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GENETIC INSTABILITY OF SUGARCANE PLANTS REGENERATED THROUGH DIRECT REGENERATION METHOD

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ABSTRACT

Sugarcane probably represents the most complex genome being studied todate. Current cultivars are derived from complex interspecific hybridization and they are highly polyloid and aneuploid with 100 to 130 chromosomes. When cells having such high aneuploid and plant plantlets regenerated from these cells their genetic fidelity become under question. Therefore, RADP (Random amplified polymorphic DNA) technique was used to detect variations in the directly regenerated plants of sugarcane. Plants directly regenerated from tissue of NIA-98 were studied for existing genetic variability with in the tissue in the form of aneuploidy. The polymorphism were observed in 39 out of 42 bands (92.8%). Polymorhism of band confirms that direct regeneration cannot maintain genetic fidelity in sugarcane but can be considered as a good source of exploring existing aneuploidy.

Key words: sugarcane, aneuploidy, RAPD.

INTRODUCTION

Sugarcane is an important industrial crop of tropical and subtropical regions and is cultivated on close to 20 million hectares in more than 90 countries. It is grown in different parts of the world from the tropics to subtropics, and accounts for around 60% of the world's sugar (Khan *et al*., 2004). It is also one of the important cash crops in many developing/developed countries, with a high trade value (Naz, 2003). The importance of sugarcane has increased in recent years because cane is an important industrial raw material for sugar industries and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed (Arencibia 1998). The intricate flowering nature of sugarcane is a major barrier for genetic improvement by conventional hybridization in Pakistan. Moreover, the perennial and highly heterozygous nature of the plant coupled with a prolonged juvenile period limits the speed of improvement using traditional methods (Khan *et al*., 2005). Besides, sugarcane is highly polyploid and aneuploid crop (Jannoo, *et al.*, 1999). Further, in vegetatively propagated plants like sugarcane it takes many years to evolve a desirable clone from an economic and commercial point of view by routine propagation methods. Therefore, application of biotechnological tools for genetic improvement of sugarcane attains greater significance, which in turn depends upon the availability of an efficient regeneration system.

Currently, a proportion part of the sugarcane plantlets are produced by tissue culture methods. The terminal portions of the stalks are collected from disease-free field plants. Shoot tips (4 cm) are then obtained by removing older surrounding leaves. After disinfection, the apical meristems are aseptically excised and placed on liquid MS medium (Murashige and Skoog, 1962) for up to 30 days. Culturing is done by transferring the plantlets to the same medium containing Kinetin, IAA and IBA to produce shoot branches, followed by subculturing for up to six months (Khan *et al.*, 2005). At a multiplication rate of about five plantlets per flask and one subculturing per month, it is feasible to obtain as many as 20,000 plantlets from a single explant.

Direct regeneration in sugarcane from immature leaves explant is very useful in sugarcane breeding programs, because of the time it saves in multiplying the promising varieties and clones and in facilitating the acquisition of large volumes of material. Direct regeneration can also be useful in eliminating pathogens (Gill *et. al.*, 2006).

Despite the advantages of *in vitro* propagation, phenotypic instability has been observed in micropropagated species, including sugarcane (Irvine *et al*., 1984; Bailey and Bechet, 1989; Irvine, 1991; Peros *et al*., 1994; Burner and Grisham, 1995; Taylor *et al*., 1995). Larkin and Scowcroft (1981) coined the term somaclonal variation to describe the occurrence of genetic variants derived from *in vitro* procedures. Factors such as explant source, time of culture, number of subcultures, phytohormones, genotype, media composition, the level of ploidy and genetic mosaicism are capable of inducing *in vitro* variability (Silvarolla, 1992).

The stimulation of shoot development is not expected to generate genetic instability, since this technique uses the normal ontogenetic route for shoot multiplication from explants. In comparison, plant regeneration produced by culturing tissue sections lacking a preformed meristem (adventitious origin) (Phillips *et al*., 1990; Phillips *et al*., 1994; Karp, 1995) or derived from callus and cell cultures (*de novo* origin) (Damasco *et al*., 1996) is more

susceptible to somaclonal variation. In the case of sugarcane, shoot tip culturing induces considerable phenotypic variability (Burner and Grisham, 1995).

Molecular markers are widely used to detect and characterize somaclonal variation at the DNA level (Ford-Lloyd *et al*., 1992; Cloutier and Landry, 1994; Barrett *et al*., 1997). Of the available techniques, RAPD is among the most useful ones (Rani *et al*., 1995; Taylor *et al*., 1995; Shoyama *et al*., 1997; Todoroviska *et al*., 1997; Rout *et al*., 1998). Changes in the RAPD pattern may result from the loss/gain of a primer annealing, caused by point mutations or by the insertion or deletion of sequences or transposition elements (Peschke *et al*., 1991).

In this study, we used RAPD to investigate the somaclonal variation in Pakistani sugarcane varieties derived from direct regeneration method by explanting immature leaves.

MATERIALS AND METHODS

Plant material

Sugarcane clones NIA-98 was used, fresh plant material of immature leaf segments was collected from 6-month-old field-grown plants (Experimental Farm of NIA, Tando Jam), RAPD analysis was conducted in hundred regenerants of NIA-98 to confirm the genetic fidelity of direct regeneration system.

DNA extraction:

DNA was extracted from fresh leaves of sugarcane varieties using DNA isolation Kit (Gentra system, Minnesota, USA.). Two hundred mg fresh leaves were ground in liquid Nitrogen; 3 ml of the cell lysis solution (Tris [hydroxymethyl] aminomethane, ethylenediaminetetra acetic acid & sodiumdodecyl sulfate) was added with leaf sample to the 15 ml centrifuge tube and incubated at 65°C for 60 minutes. A 15µl of RNase (Gentra Kit, Minnesota, USA.) solution was then added to the cell lysate and incubated at 37°C for 30 minutes. Protein precipitation solution (GENTRA Kit, Minnesota, USA.) was added and vortex for 20 seconds and the tubes were placed on ice for 30 minutes. The mixture was centrifuged at 2000 x g for 10 minutes. Supernatant containing DNA was poured in the separate 15ml centrifuge tube and DNA was precipitated by centrifuging at 2000 x g with 3 ml of isopropanol absolute. Ethanol (70%) was used to wash the pellet and the DNA samples were then hydrated with TE buffer. DNA was quantified on spectrophotometer (BIOMATE 3).

DNA amplification

Fourteen primers from Gene Link (NewYork, U.S.A), each ten bases in length, were used to amplify the DNA (Table 1). PCR reaction was carried out in 25µl reaction mixture containing 13ng of template (genomic DNA), 2.5mM $MgCl_2$ (Eppendorf, Hamburg, Germany), 0.33mM of each dNTPs (Eppendorf, Hamburg, Germany), 2.5U of Taq polymerase (Eppendorf, Hamburg, Germany) and 1µM of primer in a 1xPCR reaction buffer (Eppendorf, Hamburg, Germany). The amplification reaction was performed in the Eppendorf Master cycler with an initial denaturation for 5 min at 94°C, then 32 cycles:1 min denaturation at 94°C; 1 min annealing at 52°C; 2min extension at 72°C. Final extension was carried out at 72°C for 10 min. Amplified products were analyzed through electrophoresis on 1.5% agrose gel containing 0.5X TBE (Tris Borate EDTA) at 72 Volts for 2 hours, the gel contained 0.5µg/ml ethidium bromide to stain the DNA and photograph was taken under UV light using gel documentation system (Vilber Lourmat, France) .

Data analysis

The PCR products were electrophoresed on 1.5% agarose gels using 0.5x Tris Borate EDTA (TBE) buffer and visualized by ethidium bromide staining under UV light and photographed using Vilbour, Gel documentation System. Ten sugarcane genotypes were compared with each other using amplification profiles and band of DNA fragments were scored as presence of bands as (1) and absence of band as (0) from RAPD of amplification profile. Coefficient of similarity among cultivars was calculated according to Nei and Li (1979). A dendrogram based on these similarity coefficients was constructed by using unweighted pair group method of arithmetic means (UPGMA).

RESULTS AND DISCUSSION

Genomic DNAs of direct regenerated plantlets of NIA-98 produced multiple fragments with ten arbitrary primers. The total numbers of scorable bands were 42, out of which 39 were polymorphic and only 03 were monomorphic. Fragments ranged in size from 200 bp to 3 kb. The number of fragments produced by various primers

ranged from 1- 8, with an average of 4.3 fragments per primer. The level of polymorphism was varied with different primers. Maximum 08 bands were amplified with primer A-18 and minimum one band was amplified with primer C-07. The size of fragments ranged from 200 bp – 3.05 kbp. Similarity coefficient reflects that the micropropagated plantlets are the exact copy of its parent and all the directly regenerated plantlets irrespective of its treatments were genetically different with its parent and this difference is ranged from 23-49% (Table 2). Of the 42 loci analyzed using DNA from direct regenerants of NIA-98 plants, 39 were polymorphic, indicating an intrinsic polymorphism rate of 92.8%. Such a variation may derive from point mutations or alterations in chromosome number. The effects of pre-existing DNA polymorphism in clonally propagated plants were also taken into consideration. Plant meristems have an organized layer structure, in dicots usually three, in monocots even more. Cells in the tunica divide predominantly anticlinally, and thus do only rarely change position within the differentiated meristem. Therefore, the meristem of vegetatively propagated plants can represent a complex chimerical structure. A possible chimeric nature of the cultivar used may also be the reason for its high phenotypic instability, rather than an intrinsic genetic factor. Tissue culture was thus responsible for the generation of new variability therefore high rate of molecular polymorphism was observed. Heinz and Mee (1971) working with callus-derived cultures from sugarcane variety H50-7209, detected clones with chromosomal numbers ranging from 2n = 94 to 120. In contrast, chromosome stability was described for varieties NA56-79 (2n = 114) and Co419 (2n = 213) by Silvarolla and Aguiar-Perecin (1994), who developed a technique to obtain intact somatic metaphase sugarcane cells. Together, these observations suggest either some genotypes are more susceptible to somaclonal variation, or that the *in vitro* instability is actually a consequence of a genotype *versus* culture medium interaction.

Table 1. Sequence of the primers.

Primer	Sequence	Primer	Sequence
A-01	CAGGCCCTTC	B-10	CTGCTGGGAC
A-02	TGCCGAGCTG	B-17	AGGGAACGAG
A-03	AGTCAGCCAC	C-02	GTGAGGCGTC
A-15	TTCCGAATTT	C-05	GATGACCGCC
A-18	AGGTGACCGT	C-07	GTCCCGACGA
A-20	GTTGCGATCC	C-08	TGGACCGGTG
B-06	TGCTCTGCCC	C-09	CTCACCGTCC

Table 2. Similarity coefficient among the direct regenerants of NIA-98 calculated according to Nei and Takezaki, (1983) using power marker V3.23.

	DR1	DR2	DR3	DR4	DR5	DR6	DR7	DR8	DR9	MP	Parent
DR 1	1										
DR 2	0.70	1									
DR 3	0.65	0.67	1								
DR 4	0.49	0.74	0.70	1							
DR 5	0.67	0.56	0.60	0.44	1						
DR 6	0.51	0.40	0.49	0.33	0.65	1					
DR 7	0.58	0.79	0.70	0.63	0.72	0.60	1				
DR 8	0.60	0.53	0.44	0.42	0.60	0.58	0.56	1			
DR 9	0.47	0.44	0.44	0.42	0.56	0.72	0.60	0.72	1		
MP	0.65	0.72	0.63	0.51	0.70	0.58	0.74	0.77	0.63	1	
Parent	0.65	0.72	0.63	0.51	0.70	0.58	0.74	0.77	0.63	1	1

MP = Micropropagated plants

Thirty-nine polymorphic loci were detected in the RAPD loci analyzed. Polymorphism was calculated based on the percentage of polymorphic loci (32) out of all loci (42 x 100 regenerated plants). A large number of variant loci were observed from the beginning of the process, indicating that culturing meristems *in vitro* is stressful to the plant genome. An explanation for this finding is that the pattern observed is in accordance with the segregation of genotypes from a chimeric meristem, resulting in high polymorphisms in the initial generation, due to the breakdown of the meristematic organization, forming a heterogeneous population of homogeneous plants, but, over consecutive generations, lower polymorphisms, due to a stochastic process. *In vitro* stress may cause the genome to

respond by DNA methylation, and this may modify the RAPD profile through the insertion or excision of transposons (Hirochika *et al.*, 1996). Kovarik *et al.* (1997) suggested that epigenetic changes in the level of methylated DNA might play an important role in the mechanism of adaptation to environmental stress.

The RAPD technique reveals DNA polymorphisms as differences in the amplification patterns, and uses primers of random sequences that search for complementarity in the genome. It is suggested that RAPD bands possibly represent mainly repetitive DNA (Grattapaglia and Sederoff, 1994). Polymorphism in repetitive DNA sequences has frequently been observed during plant propagation by tissue culture (Smulders *et al.*, 1995) and undergoes more alterations than the coding sequences. *In vitro* stress may provoke changes at preferential sites, such as repetitive DNA, thereby activating transposable elements.

In conclusion, intrinsic molecular variability exists among sugarcane clones. Meristem culturing increases the rate of polymorphism, which is also influenced by the sugarcane genotype. This *in vitro* stress occurs at all stages of direct regeneration.

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