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Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas

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Summary. A RAPD marker specific to the dwarf off-type (hereafter known as dwarf) from micropropagation of Cavendish banana (*Musa* spp. AAA) cultivars New Guinea Cavendish and Williams was identified following an analysis of 57 normal (true-to-type) and 59 dwarf plants generated from several different micropropagation events. Sixty-six random decamer primers were used in the initial screen, of which 19 (28.8%) revealed polymorphisms between normal and dwarf plants. Primer OPJ-04 (5'-CCGAACACGG-3') was found to amplify an approx. 1.5 kb band which was consistently present in all normal but absent in all dwarf plants of both cultivars. Reliable detection of dwarf plants was achieved using this marker, providing the only available means of *in vitro* detection of dwarfs. The use of this marker could facilitate early detection and elimination of dwarfs from batches of micropropagated bananas, and may be a useful tool in determining what factors in the tissue culture process lead to this off-type production. Other micropropagation-induced RAPD polymorphisms were observed but were not associated with the dwarf trait.

Introduction

Somaclonal variation has been observed in many plants that have passed through a tissue culture stage. Different types of cultured tissues have been ranked in order of low to high genetic instability as follows: micropropagation from isolated shoot tips and meristems, adventitious shooting, somatic embryogenesis and organogenesis from callus, cells, and protoplasts (Scowcroft 1984). In banana (*Musa* spp.), however, micropropagation using isolated buds and meristems has resulted in the production of higher than expected numbers of somaclonal variants (hereafter referred to as off-types), with frequencies ranging from 3 to 25% (Hwang and Ko 1987, Stover 1987, Smith and Drew 1990). Off-types have been noted for plant stature (mainly dwarf), leaf variegation and thickness, and fruit bunch characters (small bunches, hairy fruit). Of these, dwarfism is by far the most common off-type, accounting for 75% of the total observed micropropagation-induced variation in the Cavendish sub-group (*Musa* spp, AAA; Stover 1987, Israeli *et al.* 1991). Fruit bunches produced by the dwarf off-types (hereafter known as dwarfs) are of inferior commercial value, causing serious economic losses to the growers.

Visual detection of dwarfs from micropropagated bananas can be undertaken 3 to 4 months after establishment in the field (Smith 1988, Israeli *et al.* 1991). Earlier detection of dwarfs in the nursery is also possible by laboriously inspecting each plant for differences in height, petiole length and leaf morphology (Smith and Hamill 1993), but is only possible if the plants are grown under optimal conditions. When grown under stressed or less-than-optimal conditions, dwarfs escape detection at high frequencies. Thus, it is important to develop a reliable technique for the early detection of dwarfs prior to planting in the field. The application of gibberellic acid (GA_3) to detect dwarfs *in vitro* has been attempted (Reuveni 1990, Damasco *et al.* 1996), and dwarfs have been shown to be less responsive to GA_3-induced leaf-sheath elongation. However, misclassification occurred in 5-10% of cases even when the screen was applied under the most stringent conditions (Damasco *et al.* 1996). Other measurements such as total plant protein have not revealed any differences between normal and dwarf plants (Reuveni *et al* 1986) and therefore have no obvious use as screening techniques for dwarfs.

Random Amplified Polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR) based technique which uses random primers to generate DNA fragments which can be used as genetic markers (Williams *et al.* 1990). Polymorphisms generated by RAPD analysis have been used for fingerprinting in many plant species including sweet potato (*Ipomoea batatas* L.; Connolly *et al.* 1994) and papaya (*Carica papaya* L.; Stiles *et al.* 1993) cultivars. RAPD analysis has also been used to classify genotypes of *Musa* representing the AA, AAA, AAB and BBB genome (Howell *et al.* 1994). Linkage of RAPD markers to specific traits such as

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disease resistance (Miklas *et al.* 1993, Penner *et al.* 1993) has been been possible. RAPD or any other PCR-based analysis would be an attractive method for detection of dwarfs produced during banana micropropagation, because it needs only small amounts of template DNA, amounts available from a small tissue sample from an *in vitro* grown plant. The technique would be rapid, with results obtained in two days; thus, dwarfs could be eliminated early in the micropropagation cycle. RAPD detection of dwarfs could also help to identify and understand the event(s) in the tissue culture process which lead to genetic change.

The following study was initiated to develop a RAPD technique for the early detection of dwarfs following micropropagation. Specifically, the aims were to (1) obtain reproducible amplifications in banana, (2) identify a RAPD marker that would differentiate dwarfs from normals, and (3) to test the commercial application of the RAPD marker.

Materials and methods

Plant materials. All plant materials were obtained from the banana collection of the Queensland Department of Primary Industries' Maroochy Horticultural Research Station, Nambour, Queensland, Australia (Table 1). Normal (true to type) and dwarf plants of Cavendish (*Musa* spp. AAA) cultivars New Guinea Cavendish and Williams were previously obtained from micropropagation in the late 1980s. These plants were characterised morphologically (Smith and Drew 1990, Daniells and Smith 1991, Smith and Hamill 1993) and maintained for several generations in the field. Dwarf Parfitt, a naturally occurring extra-dwarf Cavendish cultivar, was also included in the study to determine if the dwarf mutation generated from micropropagation is the same as that occurring in somatic mutants. Suckers were collected from these plants and micropropagated according to Drew and Smith (1990). *In vitro*, glasshouse-grown (New Guinea Cavendish C420, Williams C76 and C117 and Dwarf Parfitt) and field-grown (New Guinea Cavendish C75 and C529 and Williams C251, C271 and C117) normal and dwarf plants were analysed using RAPD. The accession number (e.g. C117) represents the original clonal plant from which original tissue cultures were initiated. In total, 116 (57 normals, 59 dwarfs) plants were used for RAPD analysis (Table 1).

DNA extraction. Total genomic DNA was extracted from fresh leaves of *in vitro*, glasshouse and field-grown plants using a modified CTAB method (Graham *et al.* 1994). Larger quantities of leaf tissues (1-2 g) were frozen in liquid nitrogen and ground in a mortar and pestle. Smaller leaf tissues obtained from *in vitro* plantlets (< 0.2 g) were frozen and ground in a microfuge tube (1.5 ml) using a micropestle. The DNA pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the amount was determined using a spectrophotometer.

DNA amplification and electrophoresis. DNA amplification reactions were performed in volumes of 25 µl containing reaction buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 2.5 to 4.0 mM $MgCl_2$ (depending on primer), 400 µM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 0.25 µM of random decamer primer (Operon Technologies, Alameda, CA, USA), 50 ng of banana genomic DNA, 1.0 unit of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) and overlaid with a drop of paraffin oil. Amplification was performed in a 480 Perkin Elmer DNA thermocycler (Perkin Elmer Corp., Norwalk, CT, USA). An initial denaturation temperature of 94°C for 5 min was followed by 45 cycles each at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. The amplification products were analysed by gel electrophoresis in 1.2 % agarose (Promega, Madison, WI, USA) containing ethidium bromide (0.25 µg ml^{-1}). The amplification products were visualized under UV light (302 nm) and photographed using 667 polaroid film (Polaroid (UK) Ltd, Hertfordshire, England).

The reproducibility of random amplification was determined by evaluating the influence of DNA and $MgCl_2$ concentration across a range of primers. DNA concentrations of 5, 10, 25, 50, 75 or 100 ng from normal New Guinea Cavendish plants were tested using primers OPA-12, OPH-09, OPJ-04 or OPJ-10 at 2.5 mM $MgCl_2$. $MgCl_2$ concentrations of 1.5, 2.0, 2.5, 3.0, 4.0 or 5.0 mM were tested using primers OPA-06, OPH-09, OPH-13, OPJ-04, OPA-10, OPA-13 or OPS-04 using 50 ng DNA from normal New Guinea Cavendish. Each DNA or $MgCl_2$ concentration was replicated three times (DNA extracted from three plants), and the experiments were repeated twice to assess reproducibility.

Identification of polymorphisms between normals and dwarfs. Initially, the usefulness of RAPD analysis in detecting polymorphisms between normal and dwarf bananas was determined using *in vitro*-grown New Guinea Cavendish C420. Sixty-six random decamer primers from kits A (OPA-01,04, 07, 09-13, 18-20), H (OPH-01-20), J (OPJ-01-15), S (OPS-01-05) and U (OPU-01-15) were used. Five *in vitro* normal and 5 *in vitro* dwarf plants were tested for each primer. The primers which initially revealed polymorphisms between normal and dwarf plants were further evaluated for markers specific to the dwarf plants using 81 micropropagated plants from New Guinea Cavendish (20 normals, 21 dwarfs) and Williams (22 normals, 18 dwarfs). The RAPD marker identified as being specific to the dwarfs was further tested on Williams C76 (10 normals, 10 dwarfs) and Dwarf Parfitt (5 plants).

Testing of the RAPD marker under commercial micropropagation conditions. The dwarf-specific marker was used to analyse micropropagated plants originating from a commercial tissue culture laboratory in southeast Queensland. A random sample of 130 plants was taken from micropropagated Williams (C594 and C595). These plants were taken at a late stage in a typical micropropagation cycle , i.e., after > 2,000 plants have been produced per original explant. The DNA from leaf tissues (0.2 g) of *in vitro* plantlets at rooting stage was extracted and analysed by RAPD as previously described.

Results

Polymorphisms in New Guinea Cavendish and reproducibility of DNA amplification

The initial 66-primer screen amplified a total of 234 products with between 1 and 10 products generated per primer (data not presented). Nineteen of the 66 primers (28.8%) revealed polymorphisms between normal and dwarf plants, with 34 polymorphic products generated. Three primers (OPJ-02, OPJ-08, OPU-04) did not amplify any products.

The DNA and $MgCl_2$ concentrations in the reaction mixture were important for successful DNA amplifications in banana. The DNA concentration influenced the number and intensity of products amplified. DNA concentrations between 25 to 75 ng resulted in good amplification, and products that were easy to score. More products were amplified at 5 to 10 ng of DNA than at higher DNA concentrations; however, bands were less intense and difficult to score (data not presented). Some products that were amplified using 25 to 75 ng DNA per reaction were not amplified using 100 ng DNA.

The number of products amplified varied widely with the concentration of $MgCl_2$. Good amplification was obtained with 2.5 to 4.0 mM $MgCl_2$. At 1.5 mM $MgCl_2$, the number of products amplified for any given primer was reduced compared to other concentrations, and the majority of primers tested gave no amplification products. In scoring for polymorphisms, only products which were reproducibly amplified in different reactions were included. Using 50 ng of banana genomic DNA as template and 2.5 to 4.0 mM $MgCl_2$ (depending on primer), field, glasshouse or *in vitro*-grown plants of a particular cultivar produced the same scorable banding pattern.

Table 1. Plant materials used in the study.

Cultivar[1]	Type[2]	Acc. No.[3]	No. of Plants Tested			Initial Explant[4]	Place of collection[5]
			In vitro	Glass-house	Field		
NGC	N	C420	14	7	-	sucker	SJRS
	D	C420	14	5	-	sucker	SJRS
NGC	N	C75	-	-	4	floral apex	RB
	D	C75	-	-	5	floral apex	RB
NGC	D	C529	-	-	2	floral apex	C
Williams	N	C76	5	5	-	floral apex	SJRS
	D	C76	5	5	-	floral apex	SJRS
Williams	D	C271	-	-	1	sucker	SJRS
Williams	N	C251	-	-	7	sucker	MC
	D	C251	-	-	2	sucker	MC
Williams	N	C117	8	3	4	sucker	K
	D	C117	9	3	3	sucker	K
DP	D	M503	2	2	1	sucker	W
Total			57	30	29	116	

[1] NGC- New Guinea Cavendish, DP - Dwarf Parfitt (naturally occurring extra dwarf cultivar)
[2] N - normal, D - dwarf
[3] Accession number of banana plant materials obtained at Maroochy Horticultural Research Station, Nambour, Australia. Each accession represents at least one independent tissue culture event.
[4] Plants from sucker were regenerated through axillary and adventitious budding while plants from floral apex tissue were regenerated from callus through adventitious budding.
[5] SJRS - South Johnstone Research Station, South Johnstone, RB - Redland Bay,
C - Cleveland, MC - Mena Creek, K - Kennedy, W- Wamuran, Queensland, Australia.

Dwarf specific marker

The profiles of amplified products from New Guinea Cavendish (C529 and C75), and Williams (C251 and C117) and their associated micropropagation-produced dwarfs were compared for identification of markers specific to dwarfs. Of the 19 primers which revealed polymorphisms in the initial primer screen using normal New Guinea Cavendish C420 and its respective dwarf, only 10 primers revealed polymorphisms for New Guinea Cavendish C75 and C529, 7 primers for Williams C251 and C271, and 4 primers for Williams C117. The majority of the polymorphisms generated were not associated with dwarfism. Six primers (OPA-06, OPH-13, OPJ-04, OPJ-10, OPJ-13, OPU-06) were able to differentiate between normal and dwarf New Guinea Cavendish C420, C75 and C529, and 2 primers (OPJ-04, OPJ-13) differentiated between normal and dwarf Williams C251 and C117 (Table 2). Of the 6 primers which differentiated normals and dwarfs of different accessions, the primer OPJ-04 was found to consistently amplify an approx. 1.5 kb band (referred to as OPJ-04_{1500}) present in all normal but absent in all dwarf plants of New Guinea Cavendish (3 accessions) and Williams (2 accessions) (Table 2, Fig.1). This primer was further tested on normal and dwarf Williams C76. The OPJ-04_{1500} band was consistently present in all normal but absent in all dwarf plants.

Polymorphisms among normal plants and among dwarf plants were also detected. The number of products amplified with the primer OPJ-04 ranged from 7 to 11 in normals and 4 to 10 in dwarfs. The naturally occurring extra dwarf cultivar, Dwarf Parfitt, gave a similar OPJ-04 RAPD profile to the normal Williams and New Guinea Cavendish plants (Fig. 1A), and band OPJ-04_{1500} was present in all individuals tested.

Testing of the RAPD marker under commercial micropropagation conditions

Micropropagated Williams (C594 and C595) produced by a commercial tissue culture laboratory revealed a high level of polymorphism with primer OPJ-04 (Fig. 2). The number of products amplified ranged from 3 to 11. Of the 130 *in vitro* plants sampled at random, 34 (23%) plants showed the absence of OPJ-04_{1500} band present in normals.

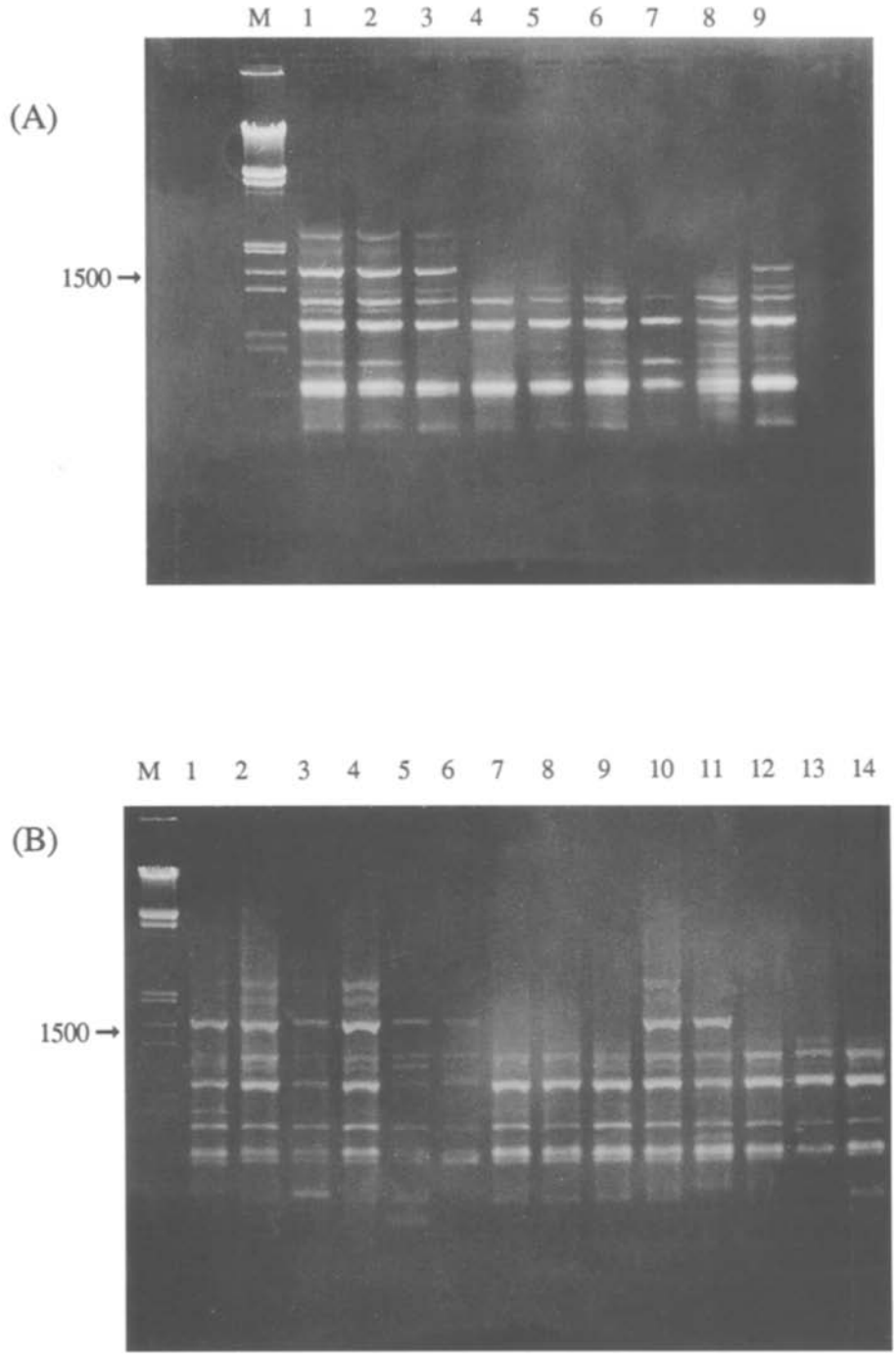

Figure 1. RAPD profile of Cavendish cultivars obtained with primer OPJ-04: (A) lanes 1-3 New Guinea Cavendish normals, 4-8 dwarfs, 9 Dwarf Parfitt, (B) lanes 1-6 Williams C251 normals, 7-9 dwarfs, 10-11 Williams C117 normals, 12-14 dwarfs, M - molecular weight marker, lambda DNA cut with *Eco*RI and *Hind*III (Progen Industries Ltd., Qld, Australia). Arrow indicates the OPJ-04_{1500} band.

Table 2. Primers which revealed polymorphisms between normal and dwarf plants of New Guinea Cavendish (NGC, 3 accessions) and Williams (2 accessions).

Primer	Approximate fragment size (kb)	Cultivar and Accession number[1]							
		NGC (C420)		NGC (C529, C75)		Williams (C251)		Willliams (C117)	
		Normal	Dwarf	Normal	Dwarf	Normal	Dwarf	Normal	Dwarf
OPA-06	0.6	-[2]	+	-	+	+	+	+	+
	1.3	-	+	-	-	-	-	-	+
OPH-13	1.4	+	-	+	-	-	-	-	-
	1.3	-	+	+	+	-	+	+	+
OPJ-04	1.5	+	-	+	-	+	-	+	-
OPJ-10	1.3	-	+	-	+	+	+	+	+
	1.4	+	+	+	+	+	-	+	+
OPJ-13	1.3	-	+	-	+	+	+	+	+
	0.7	+	+	+	+	+	-	+	-
OPU-06	0.7	-	+	-	+	+	-	-	-
OPU-08	0.8	-	+	+	+	+	-	+	+

[1]Each accession represents at least one independent tissue culture event
[2](+) presence/(-) absence of amplified band

Discussion

A RAPD marker specific to dwarf plants from micropropagation was identified after an analysis of 57 normal and 59 dwarf plants generated from several different micropropagation events. The RAPD band, OPJ-04_{1500}, was consistently present in all normals and absent in all dwarfs for all accessions of two Cavendish banana cultivars (New Guinea Cavendish and Williams). Furthermore the band was absent in all dwarfs tested regardless of initial explant source (sucker or floral apex) or mode of shoot regeneration (axillary or adventitious budding). The results therefore suggest that only one type of dwarf mutation exists in all of these micropropagated plants.

The presence of the RAPD band was tested further in micropropagated banana (cultivar Williams accessions C594 and C595) plants produced in a commercial tissue culture laboratory. Twenty-three percent (34 out of 130) of the plants sampled showed the absence of RAPD OPJ-04_{1500} band suggesting that the rate of dwarf production would be 23%. This percentage of predicted dwarf off-type is within the range expected from these materials which had undergone a higher multiplication rate (>2,000 plants per original explant) than normally performed (ca 1,000 plants per original explant) in a commercial banana micropropagation situation (F. Benson, Benson Micropropagation Pty Ltd, Pallara, Queensland personal communication). This marker presents the first available diagnostic method to reliably identify all dwarf plants *in vitro*, a situation not achievable based on morphology (Smith and Hamill 1993), even with the carefully monitored spray application the phytohormone GA_3 (Damasco *et al.* 1996).

Other DNA polymorphisms observed in micropropagated bananas were not associated with the dwarf trait and could be due to other somaclonal changes during micropropagation. The polymorphism observed with the Dwarf Parfitt (a naturally occurring extra-dwarf) and micropropagation-induced dwarfs from New Guinea Cavendish and Williams suggests that different dwarf mutations must have occurred and that the dwarf trait marker is associated with dwarfism in micropropagated Cavendish bananas only.

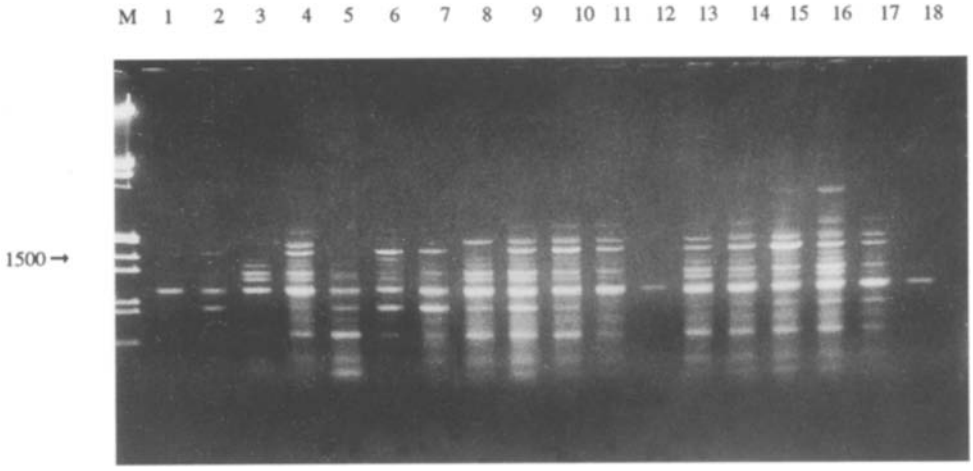

Figure 2. RAPD profile of micropropagated Williams obtained with primer OPJ-04. Lanes 1-9 Williams C594, 10-18 Williams C595, M - molecular weight marker, lambda DNA cut with *Eco*RI and *Hind*III (Progen Industries Ltd., Qld, Australia). Arrow indicates the OPJ-04_{1500} band.

The results of the present study demonstrate that RAPD analysis can be used to detect genetic variation in micropropagated Cavendish bananas. Earlier work by Rani *et al.* (1995) has demonstrated the sensitivity of RAPD for genetic analysis in micropropagated plants of poplar (*Populus deltoides* Marsh). RAPD analysis has also revealed gross genetic change in a sugarcane (*Saccharum officinarum* L) protoplast-derived callus line which had been in culture for over 2 years and had lost its ability to regenerate (Taylor *et al.* 1995). RAPD analysis has also been used to detect variation in gamma-irradiation induced mutants of the Cavendish cv Grand Naine (Kaemmer *et al.* 1992).

The use of the dwarf specific RAPD marker at the *in vitro* stage affords a reliable means for early of detection of dwarfs allowing for the elimination of dwarfs before planting of micropropagated plants in the field. In addition this RAPD marker could be particularly useful for testing the genetic integrity of *in vitro* stock materials. Those shoots with a normal banding pattern could be micropropagated further while those showing dwarf banding pattern should be discarded. The use of proliferated shoots with a normal banding pattern for further multiplication would reduce the cost of micropropagating bananas as compared to initiating micropropagation from new suckers. Further micropropagation of these normal shoots, however, could also result in production of new dwarf off-types. A final use of the RAPD marker would be to facilitate the study of factors influencing the occurrence of dwarfs during micropropagation. In banana the study of these factors is limited due to the cost and length of time in evaluating micropropagated plants. Since detection of dwarfs can be done *in vitro*, different tissue culture factors can be studied simultaneously, more plants can be evaluated, and the results can be obtained in a shorter period of time. The dwarf specific RAPD marker has been cloned and sequenced and primers homologous to this sequence have been designed for specific PCR (Damasco *et al.* in preparation).

Further work is in progress to characterise the nature of the somaclonal variation mechanism that has produced the dwarf. Of particular interest is pinpointing the locus of this genomic alteration. In addition this marker will be a useful tool in determining whether the polymorphism is pre-existing and enriched by the micropropagation technique, or if the genomic alteration is actually induced in culture.

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