



Original Contribution

MOLECULAR ANALYSIS OF MUSA MUTANTS RESISTANT TO SALINITY BY MICROSATELLITE MARKERS

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ABSTRACT

Microsatellite markers were used to investigate genetic variability within gamma irradiated clones resistant to salinity of Cavendish banana (*Musa* AAA cv. 'Dwarf Cavendish'). From the 11 microsatellite primer pairs that were tested, 8 produced specific fragments and generated a total of 29 alleles, with a mean of 3.6 alleles per locus. Ma 2/23 primer amplified two alleles, 240 and 280 bp in size, that appeared consistently in control (non-irradiated susceptible clone) but were absent in all irradiated resistant to salinity clones. Cluster analysis using Dice's coefficient and UPGMA algorithm detected genetic variation within induced mutant clones. Molecular markers also detected variation between the irradiated and non-irradiated clones. Principal coordinate analysis also confirmed this pattern of genetic diversity. These results showed the potential of mutation method as a technique for banana breeding. Moreover, SSR markers can also be utilized for detecting the genetic diversity among the banana mutants.

Key words: Banana, genetic diversity, mutation, salinity, microsatellite

INTRODUCTION

Edible bananas are derived from either *Musa acuminata* (A) or *M. balbisiana* (B), or a combination of both. The most important type of fruit commercially is the dessert banana, which is eaten fresh and makes up most of the international trade of this genus (1). 'Dwarf Cavendish' (AAA) is the most popular dessert type of bananas produced by small-scale farmers in some southern regions of Iran and consumed by the local market (2). This cultivar produces large bunches of medium-sized fruit. It is planted and better adapted to the cool climate of the subtropics than most other commercial cultivars (3). However, their productions are severely constrained by salinity in Iran (2).

Breeding strategies to improve this problem are difficult to implement because of the triploid, highly sterile and parthenocarpic of edible bananas (4, 5). Mutation induction seems to be a suitable technique to improvement vegetatively propagated banana (6). Two banana cultivars ('Klue Hom Thong KU1' and 'Novaria') derived from *in vitro* mutation induction have been released to date (7).

Induced mutants are usually differentiated from each other and from the original cultivar on the basis of morphological characteristics. However, the classification of certain accessions on this basis has been disputed (4, 8). Advances in molecular markers analysis may defeat these restrictions. In banana, several DNA marker techniques have been used to investigate genetic relationships between *Musa* accessions, and to determine differences in soma clonal variants and radiation-induced mutants (4). Microsatellites or sequence tagged microsatellite sites (STMS) are one type of PCR-based markers

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that consist of tandem reiterated, short DNA sequences (i.e., about 1 to 6 bp) motifs. The popularity of nuclear microsatellites stems from a unique combination of several important advantages, namely their co-dominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing size variation by PCR with pairs of flanking primers (9).

In the present investigation, the STMS markers in banana were used to assess genetic diversity inside the gamma irradiated bananas and also to

assess if mutation technique could be useful for genetic variation and breeding of banana germplasm.

MATERIALS AND METHODS

Plant material

Twenty two clones of *Musa* AAA (Cavendish subgroup) 'Dwarf Cavendish' including one non-irradiated clone sensitive to salinity as control (No. 1) and twenty one irradiated clones resistance to salinity (No. 2 to 22) were used in this study as previously described (2) (**Table 1**).

Table 1. Primers tested in the characterization of 22 banana clones, with original allele length; and optimized annealing temperatures

No.	Primer code	Sequence (5'→3')		Expected allele length (bp)	Annealing temperature (°C)
		Forward	Reverse		
1	STMS1FP/1RP	TGAGGCGGGGAATCGGTA	GGCGGGAGACAGATGGAGTT	126	53
2	STMS7FP/7RP	AAGAAGGCACGAGGGTAG	CGAACCAAGTCAAATAGCG	212	52
3	STMS9FP/9RP	ATGTCGCTTCGGACCAGA	GCAGGACGAAGAAGACTTACC	162	51
4	Ma 1/3	AGATGGCGGAGGGGAAGAGCCG	CCC GCCAAAAGTTAAGATC	160	49
5	Ma 1/5	AGATGGCGGAGGGGAAGAGCCG	GATCCAAGCTTATCGA	120	44
6	Ma 1/16	TTTGCCTGGTTGGGCTGA	CCCCCTTTCCTCTTTTGC	150	51
7	Ma 1/17	AGGCGGGGAATCGGTAGA	GGCGGGAGACAGATGGAGT	124	53
8	Ma 2/23	ATTCGGACAATCTTACCCA	CCCAAATCTCTCTCCCTC	250	47
9	Ma 3/60	TGGCTGACAATTACATGACA	GCGCACTGTGGTGTGT	133	48
10	Ma 3/90	GCACGAAGAGGCATCAC	GGCCAAATTTGATGGACT	140	49
11	Ma 3/130	ATTGGGGACAGGGACGAT	CCGGATCCAAGCTTATCGA	198	51

DNA extraction

Genomic DNA was extracted from 0.2 g of young leaves according to the modified CTAB method (10). Purity and quantity of the genomic DNA samples were determined with a spectrophotometer (Cecil, England) and confirmed using agarose gel electrophoresis. Only UV absorption ratios $A_{260}/A_{280} = 1.8-2$ were accepted.

DNA amplification

PCR reactions were carried out in a total volume of 15 µl containing 50 ng of template DNA, 1 U *Taq* polymerase (Fermentas, Germany), 2.5 µl 10X PCR buffer, 0.2 mM dNTP and 4 mM MgCl₂. Eleven of forward and reverse primers (Metabion, Germany) were used for the PCR (**Table I**). Amplification were performed in a thermal cycler (Biothermal, Germany) programmed for 3 min at 93°C, followed by 30 cycles of 40s denaturation at 93°C, 40s annealing at 44-53°C, 1 min extension at 72°C and a final

extension of 4 min at 72°C. The products were held at 4°C until analyzed. Modification of the annealing temperature for each primer resulted in improved band resolution. PCR amplification was confirmed by running 5 µl of PCR product on 1% agarose gels. Amplified samples were separated in 6% denaturing acrylamide gels in 1X TBE buffer at constant power of 80 W for 50 min. Silver staining was done by the procedure of Creste *et al.*, (2001) (11). Duplicate reactions were performed to ensure reproducibility. Molecular size of the amplified fragments was estimated using 100 kb DNA ladder (Fermentas).

Data analysis

Amplified fragments were scored for presence (1) or absence (0) in each clone. The genetic similarity between all the 22 clones was calculated according to the Dice coefficient. Relationships among clones were evaluated with a phenetic cluster analysis, using the unweighted pair-group method with arithmetic average

(UPGMA) clustering, and the multidimensional principal coordinate (PCO) analyses, and plotted in a phenogram using NTSYS-pc version 2.02 software. Polymorphic information content (PIC) values were calculated for each of the microsatellite loci based on following formula:

$$PIC = 2 \sum_{i=1}^{k-1} \sum_{j=i+1}^k P_i P_j (1 - P_i P_j)$$

where k represents the number of alleles, P_i and P_j are the frequencies of the i^{th} and j^{th} alleles within each locus, respectively.

RESULTS

A total of 11 primer pairs were tested to amplify specific products in PCR reactions of 22 banana clones. Of the 11 primers tested, 5 amplified products resulting in polymorphic bands; three (Ma 1/3, Ma 1/16 and Ma 3/90 primers) produced monomorphic bands; two (Ma 1/5 and Ma 1/17 primers) amplified unspecific products; and one (Ma 3/60 primer) did not amplify any products (**Table 2**). To ensure that the occurrence of null alleles was not a failure of reaction, the assays were repeated two times. The results showed that the reproducibility was 95.4-100% under the same amplification conditions.

Table 2. Microsatellite primers which produced clear and distinct amplification products used in this study

No.	Primer code	Amplification results	Number of alleles	Polymorphic alleles	Polymorphism (%)	PIC values
1	STMS1FP/1RP	Polymorphic	8	3	37.5	0.79
2	STMS7FP/7RP	Polymorphic	3	1	33.3	0.47
3	STMS9FP/9RP	Polymorphic	7	7	100	0.25
4	Ma 1/3	Monomorphic	1	0	0	-
5	Ma 1/16	Monomorphic	2	0	0	-
6	Ma 2/23	Polymorphic	5	5	100	0.48
7	Ma 3/90	Monomorphic	1	0	0	-
8	Ma 3/130	Polymorphic	2	2	100	0.85

From the eight primers that amplified polymorphic products, 29 alleles were detected, with a mean of 3.6 alleles per locus (**Table 2**). The number of alleles per locus ranged from 1 (in Ma 1/3 and Ma 3/90) to 8 (in STMS1FP/1RP). The mean polymorphism percentage was 74.1%, ranging from 33.3 to

100% for each microsatellite locus. The PIC values for the microsatellite loci ranged from 0.25 to 0.85 with an average of 0.56. Primers Ma 2/23 identified two alleles (240 and 280 bp) present only in non-irradiated clone sensitive to salinity (**Figure 1**).

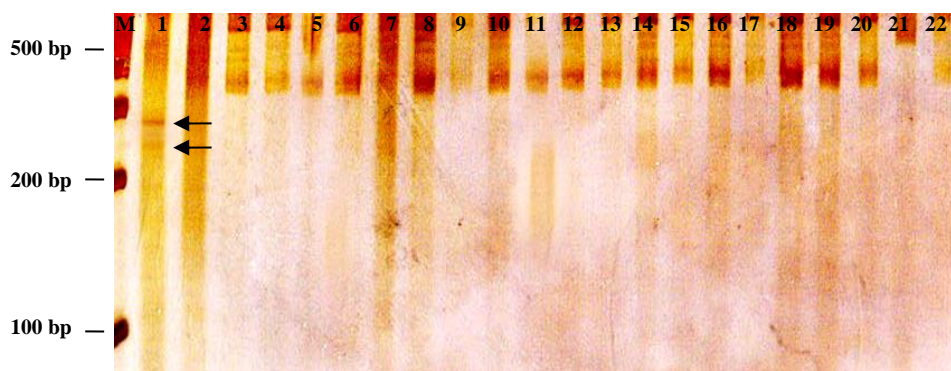


Figure 1. Banding pattern of banana clones with pair primer Ma 2/23. Arrows indicates two major alleles of 240 and 280 bp which was observed in sensitive clone but absent in all resistant clones. Lane 1 is non-irradiated clone sensitive to salinity and lanes 2-22 are irradiated ones resistant to salinity. M, 100 bp DNA ladder (Fermentas).

Table 3. Similarity of 22 clones analyzed according to the simple matching algorithm of the NTSYS program. No. 1 is non-irradiated clone sensitive to salinity and No. 2 to 22 are irradiated ones resistant to salinity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
2	0.80																						
3	0.80	1.00																					
4	0.86	0.90	0.90																				
5	0.86	0.85	0.85	0.95																			
6	0.85	0.88	0.88	0.97	0.95																		
7	0.84	0.87	0.87	0.97	0.95	0.95																	
8	0.82	0.85	0.85	0.95	0.90	0.97	0.97																
9	0.81	0.85	0.85	0.95	0.90	0.93	0.97	0.95															
10	0.81	0.85	0.85	0.95	0.90	0.93	0.97	0.97	1.00														
11	0.72	0.75	0.75	0.86	0.85	0.84	0.88	0.86	0.90	0.90													
12	0.80	0.82	0.82	0.93	0.88	0.91	0.95	0.93	0.97	0.97	0.93												
13	0.73	0.76	0.76	0.86	0.86	0.89	0.88	0.91	0.86	0.86	0.95	0.88											
14	0.79	0.81	0.81	0.91	0.86	0.93	0.93	0.95	0.91	0.91	0.91	0.93	0.95										
15	0.82	0.85	0.85	0.95	0.90	0.97	0.97	1.00	0.95	0.95	0.86	0.93	0.91	0.95									
16	0.79	0.81	0.81	0.91	0.86	0.93	0.93	0.95	0.91	0.91	0.91	0.93	0.95	1.00	0.95								
17	0.77	0.80	0.80	0.90	0.90	0.93	0.93	0.95	0.90	0.90	0.9	0.88	0.95	0.91	0.95	0.91							
18	0.80	0.83	0.83	0.93	0.88	0.95	0.95	0.97	0.93	0.93	0.88	0.95	0.93	0.97	0.97	0.97	0.93						
19	0.80	0.83	0.83	0.93	0.88	0.95	0.95	0.97	0.93	0.93	0.88	0.91	0.93	0.97	0.97	0.97	0.93	0.95					
20	0.82	0.85	0.85	0.95	0.90	0.93	0.97	0.95	0.95	0.95	0.9	0.93	0.91	0.95	0.95	0.95	0.90	0.93	0.97				
21	0.81	0.70	0.70	0.81	0.85	0.84	0.83	0.86	0.80	0.80	0.85	0.79	0.90	0.86	0.86	0.86	0.90	0.84	0.88	0.86			
22	0.81	0.70	0.70	0.81	0.85	0.84	0.83	0.86	0.80	0.80	0.85	0.79	0.90	0.86	0.86	0.86	0.90	0.84	0.88	0.86	1.00		

To estimate the similarities among clones, the simple matching coefficient gave similarity values ranging from 0.70 to 1.0 (**Table 3**). Minimum similarity coefficient was observed between clones No. 1 to 21 and 22, and No. 3 to 21 and 22. Clones No. 2 to 3, 8 to 15, 9 to 10, 14 to 16 and 21 to 22 exhibited 100% similarity and could not be distinguished with the primers used.

According to the UPGMA dendrogram, the twenty-two clones were divided into two major clusters. One cluster clearly represented the non-irradiated clone sensitive to salinity and another cluster contained all irradiated ones resistant to salinity (**Figure 2**). Within the second cluster and at a similarity coefficient of higher than 0.90, four subgroups were observed. The scatterplot produced from principal coordinate analysis distinguished four major groups in the bananas (**Figure 3**). The first and second dimensions also explained 33.8 and 22% of the variation, respectively.

DISCUSSION

Of a total of 11 primers were tested to assess the microsatellite polymorphism in 22 banana clones, five primers (45.4%) amplified scorable polymorphic products. Such lack of amplification products from banana and plantain SSR's has been reported elsewhere. Oriero *et al.*, (2006) used 44 primers to investigate genetic diversity in *Musa* accessions, while only nine primers (20%) generated amplification products (12). It may reflect divergences in the sequences flanking of the microsatellite loci or the primer-anchoring sequences flanking the microsatellite loci might differ sufficiently to restrict amplification of products (12, 13). Therefore, more primers are needed to analyze genetic variation among genotypes.

This SSR protocol is shown highly reproducible. Since the DNA sequences that flank the SSR loci are conserved, suitable primers to amplify the SSR loci were designed, resulting in highly specific PCR amplification and therefore high reproducibility (4).

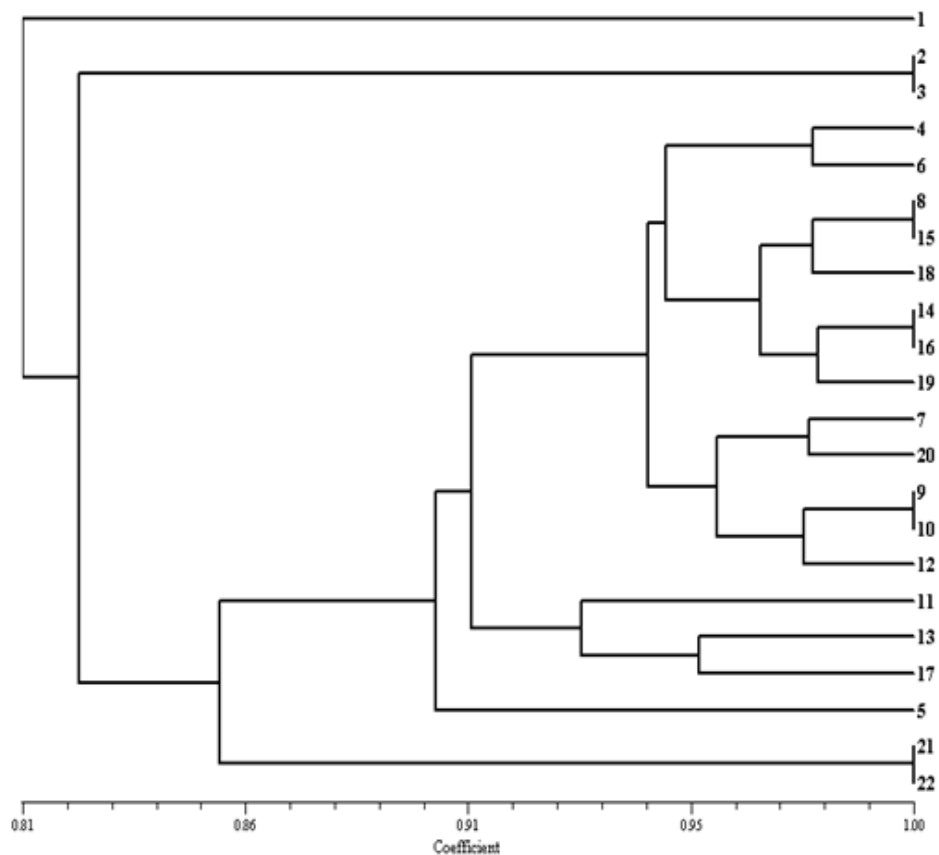


Figure 2. Dendrogram generated by UPGMA cluster analysis from the similarity matrix obtained by Dice genetic distance for 22 banana clones based on SSR markers. No. 1 is non-irradiated clone sensitive to salinity and No. 2 to 22 are irradiated ones resistant to salinity.

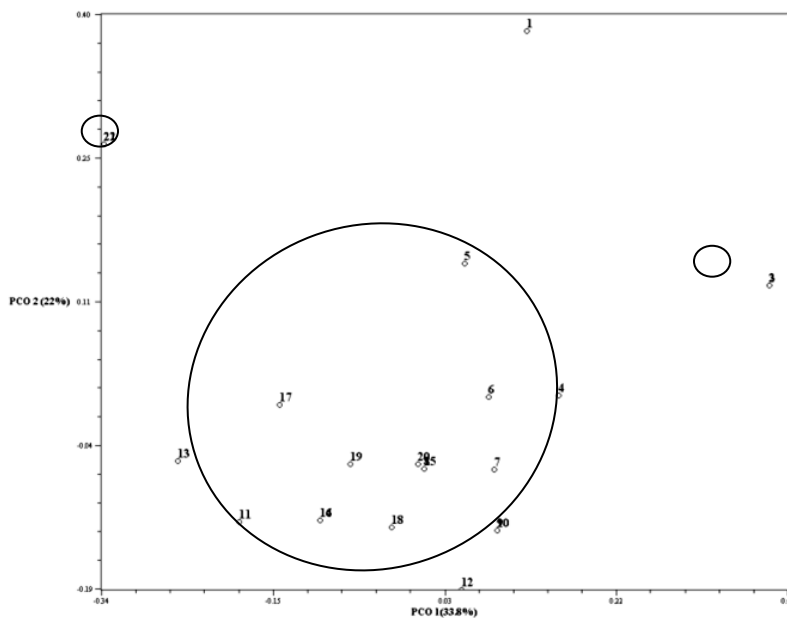


Figure 3. Dendrogram generated by UPGMA cluster analysis from the similarity matrix obtained by Dice genetic distance for 22 banana clones based on SSR markers. No. 1 is non-irradiated clone sensitive to salinity and No. 2 to 22 are irradiated ones resistant to salinity.

Primers STMS1FP/1RP, STMS9FP/9RP and Ma 2/23 presented more alleles than expected, considering the basic ploidy level (3x) of the clones. Crouch *et al.*, (1999) had also detected an unexpectedly high number of amplification from AA and AAB banana genotypes plus their progeny, and suggested that a high frequency of duplication may occur in genomes, in addition to the presence of heterozygous and/or homoeologous loci (14).

The variation in the number of repeat units is thought to be due to unequal crossing-over or slippage of DNA polymerase during replication of repeat tracts (4). Since these clones are generated common origin, the high polymorphism arise from genetic changes can also be occurred during irradiation process. Ganapathi *et al.*, (2008) and Sales and Espino (2008) reported that banana plants irradiated with gamma rays revealed variation among the clones using RAPD markers (15, 16). Pestana *et al.*, (2011) also evaluated genetic dissimilarity among putative 'Preciosa' banana mutants generated by gamma-ray irradiation, using morphoagronomic characteristics and ISSR markers (17). They found that genetic distances between the putative 'Preciosa' mutants varied from 0.21 to 0.66. Mutations are defined as heritable changes in the DNA sequence (i.e., changes at the gene, chromosome, and genome levels, including chromosomal breaks, inversion, duplications, translocations, and point mutations) that can be induced by physical or chemical mutagens. Whereas genetic diversity is essential to plant breeding, induced mutations can play an important role in development of many fruit cultivars. They change only one or a few specific traits of an elite cultivar, and can contribute to fruit improvement without upsetting neither the requirements of the fruit industry nor the consumers (7).

The clustering analysis was able to separate clones containing the non-irradiated alone, from irradiated clones. These results are in agreement with previous results for clustering clones according to the UPGMA dendrogram based on RAPD markers (2). A two dimensional plot is useful to distinguish closely related individuals into groups. Distribution of clones on the scatter plot revealed a good fitness with the clustering of clones observed in the dendrogram.

Primer Ma 2/23 could discriminate between the non-irradiated susceptible and irradiated resistant clones. Hautea *et al.*, (2004) reported out of 36 SSR primers tested, only Ma 1/3 was able to detect variation between the non-irradiated and irradiated clones, or between the clones irradiated with 3 Gy fast neutron and 40 Gy gamma rays (4). This marker can be useful for the selection of banana resistance to salinity in a breeding program, but DNA sequencing and comparing were needed to clarify the issue. Additional evidence for such association could be obtained through the analysis of more resistant and susceptible banana clones.

CONCLUSION

The study has produced promising induced mutants of Dwarf Cavendish banana cultivar, and has established microsatellite marker technique for analysis of mutation-induced changes in a breeding program.

ACKNOWLEDGMENT

We are grateful to M. Rahimi and C. Vedadi of Agricultural, Medical and Industrial Research School, Atomic Energy Organization of Iran (AEOI) for providing plant materials and useful assistance. This research was supported by AEOI.

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