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 (GA3) to detect dwarfs in vitro has been attempted (Reuveni 1990, Damasco et al. 1996), and dwarfs have been shown to be less responsive to GA3-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...
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, Daniels and Smith 1991, Smith and Harlili 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. Shoots were collected from these plants and micropropagated according to Drew and Smith (1990). In vitro, glasshouse-grown (New Guinea Cavendish C420, Williams C276 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 MgCl2, 50 mM KCl), 2.5 to 4.0 mM MgCl2 (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 MgCl2 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, OPJ-09, OPJ-04 or OPJ-10 at 2.5 mM MgCl2. MgCl2 concentrations of 1.5, 2.0, 2.5, 3.0, 4.0 or 5.0 mM were tested using primers OPA-06, OPJ-09, OPJ-13, OPJ-04, OPJ-10, OPJ-13 or OPJS-04 using 50 ng DNA from normal New Guinea Cavendish. Each DNA or MgCl2 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 (OPJ-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 MgCl2 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 MgCl2. Good amplification was obtained with 2.5 to 4.0 mM MgCl2. At 1.5 mM MgCl2, 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 MgCl2 (depending on primer), field, glasshouse or in vitro-grown plants of a particular cultivar produced the same scorable banding pattern.