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MOLECULAR CHARACTERIZATION OF SUGARCANE (*SACCHARUM OFFICINARUM* L.) GENOTYPES IN RELATION TO SALT TOLERANCE

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ABSTRACT

This study was conducted to characterize the salt tolerant and susceptible genotypes (F1s) of sugarcane by 15 ISSR primers. The amplification profile consisted of 118 fragments of size ranging from 275 bp to 2845 bp of which 28 were monomorphic and 82 were polymorphic with 69.49 % polymorphism. The number of bands generated by each primer varied from 4 (ISSR -823) to 11 (ISSR–809, ISSR–824 and ISSR-830) with an average of 8.4 fragments per primer. The percentage of polymorphic bands with different primers ranged from 50 to 88.88 %. The efficiency of ISSR marker for cultivar identification was found 6.8 % as only eight fragments are cultivar specific.PIC values were ranged from 0.47 (ISSR 830) to 0.88 (ISSR 816) primers with an average value of 0.70. Rp values were ranged from 2.40 (ISSR 823) to 11.86 (ISSR 809) primers with an average value of 6.18. The similarity coefficients detected by ISSR ranged from 0.65 to 0.84 which revealed existence of limited genetic variation among sugarcane genotypes. In the consensus tree thesalt-tolerant genotypes cluster together indicating the efficiency of ISSR markers to differentiate between tolerant and susceptible genotypes based on their similarity matrix.Our study has established that the ISSR markers are useful for studying genetic diversity among sugarcane and thereby helps in selecting superior crosses and for distribution genotypes into different groups.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a clonally propagated C_4 grass belongs to genus *Saccharum* of the family *Poaceae* with complex polyploidy genome. Sugarcane is of great importance in tropical agriculture as a source of sugar and bioethanol. Abiotic stresses such as drought and high salinity adversely affect the growth and productivity of crop plants. Substantial (about 5%) land under sugarcane cultivation (20 million hectares) is saline (Patade *et al.,* 2009). The development of stress-tolerant crops will be greatly advantageous for modern agriculture in areas that are prone to such stresses.This necessitates identification of salt tolerant parents and cross combinations that yielded higher proportion of tolerant genotypes.

Sugarcane-breeding programs take at least 12 years to develop new commercial cultivars. Marker-assisted selection can speed up breeding programs in sugarcane (Pastina *et al.,* 2012).Attempts have been made to identify salt tolerant sugarcane genotypes from available genetic resources using a multitude of biochemical, physiological and morphological indices (Saxena *et al.,* 2010, Chaum *et al.,* 2012). However, most of genotypes identified as salt tolerant were based on field trials, which are affected by many environmental factors (Chaum *et al.,* 2012).

DNA-based markers are robust, speedy; information may be obtained from little amounts of plant material at any stage of development and it is not affected by environmental conditions. Information regarding genetic diversity and genetic relationships among different genotypes is very valuable in crop improvement. Analysis of genetic diversity is useful in selecting diverse parental combinations, reliable classification of accessions, and for exact identification of variety (Zala *et al.,* 2014; Bahurupe *et al.,* 2013; Salunke *et al.,* 2012). Keeping in view all these factors objective of the present study was to investigate genetic diversity for salinity tolerance in salt tolerant varieties, salt tolerant $F1^s$ and salt susceptible $F1^s$ using ISSR primers.

MATERIALS AND METHODS

Plant material and genomic DNA isolation

Fifteen sugarcane genotypes (Three salt tolerant varieties, five salt tolerant sugarcane F_1s and seven salt susceptible sugarcane F_1s) available at Central Sugarcane Research Station, Padegaon were used as experimental material (Table 1). Total genomic DNA was extracted from three month young leaf tissue using a CTAB method (Aljanabi *et al.,* 1999).

ISSR analysis

ISSR analysis was performed using 15 ISSR primers (Table 2).Amplification reactions contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01% (v/v) gelatin, 200 μM of each dNTPs, 0.200 μM ISSR primer, 60 ng template DNA, and 1 unit *Taq* DNA polymerase in a reaction volume of 25 μl. The amplification reaction was carried out in an Eppendorf

Master Cycler Gradient thermal cycler. The reaction included an initial denaturation step of 5 min at 95°C, and then amplification reactions were cycled 45 times at 94°C for 1 min, 45°C- 55°C (depending on the primer) for 1 min and 72°C for 2 min. A final amplification was allowed for 10 min at72°C.

Upon completion of the amplification, amplification products were then resolved in 1.2% agarose gel. Gel was stained with ethidium-bromide and visualized under UV-transilluminator in gel documentation system (Flour Chem™Alpha innotech, USA). Size of the amplicons was estimated with 1 kb DNA ladder which was resolved along with amplified product. Reproducibility of the results was confirmed by repeating the amplification twice.

The clearly resolved PCR amplified ISSR bands with 15 different primers were scored manually for their presence (1) and absence (0) in the binary data sheet. Data was analyzed and similarity matrix was constructed from binary data with dice similarity coefficients which was calculated as per model suggested by Nei and Li (1979). Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) was employed for cluster analysis using the computer package NTSYSpc 2.02i (Rohalf, 1998).The polymorphism information content (PIC) values, resolving power (Rp) values were calculated.

RESULTS AND DISCUSSION

Sugarcane productivity is worldwide subjected to increasing environmental constraints, predominantly to drought and salinity. Sugarcane is a glycophyte with moderate sensitivity to salinity stress and confined to tropical and sub-tropical irrigated regions, where salinity is an ever-increasing problem. High salt levels in the soil adversely affect germination, growth rate, cane yield as well as sucrose content in cane (Patade *et al.,* 2009). Salinity is one of the most serious threats to crop production. Sugarcane has been categorized as a glycophyte (salt susceptible); because it exhibits toxic symptoms including low sprout emergence, nutritional imbalance and growth reduction, leading to low productivity, especially sugar content when cultivated in the salt-affected soils (Chaum *et al.,* 2012). The severe sensitivity of sugarcane to salinity at various growth stages is manifested by a considerable reduction in growth rate (Plaut *et al.,* 2000).

The sugarcane breeding process involves crossing superior varieties and selection among the F_1 progeny individuals with favorable allelic combinations. But the chromosomal architecture of the sugarcane hybrid makes each cross a unique, unpredictable event. This architecture, combined with the multiplicity of alleles at each locus, makes the breeding process immensely complicated (Aruda 2012). Therefore thousands of F_1 seedlings have to screen for salinity tolerance or any other character. One or two clones, out of thousands of the initial F1 seedlings, are released as commercial varieties (Matsuoka *et al.,* 2009).Sugarcane being a long duration crop, identification of suitable adaptive mechanism at early growth stage of crop could help in early screening of genotypes for salt tolerance (Satbhai and Naik 2014).

Identification of molecular markers linked to salinity tolerance will provide plant breeders a new tool for selecting cultivars with improved salt-tolerance (Saxsena *et al.,* 2010). Molecular markers allow breeders to track the genetic loci controlling salt resistance without measuring the phenotype, thus reducing time required and extensive field testing. Several types of PCR-based markers are used for assessing the genetic diversity among sugarcane varieties and hybrids which include random amplified polymorphic DNA (RAPD) (Govindaraj *et al.,* 2011), restriction fragment length polymorphism (RFLP)(Pastina et al., 2012), amplified fragment length polymorphism (AFLP) (Selvi et al., 2005), simple sequence repeat (SSR) (Devarumath et al., 2012), ISSR (Devarumath *et al.,* 2012), sequence tag micro satellite (STMS) (Singh *et al.,* 2005), target region amplified polymorphism (TRAP) (Suman *et al.,* 2012), sequence characterized amplified region (SCAR) (Srivastava *et al.,* 2012), RFLP and SSR markers derived from expressed sequence tag (EST-RFLP and EST-SSR) (Pastina *et al.,* 2012).The development of molecular markers for screening salt tolerant lines has been attempted in several crop plants but there are no reports of differentiating salt tolerant sugarcane genotype using ISSR markers.

In the current study molecular characterization of fifteen sugarcane genotypes (Three salt tolerant varieties, five salt tolerant sugarcane F_1s and seven salt susceptible sugarcane F_1s) was carried out using 15 ISSR primers in order to assess genetic diversity. Out of which 14 were polymorphic, and 1 primer was did not amplify. A representative amplification profile obtained by using random primer ISSR 807 was depicted in fig 1. The number of bands amplified with each ISSR primer along with their details is given in Table 2.

The size of the amplified DNA fragments was ranged from 275 bp to 2845 bp. A total of 82 polymorphic amplicons were observed out of a 118 amplicons generated by the 14 ISSR with 82 % polymorphism. Primers vary in their

Table 1: Sugarcane F_1s and varieties used for ISSR analyses

Salt tolerant varieties			
1.	Co86032	2.	Co 62175
3.	CoM 0265		
Salt tolerant F_1s			
4.	Co 85002 x CoT 8201	5.	Co 8371 x Co 86002
6.	Co 86002 x Co 1148	7.	Co 7201 x CoC 671
8.	Co 85021 x Co 97015		
Salt susceptible F_1s			
9.	Co 8371 x Co78201	10.	Co 95021 x Co97015
11.	Co 8371 x Co 86249	12.	ISH100 x Co 94008
13.	Co 740 x Co 775	14.	Co 8213 x CoT 8201
15.	Co 7201 x Bo 91		

Table 2: Molecular polymorphism, PIC values, Rp values and size of loci revealed by ISSR primers in 12 sugarcane F_1s and 3 varieties.

Sr. No.	ISSR primer	Polymorphic loci	Monomorphic loci	Unique loci	% polymorphism	PIC value	Rp value	Size of loci (kb)
1	807	5	1	2	62.50	0.81	5.20	0.51 to 1.93
2	809	8	3	0	72.72	0.54	11.86	0.27 to 1.57
3	810	4	3	0	57.14	0.70	3.60	0.59 to 1.97
4	811	8	0	1	88.88	0.78	6.66	0.48 to 2.60
4	812	3	3	0	50.00	0.77	2.66	0.56 to 1.64
6	816	3	2	1	50.00	0.88	2.40	0.89 to2.72
7	817	4	2	1	57.14	0.59	5.33	0.64 to 2.30
8	818	11	2	0	84.61	0.81	8.40	0.43 to 2.84
9	823	2	2	0	50.00	0.64	2.40	0.28 to 0.93
10	824	6	4	1	54.54	0.61	7.86	0.42 to 1.65
11	830	8	3	0	72.72	0.47	11.2	0.71 to 2.04
12	834	7	2	0	77.77	0.64	7.886	0.45 to 1.88
13	842	8	1	0	88.88	0.79	6.8	0.58 to 1.93
14	853	4	1	2	57.14	0.83	4.3	1.03 to 2.59
Total	82	28	8					
Average		5.86	2	0.6	69.49	0.70	6.18	0.275 to 2.85

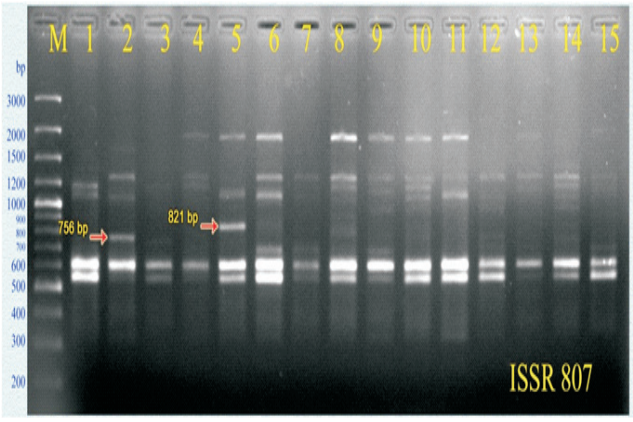

Figure 1: ISSR 807 amplification profile of fifteen sugarcane genotypes. M = marker (200 to 3000 bp), Lane 1 Co 86032, 2- CoM 0265, 3- Co 62175, 4- Co 85002 x CoT 8201, 5- Co 8371 x Co 86002, 6- Co 86002 x Co 1148, 7- Co 7201 x CoC 671, 8- Co 85021 x Co 97015, 9-Co 8371 x Co78201 , 10- ISH100 x Co 94008, 11- Co 8371 x Co 86249, 12-Co 95021 x Co97015, 13- Co 740 x Co 775, 14- Co 7201 x Bo 91, 15-Co 8213 x CoT 8201.

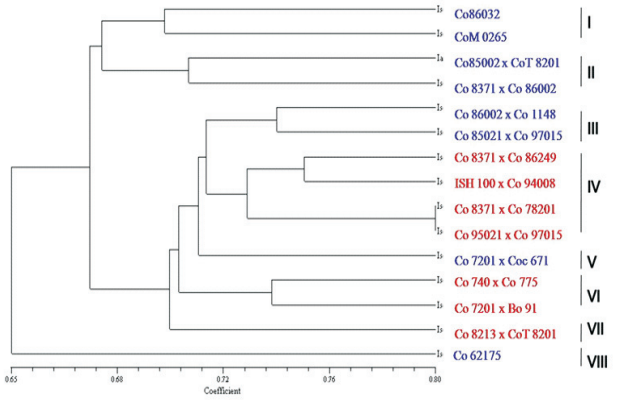

Figure 2: Consensus tree showing clustering of 15 sugarcane genotypes (salt tolerant genotypes are indicated in blue and salt susceptible genotypes are indicated in red) using ISSR analysis with NTSYS pc 2.02i software

polymorphism content with the percent polymorphism varied between 50.00 % (ISSR-812, ISSR-816 and ISSR-823) to 88.88 % (ISSR-811, ISSR-842). The average number of polymorphic bands per primer was 5.86. Earlier different levels of polymorphic bands generated with ISSR markers in sugarcane were reported by Shrivastava *et al.* (2012) 79 %;Kalwade *et al.* (2012) 84%. These findings clearly demonstrate the reliability of ISSR in analyzing genomic diversity in sugarcane.

PIC value of a marker provides an estimate of the discriminating power of the marker. In the present investigationPIC values were ranged from 0.47 (ISSR 830) to 0.88 (ISSR 816) primers with an average value of 0.70. Rp values were ranged from 2.40 (ISSR 823) to 11.86 (ISSR 809) primers with an average value of 6.18. Devarumath *et al.* (2012) reported PIC value ranged from 0.11to 0.36with an average of 0.28 and Kalwade *et al.* (2012) reported PIC value ranged from 0.19 to 0.45 with an average of 0.27.

In the present investigation the number of bands generated by each primer varied from 4 (ISSR-823) to 13 (ISSR-818) with an average of 8.4 fragments per primer. Previously Shrivastava *et al.* (2012) and Devarumath *et al.* (2012) reported an average of 7.9 and 74.8 bands, respectively.

In the present investigation the similarity coefficients range from 0.65-0.84 with an average of 0.76 which revealed existence of limited genetic variation among 15 sugarcane genotypes. Lowest similarly coefficient of 0.65 was present between genotypes Co 62175 and Co 85021 x Co 97015, while maximum similarly coefficient 0.84 was observed between genotypes Co 86002 x Co 1148 and Co 85021 x Co 97015.The limited genetic diversity among sugarcane varieties based on ISSR markers had been previously reported (Devarumath *et al.*, 2012). A close genetic relationship between parental populations is a common problem in salt tolerance breeding programs. Improving salt tolerance can be achieved by identification of the genetic distances among parental genotypes based on ISSR markers before crossing.

The genetic relationships among sugarcane cultivars and F_1S are presented in a dendrogram based on informative ISSR alleles (Fig. 2). Differential clustering in relation to salt tolerance

was observed. Among the varieties, salt tolerant sugarcane variety Co 62175 was found to be totally distinct and divergent from rest of the varieties. In the consensus tree constructed Co 62175 formed the independent cluster (Group no. 8). Remaining genotypes were divided into two clusters which were joined at 67 % level of similarity (fig 2). The first major cluster comprises sugarcane salt tolerant groups, first group consists of salt tolerant sugarcane varieties Co 86032 and CoM 265 and second group consist of salt tolerant F_1s *viz.*, Co 85002 x CoT 8201 and Co 8371 x Co86002. The second sub cluster of major cluster first consists of five groups. Third group consists of two salt tolerant sugarcane F_1 *viz.*, Co 86002 x Co 1148 and Co 85021x Co 97015. Fourth group consists of four salt susceptible sugarcane F_1 *viz.*, Co 8371 x Co 86249, ISH100 x Co94008, Co 8371 x Co 78201 and Co 95021 x Co 97015. Salt tolerant sugarcane F_1 *viz.*, Co 7201 x CoC 671 formed independent fifth group. Sixth and seventh group consists of 3 salt susceptible sugarcane F_1*viz.* Co 740 x Co 775, Co 7201 x Bo 91 and Co 8213 x CoT 8201. Cluster analysis revealed that salt susceptible sugarcane genotypes were clustered together in group number four, six and seven. Salt tolerant sugarcane genotypes exhibited more genetic diversity compared to salt susceptible sugarcane genotypes

In the present investigation we obtained 6.8% unique varietal specific bands using ISSR markers. Out of the 118 bands, only 8 fragments were specific for one cultivar. In salt tolerant F_1 Co 8371 x Co86002 specific three unique fragments were observed i.e. ISSR807-821 bp, ISSR853-2591 bp and ISSR 853-1177 bp. While ISSR 811, ISSR 807, ISSR 824, ISSR 817 and ISSR 816 primers showed unique fragment in genotype Co 86032 (2604 bp), CoM 0265 (756 bp),C0 62175 (1031 bp), Co 85021x Co 97015(2303 bp)andCo 8371 x Co 86249 (1863 bp) respectively. These unique DNA fragments seem to be useful to discriminate among cultivars and may be used to construct the s cultivar specific primers and further screening of genotypes.

ISSR patterns separated all the genotypes from each other and confirm the variability at molecular level. The consensus tree constructed showed three major clusters revealing differential clustering in relation to salt tolerance was observed.The results in the present investigation demonstrated the narrow genetic base among sugarcane cultivars and F_1S. It is recommended that genetically distinct parents should be used to enrich the existing narrow genetic base.

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