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Stability of micropropagated *Musa acuminata* cv. Grand Naine over clonal generations: A molecular assessment

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ABSTRACT

The present work was undertaken to investigate clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) regenerants from six different *in vitro* subculture generations and in the explant suckers by using ISSR and REMAP molecular markers. Both types of markers revealed high degree of monomorphism. Very low variation was observed up to the eighth subculture generation with polymorphic bands being low in both ISSR (0.96%) and REMAP (0.95%) markers. Epigenetic stability was studied by DNA methylation analysis of the eighth subculture generation samples. Single 570 bp methylation sensitive band was absent in two of the fifteen *Msp*I predigested samples, while it was present in *Hpa*II predigested and undigested samples. The results of the investigation confirmed that the micropropagation of banana up to the eighth subculture generation show low variation.

1. Introduction

Banana (*Musa* spp.) and plantain are important fruit and vegetable crop of India. It is also known as a universal fruit crop of India. Banana is a crop grown in tropical and subtropical regions of the world. Bananas provide a staple starch in some of the poorest parts of the world. In India, the area under banana was 6.47 lakh hectares with the production of 2.32 million tons in the year 2007–08. It is 29% of the world's total production of this crop (Anonymous, 2008).

In vitro propagation of banana has played a key role in obtaining a large number of homogeneous regenerated plants. Micropropagation has played a key role in *Musa* improvement programmes worldwide (Rowe and Rosales, 1996; Vuylsteke et al., 1997). As compared to the conventional propagules, micropropagated banana plants establish faster and grow more vigorously. They yield higher in shorter duration with more uniform crop cycle (Vuylsteke and Ortiz, 1996). Maximum yield gains from *in vitro* derived plants range from 20% in bananas to 70% in plantains. However, this superior field performance does not appear to be consistent and requires optimum field practices. The application of micropropagation of *Musa* spp. has been reported using different explant sources such as shoot tips meristem (Ma and Shii, 1972; Banerjee et al., 1986), and suspension culture (Roux et al., 2001).

Somaclonal variation is usually observed when plants are regenerated from cultured somatic cells, mostly during callus formation and suspension culture. However, even in absence of de-/redifferentiation stress, as during the micropropagation, off-types are observed that reduce commercial values of resultant plants. Furthermore, most of the variants are inferior to the original cultivar from which they are derived. For example, bunch and fruit of these variants are often smaller (Smith and Drew, 1990), which offsets the potential benefits of variants. Dwarfism in 'Cavendish' bananas and inflorescence variations in plantains are often observed after micropropagation of respective mother genotypes.

The appearance of somaclonal variants may not be a process limited to *in vitro* propagation but it may occur naturally in plant somatic and reproductive tissues (Cullis and Kunert, 2000). Rates of somaclonal variations in plants derived from shoot-tip culture vary from 0 to 70% according to genotype (Israeli et al., 1995; Smith, 1988; Vuylsteke et al., 1991). This genetic instability may be a risk associated with the application of *in vitro* culture techniques for germplasm handling and storage. Various factors have all been shown to influence both the quantity and the type of somaclonal variation in micropropagated banana (Smith and Hamill, 1993). These factors include genotype, origin of shoots *in vitro* (adventitious or auxiliary buds), number of subcultures, the choice of explants and the degree of dedifferentiation of the tissues in culture.

Somaclonal variation can be either genetic or epigenetic (i.e. non-heritable). There may be variability even among clonally propagated plants of a single donor clone. Somaclonal variation includes point mutation, gene duplication, changes in number and the structure of chromosomes, transposable element movement and changes in DNA methylation (Jain, 2001). Epigenetic variation is

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an important mechanistic basis of somaclonal variation in plants. Epigenetic aspects of somaclonal variation involve mechanisms of gene silencing or gene activation that are not due to changes in sequence or chromosomal aberrations.

Several methods have been used to investigate the genetic variability present in *Musa* germplasm. Development and application of technologies based on molecular markers provide tools that are able to reveal polymorphism at DNA sequence level which is adequate to detect genetic variability. ISSR and REMAP markers are preferred PCR-based markers as they are reproducible, highly polymorphic, applicable for any crop and amenable to large scale throughput demands necessary for screening large plant populations. Inter-simple sequence repeats marker involves PCR amplification of the region between two closely placed simple repeat sequences that are inversely oriented. They are identified using primers designed from within the repeated region (Zietjiewicz et al., 1994). This technique is based on PCR amplification of intermicrosatellite sequences. Retrotransposons are abundant and dispersed components of most plant genomes and comprise over 50% of nuclear DNA content in many species. Genome diversifies through the insertion of new copies, but old copy persists. Retrotransposons can be used as markers because their integration creates new joints between genomic DNA and their conserved ends. The REMAP (Retrotransposons–Microsatellite amplified polymorphism) show amplification between proximate retrotransposons and simple sequence repeats (SSR) to produce the marker bands.

DNA methylation represents conversion of cytosine to 5-methylcytosine in plant genomes (Grant-Downton and Dickinson, 2005). DNA methylation does not change the DNA sequence or its function, but changes its expression level, referred as an epigenetic change. *Hpa*II and *Msp*I isoschizomer restriction enzymes are frequently used to detect cytosine methylation. They both recognize the same sequence 5′-CCGG. Such differentially digested DNA fragments can be detected by various techniques. However, these methods are appropriate for displaying a global picture of DNA methylation changes within a genome. But they are laborious and need locus specific primers and PAGE analysis. Use of methylation sensitive restriction enzymes in combination with arbitrary primers based PCR is the simplest method of detecting methylation changes in genomic DNA (Nakamura and Hosaka, 2009).

There is lot of data available on banana somaclonal variation at the phenotypic level in micropropagated bananas. However, the basis of this variation and its extent during multiple cycles of *in vitro* culture remains unknown. In the present investigation, attempts were made to study the clonal fidelity of regenerants in banana over different clonal generations using ISSR and REMAP molecular markers. Study on epigenetic stability of micropropagated banana plants was undertaken by ISSR analysis of genomic DNA digested with methylation sensitive isoschizomers i.e. *Hpa*II and *Msp*I.

2. Material and methods

2.1. Plant material

Fifteen banana leaf samples from each of the five subculture generation (i.e. 2nd, 5th, 6th, 7th and 8th) were collected from tissue culture laboratory of State Level Biotechnology Centre, Mahatma Phule Krishi Vidyapeeth, Rahuri, India. Shoot tip explants from field suckers were established *in vitro*, multiplied on Murashige and Skoog's medium (Murashige and Skoog, 1962) + 5 mg/l BAP and rooted on MS media + 3 mg/l NAA, solidified with agar (8 g/l) at 24 ± 2 °C. Five clumps, each with 3 shoots, were transferred to fresh medium in jamjars every 3 weeks for multiplication. From each sample 100 mg plant material was used for genomic DNA isolation. Banana suckers (thirteen samples) were collected from Banana Research Station, Jalgaon, India, for micropropagation purpose and they were also used for genomic DNA isolation.

2.2. Isolation of plant genomic DNA

The genomic DNA from tender leaves (100 mg) of different clonal generations of micropropagated banana were isolated by HiPurA Plant Genomic DNA Miniprep Purification Spin kit (M/S HiMedia Ltd.) after crushing in liquid nitrogen. Concentration and purity of isolated DNA was measured using UV visible spectrophotometer (NanoDrop ND-1000, USA) at 260 and 280 nm wavelengths and by visualization under 0.8% (w/v) agarose gel electrophoresis (BioRad Sub-Cell Model 96, USA).

2.3. DNA amplification by ISSR and REMAP markers

Polymerase chain reaction was carried out in 103 samples by using 12 ISSR and 12 REMAP primer pairs (3 retrotransposons based primers and 5 ISSR primers) as per protocol (specified by Teo et al., 2005; Kalendar et al., 1999). The annealing temperature in PCR was standardized for each ISSR primer and for each combination of REMAP primer pairs. PCR regime involved initial denaturation (94 °C) for 5 min, followed by PCR amplification cycles [40 cycles (for ISSR) and 30 cycles (for REMAP), respectively] of denaturation (94 °C), annealing (as specified in Tables 1 and 2) and elongation (72 °C) and then final extension at 72 °C for 10 min. In ISSR analysis each step of this step was carried out for 30 s. In REMAP amplification, denaturation and annealing steps were carried out for 1 min each, while elongation was performed for 2 min.

2.4. Agarose gel electrophoresis and data analysis of PCR products

For ISSR and REMAP analysis 1.2% and 2% (w/v) agarose gel electrophoresis was used for PCR product profiling, respectively. The amplified PCR products were observed under UV transilluminator in gel documentation system (Flour Chem™ Alpha Innotech, USA) and image was captured. The clearly resolved PCR amplified ISSR and REMAP bands of banana samples with 12 primer/primer combinations each, were scored for their binary data. The similarity and clustering analysis were carried out using the computer package NTSYSpc 2.02i (Rohlf, 1998).

2.5. DNA methylation analysis

DNA methylation was detected by predigesting with methylation sensitive restriction enzymes *Msp*I and *Hpa*II; then PCR amplification (ISSR) and then again followed by post-digestion with the same restriction enzymes. DNA methylation was detected according to the procedure described by Nakamura and Hosaka (2009) with some modifications.

All 15 genomic DNA samples (8th clonal generation) were divided into three different lots, *Msp*I digested, *Hpa*II digested (M/S Bangalore GeNei Pvt. Ltd.) and undigested, with volume of restriction digestion setup to 40 μl. The two digestion reactions were incubated at 37 °C for 6 h. After digestion, the DNA was ethanol precipitated, dried and dissolved in 40 μl autoclaved distilled water.

All 45 samples (3 treatments × 15 original 8th clonal generation samples) were PCR amplified with ISSR marker i.e. IS-13 primer. PCR amplification products (75 μl) were ethanol precipitated, and finally dissolved in 50 μl autoclaved distilled water.

For the second restriction digestion, each of the above 45 PCR amplified reactions was further subdivided into three lots. ISSR amplification product of *Msp*I genomic DNA digestion was subdivided in three different lots again like *Msp*I digested, *Hpa*II digested and undigested respectively. The same subdivision procedure was