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Random Amplified Polymorphic DNA (RAPD) Detection of Somaclonal Variants in Commercially Micropropagated Banana (*Musa* spp. Cultivar Grand Naine)

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ABSTRACT

In a micropropagation program, it is of paramount importance to produce true-to-type planting material; somaclonal variation of any kind is undesirable. Variation among plants regenerated from tissue culture is termed 'somaclonal variation'. This article discusses various factors due to which somaclonal variations may arise. The somaclonal variation may be detected by visual screening or by using molecular markers such as Randomly Amplified Polymorphic DNA. In commercially micropropagated banana (*Musa* sp. cultivar Grand Naine) Dwarf and Giant are most common off-type plants observed. The present work was carried out to assess genetic fidelity of micropropagated plants using Randomly Amplified Polymorphic DNA. Plants showing normal and off-type morphology were selected from green house and field. Two arbitrary decamer primers have been used to amplify genomic DNA from selected plants (primers OPH-09 and OPJ-04) OPJ-04 showed polymorphism in the banding pattern as compared to normal plants. A total of 6 amplified products were obtained, out of which 5 were polymorphic.

Key words: Randomly amplified polymorphic DNA, banana, primers, somaclonal variation

INTRODUCTION

Bananas and plantains were one of the world's important food commodity and rank fourth in terms of gross value, exceeded only by rice, wheat and milk/milk products. These were important staple food crops in the humid and sub-humid tropical regions of the world. Banana has provided nourishment and a well-balanced diet to millions of people around the globe and has contributed to livelihood through crop production, processing and marketing. India have been the largest producer of banana with an annual production of 11.7 million tones on 404,000 ha, contributing to 27% of the world production and about 37% of the total fruit crop production in the country (FAOSTAT, 2006). India has a rich genetic diversity of banana with more than 90 distinct clones. Production was limited by viral and fungal diseases as well as insects and nematode pest problems. The application of classical methods of breeding for both disease and pest resistance has been resulted in limited success due to long generation times for banana and high sterility and triploidy of most cultivated banana (Sasson, 1997).

The *in vitro* amplification of DNA by the Polymerase Chain Reaction (PCR) has been proven to be a revolutionary technique in molecular biology. PCR have facilitated the in vitro amplification of DNA by using two oligonucleotide primers complimentary to opposing DNA strands. By

appropriate selection of prime sites and reaction conditions, the chain extension reactions will result in synthesis of new DNA strands for each of the primer recognition sites. By combining a thermal-stable DNA polymerase with repeated cycling of reaction conditions favoring DNA template denaturation, Followed by template prime annealing and finally chain extension, exponentially DNA amplification occurs. Beginning with as little as one molecule of template DNA, microgram quantities of a specific DNA fragments were produced in a couple of hours.

Several PCR techniques have been developed that allow amplification of targets when sequence information is limited or unknown. RAPD-PCR has been used to characterize and identify several plant varieties. Unlike conventional PCR assays which utilize two primers of a defined sequence, RAPD-PCR detects nucleotide sequence polymorphism in a DNA-amplification-based assay using only one primer of arbitrary nucleotide sequence. The short length decamer, single primer has been bound to the genome on opposite strands in an inverted orientation and produces a RAPD-PCR product that has been partial or complete homology with the arbitrary primer sequence at each end.

RAPD and Inter Genic Spacer (IGS) polymorphisms occur because of insertions, deletions and base substitutions that affect the primer-binding site and reflected as the presence of absence of bands. RAPD-PCR and IGS have the advantage of being fast, inexpensive, conservative in the use of genomic DNA, does not involve radioactive isotopes and applicable to many isolates, cultivar etc., because of random nature of the primer sequence for RAPD-PCR and specified sequence for IGS.

MATERIALS AND METHODS

Plant material: Micropropagated off type (mutant) and normal (wild) banana (*Musa* spp., cultivar Grand Naine) plants were selected in Seema Biotech, Talsande Warananagar. These selected plants were from green house which were hardened for 8 weeks. Some plants from field were also selected. The Fresh and young leaves were collected from those plants.

Extraction of genomic DNA:

- Two hundred milligram of plant tissue was ground to a fine paste in approximately 500 μL of CTAB buffer
- CTAB/plant extract mixture was transfered to a microfuge tube
- The CTAB/plant extract mixture was incubated for about 30 to 35 min at 55°C in a recirculating water bath
- After incubation, CTAB/plant extract mixture was spin at 12000 g for 10 min to spin down cell debris. The supernatant was transfered to clean microfuge tubes
- 250 μL of chloroform was added to each tube: Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, the tubes were spin at 13000 rpm for 10 min
- The upper aqueous phase only (contains the DNA) was transfered to a clean microfuge tube
- To each tube add 50 μL of 7.5 M Ammonium Acetate followed by 500 μL of ice cold absolute ethanol
- The tubes were inverted slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for overnight at -20°C after the addition of ethanol to precipitate the DNA

Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticked to the pipette and was visible as a clear thick precipitate. To

wash the DNA, the precipitate was transfered into a microfuge tube containing 500 μL of ice cold 70% ethanol and slowly inverted the tube. Repeat. (Alternatively the precipitate can be isolated by spinning the tube at 13000 rpm for a min to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70% ethanol).

- After the wash, the DNA was spin into a pellet by centrifuging at 13000 rpm for 10 min. Removed all the supernatant and allowed the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve
- The DNA was resuspended in sterile DNase free water (approximately 200 μL H_2O; The amount of water needed to dissolve the DNA can vary, depending on how much was isolated). RNaseA (10 μg mL^{-1}) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 μL RNaseA in 10 mL H_2O)
- Agarose gel electrophoresis of the DNA will show the integrity of the DNA while spectrophotometry will give an indication of the concentration and cleanliness

DNA quantification: The isolated DNA was quantified in UV spectrophotometer using absorbance ratio at 260 for DNA and 280 nm for protein.

- A 1% solution of agarose was prepared by melting 1.25 g of agarose in 125 mL of 0.5x TBE buffer in a microwave for approximately 2 min and allowed to then 2.5 μL of ethidium bromide was added, stirred to mix
- A gel was casted using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface
- The following mixtures were loaded into separate wells
    - 8 μL 100 bp ladder
    - 5 μL sample +2 μL 6x Loading Buffer
    - The gel was run for 30 min at 50 V
- The gel was then exposed to UV transilluminator
- DNA quality was confirmed, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation

RAPD PCR: PCR was performed to amplify the DNA in the sample. (35 cycles). DNA polymorphism was checked using at random two primer, opj-4 and oph-9, respectively.

RESULTS AND DISCUSSION

Optimization of RAPD protocols ensured that the RAPD profiles were reproducible. Among the 2 random primers used for the initial screening, one failed to amplify distinct bands, while the other primer provided clear and scoreable amplification products in all samples.

Primer OPJ- 04 showed amplification and polymorphism. Six major bands were observed of which five are polymorphic. Polymorphic band generated by primer OPJ-04 1600 was prominently present in dwarf type plant only (Fig. 1).

The DNA from all plants were amplified by the primer OPH-09 but no distinct bands were visible. Only one was observed which was common to all samples (Fig. 2).

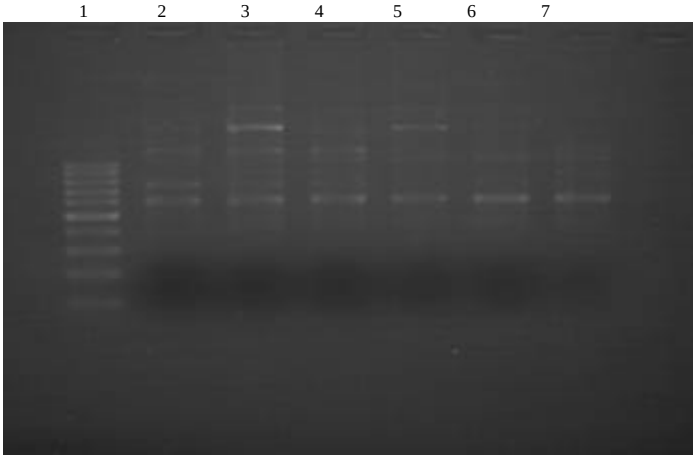

Fig. 1: Polymorphic band generated by primer OPJ-04 1600. Lane 1: Ladder, Lane 2: Normal, Lane 3: Dwarf, Lane 4: Tall, Lane 5: Mutant 1, Lane 6: Mutant 2, Lane 7: Mutant 3

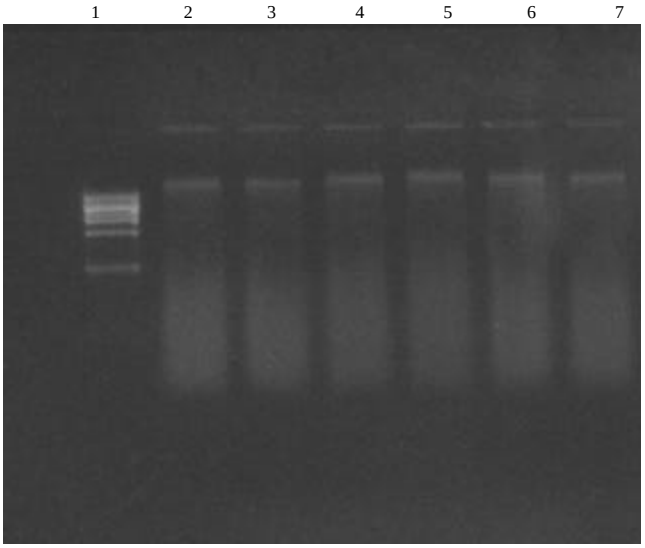

Fig. 2: Polymorphic band generated by primer OPH-09. Lane 1: Ladder, Lane 2: Normal, Lane 3: Dwarf, Lane 4: Tall, Lane 5: Mutant 1, Lane 6: Mutant 2, Lane 7: Mutant 3

The widespread use of plant tissue culture for the mass clonal propagation of *Musa* sp. is hampered by high frequency of undesirable off types. In particular in vitro micropropagation of Grand naine cultivar is prone to generation of dwarf and tall off-types. Somaclonal variants appear to be relatively stable and do not generally revert to a normal phenotype. Genetic changes resulting in these mutations are poorly characterized.

RAPD analyses are able to detect very small changes in genome of an organism. DNA markers are likely to prove important as frequency is high. RAPD markers are selected to detect variant plant as it is simple and fast. RAPD markers make it possible to differentiate between normal plant

and variant plants. The results demonstrated that RAPD analysis is useful to detection of somaclonal variant appeared during micropropagation of plant.

The results of present study demonstrated that RAPD analysis can be used to detect genetic variation in micropropagated bananas. The use of the dwarf specific RAPD marker at the *in vitro* stage affords a reliable means for early detection of dwarf allowing for the elimination of dwarf as well as tall before planting in field. In addition this RAPD marker could be particularly useful for testing the genetic integrity of in vitro stock material. Those shoots with normal banding pattern could be micropropagated further while those showing different pattern should be discarded. The use of proliferated shoots with a normal binding pattern for further multiplication would reduce the cost of micropopagating bananas as compared to initiating micropropagation from new suckers.

True totype clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem encountered with the *in vitro* culture has been the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of the *in vitro* culture of plant cells, tissues or organs. A better analysis of genetic stability of plantlets can be made by using a combination two types of markers which amplify different regions of the genome.

PCR- based techniques such as RAPD, ISSR and AFLP have been found immensely useful in establishing the genetic stability of cultivated as well as *in vitro* regeneration of plants.

In the present study, DNA extraction by CTAB method has been done. Also the conditions of PCR amplification such as magnesium chloride, template DNA, Taq polymerase concentration and annealing temperature were different and most crucial for each primer.

Dwarf somaclonal variants in *in vitro* cultures of banana (Cavendish banana), have been reported (Israeli *et al*., 1996) and such dwarf somaclones were relatively stable and did not generally revert to a normal phenotype (Ramage *et al*., 2004).

Sahijram *et al*. (2003) have analyzed somaclonal variation in micropropagated bananas *in vitro* Damasco *et al*. (1996) has done DNA (RAPD) detection of dwarf off-types in micropropaged Cavendish bananas, Smith and Hamill (1993) has done early detection of dwarf off-types from micropropagated Cavendish bananas. Aichitt *et al*. (1998) have reported the reproducibility of RAPD profiles: Effect of PCR components on RAPD analysis of date palm DNA.

Grajal-Martin *et al*. (1998) has shown the use of randomly amplified polymorphic DNA (RAPD) for the study of genetic diversity and somaclonal variation in *Musa*. Ramage *et al*. (2004) has simplified PCR test for early detection of dwarf off types in micropropagated Cavendish bananas (*Musa* spp. AAA). Williums *et al*. (1993) has done genetic analysis using with Randomly Amplified Polymorphic DNA markers. Similarly, Gubbuk *et al*. (2004) has conducted studies on identification and selection of superior banana phenotypes in the cultivar dwarf Cavendish using agronomic charachetristics and RAPD markers.

Common undesirable somaclonal variant is the mosaic type heterogeneity and such an incident of variation was has been reported in Cavendish banana (Israeli *et al*., 1996). Thus the observations made in various studies indicate that variability can occur within the clone, different explants within a plant, inter-clonal and inter varietal.

The polymorphism in DNA of normal, dwarf and mutant plant by using two primers OPJ-04 and OPH-09 has been studied and, this information can be used to develop the test for differentiation of plants on the basis of its genomic constitution.

Hence, it can be concluded that the purified DNA obtained from these methods can be used in further approaches in molecular biology. RAPD marker can be sequenced and DNA sequence can

be used to develop multiplex PCR for detection of somaclonal variants and virus indexing simultaneously.

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