression of genes located in the virulence (vir) region of *A. tumefaciens* (Zupan *et al.*, 2000; Gelvin, 2003). With the activation of the *vir* genes, the T-DNA border sequence is nick to form a single-stranded T-DNA (De la Riva *et al*., 1998) and is transported from the bacterium to the plant cell. The T-DNA is incorporated into the plant genome by recombination (Zupan *et al.*, 2000). The transformation efficiency of *A. tumefaciens* into banana has been greatly enhanced by the use of heat shock and co-centrifugation (Khanna *et al*., 2004).

Agrobacterium-mediated transformation is preferred to particle bombardment for introducing foreign genes into most plant crops because of a number of reasons (Walden and Wingender, 1995). Transformation efficiency was found to be better with *Agrobacterium*-mediated transformation than with particle bombardment (Walden and Wingender, 1995; Arinaitwe *et al*., 2004). *Agrobacterium tumefaciens*-mediated transformation further allows for foreign DNA to be integrated into a defined region in the plant genome. Transgene expression is also more stable, with fewer rearrangements (Walden and Wingender, 1995). A major shortcoming of *Agrobacterium*-mediated transformation is that *Agrobacterium* has a limited host range (Christou, 1996). Transgenic cultures can also be contaminated with *Agrobacterium* for up to a year after transformation (Christou, 1996), and require the necessary biosafety rules to prevent escape into the environment. Vector sequences outside the T-DNA borders can further be incorporated into the plant genomic DNA (Christou, 1996) and extensive testing should be performed to detect vector sequences.

Particle bombardment

Particle bombardment was developed by Wolf, Allen and Stanford in 1984 (Stanford, 1988). The DNA of the foreign gene is first coated onto microcarriers, and then directly transferred through the cells wall into the cytoplasm of intact cells by force (Gasser and Fraley, 1989) (Fig. 3). The force required for the microcarriers to enter the plant cells is achieved by speeding up a disk using high-pressure helium gas. After the device is set up and the selected pressure is obtained, the disk is fired into the stopping screen with a burst of helium gas (Kikkert, 1993). The DNA with microcarriers, which was dried on macrocarriers, is forced into the plants cells (Kikkert, 1993). Particle bombardment of embryogenic cell suspensions of Bluggoe, Williams and a plantain type, Three Hands Planty (*Musa* spp., AAB) was first performed by Sági *et al*. (1995). Grande Naine was later also transformed by means of particle bombardent (Becker *et al.*, 2000).

The main advantage of using particle bombardment for plant transformation is that a wide range of cells is receptive to the technique (Taylor and Fauquet, 2002). The technique is also easy and rapid to perform (Finer *et al*., 1992), and the DNA

integrated in multiple copies into host cells (Taylor and Fauquet, 2002). No callus or cell suspension cultures are required for particle bombardment (Christou, 1996). Host plant cells, however, can get damaged when using this technique. Unlike *A. tumefaciens*-mediated transformation, particle bombardment does not transfer the gene into a specific region of the host genome. The high cost of the equipment, required for particle bombardment can further restrict the process (Finer *et al*., 1992)

Plastid transformation

Plastid transformation involves the transfer of foreign genes into the plastome of plants by means of particle bombardment or polyethylene glycol (Van Bel *et al*., 2001). The transgene flanked by plastomic sequences is incorporated into the plastome by homologous recombination (Van Bel *et al*., 2001). There are several advantages to plastid transformation, like a high level of transgene expression and the lack of gene silencing (Daniell *et al*., 2005). At the moment, the main constraint of plastid transformation is that the technique has only been efficiently developed for tobacco plants (Daniell *et al*., 2005). To date, plastid transformation has not been reported in banana.

Other banana transformation protocols

Banana and plantain can also be genetically modified by means of protoplast culture, vacuum infiltration of *Agrobacterium* into scalps, or *Agrobacterium*-mediated transformation of thinly sliced rhizome tissue or shoot tips (Ságі *et al*., 1994; May et al., 1995; Acereto-Escoffié *et al.,* 2005; Tripathi *et al*., 2005). Ságі *et al*. (1994) transformed the protoplasts of Bluggoe bananas by means of electroporation. Technical difficulties with protoplast culture and regeneration of the protoplasts into *in vitro* plants resulted in this technique being under-utilized. Thinly-sliced wounded rhizome tissue of Grande Naine bananas has also been transformed with *A. tumefaciens* (May *et al*., 1995). Due to chimera development and low transformation efficiency, this technique is no longer preffered (Becker, 1999; Gomez-Lim and Litz, 2004). Two new transformation techniques were developed recently. Acereto-Escoffié *et al.* (2005) introduced a method based on vacuum-infiltration of

Agrobacterium into banana meristem tissue ('scalps'), whereas Tripathi *et al*. (2005) subjected wounded shoot tips to *Agrobacterium*-mediated transformation.

Promoters

An important aspect of genetic modification is whether the newly introduced genes have to be expressed constitutively or if it has to be induced in response to specific impulses. Transformation of plants with defense-related genes may lead to a defense response, such as the activation of the hypersensitive response and pathogen-related proteins. If these genes are constitutively expressed, it may eventually lead to reduction in growth due to a low "energy level" in the transgenic plant (Vandenbussche *et al*., 2004). By transforming a gene into a plant with an inducible promoter, the plant will waste less energy. The expression of transgenes in specific tissue, for instance in the root against root pathogens such as *Foc*, will not reduce energy waste by the plant, but will reduce the effect on the environment (Canevascini *et al*., 1996; Horvath *et al*., 2002).

Promoters with constitutive expression have been identified by means of T-DNA tagging using the T-DNA border-linked promoterless marker genes (Remy *et al*., 2004; Santos *et al*., 2004). Promoters have also been identified with the aid of transgenic studies of banana. The banana actin (*Act1*) promoter was expressed in banana leaf and root tissue, but at a lower rate than the maize *Ubi1* promoter (Hermann *et al*., 2001). It has also been shown that the BT6.1 promoter with an *Ubi1* intron, isolated from the banana bunchy top virus, increased the expression approximately seven to eight-fold (Dugdale *et al*., 2001). Yang *et al*. (2003) also reported that a promoter from the Taro bacilliform virus, T600, increased gene expression in banana by four times.

GENES USEFUL FOR TRANSFORMATION AGAINST FUNGAL DISEASES

Thousands of microorganisms are known to cause disease to plants, sometimes resulting in major epidemics around the world (Knogge, 1996). Protection of plants against pathogenic microorganisms is the key to sustainable crop production (Melchers and Stuiver, 2000). Genetically modified plants have been developed to

obtain broad-spectrum resistance against pathogens for the past 20 years. As there are a large number of genes that can be considered to protect plants against fungal diseases (Table 1), those with a potential relevance to banana improvement will be discussed below.

Phytoalexins

Phytoalexins are antimicrobial compounds of low molecular weight that play a key role in early protection of plants against fungal attack (Pedras and Ahiahonu, 2005). Several phytoalexins have been shown to inhibit plant pathogens (Hammerschmidt, 1999). Strategies to obtain resistance with phytoalexins can be achieved by the expression of a new phytoalexin, or the alteration of phytoalexin structure and expression (Dixon *et al*., 1995). Unfortunately, due to the complexity of phytoalexin pathways and the phytotoxicity, the chance that genetically modified plants will only contain a single phytoalexin for fungal resistance is limited (Dempsey *et al*., 1998).

Pathogenesis-related (PR) proteins

Pathogenesis-related (PR) proteins are induced in plants upon infection by a pathogen (Van Loon and Van Strien, 1999). These proteins are grouped into 14 different classes that include the chitinases, β-1,3-glucanases, peroxidases, osmotin and small cyteine-rich-proteins (Van Loon and Van Strien, 1999). PR proteins have been extensively studied in transgenic plants (Table 1). There are several reports of increase resistance of transgenic plants against fungal pathogens after transformation with PR proteins. In addition, there are various small cysteine-rich proteins that have antimicrobial activity namely defensins, lipid transfer proteins (LTP), thionins, hevein and 2S albumins. Different cysteine-rich proteins have been transformed into banana. Remy *et al*. (1998) transformed 'Three Hands Planty' bananas with the defensin gene, *Dm-AMP1,* and Williams has been transformed with a non-specific LTP (Sági, 2000). Field trials are currently conducted to determine their level of resistance.

Reactive oxygen species

The oxidative burst plays a vital role in the early phases of the defence response with radicals, including H_2O_2, O_2^- and OH^- (Lamb and Dixon, 1997). Transgenic potatoes containing a gene encoding for glucose oxidase, the enzyme that catalyses the production of reactive oxygen species, led to the constitutive expression of host defence responses such as the expression of PR proteins, salicylic acid, and lignin formation (Wu *et al.*, 1997). The transformed potatoes showed enhance resistance against pathogens. Constant high levels of H_2O_2, however, may lead to phytotoxicity (Murray *et al.*, 1999)

Ribosomal inactivating proteins

Ribosomal inactivating proteins (RIP) inhibit protein translations by modifying the 28S rRNA subunit (Cornelissen and Melchers, 1993). Expression of RIP in transgenic tobacco resulted in enhanced resistance against fungal plant pathogens but had a severe negative effect on the growth (Vandenbussche *et al.*, 2004). Plant growth abnormalities may be minimised by modification of the RIP gene (Tumer *et al.*, 1997) or by placing the RIP gene under a wound-inducible promoter (Corrado *et al.*, 2005).

Polygalacturonase-inhibiting proteins

Fungal pathogens produce endopolygalacturonase (PG) to break down the pectin in the plant cell wall (Lang and Dörnenburg, 2000). By transforming the plant with polygalacturonase-inhibiting proteins (PGIP), the PGs of fungal pathogens can be inhibited, thereby protecting the plant against the pathogen (Berger *et al.*, 2000). This technology, however, has not been widely adopted and might fail as pathogens have shown the capability to overcome PGIPs (Rose *et al.*, 2002).

Resistance genes

Plants containing resistance (R) genes that specifically recognize avirulence (Avr) proteins of a pathogen induce a range of defence responses (Hammond-Kosack and

Jones, 1997). R genes can be divided into five classes (Jones, 2000a), of which four have been transformed into plants, namely a serine-threonine kinase domain, a nucleotide binding site domain and/or a cytoplastic or extracellular leucine-rich repeat domain. Incorporation of R genes into plants leads to enhanced resistance in some cases (Jia *et al.*, 2000), but due to its specificity, cloning R genes across species may not lead to enhanced resistance (Honee, 1999). Also, pathogens may mutate and overcome the resistance provided by an introduced R gene.

Elicitors and/or induction of systemic acquired resistance

Plants recognise elicitors produced by the degradation of fungal cell wall and subsequently activate a range of plant defence responses (Nimchuk *et al.*, 2003). The expression of elicitors will not only lead to inhibition of fungal pathogens (Keller *et al.*, 1999), but bacteria and viruses can also be inhibited in multiplication.

Antibodies

By expressing an antibody, disease development or pathogen growth can be retarded (Dixon *et al.*, 1995; Peschen *et al.*, 2004). The antibody can be designed to bind to the cell surface and extracellular components of fungal pathogens (Dixon *et al.*, 1995). Expression of antibodies in a plant is a unique approach to generate resistance into transgenic plants.

Lysozyme

Lysozymes have an inhibitory effect on both fungi and bacteria by weakening the chitin and affecting the murein layer of their respective cell walls (Honee, 1999; Tripathi *et al.*, 2004). Banana rhizome tissue that was thinly sliced and wounded was transformed with the human lysozyme gene via *Agrobacterium*-mediated transformation (Pei *et al.*, 2005). Although the transformation efficiently was low, two transformed plants with high expression of the human lysozyme showed tolerance against *Foc* race 4 (Pei *et al.*, 2005). Extensive testing of these transgenic plants should be undertaken to determine the stability of the lysozyme gene under field conditions.

Non-plant antimicrobial peptides

Production of antimicrobial peptides in transgenic plants can lead to a reduction in their susceptibility to pathogens (Sági, 2000). Magainin was isolated from the African clawed frog and exhibit antimicrobial function against fungi, bacteria and protozoa (Zasloff, 1987). When the magainin analogue, MSI-99, was cloned into Rasthali (*Musa* spp., AAB) banana cell suspensions, the transformed plants showed enhanced resistance against *Foc* race 2 and moderate tolerance against *M. musicola* (Chakrabarti *et al*., 2003). Chakrabarti *et al*. (2003) further suggested that a stronger expression promoter might lead to complete resistance. Another non-plant antimicrobial peptide, cecropin, has been successfully introduced into apple and tobacco for enhanced resistance to *Erwinia amylovora* and *Fusarium solani* (Mart.) Sacc. respectively (Liu *et al*., 2001; Yevtushenko *et al*., 2005).

Inactivation or detoxification of fungal toxins

Certain plant pathogenic fungi produce phytotoxic compounds that play a role in disease development. The banana pathogen *M. fijiensis* produces 2,4,8-trihydroxytetralone, which has been shown to cause black necrosis when applied ectopically (Stierle *et al*., 1991). To counter the effect of the pathogen, a banana plant can be transformed to inactivate the toxin, or it can be cloned with toxin-insensitive targets (Dixon *et al*., 1995).

Combinatorial expression of genes

The combinatorial expression of several defence and/or resistance genes is important to protect plants against a range of diseases and pests (Melchers and Stuiver, 2000). The time that pathogens and pests take to develop resistance against single antimicrobial genes can be short. It is, therefore, of great importance that multi-action genes be introduced to achieve broad-spectrum disease resistance in transgenic plants. Genes with different mode of actions can be incorporated into the plant genome such as the transformation of rice with chitinase (enhance resistance against fungal pathogens), *Xa21* (enhance resistance to bacterial blight) and *Bt* fusion gene (enhance

resistance against insects) (Datta *et al*., 2002). Jach *et al*. (1995) reported a synergetic effect in the protection of tobacco against *Rhizoctonia solani* Kühn after transformation with several antifungal genes. More research, however, is needed into combinatorial expression of several genes with different mode of actions.

SELECTABLE AND SCREENABLE MARKERS IN TRANSGENIC PLANTS

The early detection of genetically modified cells is an important step in the transformation process, as the frequency of plant transformation events is often low (Miki and McHugh, 2004). Markers are, therefore, applied to confirm the occurrence and efficiency of the transformation process. They are also used to monitor gene expression and localization of the protein (Twyman *et al*., 2002).

Markers are grouped into selectable and screenable markers (Table 2). Selectable markers are further divided into positive and negative selection, relying on conditional or non-conditional selection (Miki and McHugh, 2004). Positive selection relies on the fact that the transformed plant gains the ability to survive while the untransformed cells dies, while negative selection is the opposite (Miki and McHugh, 2004). Conditional selection is substrate-dependent, whereas non-conditional selection systems are not dependent on the substrate (Miki and McHugh, 2004).

Conditional positive selection involves the use of genes with resistance to antibiotics, herbicides, and toxic and non-toxic metabolites to ensure growth of the transgenic plant on artificial media modified with such chemicals (Miki and McHugh, 2004). The most frequently used antibiotic marker in selection systems in transgenic plants is neomycin phosphotransferase II and hygromycin B in nuclear transformation selection, and spectinomycin in plastid transformation selection (Miki and McHugh, 2004). Phosphinothrin (active ingredient of Basta®) and glyphosate (active ingredient of Roundup®) are herbicides commonly used as selectable markers. Betaine aldehyde is a toxic substrate that is effectively used as a selectable marker in plastid transformation (Maliga, 2004). In contrast, non-toxic metabolites do not have a negative effect on the transformed cells, as in the case of toxic metabolites. β-glucuronidase, phoshomannose isomerase and xylose isomerase are non-toxic

metabolites that can be used as selectable markers (Penna *et al*., 2002). Non-conditional positive selection systems include isopentyl transferase genes, a histidine kinase homologue and hairy root-inducing genes.

Negative selection systems are used to study homologous recombination events in gene targeting, transposable elements and the processes of cell development and differentiation (Gallego *et al*., 1999). In the case of negative selection system, the transformed plant will inherit a gene that will be toxic and/or cause death to the transformed plant cell. However, after homologous recombination, the toxic gene will be lost and, therefore, the transgenic plant survives for example on cytosine deaminase (Thykjær *et al*., 1997)

Screenable markers are used to confirm the transformation process, improve transformation efficiency by selection before adding the selectable metabolite, act as transcriptional fusion markers for gene regulation, monitor gene flow monitored in field trails and to determine protein transport (Twyman *et al*., 2002; Miki and McHugh, 2004). Examples of screenable markers include β-glucuronidase (GUS), the green fluorescent protein (GFP) and luciferin (Twyman *et al*., 2002; Miki and McHugh, 2004) (Table 2).

BIOSAFETY AND ETHICS

Before genetically modified plants can be released, several biosafety issues have to be addressed (Sharma *et al*., 2002). One of the most important concerns is the containment of the transgene in the transgenic plant. Environmental groups are worried that "super" weeds can be generated when the transgene escape, in rare cases, to non-transformed plants. The effect of the transgene on non-target organisms also needs to be evaluated (Sharma *et al*., 2002). It is further argued that the introduction of foreign genes into agricultural crops might lead to allergies (Sharma *et al*., 2002). Most cultivated bananas are sterile and escape of foreign genes, therefore, is expected to be of no significance.

DISCUSSION

The intensive use of monoculture with little genetic diversity has enhanced the banana's susceptibility to increasingly aggressive pathogens. In 1960, the banana cultivar Gros Michel was decimated in Central America by *Foc* race 1 (Ploetz and Pegg, 2000), and today ***M. fijiensis*** is reducing yields around the world. No effective control strategy for many diseases and pests of banana is known (Ploetz and Pegg, 2000), and conventional breeding has a limited ability to improve banana in the short term. While resistant plants can be produced with ***in vitro*** mutagenesis, this method is hampered by a low success rate. Genetic modification, therefore, provides a strong opportunity to introduce stable resistance to diseases and pests into this important food crop relatively rapidly (Pearce, 2003).

Embryogenic cell suspensions play an important role in the transformation process. It leads to higher transformation efficiency as well as to avoid the formation of chimeras (Becker, 1999). Further research should involve the more rapid initiation of embryogenic cell suspensions with less somaclonal variation. Embryogenic cell suspensions also need to be extended to more traditional staple cultivars like the East African Highland bananas that are important to developing countries in Africa.

Bananas and plantains have been modified for disease and pest resistance in several laboratories around the world. While ***in vitro*** and greenhouse testing yielded positive results, extensive field-testing has not yet been performed. Grande Naine and Navolean (***Musa*** spp., AAB) bananas transformed with the antifungal proteins chitinase, β-1,3-glucanases and AP24 are currently being tested in Cuba (Pérez ***et al***., 2004), whereas transgenic Grande Naine plants modified with chitinase, β-1,3-glucanases and an antifungal gene from ***Capsicum anuum*** are tested in Mexico (Gómez-Lim ***et al***., 2004). The genes that were introduced into these bananas are from foreign sources, and one would hope that useful genes could soon be discovered in the great source of wild bananas available in Southeast Asia.

It is suggested that more studies involve the transformation of banana plants with genes with different modes of action to reduce resistance development. Although

only a single gene of possible resistance has been transformed into banana, further research in developing plants with multiple defense-related genes is of the utmost importance to ensure endurable resistance over the long term.

Bananas and plantains provide the ideal crop system for genetic modification. The cultivated bananas are mostly sterile and parthenocarpic, with little or no opportunity for the escape of genes in pollen. Wild banana varieties have also been identified that are immune or highly resistant to several pests and diseases, and that can serve as sources of resistance and defence genes. The introduction of genes from these wild varieties into cultivated bananas might prevent the development of allergies. It is also important that antibiotic- and herbicide-resistance markers be replaced with positive markers on non-toxic metabolites. Tissue-specific promoters have now been identified that could ensure that introduced genes are expressed only in plant parts susceptible to diseases and pests.

Genetically modified bananas would benefit both large commercial and small-scale farmers. Producers of export bananas spend million of dollars annually to control phytopathogenic fungi and nematodes with chemicals. These chemicals are not only expensive, but are harmful to the environment, and can lead to selection of resistance in pests and pathogens. Small-scale farmers are further unable to afford fungicides, nematicides and insecticides to control pests and diseases in the poorer countries of the world. Bananas that are disease and pest resistant, therefore, would provide them with a source of planting material that is affordable and safe to use.

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Table 1. Genes that can be transformed into plants for enhanced resistance to fungal diseases

Gene	**Inhibitory action**	**Examples successfully transformed in plant crops**
Phytoalexins	Inhibit fungal and bacterial growth and nematode pathogens	Stark-Lorenzen *et al.*, 1997; Leckband and Lörz, 1998; Seppänen *et al.*, 2004
β-1,3-glucanase (PR-protein)	Hydrolyses β-1,3-glucan in the fungal cell wall	Nishizawa *et al.*, 2003; Wrobel-Kwiatkowska *et al.*, 2004
Chitinase (PR-protein)	Hydrolyses chitin in the fungal cell wall	Nishizawa *et al.*, 1999; Tabaeizadeh *et al.*, 1999; Yamamoto *et al.*, 2000
Thaumatin-like (PR-protein)	Degrades fungal cell walls	Datta *et al.*, 1999
Peroxidase (PR-protein)	Polymerases the cell wall and production of lignin for plant protection	Sarowar *et al.*, 2005
Defensin (PR-protein)	Inhibits the growth of fungi as well as bacteria	Kanzaki *et al.*, 2002
Thionin (PR-protein)	Alters the membrane permeability, indirectly inhibiting macromolecule biosynthesis of fungal pathogens	Chan *et al.*, 2005
Lipid-transfer protein (PR-protein)	Unclear, but hypotheses is that the fungal cell wall is degraded	Molina and García-Olmedo, 1997
Glucose oxidase	Activates the HR and PR proteins, lignin production and production of lipid hydroperoxidase which is toxic	Wu *et al.*, 1997
Ribosomal inactivating proteins	Inhibits protein translations by modifying 28S rRNA	Tumer *et al.*, 1997; Vandenbussche *et al.*, 2004; Corrado *et al.*, 2005
Polygalacturonase-inhibiting proteins	Inhibits endopolygalacturonases of the fungal pathogen	Berger *et al.*, 2000

Gene	**Inhibitory action**	**Examples successfully transformed in plant crops**
Hevein-like proteins	Inhibit fungal growth and spore germination by binding to chitin oligomers	Kanrar *et al.*, 2002; Koo *et al.*, 2002
Resistance genes	Bind to avirulence proteins of a pathogen and induce a range of defence responses	Thomas *et al.*,1997
Harpin elicitor	Activates host defense responses	Takakura *et al.*, 2004
Antibodies	Bind to the cell surface or extracellular components of pathogens	Tavladorakii *et al.*, 1993
Lysozyme	Weakens chitinin the cell wall of fungi plus the murein layer of bacterial cell walls	Pei *et al.*, 2005
Magainin	Inhibits the growth of fungi by binding to the cell wall	Chakrabarti *et al.*, 2003
Cecropin	Inhibits the growth of fungi as well as bacteria by binding to the outer cell wall	Liu *et al.*, 2001; Yevtushenko *et al.*, 2005

Table 2. Selectable and screenable markers used in plant transformation (Miki and McHugh, 2004)

Marker	Gene	Mode of action	Genome	Reference
Selectable markers				
Antibiotic				
Neomycin Kanamycin Paramomycin G418	*Neo* *NptII* *NptI*	Inhibit protein synthesis in bacteria by binding to ribosomal subunits	Nuclear Plastid	Fraley *et al.*, 1992; Miki and McHugh, 2004
Hygromycin B	*Hph*	Hygromycin B inhibits protein synthesis in prokaryotic and eukaryotic cells	Nuclear	Han *et al.*, 2005
Spectinomycin	*AadA*	Spectomycin leads to bleaching and retarted growth	Nuclear Plastid	Shav and Maliga, 1993
Herbicides				
Phosphinothricin	*Bar* *Pat*	Phosphinothricin acetyltransferase inhibits phosphinothrin (bialaphos)(Basta®) which inhibits glutamine synthesis	Nuclear	De Block *et al.*, 1989
Glyphosate	*Epsps* *AroA*	5-Enolpyruvylshikimate-3-phosphate synthase plays an important role in biosynthesis of aromatic amino acids and is inhibited by glyphosate	Nuclear Plastid	Howe *et al.*, 2002
Toxic metabolites				
Betaine aldehyde	*Badh*	Betaine aldehyde is toxic to plant cells	Nuclear Plastid	Daniell *et al.*, 2001
Non-toxic metabolites				
Benzyladenine-N-3-glucuronidase	*GusA*	Hydrolyses benyladenine-glucuronide into cytokinin which simulate shoot regeneration	Nuclear	Penna *et al.*, 2002
Mannose	*ManA*	Mannose-6-phosphate is catalyses to fructose-6-phosphate, which can be utilized as a carbon source	Nuclear	Joersbo *et al.*, 1998

Marker	Gene	Mode of action	Genome	Reference
Xylose	*XylA*	D-Xylose is catalysed to D-xylulose, which can be used as carbon source in the pentose phosphate pathway	Nuclear	Haldrup *et al.*, 2001
Non-Conditional-positive selection				
Isopentyl transferase	*Ipt*	Catalyse the synthesis of isopentyl-adenosine-5'-monophosphate in cytokinin biosynthesis	Nuclear	Endo *et al.*, 2001
Negative selection systems				
Cytosine deaminase	*CodA*	5-Fluorocytosine is catalysed into toxic 5-fluorouracil	Nuclear Plastid	Park *et al.*, 2004
Screenable markers				
β-glucuronidase	*GusA*	Colorimetric histological assay: catalyses X-glu to produce blue colour Fluorometric *in vitro* assays: catalyses MUG to produce blue colour	Nuclear Plastid	Guivarc'h *et al.*, 1996 Jeoung *et al.*, 2002
Green fluorescent protein	*Gfp*	Absorbs blue light and fluoresces green light and is detectable using fluorescence microscopy	Nuclear Plastid	Vain *et al.*, 1998
Luciferin	*Luc*	With oxygen, ATP and magnesium, LUC protein produces light	Nuclear	Baruah *et al.*, 1999

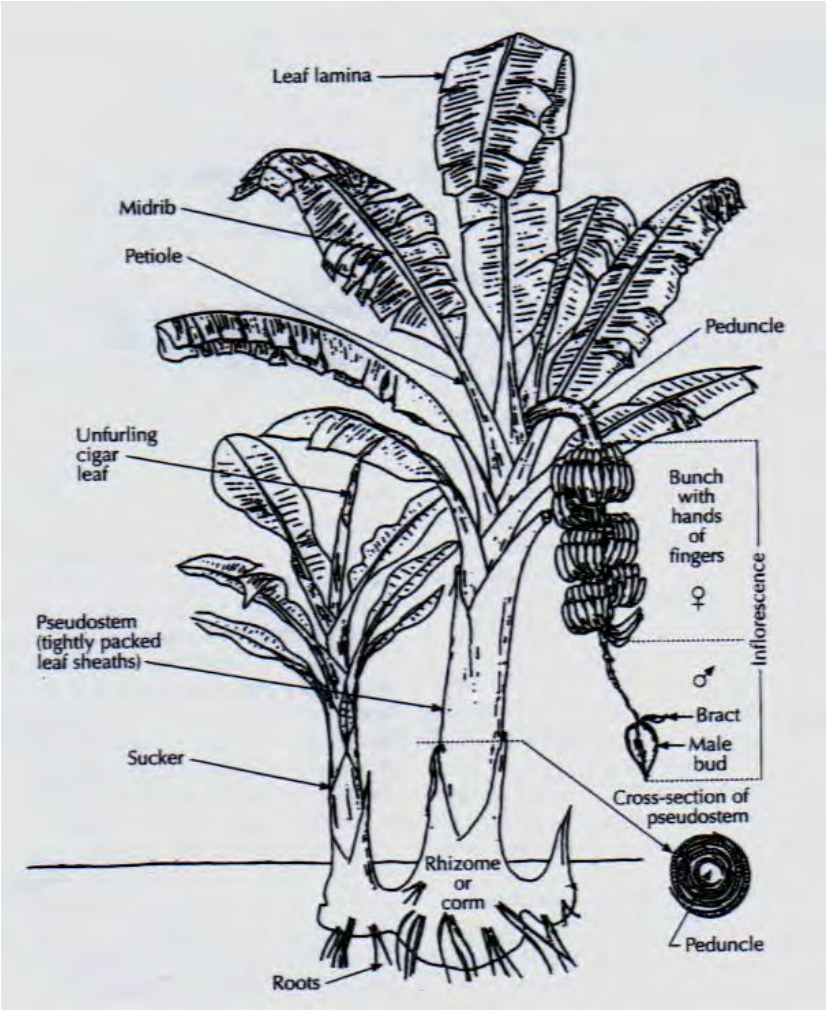

Figure 1. Diagrammatic representation of the morphology of a banana plant (Jones, 2000c).

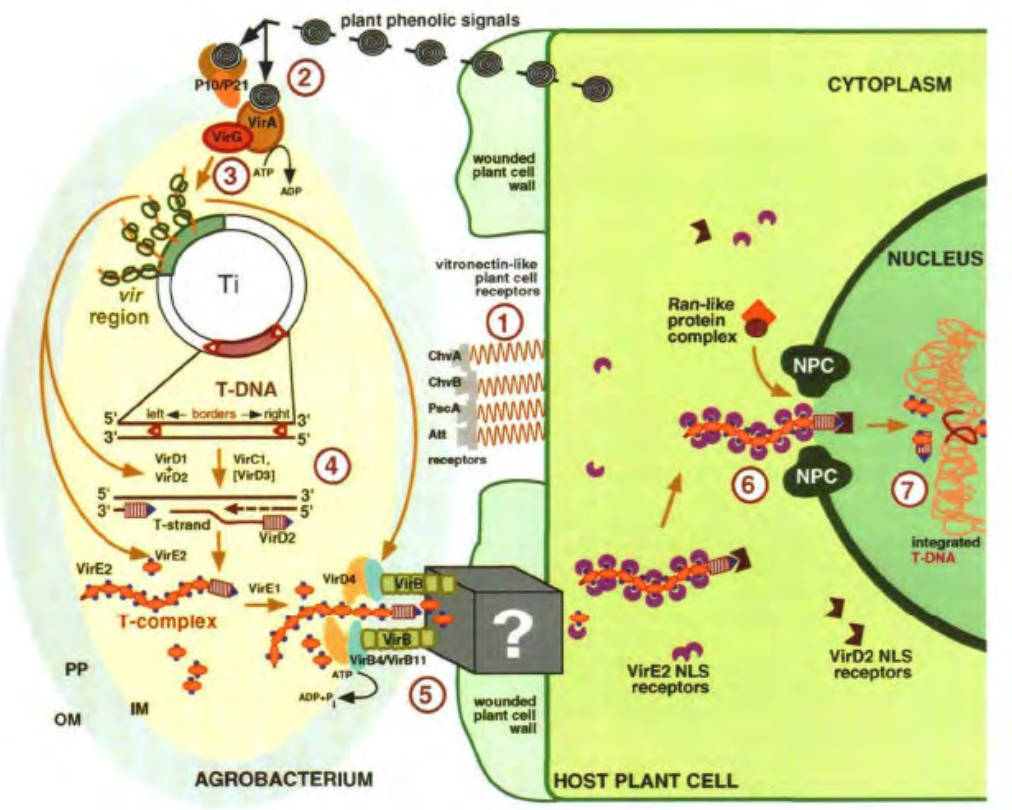

Figure 2. *Agrobacterium tumefaciens*-mediated transformation of plants cell (Sheng and Citovsky, 1996). Autophosphorylation of *VirA* results in the transfer of the phosphate to *VirG* (De la Riva *et al*., 1998). The activated *VirG* regulates the transcription of the *vir* genes (Gelvin, 2003). With the activation of the *vir* genes, *VirD1* and *VirD2* nick the T-DNA border sequence to form a single-stranded T-DNA (De la Riva *et al*., 1998). The region between the two T-DNAs is deleted and replaced by genes of interest for transformation. *VirD2* remains joined to the 5' end while *VirE2* binds the rest of the T-DNA to form a T-complex and, thereby, avoid degradation of the T-DNA with nucleases (Zupan *et al.*, 2000). *VirB* form a membrane-spanning protein channel with a T-pilus (Gelvin, 2003). *VirD2* and *VirE2* have nuclear location signal sequences for importation of the T-complex into the plant nucleus (De la Riva *et al*., 1998; Zupan *et al.*, 2000). Lastly, the T-DNA is incorporated into the plant genome by recombination. The T-DNA finds homology with a few base pairs of the plant genome, and *VirD2* join the 5' end with the 3' end of the plant DNA (Zupan *et al.*, 2000).

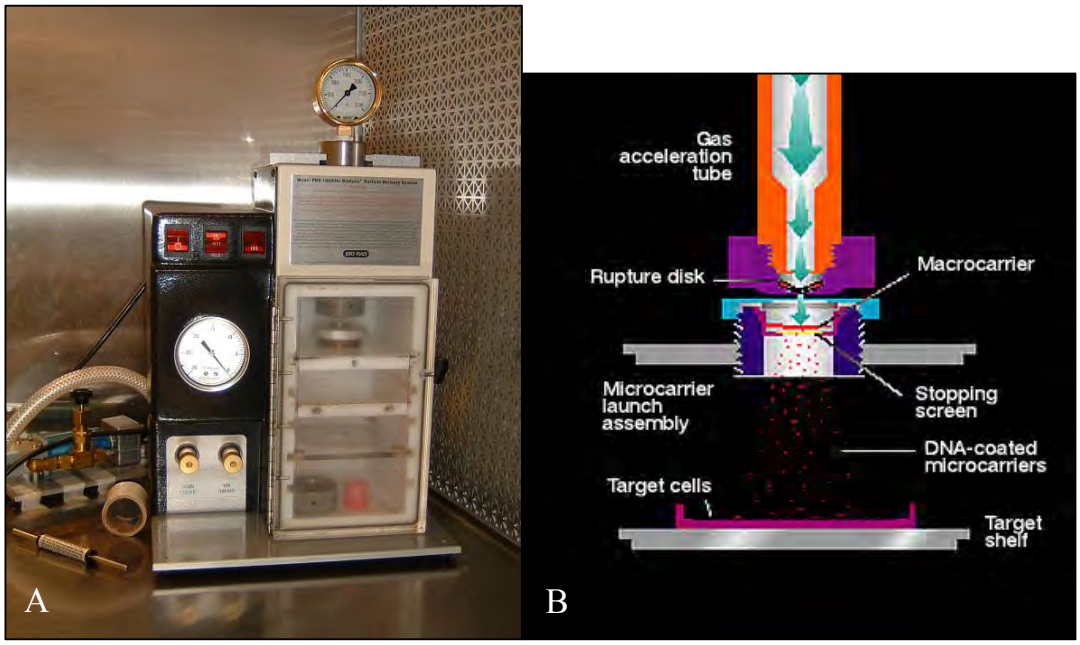

Figure 3. Transformation of cells through particle bombardment. A. Helium-driven PDS-1000/He Gun (Bio-rad, Hercules, California). B. Schematic diagram indication the action of the particle gun (Bio-rad, 2005). A rupture disk is inserted into the retaining cap whereas the macrocarrier and stopping screen is inserted into microcarrier launch assembly. The force required for the microcarriers to enter the plant cells is achieved by speeding up a disk using high-pressure helium gas. After the device is set up and the selected pressure is obtained, the disk is fired into the stopping screen with a burst of helium gas. The DNA with microcarriers, which was dried on marcocarriers, is then forced into the plants cells (Kikkert, 1993).

CHAPTER 2

Regeneration of Cavendish bananas (*Musa* spp.) by means of somatic embryogenesis

ABSTRACT

Bananas (*Musa* spp.) are an important food crop in many countries of the world. The international banana industry, however, is severely constrainted by many pests and diseases. One of the most powerful means to reduce the impact of pests and diseases is the use of somatic embryogenesis for unconventional plant improvement and the propagation of disease-free plants. Immature male flowers of Grande Naine (*M. acuminata*, Cavendish subgroup, AAA) were isolated and incubated on MA1 medium to form somatic embryos with ideal callus. Ideal callus was transferred to liquid MA2 medium, after which a heterogeneous cell suspension was formed. Non-embryogenic aggregates were removed to ensure that the cell suspension consisted of small embryogenic clusters only. Somatic embryos were obtained from the cell suspension after plating the embryogenic clusters on solid MA3 medium. The somatic embryos were transferred to MA4 medium for germination and transferred to P6 medium to develop into *in vitro* plantlets. Embryogenic cell suspensions can be used for genetic engineering of disease and pest resistant plants, *in vitro* mutagenesis, germplasm conservation and protoplast culture.

INTRODUCTION

Bananas (*Musa* spp.) are an important food crop in many tropical and sub-tropical countries of the world (Robinson, 1996). The crop serves as a staple food and source of income to millions of people in Africa, Central America and Asia (Stover and Simmonds, 1987). In South Africa, bananas are consumed as dessert bananas only, and all cultivars grown commercially belong to the Cavendish subgroup (*Musa acuminata* Colla, Cavendish subgroup, AAA). The local banana industry suffers losses because of diseases and pests, especially Fusarium wilt (caused by *Fusarium oxysporum* Schlecht f.sp. *cubense* (Smith) Snyd. and Hans) (Viljoen, 2002). There is no effective means for the conventional control of Fusarium wilt, and no resistant replacement cultivar acceptable to the local market. In other parts of the world, like Central America, black Sigatoka (caused by *Mycosphaerella fijiensis* Morelet) is a severe foliar disease in banana plantations (Carlier *et al*., 2000). Black Sigatoka is controlled by the use of fungicides that cost the banana industry in Central America approximately $49,000,000 per year (Carlier *et al*., 2000). Chemical control is further only economically feasible in highly intensive commercial production, and small-scale farmers cannot afford the fungicides (Carlier *et al*., 2000). An effective means to counter such losses to disease and pests is by means of genetic improvement of the crop (Sági, 2000).

Bananas can be improved by means of classical breeding and unconventional improvement. Since Cavendish bananas are sterile and difficult to use in conventional breeding, plant improvement for enhanced disease and pest resistance can be achieved by unconventional improvement techniques such as *in vitro* mutagenesis, protoplast culture and through genetic engineering (Crouch *et al*., 1998). Both protoplast culture (Assani *et al*., 2001) and genetic engineering (Becker *et al*., 2000; Ganapathi *et al*., 2001) require embryogenic cell suspensions as starting material. Cell suspensions are obtained from the differentiation of somatic cells that turn back to the embryogenic stage. Embryogenic cell suspensions through somatic embryogenesis will allow the development of the embryos (Fehér *et al*., 2003). Somatic embyrogenesis is a powerful tool not only for plant improvement, but also for the mass propagation of disease-free plants. Cell suspensions can be mass produced in bioreactors for the formation of somatic embryos (Kosky *et al*., 2002) and encapsulated in alginate or

agar beads (Panis and Swennen, 1993). In addition, the embryogenic cell suspensions can further be cryopreservated for germplasm conservation (Panis and Thinh, 2001).

Somaclonal variants, better known as off-types, has a major effect on *in vitro* propagation of banana (Sahijram *et al*., 2003). The frequency of off-types forming in the Cavendish group by shoot tip culture varied between 1-63% (Johns, 1994). However, Shchukin *et al*. (1997) reported that the incidence of off-types in somatic embryogenesis compared to shoot tip culture is lower. In comparison, a study by Cote *et al*. (2000) revealed that there were no significant differences in the incidence of off-types in shoot tip culture, compared to plants derived from cell suspensions.

A few techniques need to be performed for the production of a genetically modified plant with a specific trait (Escalant *et al*., 2002). The gene of interest needs to be isolated, the gene of interest regulatory elements need to be determined and inserted into a transformation vector with a selectable/screenable marker, the vector needs to be transformed into plants cells by particle bombardment or *Agrobacterium*-mediated transformation, the transformed cells need to be regenerated in the whole plants, the expression level of the gene of interest needs to be determined and, finally, the transformed plant needs to undergo field testing. The bottleneck in the establishment of a banana transformation system is the production of embryogenic cell suspensions and the regeneration of plants (Escalant *et al*., 2002; Tripathi *et al*., 2005).

For the development of a successful transformation system, a continuous supply of embryogenic cell suspensions is needed. Embryogenic suspensions can be generated from different explants, such as immature zygotic embryos (Escalant and Teisson, 1989; Navarro *et al*., 1997), basal leaf sheaths of rhizome tissue (Lee *et al*., 1997), proliferating buds ('scalps') (Dhed'A *et al*., 1991), immature male flowers (Escalant *et al*., 1994; Grapin *et al*., 1996) and female flowers (Grapin *et al*., 2000). The use of zygotic embryos as starting material is confined mainly to wild type bananas. Since Cavendish bananas are sterile, it is not possible to generate cell suspensions from seed or zygotic embryos. Transformation of thinly sliced meristem tissue often results in chimerism with low transformation efficiency (Becker, 1999, Gomez-Lim and Litz, 2004). In both scalp and flower methodologies, somaclonal variation has been reported (Schoofs *et al*., 1999; Cote *et al*., 2000). However, the use of male flowers

as starting explants has been reported to produce less somaclonal variants compared to the scalp technology, but it requires a very strict selection of plants in the field. The development of field plots with selected plants is one of the keys to the success of male flower technology.

Somatic embryogenesis of banana (*Musa* spp.) has been achieved in several laboratories around the world. Still, the technique cannot be considered a routine procedure (Schoofs *et al.*, 1999). Since this technique has several applications, the objective of this study was to establish the technology of somatic embryogenesis in Cavendish bananas in South Africa.

MATERIALS AND METHODS

Plant material used

Approximately 550 male buds (Fig. 1) were collected from Cavendish banana plants in two different districts of South Africa between May 2003 and September 2005. Buds from Grande Naine (*M. acuminata*, Cavendish subgroup, AAA) were collected in Kiepersol, Mpumulanga Province and in Letsitele, Limpopo Province. The buds were transported to the research facilities of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa.

Initiation of callus

Callus was initiated from immature male flowers (primordia) according to the method described by Stross *et al.* (2003). The bracts of the male buds were first removed by hand until the apex reached a length of approximately 30 mm. The apexes of the male buds were surface-sterilized with 70% ethanol for 1 min. The male flowers of the male buds were then carefully isolated using a scalpel and forceps under sterile conditions in a laminar flow. As the male flowers are very small and barely visible with the naked eye, a stereo microscope was utilized (Fig. 2A&B). The immature male flowers were dissected from position one to 15 and transferred to a Petri dish with sterile water droplets to avoid desiccation (Fig. 2C). Position one represents the

male flower closest to the meristem, and position 15 the male flower furthest away from the meristem.

The immature male flowers isolated from the apex of each male bud were sorted according to size and incubated in a figure six on MA1 medium in a 90-mm Petri dish (Fig. 3). MA1 medium consists of MS (Murashige and Skoog, 1962) basal medium with vitamins (Table 1) (Highveld Biological, Johannesburg, South Africa), 30 g/L sucrose (Merck, Darmstadt, Germany), 1 mg/L biotin (Sigma, Stanheim, Germany), 1 mg/L indole-3-acetic acid (Sigma), 4 mg/L 2,4-dichlorophenoxyacetic acid (Sigma), 1 mg/L naphthleneacetic acid (Sigma), 0.1 g/L malt extract (Merck), 0.1 g/L glutamine (Sigma) and 7 g/L agarose (Type II, Sigma). The pH of the medium was adjusted to 5.8±0.1 with NaOH and HCl before being autoclaved for 20 minutes at 121^{o}C. Once the male flowers were transferred onto the MA1 medium, the Petri dishes were sealed with Parafilm® and incubated in dark conditions at 25±2^{o}C for 12 months.

Production of embryogenic cell suspensions

The male flowers were examined for callus production on a regular basis, and were not sub-cultured for the duration of the experiment. Once ideal callus was produced, it was used to initiate a cell suspension according to the method described by Stross ***et al.*** (2003). The callus was transferred to 3 ml liquid MA2 medium in a 6-well plate (Adcock Ingram Scientific, Bryanston, South Africa), and placed on a rotary shaker with continuous shaking at 100 rpm in dark conditions at 25±2^{o}C. MA2 medium consisted of MS basal medium with vitamins, 45 g/L sucrose, 1 mg/L biotin, 1 mg/L 2,4-dichlorophenoxyacetic acid, 0.1 g/L malt extract, 0.1 g/L glutamine. The pH of the medium was adjusted to 5.3±0.1 with NaOH and HCl before autoclaving the medium. The medium was refreshed every 7 to 10 days. The meristem globules, cotyledonary embryos and necrotic tissue were removed from the cell suspensions.

Two to 3 months after the cell suspensions were initiated in 6-well plates, the bottom of the 6-well plate contained a layer of the banana cells. The cells were transferred to 25-ml Erlenmeyer flasks containing 5 ml of MA2 medium. The medium was refreshed every 10-14 days in the Erlenmeyer flasks by tilting the flasks and leaving

them to stand until the cells settled to the bottom of the flasks. After the cells have settled, most of the old medium was removed with an automated pipette. The settled cells were then re-suspended in fresh MA2 medium with a concentration of 3% settled cells.

Development of embryos

To verify whether the cell suspensions were indeed embryogenic, the settled cells were diluted to 3% with MA2 medium. A sample of 1 ml was then plated onto Whatman filter paper (Merck) in a 90-mm Petri dish with 25 ml MA3 medium. MA3 medium consists of SH (Schenk and Hildebrandt, 1972) basal medium, 45 g/L sucrose, 1 mg/L biotin, 0.2 mg/L naphthleneacetic acid, 0.05 mg/L zeatin (Sigma), 0.2 mg/L 2-isopenteny adenine (2iP) (Sigma), 0.1 mg/L kinetin (Sigma), 10.4 g/L lactose, 0.1 g/L malt extract, 0.23 g/L proline (Sigma), 0.1 g/L glutamine, and 3 g/L phytagel (Sigma). The pH was adjusted to 5.8±0.1 with NaOH and HCl before the medium was autoclaved. The inoculated plates were incubated in dark conditions for 1 to 3 months.

Germination of embryos

The embryos that were formed on MA3 medium were transferred to MA4 medium to germinate. MA4 contained MS basal medium with vitamins (Murashige and Shoog, 1962), 30 g/L sucrose, 2 mg/L indole-3-acetic acid, 0.5 mg/L benzylaminopurine (Sigma) and 4 g/L phytagel. The pH was adjusted to 5.8±0.1 with NaOH and HCl before the medium was autoclaved. The MA4 plates with embryos were incubated in dark conditions at 25±2^{o}C.

Development of *in vitro* plants

Germinating embryos were transferred from the MA4 plates to P6 medium and incubated at 27^{o}C with a 12-hr photoperiod to develop them into *in vitro* plantlets. P6 medium consists of MS basal medium with vitamins (Murashige and Shoog, 1962), 30 g/L sucrose, 0.175 mg/L indole-3-acetic acid, 0.227 mg/L benzylaminopurine, 10

mg/L ascorbic acid (Saarchem, Unilab, Midrand, South Africa) and solidify with 3 g/L gelrite (Sigma). Before the medium was autoclaved, the pH was adjusted to 5.8±0.1 with NaOH and HCl.

RESULTS

Initiation of callus

Three days after the male flowers were isolated from the male buds and incubated on MA1 medium, the cut side of the male flowers turned from a white to a brown-black colour due to phenolic oxidation. In addition, the male flowers swelled and turned translucent (Fig. 4A&B). The outer points of the male flowers curled up and formed a circle (Fig. 4C&D). However, approximately 50% of the male flowers in culture turned black in colour and died.

Non-embryogenic callus developed on the tip of the male flowers 30 days after incubation (Fig. 5). After 8 weeks of incubation, embryos were formed from the yellow globules (Fig. 6A & B). Callus was produced underneath the embryos 2 weeks later (Fig. 6C & D). The callus later developed into ideal callus that had individual embryos of different sizes at various stages of development with translucent small friable callus (Fig. 7). The ideal callus was obtained between position five to 10 of the male flowers. Ideal callus was observed in less than 1% of the cultures. In addition, numerous clusters of compact callus formed that varied in colour from white to yellow (Fig. 8).

Production of embryogenic cell suspensions

When ideal callus (Fig. 7) was transferred to 6-well plates it dispersed into small clumps of cells, giving the impression that it was dissolved in the medium. However, when the compact callus (Fig. 8) was transferred to 6-well plates with liquid MA2 medium (Fig. 9), no growth occurred. Within 4 days these cells became necrotic and released phenolic compounds.

Two types of cell suspensions were formed. The first type (type-A) had a white to light grey colour. Microscopic observations with a light microscope revealed that the cell suspension consisted of single cells that were highly vacuolated (Fig. 10). The second type of cell suspension (type-B) had a light yellow colour. Heterogeneous suspensions were observed under a light microscope with embryogenic clumps (Fig. 11). The embryogenic clumps contained a large nucleus with small vacuoles. Starch reserves were limited. Several flasks with embryogenic cell suspensions were obtained with a total of approximately 200 ml settle cell volume (SCV).

Development of embryos

Two weeks after the type-A cell suspension was plated onto MA3 medium the cells turned black and died (Fig. 12). No embryos formed from type-A cell suspensions. When the type-B cell suspension was plated on MA3 medium, however, numerous spherical pro-embryos were formed after 14 days (Fig. 13). The number of embryos obtained varied from $0\text{-}10^5$ for 1 ml of SCV plated. Furthermore, there were cell suspensions that consisted of a combination of type-A and type-B cell suspensions that produced necrotic cells and embryos, on MA3 medium.

Germination of embryos

Three weeks after the embryos from the embryogenic cell suspension were plated onto MA4 medium the plumule developed. Shortly after the plumule had started to form, roots developed (Fig. 14). As the time of incubation increased, the percentage of embryo germination also increased. A germination rate of up to 50% was obtained.

Development of *in vitro* plants

The germinated embryos developed into *in vitro* plantlets on P6 medium (Fig. 15). After 8 weeks, the *in vitro* plants were planted out into potting soil in 1-L plastic bags and placed under a mist sprayer for hardening-off.

DISCUSSION

In this study, somatic embryogenesis with the immature male flower of Grande Naine bananas was accomplished for the first time in South Africa. This accomplishment could contribute significantly to the development of the local banana industry. Somatic embryos could be used for genetic manipulation of Cavendish bananas for disease and pest resistance, as well as improved varieties against stresses such as cold and drought. Additionally, mass production of genetically stable banana plantlets could be achieved by means of somatic embryogenesis. Furthermore, embryogenic cell suspensions can be cryopreserved for germplasm conservation.

The composition of a banana cell suspension plays an important role in the regeneration capacity of the cell suspensions. In a study by Domergue *et al*. (2000), five different types of cell suspensions were observed under the light microscope. The type-A cell suspensions found in this study was similar to the Type I cell suspension found by Domergue *et al*. (2000). Characteristic of this type of cell suspensions is that the cells are highly vacuolated and isolated. In addition, the cell suspensions have a very low regeneration capacity, similar to what was observed in this study. The other type of cell suspension obtained in this study, Type-B, grouped with the Type III cell suspension described by Domergue *et al*. (2000). In this study, the embryogenic capacity of a Type-B was very high, as was the case with the Type III cell suspensions of Domergue *et al*. (2000).

While somatic embryogenesis has been reported for banana cultivars belonging to different genotypic groups, the frequency of the embryogenic response remains low (Daniels *et al*., 2002). Navarro *et al*. (1997) reported an embryogenic response of 2-6% for Grande Naine while Becker (1999) obtained a response of 2.2%. In this study, the frequency of the embryogenic response was below 1%. Several immature male flowers turned black and died after a few days of incubation. The reason for the low embryogenic response in this study might be contributed to the extended time between harvesting of the male buds and isolation of the male flowers. The male buds for the experiment were obtained from fields in Tzaneen and Kiepersol and transported by motor vehicle for more than 400 km to FABI in Pretoria. The isolation of male flowers at FABI took a further 2 days.

Seasonal climatic changes could also have an effect on the embryogenic response of the male buds (Escalant *et al.*, 1994). The male flowers were harvested during the summer months. Becker (1999) reported that the embryogenic response in Cavendish cultivars was higher during winter compared to summer. The reason why the embryogenic response is affected by climatic changes is still unclear. The physiological status of the plant, as well as the stage of the inflorescence's development, plays an important role in the embryogenic response (Escalant, personal communication). Healthy banana plants with good agronomical characteristics, therefore, need to be identified when male buds are harvested.

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Table 1. Components of MS (Murashige and Skoog, 1962) medium

Components		**g/L**
Inorganic salts		
Ammonium nitrate	NH_4NO_3	1.65
Potassium nitrate	KNO_3	1.9
Calcium chloride	$CaCl_2.2H_2O$	0.44
Magnesium sulphate	$MgSO_4.7H_2O$	0.37
Potassium dihydrogen orthophosphate	KH_2PO_4	0.17
Sodium dihydrogen orthophosphate	NaH_2PO_4	0.38
FeNaEDTA		0.037
Minor Elements		
Boric acid	H_3BO_3	0.0062
Manganous sulphate	$MnSO_4.H_2O$	0.0223
Zinc sulphate	$ZnSO_4.7H2O$	0.0086
Potassium iodide	KI	0.00083
Sodium molybdate	$Na_2MoO_4.H_2O$	0.00025
Cupric sulphate	$CuSO_4.5H_2O$	0.000025
Cobaltous chloride	$CoCl_2.6H_2O$	0.000025
Vitamins		
Pyridoxin.HCl		0.00050
Nicotinic acid		0.00050
Glycine		0.002

Figure 1. Banana male buds used as starting material for the initiation of callus.

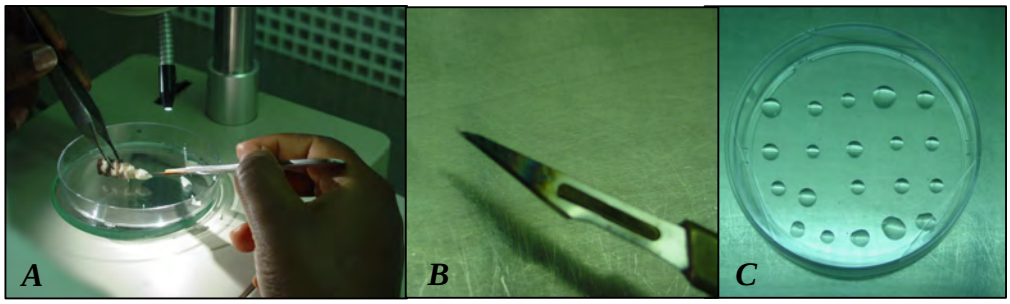

Figure 2. Isolation of the male flowers from the male buds **A**. Isolating the male flowers under a stereo microscope with a scalpel and forceps. **B**. A male flower on the tip of the scalpel blade. The male flower is very small and can hardly been seen by the naked eye. **C**. Droplets of sterile water in order that the male flowers do not desiccate while isolating the other male flowers.

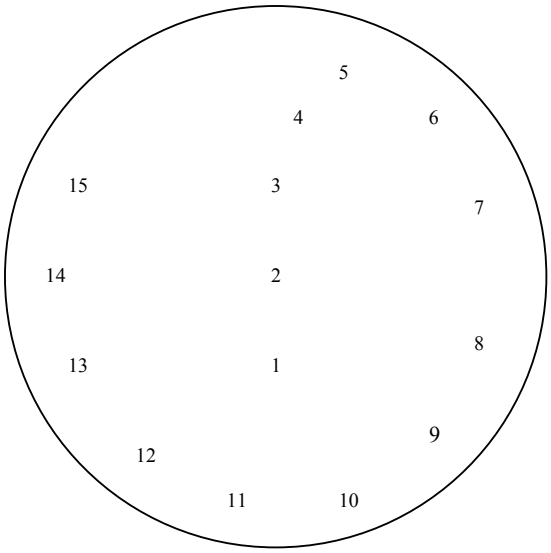

Figure 3. Diagrammatic representation of the placement of the male flowers of banana on MA1 medium.

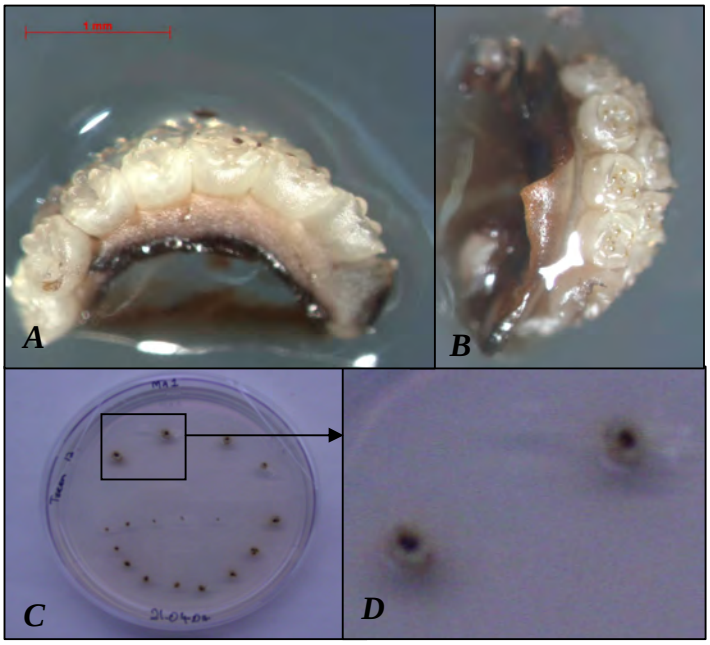

Figure 4. Male flowers of banana after a few weeks in culture. **A&B**. The male flowers swelled and turned translucent in colour. The outer tissue surrounding the male flowers turned black due to oxidization. **C**. Fifteen male flowers in a Petri dish on MA1 medium. **D**. The male flowers curled up and formed a circle.

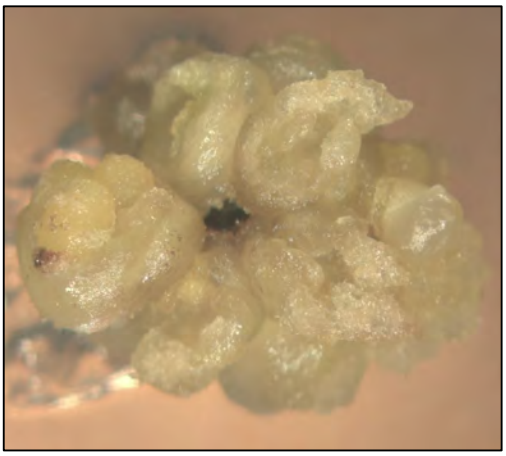

Figure 5. Non-embryogenic callus formed on the tip of the banana male flowers on MA1 medium.

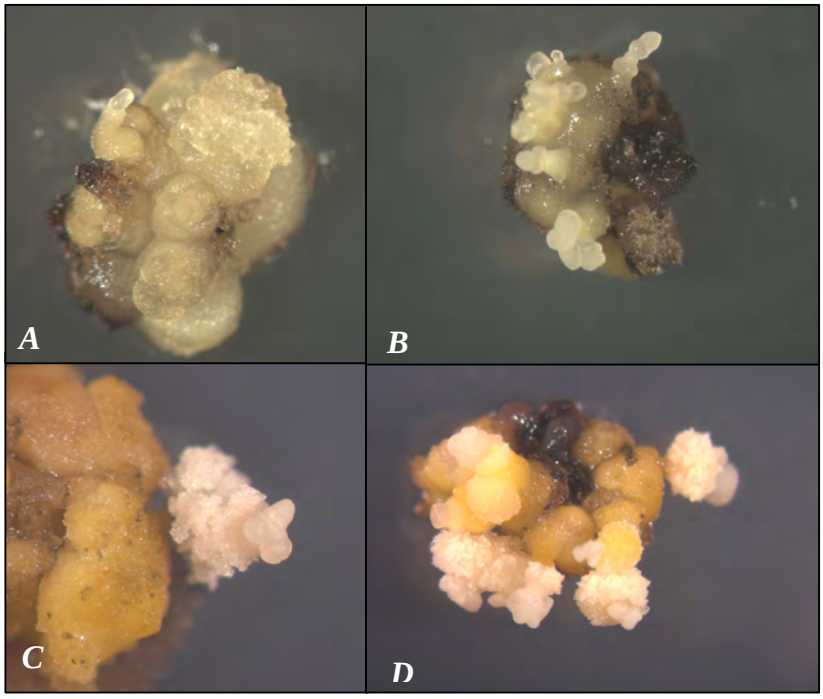

Figure 6 A & B. Embryos were formed from the yellow globules from the banana male flowers. **C&D**. Callus were formed between the embryos and the yellow globule.

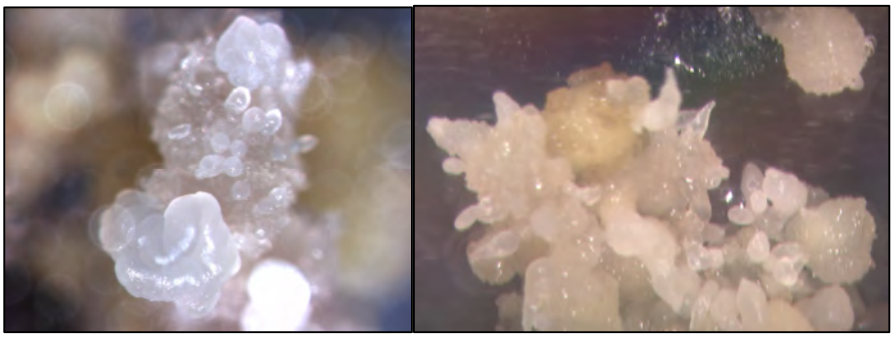

Figure 7. Ideal callus was formed from the immature banana male flowers. The ideal callus consists of friable tissue with small translucent cells. On the surface of the callus, somatic embryos at different developing stages are found. The somatic embryos varied in shape as well as in the size, but had a smooth surface.

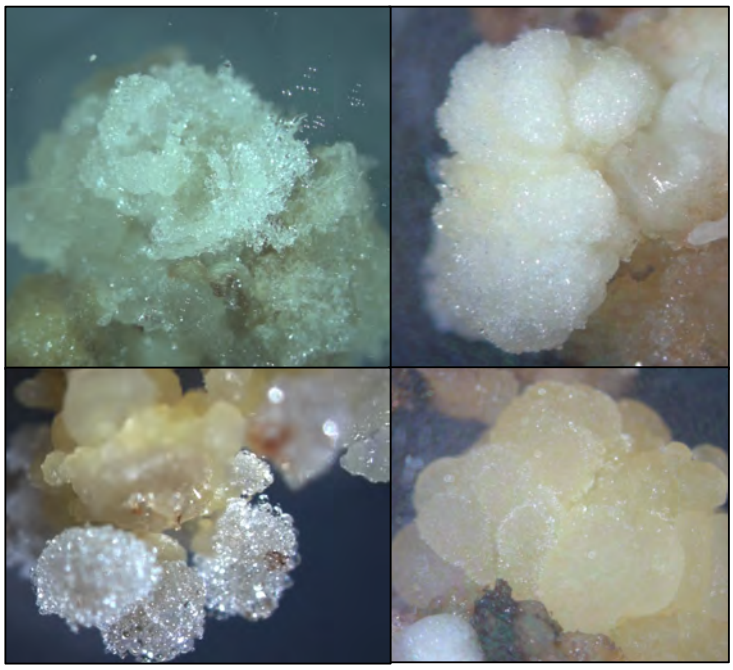

Figure 8. Compact callus formed from the banana male flowers that varied from bright white to light yellow, and with a globular shape. The compact callus was hard and solid with no friable tissue.

Figure 9. Six-well plates were used for the initiation of banana cell suspensions.

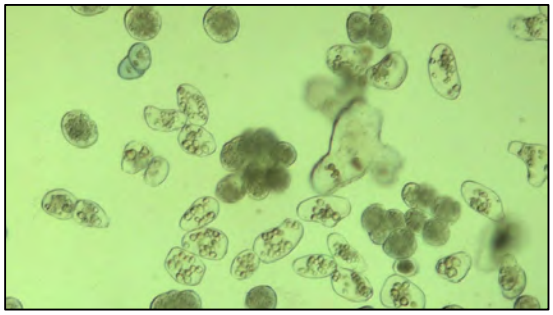

Figure 10. Type-A cell suspensions viewed under a light microscope showed highly vacuolated isolated single cells.

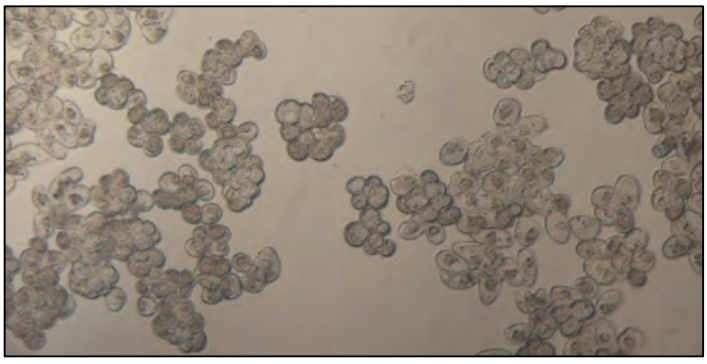

Figure 11. Type-B cell suspensions consisted of cell clumps when viewed under a light microscope.

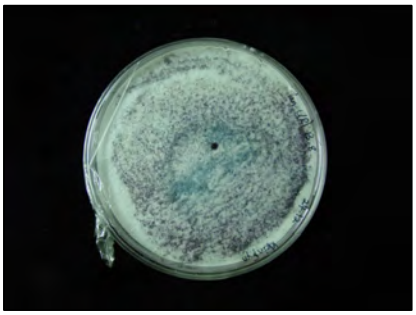

Figure 12. Type-A banana cell suspension on MA3 medium turned black and became necrotic 2 weeks after plating.

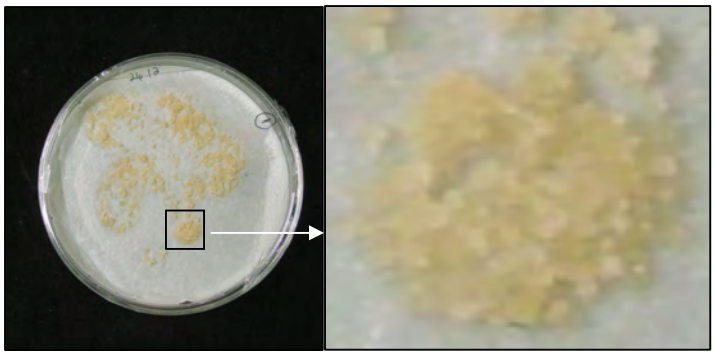

Figure 13. Embryos formed from type-B banana cell suspension 2 weeks after plating on MA3 medium.

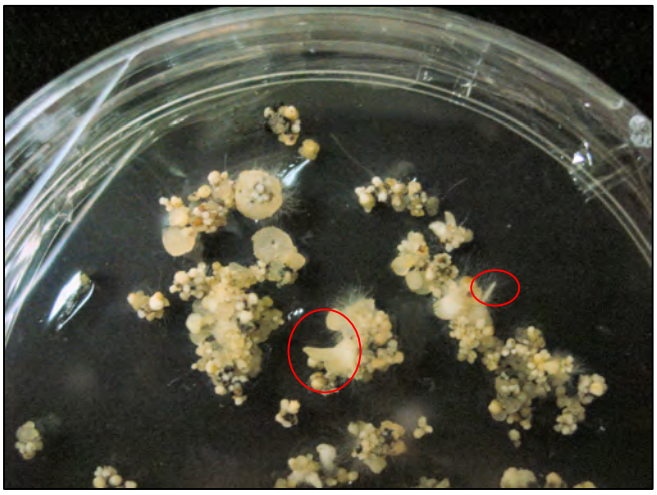

Figure 14. Germination of embryos from an embryogenic banana cell suspension. A shoot was formed in the circle on the left whereas a root formed in the circle on the right.

Figure 15. Development of *in vitro* banana plants obtained from an embryogenic cell suspension.

Chapter 3

Agrobacterium-mediated transformation of Cavendish bananas (*Musa* spp.) in South Africa

ABSTRACT

Genetic modification provides an opportunity to protect bananas and plantains (*Musa* spp.) against pathogens and pests that cannot be managed by means of conventional control strategies. Since banana production in South Africa is severely hampered by Fusarium wilt and the banana weevil, a *Agrobacterium*-mediated transformation system was established for the improvement of Cavendish banana cultivars, the only bananas produced for the fresh fruit market. Embryogenic cell suspensions from the cultivar Grande Naine were co-cultivated with *Agrobacterium* strains harbouring the plasmids pCambia1305.1, pART-TEST7 and pKYΩOCI. Antibiotic-resistant embryos derived from transgenic cell suspensions developed into banana plantlets after 12 weeks, cultivation on MA4 medium. In total, 145 putative transgenic plants were produced. Molecular analysis revealed that the *Gus* gene was integrated into the genome of transformed plants, and a histochemical GUS assay showed that the *Gus* gene was expressed in putative transgenic plants treated with X-gluc. In future, Southern blot assays will be performed to determine copy numbers of the transgenes, and the putative transgenic plants containing the *OcI* gene tested in the greenhouse and the field for resistance to the banana weevil and the burrowing nematode. The successful transformation of Grande Naine reported in this work will contribute significantly towards improving Cavendish bananas in South Africa, and offers the opportunity to modify banana and plantain varieties cultivated in Africa for enhanced disease and pest resistance.

INTRODUCTION

Bananas and plantains (*Musa* spp.) are considered one of the most important fruit crops in the world (Robinson, 1996), with a total production of 72 million tonnes in 2005 (FAO, 2006). Of these, 64% can be classified as sweet dessert bananas, and the rest as cooking bananas (Jones, 2000). Sweet dessert bananas, derived primarily from *M. acuminata* Colla, are consumed as a raw fruit or it can be cooked (Jones, 2000), while the rest of the bananas, cooking bananas, are mostly cooked and fried. Cooking bananas are eaten domestically as a staple food and serve as sources of income to millions of people in central and western Africa, South America and Southeast Asia (Robinson, 1996).

Sustainable production of bananas is often restricted by a number of important diseases and pests (Robinson, 1996). Diseases, such as Fusarium wilt (*Fusarium oxysporum* f.sp. *cubense* (Smith) Snyd. and Hans) and bacterial wilt (*Xanthomonas campestris* pv. *musacearum*) cannot be managed by conventional control methods, while subsistence farmers cannot afford the fungicides and insecticides needed for the chemical control of black Sigatoka (*Mycosphaerella fijiensis* Morelet) and insects such as the banana weevil (*Cosmopolitus sordidus* Germar). No commercially acceptable cultivar has been identified with resistance to either of these diseases, pests, and the burrowing nematode *Radopholus similis* Cobb (Tripathi *et al.*, 2004). In addition, Cavendish and East African Highland bananas (EAHB) are sterile and parthenocarpic, and are difficult to improve by means of conventional breeding. Conventional breeding programs are further hampered by long growth cycles of breeding material, low seed set and the unacceptability of hybrids by local markets (Crouch *et al*., 1998; Sági, 2000). Genetic transformation, therefore, offers an excellent opportunity for the introduction of disease and pest resistance into banana.

Different methods have been described for the genetic modification of *Musa* spp. These include electroporation of protoplasts (Sági *et al*., 1994), *Agrobacterium*-mediated transformation of wounded shoot tips and rhizome tissue (May *et al*., 1995; Tripathi *et al*., 2005), vacuum infiltration of banana meristem tissue ('scalps') with *Agrobacterium* (Acereto-Escoffié *et al.,* 2005), particle bombardment (Sági *et al*., 1995) and *Agrobacterium*-mediated transformation of embryogenic banana cell

suspensions (Engler *et al*., 2000). Some problems, however, have been encountered with these techniques. It is difficult to regenerate plants from transgenic protoplasts (Smith and Drew, 1990), and *Agrobacterium*-mediated transformation of wounded rhizome tissue has been discarded because of chimeric plants and low transformation efficiency (Becker, 1999; Gomez-Lim and Litz, 2004). Transformation of bananas is now mostly achieved by particle bombardment and *Agrobacterium*-mediated transformation of embryogenic cell suspensions. Arinaitwe *et al*. (2004) reported an increase in transformation efficiency with *Agrobacterium*-mediated transformation compared to particle bombardment of Grande Naine (*M. acuminata*, Cavendish subgroup, AAA) embryogenic cell suspensions. *Agrobacterium*-mediated transformation further offers other advantages such as stable expression of the transgene, allowing for the DNA to be integrated into a defined region in the plant genome and with fewer rearrangements (Walden and Wingender, 1995). However, the establishment of banana cell suspensions is time consuming and for some cultivars (especially EAHB), embryogenic cell suspensions have not been obtained (Tripathi *et al*., 2005).

The banana industry in South Africa is based on the production of Cavendish cultivars only (Viljoen, 2002). In the past 20 years, diseases such as Fusarium wilt and pests such as *C. sordidus* have devastated the industry. No conventional control option is available to producers, and once these pathogens and pests are introduced into specific fields, further production has to stop because of a substantial loss in yield (Viljoen, 2002). In addition, consumers in South Africa are unwilling to accept replacement cultivars. Since Cavendish bananas can not be improved by conventional breeding, and since no resistant Cavendish selection with acceptable horticultural properties could be generated by means of *in vitro* mutagenesis, genetic modification appears to be the only means to deal with the increasing losses (Viljoen *et al*., 2004). The objective of this study, therefore, was to establish the technology of genetic transformation of Cavendish bananas with *Agrobacterium*-mediated transformation in South Africa. In this endeavour, three plasmids were introduced into a Cavendish banana cultivar.

MATERIALS AND METHODS

Plant material

Embryogenic cell suspensions from the Cavendish banana cultivar Grande Naine were obtained according to the protocol described by Stross *et al*. (2003). This procedure involves the incubation of immature banana male flowers on medium containing 2,4 D which led to ideal callus. The ideal callus was transferred to liquid medium for the initiation of cell suspensions. Embryogenic cell suspensions were maintained in MA2 medium (Stross *et al*., 2003) in the dark at 25±2°C on a rotary shaker at 100 rpm. The cell suspensions were sub-cultured every 14 days for 6-12 months. *Agrobacterium*-mediated transformation was carried out 7 days after the last sub-culturing.

Agrobacterium strains and plasmids

Two *A. tumefaciens* strains and three plasmids were used for the transformation of Grande Naine cell suspensions. The *Agrobacterium* strains included AGL1 and LBA4404, and the plasmids pCambia1305.1 (CAMBIA, Canberra, Australia), pART-TEST7 and pKYΩOCI (Table 1; Fig. 1). pCambia1305.1 and pART-TEST7 were provided by J. Dale (Queensland University of Technology, Brisbane Australia), and pKYΩOCI were donated by L. Jouanin (INRA, Versailles, France). pCambia1305.1 and pKYΩOCI contain a *Gus* gene with an intron for detection of plant-specific *Gus* expression, whereas pART-TEST7 has a green fluorescent protein (GFP) as screenable marker. All the plasmids are driven with a 35CaMVS promoter.

Bacterial culture

The three strains of *Agrobacterium*, maintained in glycerol stocks at -80°C at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa, were transferred onto Luria-Bertani (LB) medium modified with antibiotics. For the strains harbouring the plasmids pCambia1305.1 and pART-TEST7, 50 mg/L kanamycin (Sigma, Stanheim, Germany) was used, and for the ones containing

pKYΩOCI, 50 mg/L rifamplicin (Sigma) and 12.5 mg/L tetracycline (USB Corporation, Cleveland, Ohio) were used. The LB medium consists of 20g/L tryptone (Merck, Darmstadt, Germany), 5 g/L yeast extract (Merck), 5 g/L sodium choride (Merck) and 15 g/L agar (Merck).

The *Agrobacterium* strains were grown for 2 days at 25±2°C. It was then transferred to 50-ml Erlenmeyer flasks, each containing 10 ml LB broth with antibiotics at concentrations described above. The *Agrobacterium* strains were incubated at 28±2°C and shaken on a rotary shaker at 160 rpm for 24 hrs. The optical density was measured with BioPhotometer (Eppendorf, Hamburg, Germany) and adjusted to 0.1 with LB broth supplemented with 20 µM acetosyringone (Acros Organics, Geel, Belgium).

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation was performed according to a modified version of the method described by Engler *et al*. (2000). Three hundred µl of embryogenic banana settled cell volume (SCV) were immersed into 3 ml of *Agrobacterium* suspension in a 25-ml Erlenmeyer flask. The suspensions were gently mixed for 3 min and left to settle for 5 min. The supernatant containing some bacterial cells (2.5 ml) was removed with a pipet and discarded. The rest of the cell suspension and *Agrobacterium* cells was transferred onto Whatman glass microfibre filters GF/A (Merck) and co-cultivated at 22±2°C on MA2 with 100 µM acetosyringone. After 3 days the glass microfiber filter papers with the cells were transferred to MA2 medium with 50 mg/L kanamycin and 200 mg/L ticarcillin (Sigma), and incubated in dark conditions at 25±2°C for 1 month. The microfibre filters with the cells were then transferred to MA3 medium (Stross *et al*., 2003) with 50 mg/L kanamycin and 200 mg/L ticarillin. After incubation for another 2 months, the embryos were transferred to MA4 medium (Stross *et al*., 2003) and incubated in the dark at 25±2°C until the plant developed. If *Agrobacterium* overgrowth was seen on the filter paper on either MA2 or MA3 medium, the filter paper was transferred to a new medium with fresh antibiotics.

Histochemical β-glucuronidase (GUS) assay

To determine the efficiency of the transformation process, a transient assay (Jefferson, 1987) was performed 3 days after co-cultivation. A small portion of the banana cells (5 µl) was incubated in 10 µl of 1 M 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) (Sigma) at 20±2°C overnight. In the morning, the cells were subjected to a colorimetric histological assay by visualization under a light microscope. In addition, putative transgenic leaf and root tissue were also subjected to a GUS assay (Jefferson, 1987) and incubated in 20 µl of 1 M X-gluc at 20±2°C overnight for colorimetric histological assay. In both cases, non-transformed Grande Naine tissue was included as a negative control.

Molecular analysis of the transgenic plants

DNA isolation

DNA was extracted from the putative transgenic banana plants by using a modified method described by Gawel and Jarret (1991). A small putative transgenic banana leaf with an approximate weight of 50 mg was ground in liquid nitrogen in a 2-ml eppendorf tube. After grinding, 700 µl of extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.1% mercaptoethanol) was added and the mixture incubated in a water bath at 60°C for 1 hr. The extraction buffer was preheated to 65°C before being added to the grounded tissue. After incubation, 600 µl chloroform:isoamyl alcohol (24:1) was added to the eppendorf tubes and mixed by inversion at room temperature for 5 min. Thereafter, the eppendorf tubes were centrifuged at 13 000 rpm for 5 min and 500 µl of the aqueous phase was transferred to new 1.5-ml eppendorf tubes. Isopropanol (500 µl) was added to the aqueous phase and incubated at -20°C for 1 hr to precipitate the DNA. The mixture was centrifuged at 13 000 rpm for 5 min to pellet the DNA. The isopropanol was then removed and the pellet washed with 1 ml of 70% ethanol. The mixture was centrifuged once more at 13 000 rpm for 5 min and the ethanol was removed. The DNA pellet was then dried in a vacuum dryer for 10 min, and dissolved in 50 µl in low TE (10mM Tris-HCl pH 8.0, 0.1 mM EDTA).

Amplification of the *Gus* gene

To determine the transformation efficiency of banana cell suspensions with the *Gus* gene, DNA of the putative transgenic banana plants was amplified with *Gus*-specific primers. For the PCR reaction, a 25-µl reaction volume was prepared with the following components: 250 ηg template DNA, 2 µl of 2.5 mM dNTP (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa), 2.5 µl of 10 x NH_4 buffer (Bioline Ltd., London, UK), 1.25 µl of 50 mM $MgCl_2$ (Bioline Ltd.), 1 µl of 10 mM GUS1 (5'ATGTTAACGTCCTGTAGAAACCC3') (Inqaba Biotechnical Industries (Pty) Ltd.), 1 µl of 10 mM GUS2 (5'TCATTGTTTGCCTCCCTGCTGC3') (Inqaba Biotechnical Industries (Pty) Ltd.), 0.5 µl of 10 mg/ml bovine serum albumin (New England Biolab Inc., Boston, USA), 0.5 U BIOTAQ™ DNA polymerase (Bioline Ltd.) and 10.75 µl sterile Sabax water (Adcock Ingram, Bryanston, South Africa). The amplification was performed in a mastercycler (Eppendorf, Hamburg, Germany) with the following parameters: one cycle at 94ºC for 5 min, 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s, and finally one cycle at 72ºC for 10 min. The positive control included the plasmid DNA isolated from *Agrobacterium* with GenElute™ plasmid miniprep kit (Sigma). The two negative controls included were non-transformed Grande Naine DNA and the use of sterile Sabax water instead of DNA. A 5-µl portion of the amplification product was run on a 1% agarose gel and compared to a 100-bp DNA marker (Fermentas, Life Sciences, Hanover, USA).

RESULTS

Selection and regeneration of transgenic plants

After 3 days of co-cultivation of *Agrobacterium* with the banana cells, bacterial growth could be seen on the edges of the glass microfibre filters, as well as on the selection medium. The banana cells also began to turn light brown in colour. The glass microfibre filters were transferred to fresh selection medium with the appropriate antibiotics. Small embryos developed after 4 weeks, and the banana cells with the glass microfibre filters as supporting material were transferred to MA3

medium. More embryos formed while the banana cells were incubated on MA3 media (Fig. 2). After a minimum of 6 weeks culturing of the embryos on MA3 medium, they were transferred to MA4 medium for germination. The embryos started to germinate after 8 weeks to form white shoots (Fig. 3). A total of 145 putative transgenic plants were obtained at a rate of approximately 50 plants from 0.3 ml SCV (Table 1; Fig. 4).

Histochemical β-glucuronidase (GUS) assay

Random blue spots developed in banana cells after co-cultivation in X-gluc (Fig. 5). A blue discolouration of transgenic plant material became visible when the putative transgenic banana roots and leaves were incubated in X-gluc for 15 min. The root and leaf tissue turned completely blue when incubated overnight (Fig. 6), while the negative control and non-transformed Grande Naine tissue remained without any colour change.

Molecular analysis of the transgenic plants

PCR analysis of the putative transgenic banana plants revealed that the *Gus* gene was successfully introduced into the genome of the banana plant (Fig. 7). A band of 620 bp in size was obtained following GUS gene amplification. Additional bands of unexpected sizes were also produced that might be the result of non-specific binding to the plasmid. In the negative control, no bands were detected.

DISCUSSION

Genetic transformation offers an opportunity to produce banana plants with unique characteristics such as resistance to pests and pathogens (Chakrabarti *et al*., 2003; Atkinson *et al.*, 2004; Pei *et al*., 2005), an increased nutritional value of the fruit, reduced abiotic stress, the production of secondary metabolites (Kumar *et al*., 2005) and the better understanding of plant-pathogen interaction (Balint-Kurti *et al*., 2001). The South African banana industry is challenged by several important diseases and pests, such as Fusarium wilt and the banana weevil, and abiotic stresses such as cold

and drought, that have caused extensive damage to plantations in the past (Viljoen *et al*., 2004). Since the Cavendish cultivars constitute 95% of all bananas produced in South Africa (Viljoen, 2002), the establishment of a national transformation unit has become essential to improve local cultivars against these biotic and abiotic stresses. This study represents the first report of the genetic modification of Cavendish bananas in South Africa, and would contribute substantially to the future unconventional improvement of bananas in the country. Since banana serves as a staple food crop in many African countries, our ability to transform bananas should in future be extended to include banana and plantain varieties of importance to Africa, such as the EAHB. The process of transforming the EAHB is currently hampered by the unavailability of embryogenic cell suspensions.

Banana transformation has been performed at several research laboratories, but transformation efficiency remains low (Khanna *et al*., 2004). May *et al*. (1995) reported an average of one regenerated shoot per rhizome slice tissue incubated on selective medium. A higher efficiency was obtained by transforming embryogenic cell suspensions. Transformation of embryogenic cells suspensions with particle bombardment gave rise to 10 transgenic plants from 90 bombardments (0.04 ml SCV per bombardment), whereas Ganapathi *et al.* (1999) reported that the transformation of embryogenic cell suspensions with *Agrobacterium* resulted in 40 transgenic plants from a 0.5 ml packed cell volume. Similar results were obtained in this study, with 50 transformed plants recovered from 0.3 ml SCV. The highest transformation efficiency regarding embryogenic cell suspensions was reported by Khanna *et al*. (2004), who obtained 65 transgenic plants from 0.05 ml SCV.

One of the most import aspects of the genetic modification of plant crops is to ascertain the genetic stability of the transgenic plants. The number of off-types and mutants in banana depends on the plant selected, the age of the male flower, and the season of the year (Escalant, personal communication). Houllo-Kido *et al*. (2005) reported that particle bombardment reduced the regeneration rate of banana plants. Several high-yielding Cavendish selections are available at tissue culture companies in South Africa (Viljoen, personal communication), and the ability of these selections to produce stable transgenic plants needs to be determined. Amplified fragment length polymorphisms were successfully used in rice to determine the genomic changes after

Agrobacterium-mediated transformation (Labra *et al*., 2001). In future, detection of polymorphisms in the genome of transformed banana plants in comparison to non-transformed plants should be performed.

Further testing of the putative transgenic Cavendish plants is required. Southern blots should first be performed to determine the copy number of the transgenes (Register, 1997). Transgenic plants expressing the *OcI* gene then needs to be screened for resistance to the banana weevil and the burrowing nematode in the glasshouse before useful transplants can be tested in the field. Grande Naine, have previously genetically engineered with a variant of the rice cystatin gene by means of *Agrobacterium*-mediated transformation of the embryogenic cell suspension, showed greater resistance against the burrowing nematode (Atkinson *et al.*, 2004).

New transformation technologies, such as plastid transformation of cell suspensions (Langbecker *et al*., 2004), vacuum infiltration of scalps (Acereto-Escoffié *et al.*, 2005) and *Agrobacterium*-mediated transformation of shoot tips (Tripathi *et al*., 2005) could also be tested at our laboratories. Plastid transformation offers the advantage of higher levels of expression of the transgenes without gene silencing (Daniell *et al*., 2005), but has only been optimized for the genetic modification of tobacco (Daniell *et al*., 2005). Vacuum infiltration increases the transformation efficiency (Acereto-Escoffié *et al.*, 2005), and transformation of shoot tips provides a more rapid technique to obtain genetically modified banana (Tripathi *et al*., 2005).

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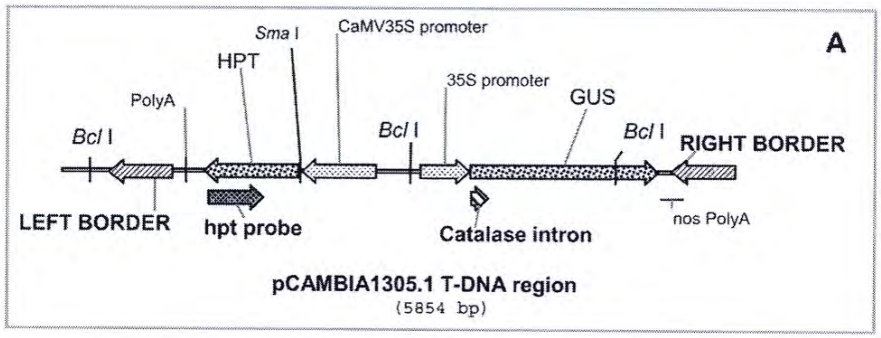
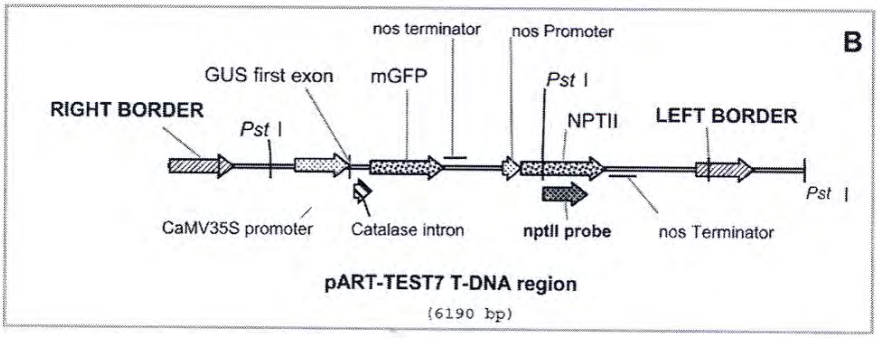
Table 1. The number of putative transgenic banana plants obtained after *Agrobacterium*-mediated in transformation of Grande Naine (*Musa acuminata*, Cavendish subgroup, AAA)

Strain	**Plasmid**	**Number of putative transgenic plants**
AGL1	pCambia1305.1	78
AGL1	pART-TEST7	18
LBA4404	pKYΩOCI	49





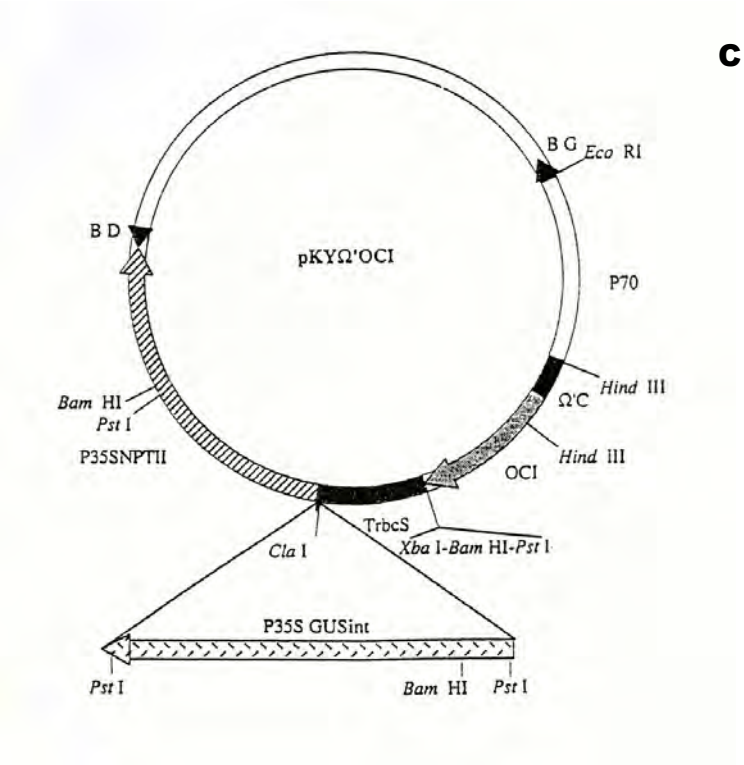


Figure 1. Schematic representation of the gene constructs used in banana transformation. A. pCAMBIA1305.1 (Khanna *et al*., 2004), B. part-TEST7 (Khanna *et al*., 2004), and C. pKYΩOCI.

Figure 2. Development of embryos from banana cell suspensions on MA3 medium containing kanamycin and ticarcillin following *Agrobacterium*-mediated transformation.

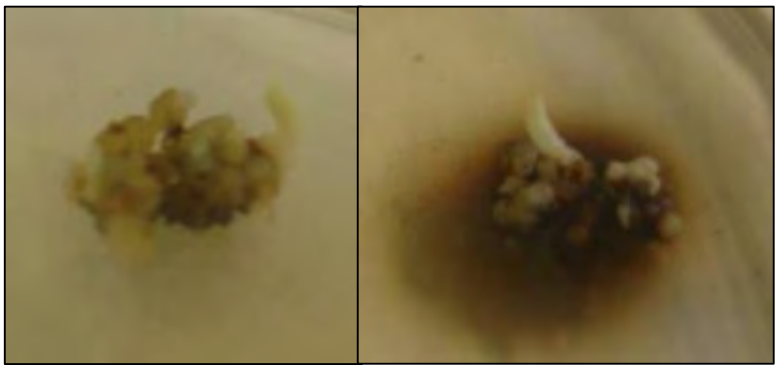


Figure 3. Shoot development of the germinating banana embryos on MA4 selection medium containing kanamycin and ticarcillin.

Figure 4. *In vitro* growth of putative genetically modified Cavendish banana plantlets on MA4 medium containing kanamycin and ticarcillin.

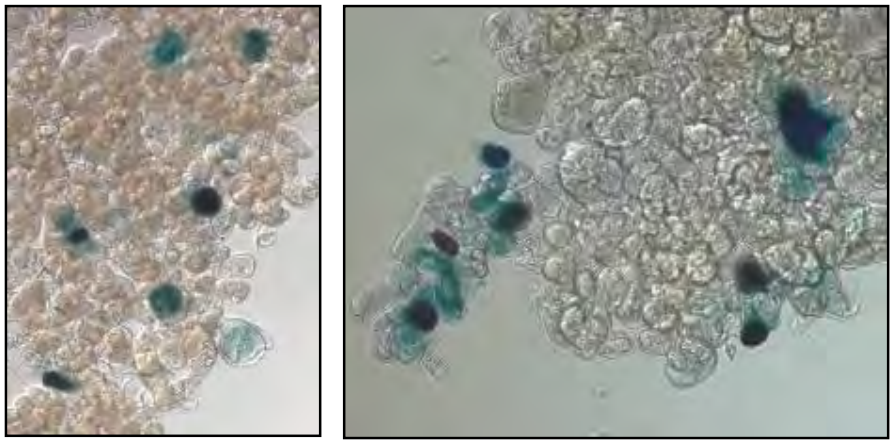


Figure 5. Blue discolouration of banana cells 3 days after *Agrobacterium*-mediated transformation with the *Gus* gene.

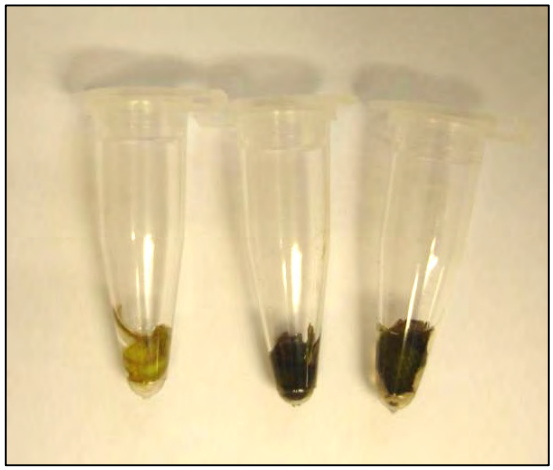


Figure 6. Discolouration of putative transgenic banana leaf tissue modified with the *Gus* gene, following incubation in X-gluc. The negative control (non-transformed Grande Naine) is on the left, and the two putative transgenic banana leaves in the middle and on the right.

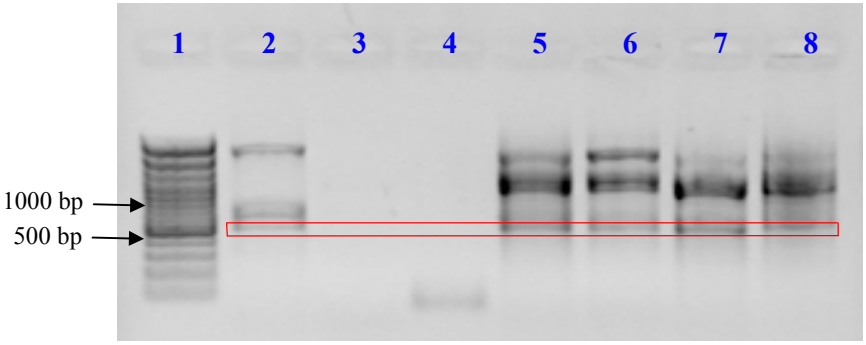


Figure 7. Agarose gel with the amplification product of the *Gus* gene from putative transgenic banana leaves. Lane 1: 100 bp DNA marker. Lane 2: Positive control. Lane 3: Negative non-transformed Grande Naine control. Lane 4: Negative water control. Lane 5-8: Putative transgenic plants.

Chapter 4

Molecular fingerprinting of Cavendish bananas (*Musa* spp.) using amplified fragment length polymorphisms

ABSTRACT

Molecular characterization plays an important role in the classification and detection of somaclonal variants in banana plants. Several molecular techniques have been employed to compliment the classification system, which previously was only based on plant morphology. The objective of this study was to use a highly discriminative fingerprinting technique, amplified fragment length polymorphisms (AFLPs), to facilitate the differentiation between closely related cultivars within the Cavendish banana subgroup. AFLP profiles of eight banana varieties, cultivars and hybrids were generated on a Licor analyser using seven different primer combinations. Results showed that the banana plants were subdivided in clades according to their genomic composition. More importantly, the AFLP technique was able to separate the different cultivars within the Cavendish subgroup. This technique could potentially be applied to accurately detect somaclonal variants in banana arising from micropropagation and genetic modification.

INTRODUCTION

Cultivated bananas (*Musa* spp.) belong to the section Eumusa, and consist of sweet dessert and cooking banana varieties (Robinson, 1996). These varieties were formed by means of interspecific hybridization between *Musa acuminata* Colla and *M. balbisiana* Colla (Stover and Simmonds, 1987). *Musa acuminata* originated in Southeast Asia and contributes the "A" genome to interspecific hybrids, while *M. balbisiana* originated in the Indian subcontinent and contributes the "B" genome (Stover and Simmonds, 1987). From hybridization, diploid, triploid and tetraploid bananas can be produced. Dessert bananas are mostly triploid and consist of three sets of chromosomes of *M. acuminata* (AAA), although there are notable exceptions such as the Lady Finger bananas (AAB), and Goldfinger bananas (AAAB) (Jones, 2000). Cooking bananas are mostly triploid and tetraploid, and consist of chromosomes from both *M. acuminata* and *M. balbisiana* (AAB, ABB, AAAB, AABB). Most of the wild type bananas are diploid (AA, BB). Somatic mutations also occur in banana clones and result in phenotypically different forms, called cultivars.

Banana varieties and cultivars can be separated from each other by using a classification system based on 15 morphological features selected to differentiate between the wild species of *M. acuminata* and *M. balbisiana* (Simmonds and Shepherd, 1955). Classification of bananas, however, is often complicated by ploidy and the designation of different local names to the same cultivar in different countries (Jones, 2000). The accurate classification of bananas is a prerequisite for the proper identification, collection, conservation and breeding of banana (Bhat *et al.*, 1995). To compliment morphological classification, several molecular techniques have now been employed. These include isozyme analysis (Jarret and Litz, 1986), random amplified polymorphic DNAs (RAPDs) (Howell *et al*., 1994), restriction fragment length polymorphisms (RFLPs) (Gawel *et al.*, 1992), amplified fragment length polymorphisms (AFLPs) (Loh *et al.*, 2000), rRNA gene spacer length polymorphisms (Lanaud *et al*., 1992) and short sequence repeats (SSRs) (Kaemmer *et al*., 1997).

AFLPs and SSRs are the most promising techniques to differentiate between closely related banana cultivars. SSRs have often been used for studies on genetic diversity within and between populations, but are expensive to develop, and require technical

expertise to interpret (Kubik *et al.*, 2001; Bornet *et al.*, 2002). AFLPs, in contrast, are easy to perform, robust and a high level of polymorphism is detected without prior sequence knowledge (Savelkoul *et al.*, 1999). AFLP is not only a powerful technique, but also has a wide range of applications. AFLPs have been used in other clonal crops as a fingerprinting technique, for example in date palm (Diaz *et al.*, 2003), and to determine the level of diversity, for example in Enset (Negash *et al.*, 2002). In banana, AFLPs have also been utilized to determine tissue culture-induced mutations (Singh *et al.*, 2002), and in sugarcane to detect genetic changes due to transformation (Arencibia *et al.*, 1999).

The most important dessert banana grown is the Cavendish banana (***M. acuminata***, AAA), which constitutes approximately 43% of all bananas produced worldwide (Jones, 2000). For the export market, almost 16 million tonnes of Cavendish bananas are produced with an estimated value of US$5.2 billion (FAO, 2006). Despite this, only 14% of Cavendish banana fruit is exported. The remainder is consumed locally as a cash crop for resource poor farmers (Robinson, 1996). Cavendish bananas are sterile and, therefore, clonal selections with good cultivation properties are derived from the same predecessor. Unfortunately, these bananas are also highly susceptible to many diseases and pests (Robinson, 1996). The only way to effectively reduce potential losses involves the development of resistant plants. Such resistant plants can be obtained by means of conventional improvement, but because of the high sterility of the triploid cultivars, this method requires several years of breeding before new hybrids are developed. Unconventional improvement methods such as ***in vitro*** mutagenesis and genetic modification are an important alternative (Crouch *et al.*, 1998).

Molecular characterization of banana plants plays an important role in the classification and detection of somaclonal variation in banana plants. Molecular techniques could also be considered to determine the effect of genetic transformation on the genome of banana plants. The objective of this study was to determine the potential of AFLPs to differentiate closely related banana cultivars, varieties and hybrids within the Cavendish banana subgroup.

MATERIAL AND METHODS

Plant material

Eight banana varieties, cultivars and hybrids were selected for molecular identification. These included the Cavendish cultivars (*M. acuminata*, AAA) Williams, Grande Naine and Giant Cavendish, and a Fusarium wilt tolerant selection, GCTCV-218; the Calcutta variety *M. acuminata* subsp. *burmanicoides*, AA); the Bluggoe cultivar (*Musa* spp., ABB); and the tetraploid AAAB hybrids FHIA 18 and High Noon (SH 3640/10). For each of the cultivars, varieties and hybrids, three tissue culture plants were randomly selected from the germplasm collection of either DuRoi Laboratories (Tzaneen, Limpopo Province, South Africa), or the Forestry and Agricultural Biotechnology Institute (FABI), at the University of Pretoria, Pretoria, South Africa.

DNA isolation

DNA was extracted from banana leaves by making several modifications to the method previously described by Gawel and Jarret (1991). Briefly, 300 mg of leaf tissue from a curled cigar-roll leaf was grinded in liquid nitrogen in a 2-ml eppendorf tube, 700 µl of warm extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.1% mercaptoethanol) was added, and the suspension incubated at 60^{o}C for 1 hr. After incubation, 600 µl chloroform:isoamyl alchol (24:1) was added and mixed by inversion at room temperature for 5 min. The mixture was then centrifuged at 13 000 rpm for 5 min. After centrifugation, the supernatant was transferred to a new tube and the DNA precipitated by the addition of 500 µl ice-cold isopropanol and further incubation at -20^{o}C for 1 hr. The precipitated DNA was collected by centrifugation and washed with 70% ethanol. The pellet was dried in a vacuum dryer for 10 min. The pellet was resuspended in 200 µl low TE buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA). RNA was removed from the solution by treatment with *RNase*I (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) and incubation at room temperature overnight.

Due to the high concentration of phenolics in the banana plant cells, the DNA pellet had a brownish colour, and an additional phenol/chloroform extraction was performed. This was done by the adding of 200 µl phenol and 200 µl chloroform to the resuspended DNA solution, and centrifugation at 10 000 rpm for 5 min. The aqueous phase was transferred to a new tube and further extracted with 400 µl chloroform before centrifugation at 10 000 rpm for 5 min. The additional chloroform extraction was repeated three times. The DNA was precipitated with 2 x volumes ethanol and 0.1 x volumes sodium acetate (pH 5.5) and incubated at -20^{o}C for 1 hr. The DNA was collected by centrifugation at 10 000 rpm for 30 min. The ethanol was removed and the pellet washed with 70% ethanol, vacuum dried for 10 min, and resuspended in 50 µl low TE. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA) and verified by running the DNA samples on a 0.8% agarose gel (Roche Molecular Biochemicals, Manheim, Germany) with a λ DNA marker (Fermentas, Life Sciences, Hanover, USA). The DNA concentrations were adjusted to 30 ng/µl with low TE.

AFLP procedure

The AFLP procedure was performed according to the procedure described by Vos *et al*. (1995), with slight modifications.

DNA digestion and adapter ligation

Approximately 360 ng DNA was digested with 12 U EcoRI (Fermentas) and 8 U MseI (New England Biolab Inc., Boston, USA) at 37^{o}C for 3 hrs. The restriction enzymes were deactivated by heating to 70^{o}C for 15 min. EcoRI (5'CTCGTAGACTGCGTACCCTGACGCATGGTTAA3') and Mse (5'GACGATG AGTCCTGAGTACTCAGGACTCAT3') adaptors (Inqaba Biotechnical Industries (Pty) Ltd.) were ligated to the restricted fragments with 1 U T4 DNA ligase (Roche Molecular Biochemicals). To ensure complete ligation the samples were incubated overnight at 37^{o}C. The ligation mixture was diluted 10-fold by the addition of 5 µl ligation mixture to 45 µl low TE.

Pre-selective amplification

Pre-selective amplification was carried out in a 30-µl reaction volume with the following components: 3 µl diluted ligation mixture, 2.4 µl of 2.5 mM dNTP (Inqaba Biotechnical Industries (Pty) Ltd.), 3 µl of 10 x PCR buffer with $MgCl_2$ (Roche Molecular Biochemicals), 0.9 µl of 10 mM EcoRI primer + A (5' GACTGGGTACCAAATCA3') (Inqaba Biotechnical Industries (Pty) Ltd.), 0.9 µl of 10 mM Mse primer + C (5'GATGAGTCCTGAGTAAC3') (Inqaba Biotechnical Industries (Pty) Ltd.), 2 U *Taq* polymerase (Roche Molecular Biochemicals) and 19.4 µl sterile Sabax water (Adcock Ingram, Bryanston, South Africa). The pre-selective amplification was performed using a mastercycler (Eppendorf) with the following temperature cycling conditions: one cycle at 72ºC for 30 s, 30 cycles of 94ºC for 30 s, 56ºC for 30 s, and 72ºC for 60 s with 1 s/cycle increase, and finally one cycle at 72ºC for 2 min. A 10-µl portion of the pre-selective amplification was run on a 1.5% agarose gel with a 100-bp DNA marker (Fermentas) to determine the intensity and range of the amplification products. The pre-selective amplification mixture was diluted between 10- and 50-fold with low TE depending on the intensity of the smear.

Selective amplification

For selective amplification the following components were added together to reach a total volume of 20 µl: 5 µl diluted pre-selective amplification mixture, 1.6 µl of 2.5 mM dNTP, 2 µl of 10 x NH_4 buffer (Bioline Ltd., London), 1.5 µl of 50 mM $MgCl_2$ (Bioline Ltd.), 1 µl of 10 mM EcoRI primer + 3 (LICOR, Lincoln, NE, USA), 1 µl of 10 mM Mse primer + 3 (Inqaba Biotechnical Industries (Pty) Ltd.), 2 U BIOTAQ™ DNA polymerase (Bioline Ltd.) and 7.78 µl sterile Sabax water (Adcock Ingram). The EcoRI primers were labeled with infrared dye IRDye™ 700 or IRDye™ 800 (LICOR). Seven primer combinations were used in this study: E-ACT/M-CAG; E-ATC/M-CCT; E-ACA/M-CTC; E-AAA/M-CCC; E-ACA/M-CAG; E-ACG/M-CCA; and E-AAA/M-CAA. E and M indicates EcoRI and MseI, respectively, while the letters after the hyphen show the selective bases on the primer sequence. PCR conditions were set at 13 cycles of 94ºC for 30 s, 65ºC-0.7ºC/cycle for 30 s, 72ºC for

60 s, 23 cycles of 94ºC for 30 s, 56ºC for 30 s, 72ºC for 60 s with 1 s/cycle increase, and one cycle at 72ºC for 60 s. The selective amplification was performed in a mastercycler. Half the volume (1:2) of formamide loading dye (Roche Molecular Biochemicals) was added to the selective amplification products before gel analysis.

Gel analysis

Detection of AFLP fragments was performed on a LICOR® 4200 automated DNA sequencer (LICOR) according to Myburg *et al*. (2001) with minor changes. The selection amplification fragments were denatured for 3 min at 90ºC and, thereafter, placed on ice until loading. AFLP fragments were run on a 25-cm gel with 8% Long Ranger polyacrylamide gel solution, 7.0 M urea and 0.8 x TBE (71.2 mM Tris, 71.2 mM boric acid, 1.6 mM EDTA) with a 50-700 bp size marker (LICOR). Of each sample, 0.8 µl was loaded in 64-well combs (LICOR). The polyacrylamide gel was run for 4 hrs with the following parameters: 1500 V, 35 mA, 35 W, 45ºC, motor speed 3 and signal filter 3.

Data analysis

The TIFF file generated from the LICOR® 4200 automated DNA sequencer was imported for data analyses in Bionumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). The following conditions were used: minimum profiling parameter of 5.0-5.2% and gray-zone parameter of 0% relative to the maximal value, a minimum area parameter of zero, and a shoulder sensitivity parameter of zero. Band matching was performed with a 1% optimization parameter and a 1% band tolerance. Only fragments between 50-700 bp were considered for analysis.

Two different trees were generated. Firstly, the relationship among banana cultivars were visualised by means of cluster analysis using the average of the combined trees with the unweighted pair-group method of arithmetic averages (UPMGA). Secondly, the band matching profiles were exported and further analysed using Phylogenetic Analysis Using Parsimony version 4.0b10 (Swofford, 1999). A phylogram was constructed with Neighbour-Joining as a tree-building algorithm, Tree-Bisection-

Reconnection used as branch-swapping algorithm, and bootstrap analyses with 1000 replicates were performed for confidence support (Felsentein, 1985).

RESULTS

High quality genomic DNA was isolated from banana leaf tissue with concentrations varying between 300-1000 ng/µl (Fig. 1). Amplification of the ligated fragments produces a smear of between 100-1100 bp, with highest intensity at 500 bp (Fig. 2). The high-resolution polyacrylamide gels that were obtained yielded distinct fingerprints for each of the different primer combinations (Fig. 3). In total, the seven primer combinations generated 887 bands with an average of 126 bands per primer pair, and 638 unique bands were produced (Table 1).

The cladogram calculated from the similarity matrix by comparison, using the average for five different primer pairs with UPGMA analysis, revealed three major clades (Fig. 4). As expected, the banana individuals grouped according to their respective cultivars, varieties and hybrids. Three different clades were: Clade 1, which included Bluggoe, FHIA 18 and High Noon; Clade 2, which included Calcutta; and Clade 3, which included the four Cavendish cultivars. Grande Naine had a 62% similarity level with Williams. In addition, GCTCV-218 displayed a 45% similarity level to the other Cavendish cultivars examined. The three Cavendish cultivars, Giant Cavendish, Grande Naine and Williams shared a 42% similarity level.

Distance analyses with Neighbour-Joining as a tree-building algorithm, Tree-Bisection-Reconnection used as branch-swapping algorithm and bootstrap analyses with 1000 replicates, divided the banana varieties in four major clades. Clade 1 contained the three leaf DNA samples from Bluggoe, and this clade was distinctly separated from the other cultivars with a bootstrap support of 100% (Fig. 5). Clade 2 included Calcutta and the four Cavendish cultivars. In this clade, the Cavendish subgroup was clearly separated from Calcutta, and subdivided according to their respective cultivars, namely Grande Naine, Williams, Giant Cavendish and GCTCV-218. High Noon was contained in Clade 3 with a bootstrap support value of 100%, and Clade 4 contained FHIA 18 supported by a bootstrap score of 100%.

DISCUSSION

In this study, eight banana varieties, cultivars and hybrids were subdivided according to their genomic composition. These results correspond with earlier studies, where Ude *et al*. (2002) reported that ***M. acuminata***, ***M. balbisiana***, and their hybrids grouped according to their genomic make-up. Contrary to this observation, Loh ***et al.*** (2000) clustered Grande Naine, Pisang Rastali (*Musa* spp., AAB) and Pisang Awak (*Musa* spp., ABB) together, while Dwarf Cavendish (***M. acuminata***, AAA) and Pisang Assam (*Musa* spp., AAB) formed another cluster. Their explanation was that cultivars with similar genomic backgrounds and horticultural qualities might have arisen during separate events, and not necessarily from an original ancestor. In this study, the Cavendish cultivars clustered together, and separate from those with a different genomic composition. The true nature of evolution of bananas within genomically defined units and according to their fruit qualities should be further substantiated in a more extensive investigation than the current one or that of Loh *et al*. (2000).

The AFLP technique was useful in differentiating cultivars within the Cavendish subgroup of bananas. Ude *et al*. (2003) reported that AFLPs have the ability to differentiate between plantain varieties, while RAPDs detected less polymorphisms. Similarly, Visser (2000) was unable to distinguish among Cavendish cultivars with RAPDs when a single primer was used. AFLP analysis was also able to detect variation among DNA samples from different plants within a cultivar. This makes us believe that they could be applied to detect genomic changes due to genetic modification, such as had been shown in sugarcane (Arencibia *et al*., 1999) and rice (Labra *et al*., 2001). Tissue cultured plants are vulnerable to somaclonal variation (Sahijram *et al*., 2003), and dwarf mutants have previously been identified using AFLPs (Engelborghs *et al.*, 1998). By selecting several primer combinations, the resolution of AFLP analyses could be improved (Kashkush *et al*., 2001), an approach that could be useful for both the accurate detection of somaclonal variants due to micropropagation and genetic modification.

The two different phylogenetic trees that were produced from the different analyses programs revealed some interesting information. It was observed that High Noon

grouped with FHIA 18 after a cladogram was generated with UPGMA analysis. This differed from the phylogram obtained with Neighbour-Joining, Tree-Bisection-Reconnection and bootstrapping with 1000 replicates. The UPGMA analyses grouped High Noon and FHIA 18, both AAAB banana hybrids, together. This is in contrast to the Neighbour-Joining analyses, which grouped High Noon closer to Calcutta and the Cavendish subgroup than to FHIA 18. This inconsistency could be explained by the fact that the UPGMA method is based on the assumption that the same rate of evolution applies to each branch, while the Neighbour-Joining matrix is constructed to adjust for differences in the rate of evolution of each taxon (Leitner *et al.*, 1996).

The current investigation showed that AFLP analysis offers a technology with several advantages for the improved classification of cultivated bananas when compared to other fingerprinting techniques. Isozymes are easy to perform, but only detect a low polymorphic level between closely related banana cultivars (Gawel and Jarret, 1991). RAPDs were used to distinguish CIEN BTA-03 (Williams mutant plant resistant to Yellow Sigatoka obtained through *in vitro* mutagenesis) from its susceptible Williams mother plants (Vidal and Garcia, 2000), but failed to differentiate between closely related Cavendish cultivars when using a single primer (Visser, 2000), or between cooking and beer bananas (Pillay *et al.*, 2001). The technique has a further disadvantage in that it is not reproducible in different laboratories (Kaemmer *et al.*, 1997). RFLPs are useful in genetic studies but are expensive, technically demanding, time consuming, and high concentrations of pure DNA are needed (Ude *et al.*, 2002). AFLP analyses, in contrast, is reproducible, analysis the entire genome, and discriminates between closely related cultivars, varieties and hybrids of this clonal crop plant.

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Table 1. Number of bands generated by means of AFLP for eight banana cultivars, varieties and hybrids using seven different primer combinations

Primer combination	Total number of bands between 50-700 bp	Total number of unique bands between 50-700 bp
E-ACT/M-CAG	134	102
E-ATC/M-CCT	155	128
E-ACA/M-CTC	108	69
E-AAA/M-CCC	120	75
E-ACA/M-CAG	121	87
E-ACG/M-CCA	133	103
E-AAA/M-CAA	116	74
Total	887	638

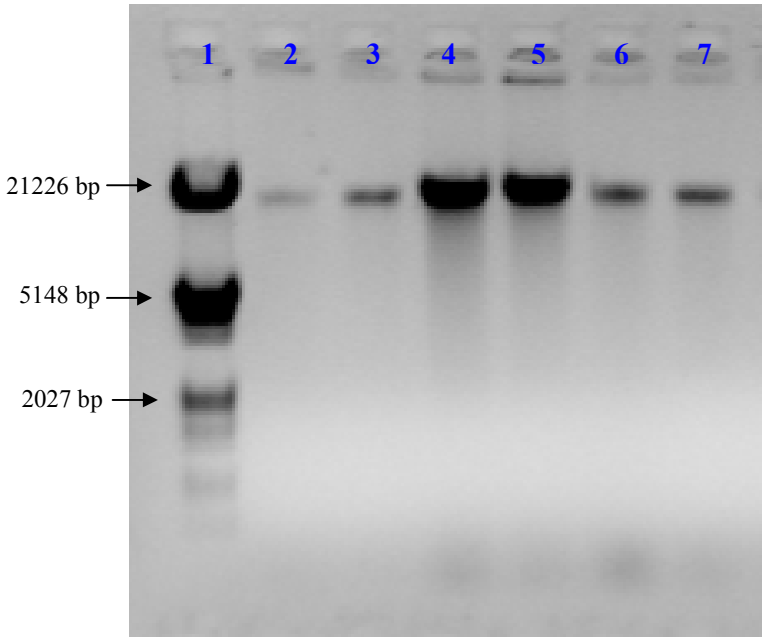

Figure 1. Agarose gel containing DNA fragments extracted from banana leaf tissue. Lane 1: λ DNA marker. Lane 2-7: DNA from six different banana varieties, cultivars and hybrids.

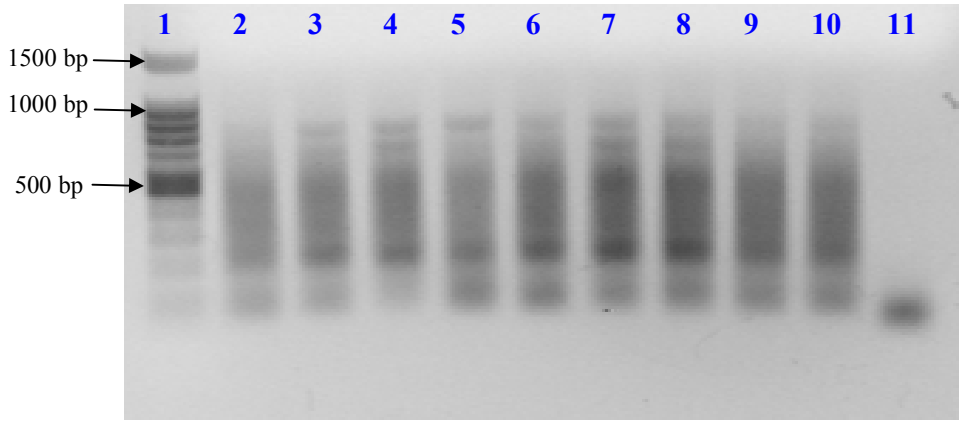

Figure 2. Agarose gel with pre-selective amplification of DNA fragments from banana leaves. Lane 1: 100 bp DNA marker. Lane 2-10: Pre-selective DNA fragments from nine different banana samples, varieties and hybrids. Lane 11: Negative control.

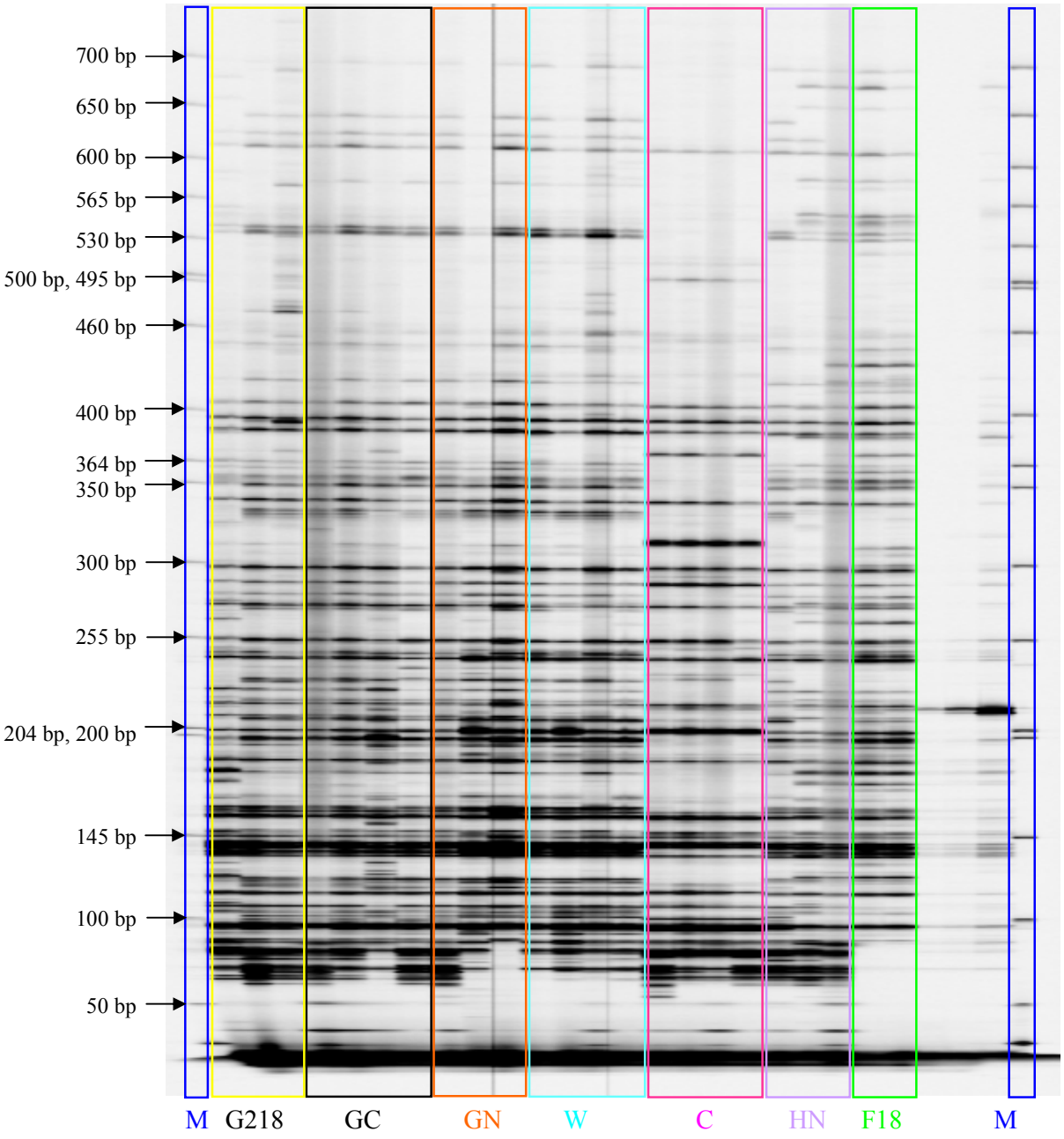

Figure 3. Polyacrylamide gel with AFLP fragments of different banana cultivars, varieties and hybrids, with E-AAA and M-CCA as primer combination. Lane 1, 50-700 bp sizing marker; lanes 2-4, GCTCV-218; lanes 5-8, Giant Cavendish; lanes 9-11, Grande Naine; lanes 12-14, Williams; lanes 15-18, Calcutta; lanes 19-22, High Noon; lanes 23-25, FHIA 18, lane 29, 50-700 sizing marker.

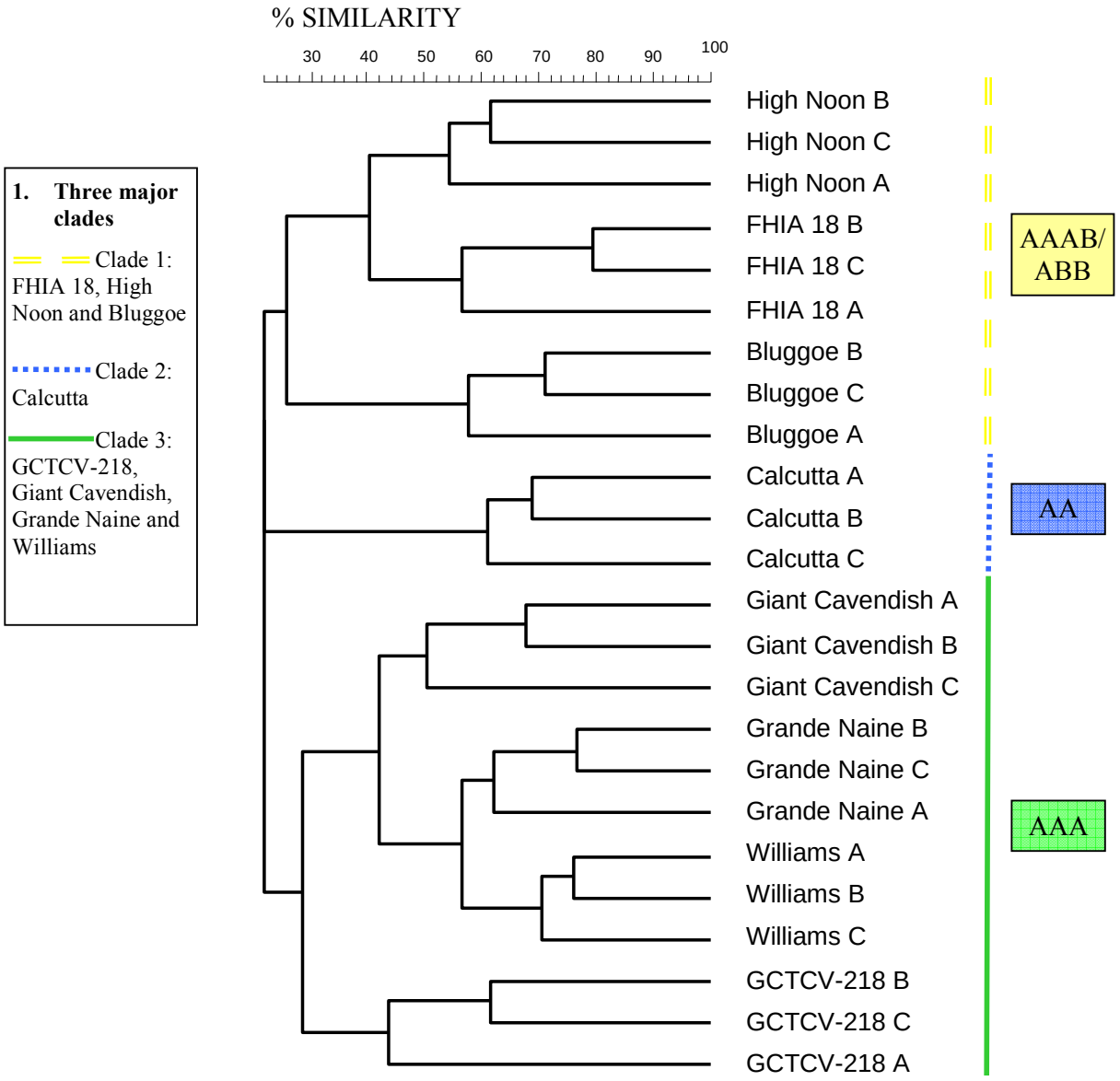

Figure 4. Cladogram generated using AFLP analyses of 24 *Musa* DNA samples representing eight different banana cultivars, varieties and hybrids. The cladogram is based on the average of five primer combinations with the unweighted pair-group method of arithmetic averages. The five primer combinations were E-ACT/M-CAG, E-ATC/M-CCT, E-AAA/M-CCC, E-ACA/M-CAG and E-AAA/M-CAA.

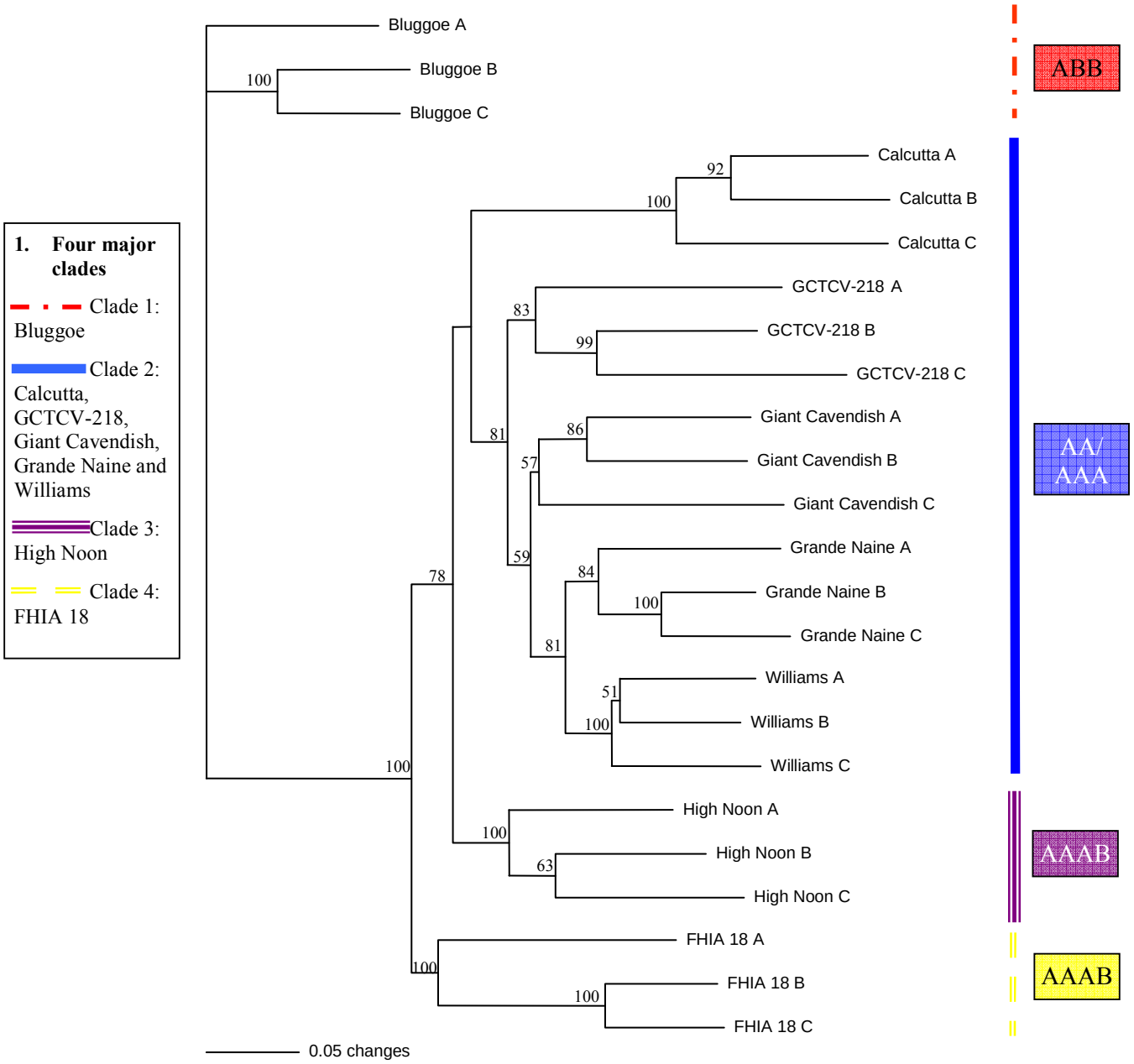

Figure 5. A phylogram representing eight banana cultivars, varieties and hybrids generated with five primer combinations, E-ACT/M-CAG, E-ATC/M-CCT, E-AAA/M-CCC, E-ACA/M-CAG and E-AAA/M-CAA, with Neighbour-Joining as tree building algorithm, Tree-Bisection-Reconnection used as branch-swapping algorithm bootstrap of a 1000 replicates. Bootstrap values obtained for analyses with 1000 replicates are indicated above the nodes.

SUMMARY

Bananas and plantains (*Musa* spp.) are cultivated commercially as a dessert fruit and by small-scale farmers as a staple food crop in many countries of the world. Sustainable production, however, is threatened by a number of diseases and pests such as Fusarium wilt (*Fusarium oxysporum* f.sp. *cubense*), black Sigatoka (*Mycosphaerella fijiensis*), the banana weevil (*Cosmopolitus sordidus*) and the burrowing nematode (*Radopholus similis*). Many diseases and pests of banana cannot be managed by conventional control methods, and replacement cultivars are often not acceptable to local industries. Resistance can be introduced into banana by means of conventional and unconventional improvement methods. Conventional breeding programs have many limitations, due to sterility of cultivated bananas, long growth cycles, low seed set and hybrids that are often not accepted by consumers. Unconventional improvement for enhanced resistance involves methods such as *in vitro* mutagenesis, protoplast culture, and genetic modification. During genetic modification, foreign genes are introduced into banana by means of *Agrobacterium*-mediated transformation or by particle bombardment.

One of the most powerful means to reduce the impact of pests and diseases is the use of somatic embryogenesis for unconventional plant improvement and the propagation of disease-free plants. In this thesis, immature male flowers of Grande Naine (*Musa acuminata*, Cavendish subgroup, AAA) were isolated and incubated on MA1 medium to form somatic embryos with ideal callus. When ideal callus was transferred to liquid MA2 medium, a heterogeneous cell suspension was formed. Non-embryogenic aggregates were removed to ensure a cell suspension constituted of small embryogenic clusters only. Somatic embryos were obtained from the cell suspension after plating the embryogenic clusters on solid MA3 medium. These somatic embryos were transferred to MA4 medium for germination, and to P6 medium to develop into *in vitro* plantlets. Embryogenic cell suspension can be used for genetic engineering of disease and pest resistant plants, *in vitro* mutagenesis, germplasm conservation and protoplast culture.

An *Agrobacterium*-mediated transformation system was established for the improvement of Cavendish banana cultivars, the only bananas produced for the fresh

fruit market in South Africa. Embryogenic cell suspensions from the cultivar Grande Naine were co-cultivated with *Agrobacterium* strains harbouring the plasmids pCambia1305.1, pART-TEST7 and pKYΩOCI. Antibiotic-resistant embryos derived from transgenic cell suspensions developed into banana plantlets 12 weeks after cultivation on MA4 medium. In total, 145 putative transgenic plants were produced. Molecular analysis revealed that the *Gus* gene was integrated into the genome of transformed plants, and a histochemical GUS assay showed that the *Gus* gene was expressed in putative transgenic plants. In future, southern blot assays will be performed to determine copy numbers of the transgenes, and the putative transgenic plants containing the *OcI* gene tested in the greenhouse and the field for resistance to the banana weevil and the burrowing nematode. The successful transformation of Grande Naine reported in this work will contribute significantly towards improving Cavendish bananas in South Africa, and offers the opportunity to modify banana and plantain varieties cultivated in Africa for enhanced disease and pest resistance.

A highly discriminative fingerprinting technique, called amplified fragment length polymorphisms (AFLPs), was used to differentiate between closely related species within the Cavendish banana subgroup. AFLP profiles of eight banana varieties, cultivars and hybrids were generated on a Licor analyser using seven different primer combinations. Results showed that the banana plants were subdivided in clades according to their genomic composition. More importantly, the AFLP technique was able to separate the different cultivars within the Cavendish subgroup. Hopefully this technique could eventually be applied to accurately detect somaclonal variants in banana due to micropropagation and genetic modification.

OPSOMMING

Piesangs en planteins word kommersieël verbou as ’n soet, nagereg vrug en ook deur klein-skaalse boere as ’n stapelvoedsel geproduseer. Volhoubare verbouing word egter deur verskeie siektes en peste bedreig naamlik Fusariumverwelk (*Fusarium oxysporum* f.sp. *cubense*), swartsigatoka (*Mycosphaerella fijiensis*), piesangnuitkewer (*Cosmopolitus sordidus*) en die booraalwurm (*Radopholus similis*). Verskeie siektes en peste van piesangs kan nie deur konvensionele beheer metodes beheer word nie en vervangingskultivars word gewoonlik nie op die lokale industrie aanvaar nie. Weerstand in piesangs kan verkry word deur konvensionele en nie-konvensionele metodes. Konvensionele teel programme het baie perke soos steriliteit van verboude piesangs, lang groei siklusse, lae saad opbrengs, en hibriede word baie keer nie deur die verbruikers aanvaar nie. Nie-konvensionele metodes vir verhoogde weerstand sluit in: *in vitro* mutagenese, protoplast kulture en genetiese modifikasie. Tydens genetieses modifikasie word uitheemse gene in piesang geïnkoporeer met behulp van *Agrobacterium* transformasie of deur partikel bombardering.

Een van die kragtigste tegnieke om die invloed van pestes en siektes te verminder, is deur die gebruik van somatiese embriogenese met behulp van nie-konvensionele plant verbeterings en vermedering van siekte vrye plante. In hierdie tesis was onontwikkelde manlike blomme van Grande Naine (*Musa acuminata*, Cavendish subgroep, AAA) geisoleer en geinkubeer op MA1 medium om sodoende somatiese embrios met ideale kallus te vorm. Wanneer die ideale kallus na vloeibare MA2 medium oorgedra was, het ’n heterogeniese selsuspensie gevorm. Nie-embriogeniese aggregate was verwyder om sodoende ’n selsuspensie te verkry wat aleenlik bestaan uit klein embriogeniese groepe selle. Somatiese embrios is verkry van die selsuspensie deur die embriogeniese groepe selle uit te plaat op soliede MA3 medium. Hierdie somatiese embrios is oorgedra na MA4 medium vir ontkieming en daarna na P6 medium vir die ontwikkeling tot *in vitro* plante. Embriogeniese selsuspensies kan gebruik word vir genetiese transformasie vir siekte en pes weerstandbiedende plante, *in vitro* mutagenese, kiemplasma storing en protoplast kulture.

’n *Agrobacterium* transformasie sisteem is geïmplimenteer vir die verbetering van Cavendish piesang kultivars. Cavendish piesang kultivars is die enigste piesangs wat

vir die vars vrugte mark in Suid-Afrika geproduseer word. Embriogeniese selsuspensies van die kultivar, Grande Nain, was saam met *Agrobacterium*- wat verskille plasmiede bevat - naamlik pCambia1305.1, pART-TEST7 and pKYΩOCI, geinkubeer. Antibiotika weerstandbiedende embrios wat verkry is deur transgeniese selsuspensies het ontwikkel in piesang plante na 12 weke van inkubasie op MA4 medium. In totaal was 145 moontlike transgeniese plante geproduseer. Molekulêre analise het getoon dat die *Gus* geen geïntegreer was in die genoom van die getransformeerde plante en 'n histochemiese GUS-toets het getoon dat die *Gus* geen uitgedruk word in vermoedelike transgeniese plante. In toekomstige studies sal die Southern blot toets gebruik word om die aantal kopieë van die transgeen te bepaal. Vermoedelike transgeniese plante wat die *OcI* geen besit sal in die glashuis en in die veld proewe getoets word vir weerstand teen die piesang kewer en die booraalwurm. Die suksesvolle transformasie van Grande Naine in hierdie tesis sal verder betekenisvolle bydrae lewer tot die vebetering van Cavendish piesangs in Suid-Afrika en bied ook die geleentheid om piesangs en plantains, wat geproduseer word in Afrika vir weerstand teen siekte en peste, te transformeer.

'n Hoogs gediskrimineerde vingerafdruk tegniek naamlik amplifiseerde fragment lengte polimorfisme (AFLP) is gebruik om tussen naas gewante spesies in die Cavendish subgroep te onderskei. AFLP profiele van agt piesang variëteite, kultivars en hibriede is genenereer op 'n LICOR analiseerder met behulp van sewe verskillende voorvoerders kombinasies. Resultate het getoon dat die piesang plante onderverdeel word in subgroepe op grond van hulle genomiese samestelling. Meer belangrik het die AFLP tegniek die vermoë gehad om die verskillende kultivars binne die Cavendish subgroep van mekaar te skei. Hopelik kan hierdie tegniek ook gebruik word om somaklonale variante in piesang te identifiseer as gevolg van weefselkultuur en genetieses modifikasie.