IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Identification of Molecular Markers to Detect Somaclonal Variations in Banana cv. Grand Naine (AAA) Using Sequence Related Amplified Polymorphism (SRAP) Molecular Marker Technique

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Abstract: The present study was designed with the main aim of detection of variations of somaclones in the banana cv. Grand Naine (AAA) using sequence related amplified polymorphism (SRAP) molecular marker technique. Matured leaves that are free from damage caused by pests or disease were collected in brown covers from different field places Karnataka state. Isolation of genomic DNA was carried out by cetyl trimethyl ammonium bromide. The integrity and purity of DNA and visualization of amplified products was done by electrophoresis and quantified. PCR amplification of DNA from banana somaclones using SRAP markers were carried out using Taq DNA polymerase and short stretch of oligonucleotides (primers), which are specific to the DNA to be amplified. Then the SRAP, markers are used to assess the genetic variation in somaclonal variants of banana cv. Grand Naine (AAA). The amplified products of SRAP markers were separated using agarose gel electrophoresis technique. out of 44 primers combination used, only 8 combinations showed polymorphism. The 8 primer combinations showed very good intense polymorphic bands. SRAP primer combinations EM3+ME3, EM2+ME6, EM5+ME5, EM7+ME7 showed clear polymorphism. In conclusion, overall 19.4% polymorphism between dwarf off type and the normal banana cv. Grand Naine (AAA) plants was obtained.

Index Terms **- SRAP, banana cv., Grand Naine (AAA), Polymorphism, Molecular markers.**

I. INTRODUCTION

Bananas are the most common fruit crop in tropical and subtropical regions [1]. Micropropagative banana planting materials have many advantages compared with vegetative propagation. Somatic clonal mutations, whether genetic or epigenetic, are considered to be an integral part of the *in-vitro* regeneration process. Somatic clonal variants are an important source of genetic variation, especially in vegetatively propagated plants, and have been used as tools for plant improvement and new variety development [2-3]. The important characteristics of selection *in-vitro* propagation are its huge ability to reproduce in a relatively short period of time, the production of healthy and disease-free plants, and the ability to produce propagules throughout the year [5]. These variations are often undesirable in a tissue culture industry where the main aim is production of "true to type" plants. Characterization of off-type variants for traits superior to mother plants in terms of yield, fruit quality, resistance to biotic or abiotic stresses and higher regeneration efficiency in tissue culture media might lead to development of new cultivar. Dwarf Cavendish, Grand Naine Israel, Lancefield, Chinese Cavendish are some proven examples in which somatic clone variants/mutants have been characterized, selected and released as new varieties in different regions of the world.

However, some somaclonal variants also show inferior traits compared with the female parent plant, and cause huge economic and yield losses to farmers and tissue culture industries. Therefore, it is important to characterize, select, and evaluate somaclonal variants to detect the presence of excellent traits and atypical traits observed in-vitro or during farmer's field regeneration. Morphological description, physiological monitoring, karyotype, biochemical estimation, and field evaluation are commonly used to describe and

characterize such soma clonal variants. Since most of these traits are controlled by many genes with additional or environmental interactions, they are still highly subjective and difficult to identify. Recently, molecular identification and characterization techniques are being used efficiently and effectively manage plant genetic resources. The genetic consistency of plants grown in-vitro is also a prerequisite for the production of high-quality banana planting materials [6].

Somaclonal variation arising from in-vitro production of plantlets is associated with DNA novel and heritable phenotypic variation which could be epigenetic or genetic [7]. The present day molecular markers have complemented traditional methods to detect genetic variants, monitor genetic fidelity and varietal development of asexually propagated plants. Thus, DNA markers based characterization of somaclonal variants showing discrete elite or off-type characters followed by identification of markers associated to superior traits (if any) would be extremely beneficial. With this scenario, the present study was undertaken with main aim of detection of variations of somaclones in the banana cv. Grand Naine (AAA) by using sequence related amplified polymorphism (SRAP) molecular marker technique.

II. MATERIALS AND METHODS

Source of Planting Materials

Matured leaves that are free from damage caused by pests or disease were collected in brown covers from different fields places like Anekal, Kadur, Hulimavu, Hesaraghatta. Source of these micropropagated planting materials were from Khodays Biotech, MSR Biotech, Green earth, Ramco Biotech companies.

Isolation of Genomic DNA by CTAB Method (Cetyl Trimethyl Ammonium Bromide)

This is an efficient method for isolating plant genomic DNA from leaf tissues. It provides high quality preparation of high molecular DNA. CTAB is used to liberate the nucleic acid from cell which was further purified by phenol-chloroform to remove proteins and other contaminating plant debris. The protocol for isolation of genomic DNA was carried according to Porebski et al. 1997 [8].

PCR Amplification of DNA

Polymerase chain reaction (PCR) is a very simple method for in-vitro amplification of specific nucleic acids using Taq DNA polymerase and short stretch of oligonucleotides (primers), which are specific to the DNA to be amplified. It is carried out in three steps at discrete temperatures. a) Heat denaturation of template DNA at 94-98°C, b) Annealing of primers to template DNA at 35-55°C and c) Primer extension from their 3' ends at 72°C. These steps are repeated as cycles (30 to 40). The length of the product generated during the PCR is equal to the sum of the length of the primers plus the distance in the target sequence [9].

SRAP to Detect Genetic Variation in Micropropagated Banana cv. Grand Naine

SRAP is a new and useful molecular marker system to target coding sequences and results in the identification of a number of co-dominant markers. SRAP is based on two primer amplification where the primers are 17-18 nucleotides long. Primer consists of a core sequence of 13 or 14 bases, where the 5' most 10 or 11 bases are non-specific followed by the sequence of CCGG in the forward primer and AATT in the reverse primer. The core sequence is followed by 3 selective nucleotides at 3' end of each primer. SRAP is reproducible, simple and faster than amplified fragment length polymorphism (AFLP) [10]. Around 44 SRAP primer combinations were used to check the genetic variation between dwarf-off type and normal banana plant. The list and sequence of SRAP primers used in our research investigation as represented in Table 1.

Table 1: SRAP Sequences Used for Screening

Forward Primers	
Me1	TGA GTC CAA ACC GGA TA
Me2	TGA GTC CAA ACC GGA GC
Me3	TGA GTC CAA ACC GGA AT
Me4	TGA GTC CAA ACC GGA CC
Me5	TGA GTC CAA ACC GGA AG
Me6	TGA GTC CAA AC GGA CA
Me7	TGA GTC CAA ACC GGA CG
Me8	TGA GTC CAA ACC GGA CT
Me9	TGA GTC CAA ACC GGA GG
Me10	TGA GTC CAA ACC GGA AA
Me11	TGA GTC CAA ACC GGA AC
Me12	TGA GTC CAA ACC GGA GA
Me13	TGA GTC CAA ACC CGA AG
Reverse Primers	
Em1	GAC TGC GTA CGA ATT AAT
Em2	GAC TGC GTA CGA ATT TGC
Em3	GAC TGC GTA CGA ATT GAC
Em4	GAC TGC GTA CGA ATT TGA
Em5	GAC TGC CTA CGA ATT AAC
Em6	GAC TGC GTA CGA ATT GCA
Em7	GAC TGC GTA CGA ATT CAA
Em8	GAC TGC GTA CGA ATT CAC
Em9	GAC TGC GTA CGA ATT CAG
Em10	GAC TGC GTA CGA ATT CAT
Em11	GAC TGC GTA CGA ATT CTA
Em12	GAC TGC GTA CGA ATT CTC
Em13	GAC TGC GTA CGA ATT CTG
Em14	GAC TGC GTA CGA ATT CTT

Resolving DNA Amplification Products

The DNA fragments were resolved based on their molecular weight using agarose gel electrophoresis. The size of the fragment was determined by comparing mobility of DNA fragments of known size and mobility, such as 1 kilo base pair DNA ladder (marker). After separation, DNA was visualized under transilluminated UV light (302 nm) after staining the gel with ethidium bromide.

III. RESULTS

Occurrence of somaclonal variation in micropropagated banana is a serious problem facing the tissue culture industry. In the present study, involves identification of field grown dwarf off-types with molecular marker techniques which would help in elimination of such types from micropropagated banana. Experiments were conducted to identify markers specific to the variants using SRAP molecular marker technique. Morphological variants like dwarf off-type without bunch, bunch variants were chosen for marker identification as they were causing serious economic loss to the farmers.

44 primer combinations of SRAP were screened to investigate the genetic variations between dwarf off-type and normal banana plants. 8 combinations showed inconsistency in amplification.

The results of combinations of SRAP primer used and the number of bands (MB & PB) obtained were depicted in Table 2 and 3, and the band profile of SRAP was as shown in Plate 1 (Figs. a-d). The results revealed that out of 44 primers combination used, only 8 combinations showed polymorphism. The 8 primer combinations showed very good intense polymorphic bands. SRAP primer combinations EM3+ME3, EM2+ME6, EM5+ME5, EM7+ME7 showed clear polymorphism (Plate 1, Fig. b-d).

Table 2: Monomorphic and polymorphic bands amplified by SRAP primers for normal and dwarf off-type of banana cultivar Grand Naine

S. No.	SRAP Primer Combi nations	No. of MB	No. of PB	No. of PB in true to types banana	No. of PB in dwarf off types banana	No. of MB + PB	Polymorphism (%)
1.	Em8 & Me5	1	1	0	1	2	3.30
2.	Em7 & Me5	2	1	0	1	3	3.30
3.	Em8 & Me4	1	3	0	3	4	10.00
4.	Em2 & Me6	4	2	2	0	6	6.60
5.	Em5 & Me5	3	1	1	0	4	3.30
6.	Em3 & Me3	1	2	0	2	3	6.60
7.	Em2 & Me4	2	2	0	2	4	6.60
8.	Em2 & Me5	2	2	0	2	4	6.60
	Total	16	14	3	11	30	

MB: Mono-morphic bands; PB: Poly-morphic bands

Table 3: Monomorphic bands amplified by SRAP primers for normal and dwarf off-type banana cultivar Grand Naine

S. No.	SRAP Primer Combinations	No. of	S. No.	SRAP Primer Combinations	No. of MB
1	Em and Me5	1	15	Em10 and Me2	3
2	Em13 and Me5	1	16	Em2 and Me3	3
3	Em14 and Me5	1	17	Em10 and Me10	3
4	Em7 and Me4	2	18	Em1 and Me10	2
5	Em8 and Me1	1	19	Em1 and Me2	2
6	Em13 and Me1	1	20	Em7 and Me2	2
7	Em7 and Me7	2	21	Em11 and Me3	2
8	Em4 and Me4	1	22	Em4 and Me2	1
9	Em1 and Me1	2	23	Em2 and Me10	1
10	Em9 and Me9	2	24	Em2 and Me12	2
11	Em8 and Me8	4	25	Em2 and Me10	3
12	Em2 and Me2	3	26	Em2 and Me8	2
13	Em9 and Me9	2	27	Em2 and Me5	2
14	Em8 and Me2	2	28	Em2 and Me7	2

MB: Mono-morphic bands

Total No. of Monomorphic bands obtained through SRAP primers = 72
Total No. of Polymorphic bands obtained through SRAP primers = 14
% of Polymorphism observed by using SRAP primers = 19.4

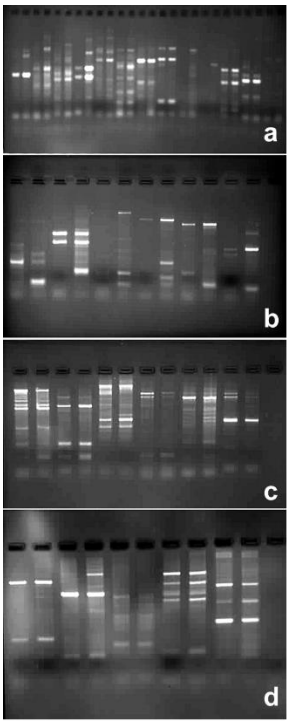

Plate-1

Figs. a-d Gels showing amplification of DNA by SRAP primers in true-to-type and dwarf off-type *in-vitro* propagated banana plants cv. Grand Naine.

a. Gel showing initial screening with SRAP primers having combinations. EM3 + ME3, EM9 + ME2, ME 11 + ME 2, EM8 + ME2, EM4 + ME2, EM10 + ME2, EM2 + ME3, EM10 + ME10, EM1 + ME4, EM1 + ME10, EM7 + ME2, EM1 + ME2.

b. Gel showing amplification of DNA using SRAP primer combinations, EM8 + ME5, EM7 + ME5, EM4 + ME5, EM1 + ME5, EM13 + ME5, EM14 + ME5 (polymorphic band with EM8 + ME5, EM7 + ME5).

c. Gel showing amplification of DNA using SRAP primer combinations, EM2+ME6, EM10+ME10, EM5+ME5, EM7+ME7, EM4+ME4, EM1+ME1 (polymorphic bands were seen with EM2+ME6).

d. Gel showing amplification of DNA using SRAP combinations EM9 + ME9, EM3 + ME3, EM11 + ME11, EM8 + ME8, EM2 + ME2, EM8 + ME8, EM2 + MF2 (polymorphic bands was generated by EM3 + ME3).

IV. Discussion

As per INIBAP, the occurrence of somaclonal mutation is a serious obstacle in in-vitro management and improvement of *Musa* [11]. The Somaclonal variant is usually poor. Due to low yield, coupled with the loss of time to plant and propagate plants, field space and other resources, farmers may suffer serious economic losses [12]. The large-scale production of commercial superior plants by *in-vitro* micropropagation is technically applicable to several species. A common problem encountered when growing plants by tissue culture is the development of tissue cultured induced genomic polymorphisms in which genetic changes in the nuclear, mitochondria, chloroplast genomes result in the lack of homogenecity among the regenerants and the production of inferior plants that are not true-to-type (off-types) with little commercial value. Many characteristics are only expressed in more mature stage of plant development and not in young stages. Off-types such as dwarfs, thicker leaves are difficult to identify at both tissue culture and nursery stage [13].

Present requirement for confronting somaclonal variation is the early stage markers that could be applied in-vitro to identify variation as soon as it occurs. In this direction molecular markers have particularly been suggested to be useful for early stage confirmation of genetic fidelity in micropropagated elite species [14]. The aim of the present study was to provide polymorphic markers for detection of somaclonal variations in tissue cultured banana cv. Grand Naine. Although there are many references dealing with banana tissue culture [15-17], the analysis of tissue culture derived plants for somaclonal variations of Indian cvs. are minimum [18,19]. Therefore, study was undertaken to isolate molecular markers for early detection of plants that are not true-to-type. More particularly the work relates to the use of such genetic markers as a diagnostic and quality control tool for monitoring the development of genetic polymorphisms arising during tissue culture regeneration of plants.

Our morphological study of variants in the field revealed dwarf off-type was the most common somaclonal variant in the field, and economically affecting variants like dwarf non-flowering and malformed fingers (small fingers with bitter taste) were also in more numbers and studied for molecular characterization. Many authors have also reported dwarf off-type variant to be the common variant in micropropagated bananas [20,21]. In oil palm non-flowering micropropagated variant has caused enormous loss to the producers [22], along with 10% of regenerants showing mantled flowering in oil palm [23]. Similar non-flowering variant has been recorded in this study and isolation of molecular markers were also tried for such variant along with malformed fingered bunch and bunch variants. Early detection of these variants would help to discard these inferior variants from the nursery fields.

SRAP are the markers which targets coding sequence and results in the identification of number of codominant markers. 44 primers combination have been used in this study to detect genetic fidelity in dwarf off-type micropropagated plant. In the initial screening 8 SRAP primer combinations showed inconsistency in band pattern. This is because of heterozygosity or amplification of similar sequences in two separate genomic regions [24]. Out of 44 primers 14 primer combination showed monomorphic bands and 8

primer combination showed 14 polymorphic loci. The percentage of polymorphic loci is 19% between dwarf off type and true-to-type Grand Naine plants. According to Budak et al SRAP markers preferentially amplify open reading frames and can be used for estimating genetic diversity [24]. Several research studies reported in literature used other markers to identify somaclonal variations in banana. Engelbarghs and Remy, used fluorescent AFLP to detect genetic variation between normal and dwarf micropropagated banana cv. [25]. Gimenez et al characterized micropropagated banana cv. Variants, and identified polymorphism in the microsatellite regions of micropropagated plants [26]. Furthermore, James et al used Methylation Sensitive Amplification Polymorphism (MSAP) technique and AFLP technique, and identified significant levels of DNA polymorphism in micropropagated Grand Naine bananas [27].

As banana and plantain cvs. are largely sterile and vegetatively propagated, analysis of somaclonal variants by classical genetic technique is not possible therefore in order to understand genetic basis of somaclonal variation in Musa cvs. molecular techniques are essential. From this study it was evident that tissue culture of bananas increases the rate at which genetic change occurs. Almost all well characterized banana somaclonal variants have poorer agronomic characteristics than their parent clone, one for example is non flowering dwarf off type. This kind of obvious phenotypic change can be negative. The underlying basis for accelerated rate of genetic change in tissue cultured bananas is of both applied and basic interest. We have characterized polymorphic bands for dwarf off type which can be used to detect dwarf off types at the early stage of *in-vitro* growth of banana cvs..

Thus economic consequences of somaclonal variation can be enormous in horticultural crops like banana, in the light of the various factors (genotype, ploidy, culture, type, explant) that lead to somaclonal variation of divergent genetic changes at the cellular and molecular levels. Hence genetic analysis of micropropagated plants using multidisciplinary approach is mandatory.

V. CONCLUSION

In our study the potential molecular marker like SRAP were used to identify the genetic variations that has resulted in banana cv. Grand Naine (AAA). The findings delineated that out of 44 primers 14 primer combinations showed monomorphic bands and 8 primer combination showed 14 polymorphic loci. The percentage of polymorphic loci was 19.4% between dwarf off type and true-to-type banana cv. Grand Naine (AAA). SRAP markers tried clearly showed polymorphism between the dwarf off types and the normal banana cv. Grand Naine (AAA). However, this is a preliminary report on limited number of individuals, and hence a large number of individuals needs to be tested before endorsing the relationship between these markers to a specific genotype.

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