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IN VITRO MUTAGENESIS IN BANANA AND VARIANT SCREENING THROUGH ISSR

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Abstract

An efficient culture medium for *In vitro* clonal propagation of banana cultivars was established. Meristamtic tips of Basrai and GCTCV-215 were irradiated with different doses of gamma rays viz., 0, 10, 20, 30 and 40Gy through cobalt 60 source. Eleven random ISSR primers were used to study genetic variation among the radiated population. ISSR primers produced 37 bands in all the clones studied. Two monomorphic and 35 polymorphic bands were observed during the study. The number of fragments produced by the primers ranged from 2-5, with an average of 3.45 fragments. Fragments size ranged from 198bp-1.842kb. Genetically most similar genotypes were G-215MP3 and G-215MP4 (89.8%) while most dissimilar genotypes were Basrai and G-215MP2 (38.5%). On the basis of similarity matrix clones could be divided into three clusters and two groups.

Introduction

Banana is one of the most important staple fruits in the world and major export commodity for many tropical and sub-tropical countries. In Pakistan it is grown extensively in lower part of Sindh (Khatri *et al*., 2009). During 2007-08, banana was cultivated on an area of 32.9 thousand hectares with a production of 127 thousand tons (Anon., 2009). In the last ten years, banana production decreased continuously in Pakistan due to spread of Banana bunchy top virus. Approximately 98% area was covered by a variety Basrai (Bloach & Siddique1991, Khatri *et al*., 1997). This created the monoculture, that resulted in no genetic buffering and thus facilitating diseases spread (Khushk *et al*., 1993).

Bananas are propagated vegetatively through suckers. Since most of the edible bananas are triploid due to which they become sterile and parthenocarpic fruit development hampered banana improvement through conventional breeding methods. Mutation breeding and biotechnological methods can offer as useful tools for banana improvement. The extensive work on *in vitro* propagation of banana (Banerjee & De Langhe, 1985; Wong, 1986; Novak *et al*., 1989; Suprasanna *et al*., 2002 and 2008) provided opportunity for *In vitro* mutagenesis and selection in different banana cultivars. Somaclonal variation is the source of inducing genetic variability in vegetatively propagated crops like banana and generated variation has been used to obtain superior quality banana clones (Maria & Garcia, 2000; Asif *et al.*, 2004; Hwang & Ko, 2004). Clonal variation is the manifestation of epigenetic influence or genetic variability induced by *In-vitro* mutagenesis and created diverse population carrying interesting heritable traits (Soniya *et al*., 2001).

Molecular technology has been used to investigate the genetic polymorphism because it provides more accurate results as compared to other methods. Molecular techniques have been used to analyze genetic information in fruit crops such as isozyme (Bhat *et al*., 1992), RFLP (Gawel & Jarret, 1991), RAPD (Howell *et al*., 1994, Khatri *et al*., 2009), SSR (Eiadthong *et al*., 1999; Grapin *et al*., 1998) and AFLP (Wong *et al*., 2002). ISSR (inter-simple sequence repeat) is another molecular technique developed by Zietkiewicz *et al*., (1994) and it can screen 100 to 3,000 bp DNA fragments. ISSR is amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18bp). The main advantage of ISSR is that it is randomly distributed throughout the genome and results are reproducible.

The present study reports the use of ISSR markers in studying the genetic variability among the population of Basrai and GCTCV-215 regenerated through *In-vitro* mutagenesis.

Materials and Methods

***In-vitro* mutagenesis:** Explant consist of meristem tips of banana derived micropropagation technique were isolated and irradiated with different doses of gamma rays viz., 0, 10, 20, 30 and 40Gy through cobalt 60 source. After irradiation the shoot tips were cultured on MS medium containing 20µg/l BAP for proliferation to raise M_1V_1. After 21 days the individual plants were dissected and cultured on the same medium up to M_1V_4. Then the plantlets were cultured for rooting on MS medium with 1mg/l IBA. The well developed plants were acclimatized and transferred to field for evaluation.

DNA isolation: DNA was extracted from the young leaves of mutants and their parents through DNA isolation kit (Gentra system, Minnesota, USA) (Khan *et al*., 2009). Isolated DNA was quantified on spectrophotometer (Bio-mate 3), at absorbance of 260/280nm. The quality of DNA was further checked on 0.8% agarose gel.

Polymerase chain reaction: PCR was optimized for genetic diversity in banana using ISSR markers. The amplification reaction was carried out in 25µl reaction volume containing 1X PCR buffer, 0.5mM dNTPs, 2.5mM Mg++, 1µM primer, 0.25 U Taq DNA polymerase and 50ng of template DNA. The amplification profile consisted of initial denaturation of the template DNA at 94^{o}C for 4 min, followed by 10 cycles of denaturation at 94^{o}C for 45 sec; touchdown one-degree decrement for annealing temperature started with 5^{o}C above temperature for each primer for 30 sec and 72^{o}C extension for 2 min. Followed by 25 cycles of 94^{o}C for 45 sec, last annealing temperature for 30 sec., and 72^{o}C for 2 min and final extension of 72^{o}C for 7 min.

The amplification products were visualized in an ultra transilluminator, after horizontal electrophoresis in 1.5% agarose gel, using the TBE buffer, stained with Ethidium bromide.

Data analysis: Presence of band on gel was scored as (1) and absence of band as (0) for the analyses. The similarity was calculated according to Nei & Li (1979). Similarity coefficient was utilized to generate a dendrogram by means of Un-weighted Pair Group Method of Arithmetic means (UPGMA).

Results

Genomic DNA produced multiple fragments with 11 random primers. The total number of the amplified DNA products (bands) yielded across the set of banana mutants along with their parents was 37, out of which 35 were polymorphic and 02 were monomorphic. The number of fragments produced by the primers ranged from 2-5, with an average of 3.45 fragments. Fragments size ranged from 198bp to 1.842kb. Maximum 05 polymorphic bands were amplified with primer (OSP) 834, 840 and 845 whereas minimum one monomorphic band was amplified with primer (OSP) 881 (Table 1).

Table 1. Sequences of primers and amplified products.

Primer name	Primer sequence	Range of amplified prod.	Polymorphic bands	Monomorphic bands	Total no of bands
OSP 810	GAGAGAGAGAGAGAGAT	298 bp-860 bp	03	01	04
OSP 812	GAGAGAGAGAGAGAGAA	198 bp-989 bp	02	NIL	02
OSP 814	CTCTCTCTCTCTCTCTA	258 bp-1.0 kb	03	NIL	03
OSP 815	CTCTCTCTCTCTCTCTG	222 bp-790 bp	02	NIL	02
OSP 822	TCTCTCTCTCTCTCTCA	363 bp-1.19kb	02	NIL	02
OSP 834	AGAGAGAGAGAGAGAGYT	453 bp-1.31kb	05	NIL	05
OSP 840	GAGAGAGAGAGAGAGAYT	516 bp-1.4 kb	05	NIL	05
OSP 845	CTCTCTCTCTCTCTCTRG	336 bp-1.842 kb	05	NIL	05
OSP 852	TCTCTCTCTCTCTCTCRA	523 bp-898 bp	02	NIL	02
OSP 876	GATAGATAGACAGACA	275 bp-1.1 kb	03	NIL	03
OSP 881	GGGTGGGGTGGGGTG	302 bp-976 bp	03	01	04
			35	02	37

Primer OSP 810 (Fig. 1) amplified four bands with the range of 298-860bp in which three were polymorphic and one monomorphic. Five bands were amplified by primer OSP 840 (Fig. 2), whereas mutant BMP3, G-215MP2 and G-215MP4 did not have the corresponding sequence in their genome therefore no amplication was observed. Primer OSP 881 (Fig. 3) amplified four bands in which one is monomorphic (848bp) and three were polymorphic, ranged between 302-976bp. Specific band (976bp) was observed in BMP1, BMP2, BMP4 and G-215MP1.

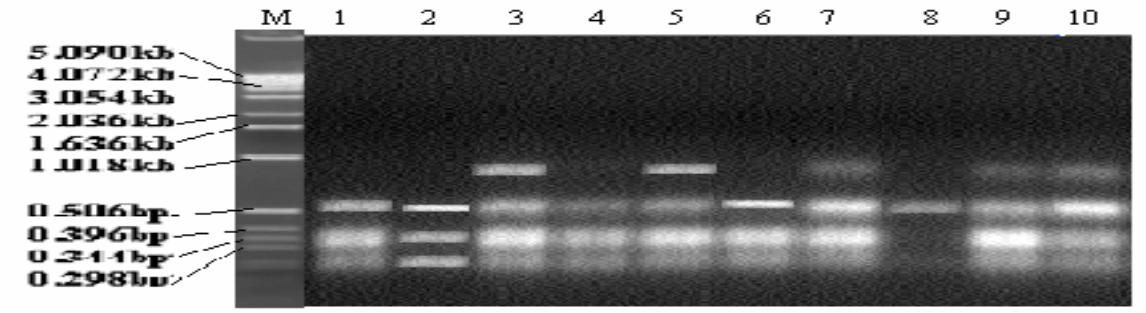

Fig. 1. ISSR profile of banana clones using primer OSP 810, M=DNA marker, 1=Basrai, 2=BMP1, 3=BMP2, 4=BMP3, 5=BMP4, 6=GCTCV-215, 7=G-215MP1, 8=G-215MP2, 9=G-215MP3, 10=G-215MP4.

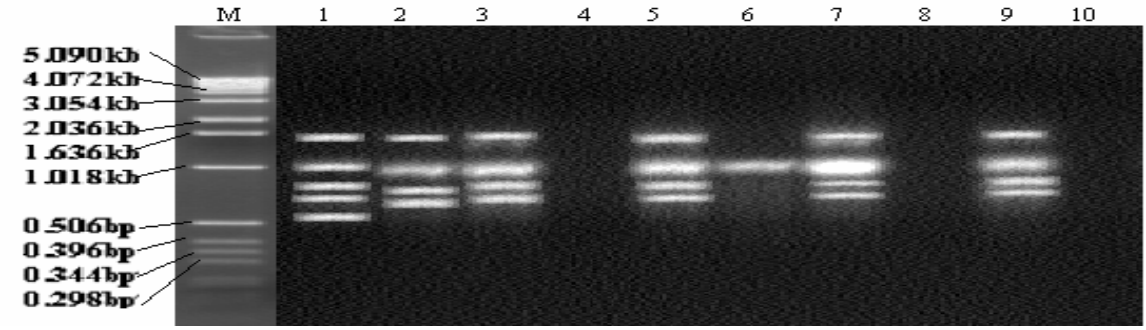

Fig. 2. ISSR profile of banana clones using primer OSP 840, M=DNA marker, 1=Basrai, 2=BMP1, 3=BMP2, 4=BMP3, 5=BMP4, 6=GCTCV-215, 7=G-215MP1, 8=G-215MP2, 9=G-215MP3, 10=G-215MP4.

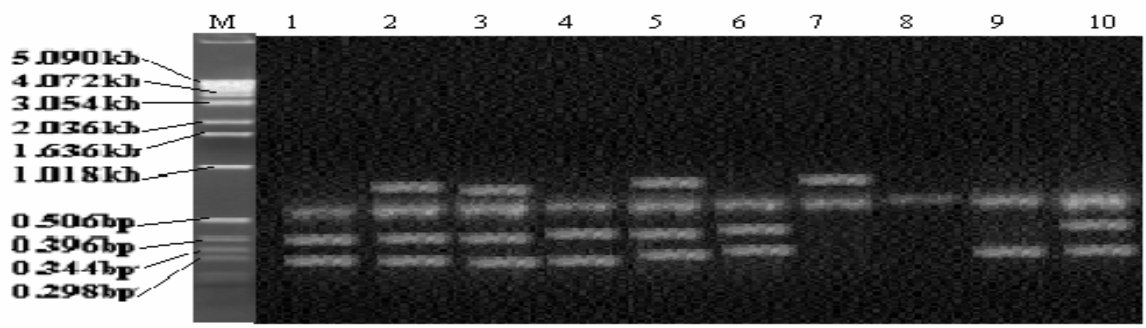

Fig. 3. ISSR profile of banana clones using primer OSP 881, M=DNA marker, 1=Basrai, 2=BMP1, 3=BMP2, 4=BMP3, 5=BMP4, 6=GCTCV-215, 7=G-215MP1, 8=G-215MP2, 9=G-215MP3, 10=G-215MP4.

Primer OSP 881 produced 75% polymorphic bands and 25% monomorphic bands, while primer OSP 812, primer OSP 815, primer OSP 822 and primer OSP 852 produced 100% polymorphic bands (Table 1). Genetically most similar genotypes were G-215MP3 and G-215MP4 (89.8%) while most dissimilar genotypes were basrai and G-215MP2 (38.5%) (Table 2).

On the basis of similarity matrix tested banana population could be divided into three clusters, designated A to C (Fig. 4). Cluster 'A' comprises of the G-215MP3 and G-215MP4 and Cluster 'B' contained predominantly BMP2 and BMP3 showing more genetic similarity among each other and forming a group 'One' with distinct genotype GCTCV-215. Mutants BMP4, G-215MP1, BMP1 and Basrai are in cluster 'C' and a distinct clone G-215MP2 is showing 55% similarity with cluster 'C' and forming a group 'Two'.

Table 2. Similarity co-efficient among the banana clones calculated according to Nei & Li's coefficient.

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
L1	1									
L2	0.606	1								
L3	0.661	0.611	1							
L4	0.588	0.568	0.805	1						
L5	0.674	0.778	0.729	0.626	1					
L6	0.618	0.583	0.623	0.592	0.641	1				
L7	0.687	0.747	0.629	0.574	0.805	0.582	1			
L8	0.385	0.4	0.391	0.461	0.428	0.528	0.446	1		
L9	0.686	0.719	0.802	0.727	0.829	0.668	0.718	0.476	1	
L10	0.603	0.651	0.745	0.788	0.764	0.624	0.65	0.537	0.898	1

1=Basrai, 2=BMP1, 3=BMP2, 4=BMP3, 5=BMP4, 6=GCTCV-215, 7=G-215MP1, 8=G-215MP2, 9=G-215MP3, 10=G-215MP4

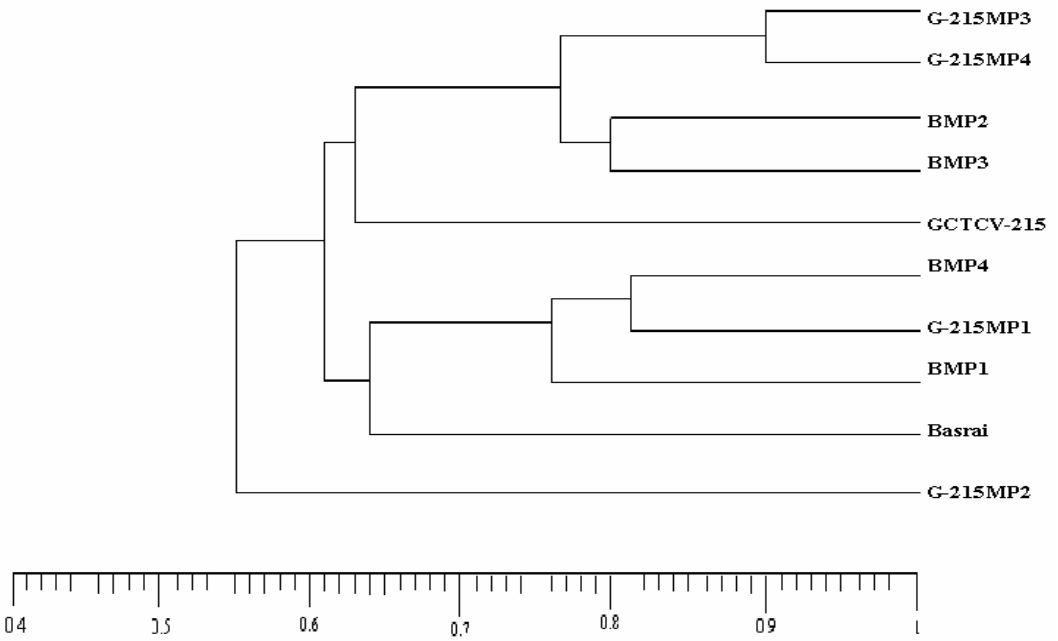

Fig. 4. Dendrogram of ten banana clones developed from ISSR data using un-weight pair group method of arithmetic means (UPGMA).

Discussion

Inter simple sequence repeat (ISSR) marker have more potential to screen the genetic diversity. For these purpose 11 ISSR primers has been selected to estimate the level of genetic diversity in eight mutants of Basrai and GCTCV-215 along with parents. Although mutants are usually differentiated from the original plant by phenotypic analysis, but it can be severely limited by the large size of mutagenized populations, particularly in the case of banana and developmental and environmental effects. The application of molecular markers may overcome this limitation (Hautea *et al*., 2004). DNA amplification of polymorphic markers (presence and absence of bands) could be the result of base deletion or insertions at priming sites (Muhammad & Othman, 2005). In this study, ISSRs were chosen because it amplify different region of the genome allowing better analysis of genetic stability/variation of clones, as well as their simplicity, reproducibility and cost-effectiveness (Racharak & Eiadthong, 2007). Earlier studies were implemented RAPD in analyzing banana genotypes (Hautea *et al*, 2004; Finalet *et al*., 2000; Mathius & Haris, 1999, Khatri *et al*., 2009). However, in this study we were able to examine efficiently and differentiate genotypic changes in banana created by gamma rays with the help of ISSR markers.

The specific band 516 bp was observed in Basrai with primer OSP 840, indicating the specificity of clone. The presence of specific bands/loci in the parental banana clones and loss of them in the regenerated parental plantlets indicates the loss of certain loci during tissue culture due to *In-vitro* mutagenesis and produce the somaclonal variation According to Hautea *et al.,* (2004) and Newbury *et al*., (2000), occurrence of specific bands/loci in the regenerated plants and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants. Such specific loci are of high importance in the genetic identification of the somaclones from each other (Uma *et al*., 2006).

The absence of a band/ loci in one of the genotypes indicates genetic changes of the plants brought about during *In-vitro* mutagenesis (Eiadthong *et al*., 1999; Soniya *et al.,* 2001). Even single base change at the primer annealing site is manifested as appearance or disappearance of ISSR bands (Creste *et al*., 2003), it could be suggested that tissue culture conditions have induced varied amount of genetic changes in different regenerated plants (Newbury *et al*., 2000). Some of these changes appeared identical in different plants as represented by appearance of non-parental bands. The reason for such commonness of genetic variation in these plants could be because they were all derived from the same callus (Soniya *et al.,* 2001). The variations observed in the ISSR pattern may be due to different causes including loss/ gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke *et al*., 1991; Muhammad & Othman, 2005).

Explant source is also considered as one of the critical variable for somaclonal variation (Godwin *et al.,* 1997; Grapin *et al*., 1998). Since explants may present dissimilar regeneration rates, selection procedures can differ among different explants types. Jong & Custers, (1986), reported that plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex-induced calli. Therefore it may be suggested that different sources of explants may be tried in banana and compare the level of genetic variation obtained. The present finding indicates the possible use of *In-vitro* mutagenesis as a source for inducing genetic variation in banana cultivars which may be used in planning breeding program of banana.

Grouping of the parental cultivar and their mutants indicate the genetic distinctness of the clones studied as they are placed in different clusters/groups far from each other (Fig. 4 & Table 2). The mutant plants MP3 and MP4 of GCTCV-215 show comparatively lower degree of genetic difference from the parental plants as they are placed in the cluster 'A' much closer to each other compared to the rest of banana clones. A distinct mutant G-215MP2 shows high degree of genetic difference from its parental plants as well as rest of the clones. It is concluded that distinct banana mutants could be used for breaking the monoculture of Basrai variety in province of Sindh, Pakistan and this information will be helpful for breeders to produce distinct clones.

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