Molecular Analysis of Somaclonal Variation in Tissue Culture Derived Bananas Using MSAP and SSR Markers

Emma K. Sales, Nilda G. Butardo

Abstract—The project was undertaken to determine the effects of modified tissue culture protocols e.g. age of culture and hormone levels (2,4-D) in generating somaclonal variation. Moreover, the utility of molecular markers (SSR and MSAP) in sorting off types/somaclones were investigated.

Results show that somaclonal variation is in effect due to prolonged subculture and high 2,4-D concentration. The resultant variation was observed to be due to high level of methylation events specifically cytosine methylation either at the internal or external cytosine and was identified by methylation sensitive amplification polymorphism (MSAP). Simple sequence repeats (SSR) on the other hand, was able to associate a marker to a trait of interest.

These therefore, show that molecular markers can be an important tool in sorting out variation/mutants at an early stage.

Keywords—Methylation, MSAP, somaclones, SSR, subculture, 2,4-D.

I. INTRODUCTION

PLANT tissue culture is one of the most popular means of mass propagation in asexually propagated plants including bananas. It has also been used as a tool for crop improvement in several crop breeding programs. However, one of the constraints of *in-vitro* culture propagation is the appearance of problematic off-types that diminish the commercial value of resultant plants [1]. These variations termed as somaclonal variation (variations in plants regenerated from tissue culture) had been reported in crop plants and are abundant in bananas [2]-[5]. Moreover [6] and [7] reported that somaclonal variation in plants derived from shoot tip culture vary from 0-70% depending on the genotype.

According to [8], the mechanism of somaclonal variation involves extensive genomic flux or altered methylation patterns and that it can be inherited. Moreover, epigenetic factors are also involved, hence making it more complex [9]. Somaclonal variation arising from *in-vitro* propagation is associated with DNA novel and heritable phenotypic variation [10]. Several researchers had reported that methylation patterns vary among *in-vitro* regenerated plants and their progeny e.g. Rice [11], Corn [12], [13], Oil Palm [11], Bananas [14].

E.K. Sales is with the Genetics Molecular Biology Laboratory, University of Southern Mindanao, 9407 Kabacan, Cotabato Philippines She is the Director of USM CHED Higher Education Regional Research Center (Telefax: 064-248-2610; e-mail: eperezkalaw@gmail.com).

N.G. Butardo is with the Genetics Molecular Biology Laboratory, University of Southern Mindanao, 9407 Kabacan, Cotabato Philippines (Tel: 064-248-2610; e-mail: nildabutardo@yahoo.com).

Owing to the sterility and its being vegetatively propagated classical genetic techniques is very difficult to apply in analyzing and detecting somaclonal variants in banana. Therefore, molecular technique had been developed and used for to understand the genetic and epigenetic basis of somaclonal variation. Included among these are several molecular markers had been utilized on *Musa*. These workers include Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphic (AFLP) and Simple Sequence Repeats (SSR) or microsatellites. The development and application of technologies based upon molecular markers provide the only tools that are able to detect polymorphism at the DNA sequence level where detection occurs. Likewise, they have been used to find out whether or not additional variation originates during storage and conservation [15]. DNA methylation is a biochemical process which is important for normal development in higher organism. This methylation events are usually analyzed using the methylation sensitive amplification polymorphism protocol/technique.

Methylation sensitive amplification polymorphism is a molecular technique that highlights DNA methylation variation in somaclones compared to mother clones and can be a powerful tool for genotypic characterization [16]. In *Musa*, [14] reported the usefulness of MSAP in detecting DNA methylation events in micropropagated banana plants, while [17] reported genomic DNA methylation-demethylation as a marker for aging and reinvestigation of pine tree (*Pinus radiata*).

Another useful marker is simple sequence repeats (SSR). SSRs are among several molecular markers that can be used to characterize and asses' genetic variability. They are highly polymorphic, multi-allelic, co-dominant, reproducible, easy to interpret and amplified via polymerase chain reaction [18]. It has been reported to be abundant and randomly dispersed throughout the genomes of many plant species [19]. Moreover, microsatellites appear to be powerful and reliable markers for genetic analysis, hybrid fingerprinting and marker assisted breeding in Musa [19]. Moreover, B genome derived SSR markers was able to detect genetic variability among *Musa* genotypes in Ibadan, Nigeria [20]. It has also been successfully applied in the molecular genotyping of several crops e.g. rice [21], cereals [22], grapevines [23], cacao [24].

The study was undertaken to determine the utility of molecular markers (MSAP and SSR) in identifying

World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:8, No:6, 2014

variants/somaclones and sort out at an early stage the offtypes and determine the underlying mechanism behind the variation.

II. MATERIALS AND METHODS

A. Plant Materials

Corms were obtained from a plantation in Magpet, North Cotabato, Philippines and were used for *in vitro* propagation of somaclones. Shoot tip cultures derived from the said 'Lakatan' corms were established using standard tissue culture procedures for disinfection, culture initiation and maintenance [25].

Shoot tips were inoculated onto full strength [26] medium solidified with 7% agar where varied levels of 2,4-D concentrations (3, 6, 9, 12 µM/li respectively) was supplemented. Cultures were maintained at 27^0C: 24 hr white light was provided by a 40 watt fluorescent lamp.

After 21 days, the initial cultures were further subdivided into 2-3 sections. Every 21 days thereafter the cultures were transferred to a fresh medium. The cultures were maintained until the 15th subculture to determine the effect of age of culture in the induction of somaclonal variation in vitro. Beginning at the 3rd cycle until the 11th - 13th cycles, observations and data on number of days to shoot initiation and number of shoots developed were recorded. Plantlets derived per treatment combination were planted in the greenhouse for four weeks. Evaluation of the pre and post harvest traits were carried in the field and association between molecular markers viz-a-viz morphological trait was observed.

B. Molecular Analysis

Genomic DNA was extracted from samples for every treatment combinations (starting from subcultures 4 until subculture 11 and different 2,4-D concentrations) using the modified CTAB extraction protocol [27].

To detect MSAP, two digestion reactions were set up at the same time for each genomic DNA sample. In the first reaction 200 ng of the genomic DNA was digested with 5 U of EcoR 1 (Gibco BRL) plus 5 U of Msp 1 (Gibco BRL) in a final volume of 50 µl containing 50 mMTris-HCl (pH 8.0), 10 mM MgCl2 and 50 mM NaCl for 5 h at 37^0C. The second digestion reaction was carried out as above; however, Hpa II (Gibco BRL) was used instead of Msp 1. The digestion reactions were then ligated to the adapters by adding 10 µl of ligation mixture containing 1 z T4 DNA ligase buffer (Gibco BRL), 10 U T4 DNA ligase (Gibco BRL), 5 pmol EcoR 1 adapter and 50 pmol Msp i-Hpa II adapter (Table I). The ligation reaction was incubated at 23^0C for 5 h. The digestion and ligation reactions were stopped by incubating at 65^0C for 10 min.

The preamplification reaction was performed by using 5 µl of the above ligation product with 75 ngEcoR 1 primer with one selective base (Eco R1 + 1), 75 ngMsp 1-Hpa II primer with no selective base (Msp 1-Hps II + 0), 1 x PCR buffer (Gibco BRL), 1 U Taq polymerase (Gibco BRL), 1.5 mM $MgCl_2$ and 0.4 mMdNTP's in a final volume of 50 µl. Reaction conditions were 20 cycles of 94^0C for 1 min, 56^0C for 1 min and 72^0C for 2 min.

Selective amplification was conducted in volumes of 20 µl. For selective amplification the preamplified mixtures were diluted 1:25 from their original volume with TE. A volume of 5 µl of these diluted samples was mixed with 30 ng of Eco R 1 with two selective bases, 10 ng of Msp 1-Hpa II primer with two or three selective bases end-labelled with 0.8 uCi [α^{32} P]ATP, 1 x PCR buffer, 0.5 U Taq DNA polymerase and 1.5 mM $MgCl_2$. Fragments present in these mixtures were amplified for 1 cycle of 94^0C for 30 s, 65^0C for 30 sand 72^0C for 1 min in the following 12 cycles the annealing temperature of 65^0C was lowered by 0.7^0C each cycle, followed by 24 cycles with the following program: 94^0C for 30 s, 56^0C for 1 min and 72^0C for 2 min with a final extension at 72^0C for 5 min. All amplification reactions were carried out in a PTC 200 Thermal cycles (Waltham, MA, USA).

C. SSR Analysis

Forty (40) Simple Sequence Repeats (SSR) primers', specific for bananas were screened for their utility in differentiating the somaclones. Amplification of SSRs was performed on 12.5 µl PCR reactions containing 20-25 ng genomic DNA, 0.4 µM each of forward and reverse primers, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mMdNTPs and 0.1 U of Taq Polymerase. PCR was performed under these conditions: initial denaturation at 94^0C for 4 min followed by 35 cycles of denaturation at 94^0C for 30 sec, 54^0C annealing for 30 sec (Tm depends on the primers used) and 72^0C for a minute. Final extension was at 72^0C for 10 min and incubation at 10^0C. PCR amplification was evaluated using 3% agarose gel electrophoresis and visualized with ethidium bromide. All primers yielding amplification were determined and characterized based on intensity, resolution of bands, and number of bands produced. Those primers yielding at least 3 to 5 amplification products of good intensity and resolution were tested further in replicated trials. Those primers revealing polymorphic patterns were determined and the extracted DNA data were subsequently analyzed statistically using the NTSYS pc software package version 2.01 [28]. A dendrogram was constructed based on the matrix of similarity using the Unweighted Pair Group Method of Arithmetic Average (UPGMA). Specifically, results of PCR amplification using specific primers were scored as presence (1) or absence (0) of the amplified fragment. Genetic similarities were evaluated using the unweighted pair grouping with arithmetic average (UPGMA). A dendrogram of genetic similarity was generated with the NTSYSPc Version 2.10z (1986-2002 Applied Biostatistics, Inc.)

III. RESULTS - MOLECULAR EVALUATION

A. MSAP Analysis

DNA methylation is a biochemical process that is important for normal development in higher organisms. This methylation events are usually analyzed using the methylation sensitive amplification polymorphism protocol/technique. For this project, MSAP analysis was done in DNA of tissue cultured plantlets using the candle or cigar leaf stage. This was carried

World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:8, No:6, 2014

out to determine the methylation events in their genetic make-up. Fig. 1 shows an example of an MSAP profile generated from the analysis. Table I shows the list of adapters and primers used in the MSAP analysis. For the methylation analysis, out of the 42 MSAP primer combinations tested/screened, 6 primer combinations were found to be informative based on their amplification and intensity. Table II shows 15,993 MSAP fragments of which 277 were methylated fragments (CpG methylation) while the hemi-methylated (non- CpG methylation); was 188. Out of these methylated and hemi-methylated fragments, 66 loci showed polymorphism for the treatment combinations corresponding to a genetic variation of 16%. Specifically, the number of loci counted for each primer combination vis-a-vis treatment combinations varied from 23 to 189 (Table III). The identified type of methylation events that occurred was based on methylation sensitivities of 2 isoschizomers (EcoRI/MspI and EcoRI/HpaII). Presence of MSAP fragments in both EcoRI/MspI and EcoRI/HpaII profiles indicated non-methylated loci. CpG-methylated loci on the other hand, were characterized by the presence of EcoRI/MspI fragment andabsence of HpaII fragment in the same locus. The opposite pattern, fragments present in EcoRI/MspI digestions were counted as hemi-methylated loci representing methylation on external cytosine i.e. non-CpG methylation (Salmon et al. 2008). To investigate somaclonal variation, variation in methylation was evaluated among the samples. A sample result (Table III) shows that, variation in the DNA methylation occurred in the *in-vitro* cultures and that the different treatments showed variation in the number and pattern of methylation events. Differences in the polymorphic loci between control and different treatment combinations were also noted. As mentioned earlier, the 6 primer combinations generated 66 polymorphic MSAP fragments. This is an indication that polymorphic band is due to variation in methylation events/patterns.

Results also reveal higher incidence of non-methylated loci over that of hemi and internal methylation for all the treatments. Some results also showed varied occurrence of internal methylation due to higher levels of 2,4-D (i.e. 6-12 µM) for some primer combinations and prolonged subculture.

It is also noteworthy to mention that some treatments generally gave higher frequency of methylation than the untreated samples (with few exceptions). General observations of the results show differences in the number of bands and number of methylated bands for all the treatment combinations. However, a general trend was not observed for most of the oligonucleotide combinations. For the primer combination, EAC/MHCA, subculture 11 generated the most number of bands for all the 2,4-D treatments. However, this was not observed for the number of methylated bands. It can also be noted that in some cases, no methylation event happened at some treatment combinations. This can be an indication of hyper-methylation hence less epigenetic diversity.

TABLE I
LIST OF ADAPTERS AND PRIMERS USED FOR MSAP ANALYSIS

Adapters/primers	DNA Sequence
EcoR1 adapter	5' – CTCGTAGACTGCGTACC – 3'
	3' – CATCTGACGCATGGTTAA – 5'
EcoR1 + 1 primer	5' – GCATGCGTACCAATTCA – 3'
EcoR1 + 2 primers	5' – GCATGCGTACCAATTCAC – 3'
	5' – GCATGCGTACCAATTCAG – 3'
	5' – GCATGCGTACCAATTCTG – 3'
MspI-HpaII adapter	5' – CGACTCAGGACTCAT -3'
	3' – AGCGATGAGTCCTGAGT – 3'
MspI-HpaII + 0 primer	5' – GATGAGTCCTGAGTCGG -3'
MspI-HpaII + 2 primers	5' – GATGAGTCCTGAGTCGGCA -3'
	5' – GATGAGTCCTGAGTCGGGT -3'
MspI-HpaII + 3 primers	5' – GATGAGTCCTGAGTCGGATG -3'
	5' – GATGAGTCCTGAGTCGGCAT -3'
	5' – GATGAGTCCTGAGTCGGCTC -3'

TABLE II
SUMMARY OF METHYLATION AMPLIFICATION OF THE TISSUE CULTURE DERIVED PLANTLETS FROM PROLONGED SUBCULTURE AND LEVELS OF 2,4-D TREATMENTS

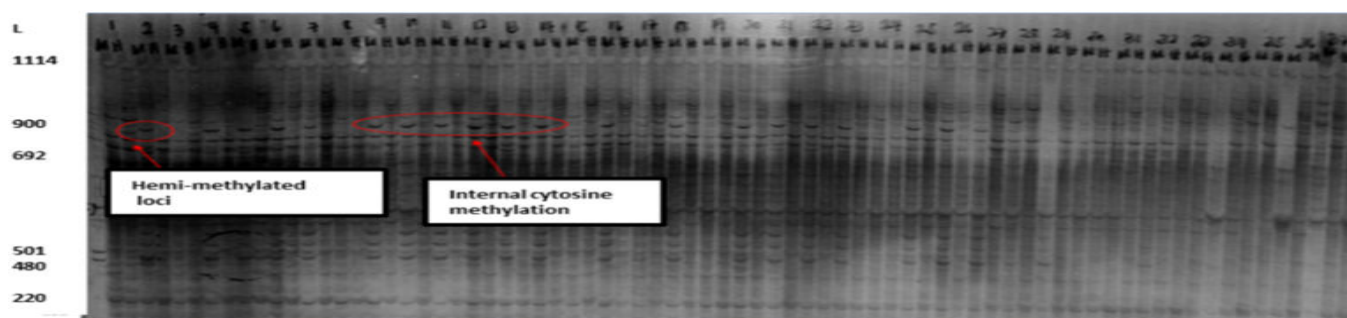|	
Total MSAP Fragments detected by the six primer combinations	15,993
Total Methylated Fragments (6 primers)/Internal	277 (1.73%)
Total Hemi-methylated Fragments	188 (1.18%)
Total Non-methylated Fragments	15,451 (96.61%)
Total Polymorphic DNA Methylation events	66
% Variation	16 %

Fig. 1 MSAP profile of selected banana somaclones at different subcultures supplemented with high 2,4-D levels using the primer combination E-TG/MH-CTC

Moreover, comparison between the treated and untreated samples is shown in Table IV. In general, divergences of occurrence of methylation events among the tissue culture derived plantlets were evident. Specifically, each of the treatment combinations (2,4-D level and subculture) showed varied results. For the untreated plantlets, a total of 49 methylation events were generated, where 29 were CpG methylation, 15 were hemi methylation (non-CpG methylation) and 5 were demethylation. Noteworthy is that, 3 of these DNA methylation events are polymorphic. For the treated samples on the other hand, MSAP profiles showed a total of 356 methylation events where 191 were CpG methylation (full methylation at the internal cytosine), 45 were hemi methylation (methylation at the external cytosine) and 45 were demethylation. In previous studies of tissue culture induced mutation in coffee, [16] reported higher methylation in somaclones was due to more polymorphic bands in tissue culture induced variation in barley, [30] also reported that variation was due to nucleotide mutations and changes in methylation state. Therefore, data shows that total methylation events were much higher in the 41 somaclones compared to the control. This was mainly due to a higher level in full methylation of the internal cytosine as most of the bands were produced from a cleavage by Msp 1, but not Hpa II. This result is in agreement with the results of [16] in grapevine. However, in the case of tissue culture in wild barley, [31] reported a significant decrease in the cytosine methylation levels at the CCGG sites. On the other hand, hemi methylation of external cytosine of the selected somaclones was significantly lower than the untreated samples. This modification, in the cytosine methylation indicates that epigenetic alterations might have played an important role that resulted to somaclonal variation. This result is in agreement with results of [32] and [12] where they suggested that epigenetic alterations play an important role in the cytosine modifications. Moreover, [29] and [33] pointed out the possible interactions of both epigenetic and genetic changes induced by the plant tissue culture process.

Moreover, the results of the study revealed alterations of methylation patterns at different loci and regenerants were distinct from the donor plants/control. Altogether, MSAP analysis suggests modification in cytosine methylation and alterations in methylation patterns as affected by age and hormone level.

B. SSR Analysis

In order to determine the differences in the genetic constitution of these selected somaclones, 40 SSR markers were used to discriminate one variant from the others. Out of the 40 SSR primers used, only 10 gave scorable bands e.g. observed (Ho) and expected heterozygosity (He), and power to discriminate (PD) (Table V). Fig. 2, on the other hand, is an example of the SSR profile of the selected 'Lakatan' somaclones. Results (Table V) shows that allele size ranged from 26-1140 bp. Observed heterozygosity (H_o) ranged from 0.73-0.98 while expected heterozygosity (H_E) ranged from 0.52- 0.94 and PD ranged from as low as 0.06 to as high as

0.85. Noticeably, primer Ma 3/60 had high H_o and H_E values but its power to discriminate was very low (0.06). This primer, although, was able to generate 2 polymorphic genotypes, all genotypes; however, had the same allelic size or has the same banding pattern. This case is true to all primers with high H_os and H_Es but low PDs. Primers Ma 119 and Ma 364 were the most discriminating primers. They were able to detect substantial gene diversity from all plant samples evaluated. This was validated by their PD values of 0.85 and 0.84, respectively.

TABLE III
SAMPLE MSAP METHYLATION PATTERNS OBSERVED PER PRIMER COMBINATION IN TISSUE CULTURE-DERIVED 'LAKATAN' TREATED WITH PROLONGED SUBCULTURES AND 2,4-D LEVELS

Primer Combinations	Treatments	SUB-CULTURE						
		5	6	7	8	9	10	11
AC-CA	Control	5						
	3uM 2,4-D/Li	6	1	0	4	0	0	2
	6uM 2,4-D/Li	7	4	0	0	0	0	2
	9uM 2,4-D/Li	5	0	0	2	0	0	0
	12uM 2,4-D/Li	2	0	0	0	0	4	9
AC-ATG	Control	1						
	3uM 2,4-D/Li	5	1	0	4	2	1	6
	6uM 2,4-D/Li	2	2	0	0	1	1	3
	9uM 2,4-D/Li	3	0	0	2	0	2	2
	12uM 2,4-D/Li	0	0	3	0	0	2	1
AC-CAT	Control	2						
	3uM 2,4-D/Li	3	5	0	4	0	3	4
	6uM 2,4-D/Li	0	2	0	4	0	2	3
	9uM 2,4-D/Li	4	2	0	3	0	4	2
	12uM 2,4-D/Li	4	4	0	4	0	4	0
AC-CTC	Control	1						
	3uM 2,4-D/Li	0	2	0	6	0	1	1
	6uM 2,4-D/Li	3	3	0	2	0	3	1
	9uM 2,4-D/Li	7	0	0	5	0	0	7
	12uM 2,4-D/Li	5	0	0	1	0	0	6
AC-GT	Control	3						
	3uM 2,4-D/Li	5	3	0	6	0	8	8
	6uM 2,4-D/Li	1	2	0	6	0	8	7
	9uM 2,4-D/Li	3	2	0	1	0	8	6
	12uM 2,4-D/Li	2	2	0	6	0	4	0
TG-ATG	Control	3						
	3uM 2,4-D/Li	6	6	3	6	0	8	1
	6uM 2,4-D/Li	3	1	4	4	0	7	3
	9uM 2,4-D/Li	4	6	0	4	3	8	4
	12uM 2,4-D/Li	4	6	4	4	0	8	2

TABLE IV
COMPARISON OF METHYLATION EVENTS BETWEEN THE UNTREATED SAMPLES AND TISSUE CULTURE DERIVED PLANTLETS TREATED WITH LONG CULTURE PERIOD AND 2,4-D CONCENTRATIONS

	Total methylation events	Full methylation	Hemi methylation	Demethylation	Non-methylation
Control	49	29	15	5	3,137
Treated Samples	356	191	45	45	12,856

Ten (10) SSR markers (Table VI) were able to generate a total of 61 polymorphic genotypes at each loci and 60 observed heterozygous alleles from all selections. A total of 2,377 fragments were amplified and/or detected by these primers from the 40 selected variants giving an average of 237.7 fragments/ primer. Unweighted Pair Group Method using arithmetic average (UPGMA) by NTSYSpc Version

World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:8, No:6, 2014

2.01 software by Rohlf (1997) [28] was performed to calculate hierarchical clustering of 40 selected somaclones on the basis of their shared allele distances. A dendrogram was constructed for these selected somaclones (Fig. 3). At 0.90 similarity coefficient, somaclones can be grouped into10 clusters/groups. The dendrogram shows that C7T4#25, C13T2#30 and C13T4#13 were individually grouped indicating individual traits specific for each. This is also an indication of their genetic differences from the other selections. C7T4#25 with a heavier bunch (15.43 kg) and with 20 fingers per hand weighing an average of 2.20 kg was found to be the most genetically distant somaclone. The variation observed in the SSR pattern maybe due to different causes,either, methylation, loss or gain of nitrogen base or point mutation. Epigenetic component of the genome on the other hand cannot be disregarded to have a role on this variation.

Based on the 10 informative SSR markers, a genotypic constitution of 17 somaclones (with positive traits) was established. Some of the markers generated specific genotype for each somaclone carrying the most superior traits over that of other selected somaclones and untreated samples.

Table VII shows the specific SSR markers that were able to differentiate and generate specific phenotype for a trait of interest. For example, marker Ma1/27 generated a unique genotype for somaclone C7T3#21 with heaviest fruit.

For the heaviest hand (C7T4#25), primers Ma1/27 and 1/24 (genotype A and B, respectively) were able to detect unique genotypes for this trait. Ma1/16 on the other hand, was able to generate specific genotype for 4 somaclones [longest fruit (C8T1#25)], heaviest bunch (C11T1#31), more number of hands (C11T2#38) and longer days to 50% yellowing (C14T5#28)]. Moreover, Ma 1/19 generated specific genotype for 3 somaclones corresponding to heaviest hand (C7T4#25), more number of hands and fingers (C11T2#15), and longer days to 50% yellowing (C14T5#48). This therefore suggests that the abovementioned SSR markers are useful in sorting of improved somaclones with subtle changes. Likewise, the result suggests/implies that SSR markers can serve as a marker for some post harvest traits e.g. yield and shelf life of bananas, in this case. Nevertheless, the trueness to type and stability should be confirmed in succeeding generations to rule out epigenetic variation.

All of the selected somaclones (Table VIII) are significantly better than the control in terms of the selected traits. Also, the summary of the character traits of Lakatan somaclones in association with MSAP markers used is presented in Table IX.

TABLE V
ALLELE SIZE, OBSERVED HETEROZYGOSITY, EXPECTED HETEROZYGOSITY AND POWER OF DISCRIMINATION OF SSR PRIMERS USED TO EVALUATE 40 SELECTED BANANA SOMACLONES

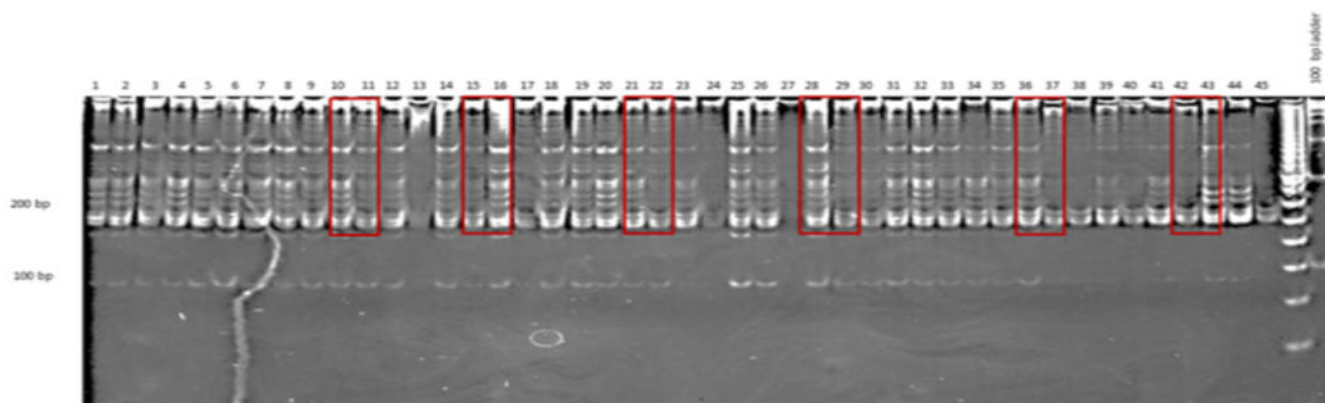| SSR name	Primer sequence	Allele size range (bp)	H_o	H_E (PIC)	PD
1. Ma 3/2	GGA ACA GGT GAT CAA AGT GTG A (F) TTG ATC ATG TGC CGC TAC TG (R)	81-597	0.98	0.89	0.65
2. Ma 1/27	TGA ATC CCA AGT TTG GTC AAG (F) CAA ACA CAT GTC CCC ATC TC (R)	26-900	0.95	0.78	0.48
3. Ma 1/18	TTT GCC TGG TTG GGC TGA (F) CCC CCC TTT CCT CTT TTG C (R)	126-134	0.98	0.70	0.22
4. Ma 3/60	TGG CTG ACA ATT ACA TGA CA (F) GCG CAC TGT GGT GTG T (R)	83-140	0.85	0.75	0.06
5. Ma 1/24	GAG CCC ATT AAG CTG AAC A (F) CCG ACA GTC AAC ATA CAA TAC A (R)	76-144	.90	0.49	0.18
6. Ma 1/16	TTT GCC TGG TTG GGC TGA (F) CCC CCC TTT CCT CTT TTG C (R)	133-1140	0.73	0.86	0.79
7. Ma 1/19	ATT GGG CAG GCA TCA AGT AC (F) GCA ATG GTG CTA CCC ACC (R)	146-900	0.78	0.89	0.85
8. Ma 3 64	CAA CAG CTC TCG CACA TTC (F) AAC CTT TAA TGT ATC TGC (R)	128-1028	0.90	0.92	0.84
9. Ma 3/90	GCA CGA AGA GGC ATC AC (F) GGC CAA ATT TGA TGG ACT (R)	87-583	0.97	0.94	0.72
10. Ma 1/5	AGA TGG CGG AGG GAA GAG CCG (F) GAT CCA AGC TTA TCG A (R)	149-588	0.95	0.80	0.63

Fig. 2 SSR Profile of the selected 'Lakatan' somaclones.

World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
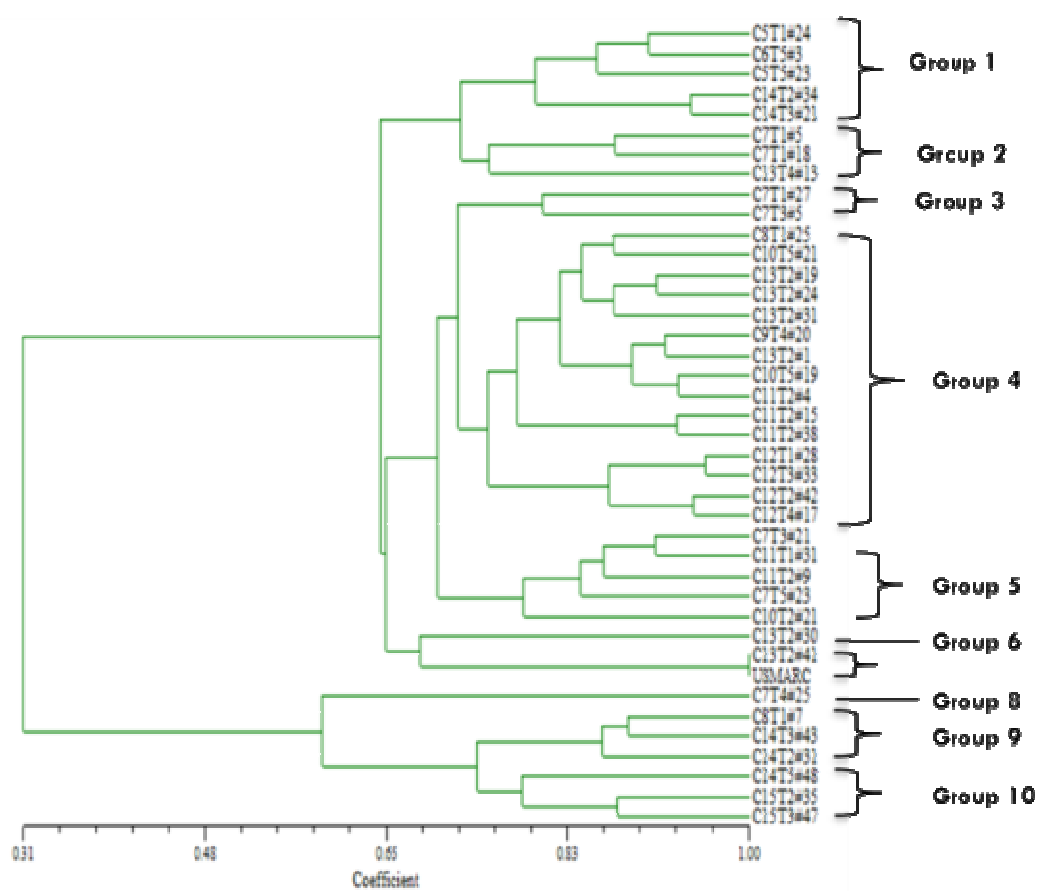Vol:8, No:6, 2014

Fig. 3 Tree plot of 40 selected 'Lakatan' somaclones clustered b UPGMA [28]

TABLE VI

NUMBER OF BAND AMPLIFICATIONS, POLYMORPHIC AND HETEROZYGOUS LOCI, AND TOTAL FRAGMENTS AMPLIFIED BY SSR MARKERS USED TO EVALUATE 40 SELECTED 'LAKATAN' SOMACLONES

Primer Name	# of genotypes amplified	# of polymorphic loci	Heterozygous loci	Total # of fragments amplified
1. Ma 3/2	10	10	10	315
2. Ma 1/27	3	3	3	148
3. Ma 1/18	2	2	2	116
4. Ma 3/60	2	2	2	134
5. Ma 1/24	2	2	1	74
6. Ma 1/16	7	7	7	204
7. Ma 1/19	11	11	11	246
8. Ma 3 64	13	13	13	406
9. Ma 3/90	8	8	8	565
10. Ma 1/5	3	3	3	169
Total	61	61	60	2,377

TABLE VII

CHARACTER TRAITS OF THE 'LAKATAN' SOMACLONES IDENTIFIED BASED ON THE SSR PRIMERS USED

Character Trait	Somaclone identity	Unique/Specific Marker
highest dry matter content	C6T5#3	Ma 1/18
heaviest fruit	C7T3#21	Ma 1/27
heaviest hand	C7T4#25	Ma 1/27
		Ma 1/24
longest fruit	C8T1#25	Ma 1/16
highest TSS	C-T4#20	Ma 1/19
highest pH and longer shelf life	C10T2#21	Ma 3/90
early maturing	C10T5#1-	Ma 3/2
heaviest bunch, more # of hands	C11T1#31	Ma 1/19
more # of hands and fingers	C11T2#15	Ma 1/19
	C11T2#38	Ma 1/16
more # of Hands	C11T2#4	Ma 1/19
	C13T2#31	Ma 1/16
highest fruit girth	C14T3#21	Ma 3/90
longer days to 50% yellowing	C14T5#48	Ma 1/16
		Ma 1/19
		Ma 3/90

TABLE VIII
SUMMARY OF THE SELECTED SOMACLONES AND THE CORRESPONDING
CHARACTER TRAITS EXHIBITED EXPRESSED IN RESPONSE TO PROLONGED
SUBCULTURE AND 2,4-D LEVEL

Somaclones	Weight of bunch	Somaclones	Weight of finger	Somaclones	More number of fingers/ hand
C11T1#31	**20.78kg**	**C7T1#5**	**140g**	**C11T2#15**	**21**
C7T1#18	17.20kg	**C12T2#42**	**140g**	**C11T2#38**	**21**
C7T1#27	17.00kg	C10T2#21	121g	C7T3#5	20
C11T2#15	18.63kg	C11T1#31	120g	C7T4#25	20
C13T2#1	19.15kg	C12T4#17	120g	C11T2#9	20
C13T2#31	17.80kg	C14T3#21	120g	C13T2#19	20
C13T2#19	17.00kg	C14T3#43	120g	**Control**	**16**
C14T3#21	17.00kg	**Control**	**80g**		
Control	**12.10kg**				

TABLE IX
RECOMMENDATIONS SUMMARY TABLE FOR THE CHARACTER TRAITS IN
ASSOCIATION WITH MSAP MARKERS

Character traits	Primer	Size of bp
More number of fingers	E-TT/MH-CAT	690
		495
	E-TT/MH-ATG	148
More number of hands	E-TT/MH-CAT	690
	E-TT/MH-ATG	148
Heaviest weight of hand	E-TT/MH-CAT	690
High dry matter content	E-TT/MH-CAT	690
		495
	E-TT/MH-ATG	148
High TSS	E-TT/MH-CAT	495
	E-AC/MH-GT	37
	E-TT/MH-ATG	148
Longer shelf life	E-TT/MH-CAT	690
	E-AC/MH-GT	77
Early flowering	E-TT/MH-ATG	148
Highest fruit girth	E-TT/MH-CAT	690
	E-TT/MH-CAT	495

IV. SUMMARY AND CONCLUSION

The project employed modified tissue culture protocols such as prolonged subculture and hormone levels (2,4-D) to determine the rate and level of somaclonal variation that can be generated.

Based on the results, tissue culture protocols can be used as a tool for improvement of asexually propagated crops where conventional breeding is difficult if not impossible. In this particular case, age of culture (prolonged subculture) and high concentration of hormone (2,4-D) facilitated the production of novel phenotypes (both positive and negative traits) leading to the production of superior 'Lakatan' mutants in terms of yield e.g. bunch weight, number of hands, weight of hand, shelf life, etc.

From these, somaclones were identified and selected. The said selected somaclones if proven to carry stable traits can be submitted to the NSIC for evaluation and accreditation as a new strain of 'Lakatan.' This then, eventually will be distributed to farmers as a new 'Lakatan' strain with improved qualities.

In terms of protocols for sorting of off types, molecular markers e.g. Methylation Sensitive Amplification Polymorphism (MSAP) and Simple Sequence Repeats (SSR) markers were found to be efficient technologies that can identify the variants and explain the underlying mechanism behind the resulting variations.

As an alternative technology, plant improvement through somaclonal variation can support conventional breeding of new superior variants with a better performance. Likewise, induced mutation could increase genetic variation in 'Lakatan' banana which eventually could give advantage in providing farmers with superior strains of interest, in this case 'Lakatan'. In vitro culture (prolonged subculture and 2,4-D levels) effectively produce new somaclones with desired characters.

V. RECOMMENDATIONS

In vitro culture techniques, although a means to propagate true to type planting materials can also be used as a tool for genetic improvement of sterile or asexually propagated crops. This study proved that genetic manipulation can be done through modification of tissue culture protocols such as prolonged subculture (age) and 2,4-D levels (hormone concentration.) Moreover, molecular markers i.e. MSAP and SSR markers can be used to determine the underlying mechanisms that brought about such variations (whether positive or negative traits). SSR in particular can assist breeders in identifying and sorting the traits of interest.

The results of this study can therefore be used in assisting tissue culture laboratories in ensuring the trueness to type of their plantlets by limiting their subculturing protocol and use of hormones. On the other hand, genetic improvement can be pursued through induction of somaclonal variation in targetting novel and superior traits.

In this study, although there is an evidence of differences between regenerants and explants types, there is a need to consider further evidence to fully ascertain the effect of the hormones and age of subculture on the extent of DNA methylation polymorphism using other methylation sites such as the trinucleotide C x G [14].

ACKNOWLEDGMENT

REFERENCES

[1] Cullis, C.A. 2005. Mechanisms and control of rapid genomes changes in flax. Annals of Botany 95:201-
[2] Hwang, S.C. and Tang, C.Y. 1995. Somaclonal Variation and its use in Taiwan. (Paper presented to MARDI. International Banana Workshop: New frontiers in Resistance Breeding for Nematodes Fusarium and Sigatoka. Serdang, Malaysia 2-5 Oct.1995)

World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:8, No:6, 2014

[3] Reuveni, O., Y. Israeli. 1990. Measures to reduce somaclonal variation in in vitro propagated bananas. ActaHorticulturae. 275:307-313.

[4] Vuylsteke, D., Swennen, R. and De Langhe, E. 1991.Somaclonal Variations in Plantains (*Musa* spp., AAb group) derived from Shoot-tip Culture. Fruits: 46 (4): 429-439.

[5] Daniells, J.W. and Smith, M.K. 1993. Somatic mutation of bananas - their stability and potential. p.162-171. In: R.V. Valmayor, S.C. Hwang, R. Ploetz, S.W. Lee and N.V. Rao (eds.), Proceedings International Symposium on Recent Developments in Banana Cultivation Technology. INIBAP/ASPNET, Los Banos, Philippines.

[6] Israeli, Y., Lahav, E. and Reuveni, O. 1995. *In vitro* Culture of Bananas. In: Bananas and Plantains. S. Gowen. Lomndon, Chapman and Hall.Pp.147-178.

[7] Smith, M.K. 1998. A Review of Factors Influencing the Genetic Stability of Micropropagated Bananas. Fruits 43 (4):219-223.

[8] Scowcroft, W.R., S.A. Ryan, R.I.S. Brettel and P.J. Larkin. 1985. Biotechnology in International Agricultural Research, IRRI, Manila. Pp:99-109.

[9] Kaeppler, S.M., H. Kaeppler, Y. Rhee. 2000:Epigenetic aspects of somaclonal variation in plants. Plant Mol Bio Rep 43:179-188.

[10] Lukens, L.N., S. Zhan. 2007: The plant genome's methylation status and response to stress:implications for plant improvement. CurrOpin Plant Biol 10:317-322.

[11] Jaligot, E., A. Rival, T. Buele, S. Dussert, J.L. Verdeil. 2000:Somaclonal variation in oil palm (*Elaeisguineensis*Jacq.):the DNA methylation hypothesis. Plant Cell Rep,19:684-690.

[12] Brown, P.T.H., E. Gobel, H. Lorz. 1991:RFLP analysis of Zea mays callus culture and their regenerated plants. TheorAppl Genet, 81:227-232.

[13] Kaeppler, S.M., and R.L. Phillips. 1993. DNA methylation and Tissue Culture-Induced Variation in Plants. In Vitro Cell Dev. Biol 29P:125-130.

[14] Peraza-Echeverria, S.P., VAH Valencia, and A.J. Kay. 2001: Detection of DNA methylation changes in micropropagated banana plants using methylation-sensitive amplification polymorphism (MSAP). Plant Science. Vol. 161, No. 2:359-367

[15] Sahjiram, L. J. R. Soneji and K.T. Bollama, 2003. In-vitro Cellular & Dev. Biology. Plant. 39 (6) pp 551-556.

[16] Schellenbaum. P., V. Mohler, G. Wenzel and B. Walter. 2008. Variation in DNA methylation patterns of grapevine somaclones (Vitisvinifera L.). BMC Plant Biology 2008,8:78.

[17] Fraga, M.F., M.L. Centeno, A.E. Valdes, P. Moncalean, B. Fernandez, M.J. Canal and R. Rodrigeuz. 2000. Genomic DNA methylation and polyamines titer as key processes in plant ageing: applications for clonal multiplication of mature Pinusradiata trees. In Applications of Biotechnology to Forest Genetics. Eds. S. Espinel and E. Ritter, Neiker, Victoria, pp 495-506.

[18] Crouch, J.H., H.K. Crouch, H. Constandt, A. Van Gysel, P. Breyne, M.V. Montager, R. L. Jarret, and R. Ortiz. 1999. Comparison of PCR based molecular marker analysis of Musa breeding populations. Mol. Breeding 5: 233-244.

[19] Crouch, J.H., H.K. Crouch, R. Ortiz and R.L. Jarret. 1997. Microsatellite markers for molecular breeding of *Musa*.InfoMusa 6:5-6.

[20] Oriero, C. E., Odunala, O. A., Lokko, Y. &Ingelbrecht, I. 2006. African J of Biotech. 5(2): 126-128.

[21] Pessoa-Filho, M.A.C., A. Belo, A.A.N. Alcochete, P.H.N. Rangel, M.E. Ferreira. 2007. A set of multiplex panels of microsatellite markers for rapid molecular characterization of rice accessions. BMC Plant Biology, 7:23.

[22] Hayden, M.J., T.M. Nguyen, A. Waterman, G.L. McMichael, K.J. Chalmers. 2007. Application of multiplex-ready PCR for fluorescence-based SSR genotyping in barley and wheat. Molecular Breeding 21:271-281.

[23] This, P. A. Jung, P. Boccacci, J. Borrego, R. Bott, L. Costantini, M. Crespan, G.S. Dangl, C. Eisenheld, F. Ferreiza-Monteiro, S. Grando, J. Ibanez, Lacombe, R. Laucon, R. Magalhaes, C.P.Meredith, N. Milani, E. Peterlunger, F. Regner, L. Zulini, E. Maul. 2004: Development of standard set of microsatellite reference alleles for identification of grape cultivars. TheorAppl Genet 2004.109:1148-1158. PubMed.

[24] Zhang, D., S. Mischke, R. Goenaga, A.A. Hemeida, S'A. 2006. Accuracy and reliability of high-through put microsatellite genotyping for cacao clone identification, Crop Sci 46:2084-2092.

[25] Hwang, S.C. and Ko, W.H.1990. Selection of Improved Cavendish Banana Mutants Resistant to Race-4 of *Fusarium oxysporum* F. sp. *cubense.* Acta Horticulture 275:417-423.

[26] Murashige, T., Skoog, F.A. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. PhysiologiaPlantarum, v.15, p.473-497, 1962.

[27] Gawel, N. and Jarret, L. 1991. A Modified CTAB DNA Extraction Procedure for *Musa* and *Ipomea*.PlantBiol.Mol.Reports. 9(3) 262-266

[28] Rohlf, F.J. 1997, NTSYSpc:numerical taxonomy and multivariate analysis system, Version 2.02. Setauket (New York):Exeter Publishing.

[29] Salmon, A., J. Clotault, E. Jenczewski, V. Chable, M. Manzaneres-Daulex. 2008. Brassica oleracea displays a high level of DNA methylation polymorphism. Plant Sci 174:61-70.

[30] Bednarek, P.T., Orlowska, R., Koebner, R.M.D., Zimny, J.:Quantification of the tissue-culture induced variation in barley (Hordeumvulgare L.) BMC Plant Biol 2007, 7:10.

[31] Li J., H. Yan, K. Wang, W. Tan, X. Zhou. 2007. "Hairpin fluorescence DNA probe for real-time monitoring of DNA methylation". Anal. Chem. 79(3):1050-1056.doi:10.1021/ac061694i. (http://dx.doi.org/10.1021%2Fac061694i). PMID 17263334 (//www.ncbi.nlm.nih.gov/pubmed/17263334).

[32] Brown, P.T.H., Kyozuka, J., Sukekiyo, Y., Kimura, Y., shimamoto, K., Lorz, H.1990. Molecular Change in Protoplast-derived rice plants. Mol Gen Genet. 223:324-328.

[33] Gou, W.L., Wu, R., Zhang, Y.F., Liu, X.M., Wang, H.Y., Gong, L., Zhang, Z.H., Liu, P. 1997. Tissue-culture-induced locus specific alteration in DNA methylation and its correlation with genetic variation in CodonopsisLanceolata Beth. et. Hook.f. Plant Cell Tiss Org Cult, 90:1297-1307.