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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF SUGARCANE GENOTYPES FOR THEIR SALINITY AND SUSCEPTIBILITY USING TRAP MARKERS

[1,2]Forough Jomhe Farsangi, [1]Avinash S. Thorat and *[1,2]Rachayya M. Devarumath

[1]Molecular Biology and Genetic Engineering Section, Vasantdada Sugar Institute, Manjari (Bk), Pune, India-412307
[2]Department of Biotechnology, Savitribai Phule Pune University, Pune, India-411007

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ABSTRACT

Sugarcane is a cash crop in India and salinity affects its productivity and crop growth. Identification of molecular markers linked to salinity tolerance traits has provided plant breeders a new tool for selecting cultivars with improved salt-tolerance and hence, molecular characterization of eighteen sugarcane genotypes of salinity tolerant and susceptible was carried out using thirty combinations of 5 TRAP markers related salinity tolerant ESTs and 3 arbitrary primer combinations. Out of 124 alleles, 81 (65%) were found polymorphic and The PIC values were ranged from 0.10 to 0.41 with an average of 0.30 furthermore Rp value varied from 0.33 to 4.33 with an average of 1.48. Also, the range of polymorphism was found about 25% to 100%. The genetic similarity coefficients ranged from 0.53-0.91 with an average of 0.72 which revealed the existence of limited genetic variation among 18 sugarcane genotypes.

Abbreviations:
ESTs: Expressed sequence tag
MA: Monomorphic Alleles
P: Percentage Polymorphism
PA: Polymorphic Alleles
PCR: Polymerase chain reaction
PIC: Polymorphic Information Content
PIC: Polymorphism Information Content
RAPD: Random Amplified Polymorphic DNA
Rp: resolving power
TA: Total Alleles
TM: Temperatures of primers
TRAP: Tartrate-resistant acid phosphatase

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INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a member of the Poaceae family and imperative cash crop. Sugar production contributes around 70–80% globally and 100% in India (Thorat, 2017 and Breusegem, 2001). Sugarcane is a tropical plant and grows well under tropical condition all over the world. Nearly 120 countries produce sugar from sugarcane, 57 countries produce, sugar from sugar beet and 12 countries produce it from both these crops. India is the second biggest sugarcane producer next to Brazil and contributes around 15% production. However, in India, it is cultivated under a wide range of agro-climatic conditions. Abiotic stress is the most harmful factor concerning the growth and productivity of crops worldwide (Breusegem, 2001 and Gao, 2007). Excess amount of salt in the soil harmfully affects plant growth and development. Nearly 20% of the world's cultivated area and nearly half of the world's irrigated land are affected by salinity (Zhu, 2001). About 12 million hectare of land has been affected by saline and alkaline conditions in India (Yadav, 1984). Salinity in agricultural fields, a major environmental stress, is a severe constraint on crop growth and productivity in many regions, and the situation has become a global concern.

Corresponding author: [1,2] **Rachayya M. Devarumath**
[1]Molecular Biology and Genetic Engineering Section, Vasantdada Sugar Institute, Manjari (Bk), Pune, India-412307.
Department of Biotechnology, Savitribai Phule Pune University, Pune, India-411007.

Table 1. Details of salinity tolerant and susceptible sugarcane genotypes used in TRAP marker study

Sr. No.	Genotype	Parentage	Remarks
1	Co 85004	Co 6304 × Co 740	Susceptible
2	Co 419	PoJ 2878 × Co 290	-
3	Co 7704	Co 740 × Co 6806	-
4	Co 86032	Co 62198 × CoC 671	-
5	Co 8014	Co 740 × Co 6304	-
6	Co 92008	CoC671 self.	-
7	Co 775	PoJ 2878 × Co 371	-
8	Co C671	Q 63× Co 775	-
9	Co 9805	Co8371PC	-
10	Co 94004	Co 62175 × Co 86250	Salinity tolerant
11	ISH 176	Co 6806 × Khakai	Salinity tolerant
12	Co 89027	CoC 671 × Co 6806	Susceptible
13	Co 62175	Co 957 × Co 419	Salinity tolerant
14	Co 87002	Co 7704 × CoC 671	Susceptible
15	Co 93017	Co 6806 × Co 7717	Susceptible
16	ISH 135	Co 62174 × SES 51513	Salinity tolerant
17	Co 89010	Co 6304 × Co 775	Susceptible
18	CoM 0265	Co 87044GC	Salinity tolerant

Table 2. List of TRAP primers and their sequence information

Sr. No.	Primer	Function	Primer sequence	Tm Cº.
1	TRAP1	Metallothionein Cellular defense	Fp:TCCTTTCAAGTGCCTAGTTTTC Rp:AAAGCACGACAAGTCTCTAAG	52 ºC
2	TRAP2	Protein Cellular Defense	Fp:TCCAGCTCAATCTCGCAAG Rp:CAGGAGCGACAGACATAC	54 ºC
3	TRAP3	Hypothetical Protein	Fp:AGCATGAGCCACTATGAGAAG Rp:GGGATGACACCAGAAGGAGA	54 ºC
4	TRAP4	Protein Transport facilitation	Fp:CGGGATATTGCTTCACTGC Rp:AGTCCATCTCCATGTGTGTC	55 ºC
5	TRAP5	Plastoquinone oxidoreductase subunit 5 Energy	Fp:AAGAATAGGTTTGGTGAATCGG Rp:ACGCGGGATAATCGTCACTA	52 ºC
6	Arb1	-	GACTGCGTACGAATTAAT	
7	Arb2	-	GACTGCGTACGAATTGAC	
8	Arb3	-	GACTGCGTACGAATTTGA	

Note: Tmº= melting temperature in degree Celsius

It has been reported that around 5% of the cultivated land is affected by salinity (Lee, 2009). Sugarcane being a glycophyte shows high sensitivity to salinity at various growth stages, like majority of other crop species and cannot tolerate high salinity at early stages of growth affecting its normal physiology and entire metabolic balance even at cellular levels through osmotic and ionic adjustments that result in reduced biomass production (Nimbalkar, 2008). Identification of molecular markers linked to salinity tolerance traits has provided plant breeders a new tool for selecting cultivars with improved salt-tolerance. Thus, understanding the molecular approaches on salinity stress will be helpful in developing selection strategies for salinity tolerance (Saxena, 2010). TRAP is a simple PCR based marker system using EST database sequence information to generate polymorphic markers targeting candidate genes (Hu, 2003). The technique involved in designing a fixed primer from EST sequences or genes of interest and an arbitrary primer reported from earlier studies (Li, 2001). The method involved in designing a fixed primer of about 18 nucleotides from EST sequences or genes of interest and an arbitrary primer about the same length is designed with either an AT- or a general collection (GC)-rich motif to anneal with an intron or exon, respectively (Hu, 2003; Li, 2001). In view of this, we propose to employ the TRAP technique for identifying potential salt tolerance candidate genes and their validation on selected tolerant and susceptible sugarcane genotypes.

MATERIALS AND METHODS

Fresh plant material (young leaves) of 18 sugarcane genotypes were collected from sugarcane research station of Vasantadada Sugar Institute, Manari (Bk), Pune (VSI), (N 18°51' 78 and E 73°97') and Sugarcane breeding Centre, Amboli (N 15°96' and E 74°00') (Table 1). Collected leaves were frozen in liquid nitrogen and stored in -80°C for preservation. Total genomic DNA was isolated from each stored sample by using modified CTAB method as per Aljanabi et al. (Aljanabi, 1999), and quantified by UV spectrophotometer and subsequently quality of the DNA checked on 0.8% agarose gel electrophoresis Fig. 1. Details of the sugarcane genotypes were given in the Table 1.

TRAP Markers PCR and Electrophoresis of PCR Product

Forward primers in combination with arbitrary reverse primers of TRAP as mentioned in the Table 2 were selected for amplification. The TRAP primers were 18 nucleotides long and were designed in the present study based on the (Li, 2001), findings, which were adapted from (Hu, 2003; Khan, 2011). The PCR amplification was carried out, by using a reaction mixture containing, 10 × PCR buffer with 10 mM Tris HCl (pH 8.3), 2.0 mM MgCl2, 100 µM each dNTP, 0.5 µM fixed forward and arbitrary reverse primer, 50 ng of template DNA, and 1U of Taq polymerase. PCR was performed in Thermal cycler (Applied Biosystem) at initial temperature of 94°C (4.30 min, 1 cycle), followed by 35 cycles of 1 min at 94°C, 1 min at 52-55°C, 2 min at 72°C and final extension cycle of 7 min at 72°C. PCR products of TRAP were resolved on 1.5 % (w/v) agarose gel with 1× TBE buffer, stained with ethidium bromide (EtBr) and documented under UV trans-illuminator (UViTech). A 100 bp DNA molecular marker was used to estimate the amplicon size.

Table 3. Molecular characterization of 18 sugarcane genotypes using TRAP markers

Markers name	Tm C°.	TA	PA	MA	%P	PIC	RP	AR bp.
TRAP1F+Arb1	52	3	3	0	100	02	055	~100-1500
TRAP1F+Arb2	52	4	4	0	100	02	110	~100-1300
TRAP1F+Arb3	52	8	8	0	100	03	433	~200-600
TRAP1R+Arb1	52	6	6	0	100	03	288	~100-500
TRAP1R+Arb2	52	7	7	0	100	03	322	~100-650
TRAP1R+Arb3	52	2	2	0	100	03	088	~150-750
TRAP2F+Arb1	54	4	4	0	100	03	211	~100-400
TRAP2F+Arb2	54	4	4	0	100	02	122	~100-300
TRAP2F+Arb3	54	2	2	0	100	02	211	~100-800
TRAP2R+Arb1	54	5	4	1	80	01	055	~100-400
TRAP2R+Arb2	54	2	2	0	100	02	044	~100-600
TRAP2R+Arb3	54	4	2	2	50	02	133	~100-500
TRAP3F+Arb1	54	4	4	0	100	02	100	~110-1600
TRAP3F+Arb2	54	3	1	2	333	02	055	~150-500
TRAP3F+Arb3	54	4	2	2	50	03	144	~100-400
TRAP3R+Arb1	54	4	1	3	25	03	100	~100-400
TRAP3R+Arb2	54	4	2	2	50	01	033	~100-800
TRAP3R+Arb3	54	4	1	3	25	03	177	~100-700
TRAP4F+Arb1	55	5	3	2	60	04	288	~200-700
TRAP4F+Arb2	55	3	1	2	333	03	077	~250-700
TRAP4F+Arb3	55	5	2	3	80	03	188	~150-700
TRAP4R+Arb1	55	4	2	2	50	02	078	~130-900
TRAP4R+Arb2	55	4	2	2	50	01	033	~100-700
TRAP4R+Arb3	55	3	1	2	333	03	033	~100-800
TRAP5F+Arb1	52	4	2	2	50	02	100	~200-1500
TRAP5F+Arb2	52	4	3	1	75	04	233	~150-700
TRAP5F+Arb3	52	3	1	2	333	04	166	~250-1500
TRAP5R+Arb1	52	8	3	5	375	03	366	~100-1500
TRAP5R+Arb2	52	4	1	3	25	03	111	~100-600
TRAP5R+Arb3	52	3	1	2	335	02	088	~300-500
Total		124	81	43	1974	77	444	
Average		41	3	14	66	03	148	

***Note*:** TM: Temperatures of primers. TA: Total Alleles. PA: Polymorphic Alleles. MA: Monomorphic Alleles. P: Percentage Polymorphism. PIC: Polymorphic Information Content

Data Analysis

Amplified products with reproducible and distinct bands were counted as the identical band and DNA bands of each TRAP marker transformed in the form of binary numbers 1 and 0. The number 1 indicates the present of DNA bands and 0 indicating no DNA bands. Only clearly resolved bands were considered in the present study. The genetic similarity (GS) among the genotypes was calculated by Jaccard's similarity coefficient and the dendrogram was constructed based on similarity coefficient generated Fig.2 using the un-weighted pair group method with arithmetic average (UPGMA), and all statistical analysis was done by using NTSYS (version 2.02). The allelic diversity at a given locus was measured by Polymorphism Information Content (PIC). The ability of primer to distinguish between genotypes was assessed by calculating their resolving power (Rp).

RESULTS AND DISCUSSION

PCR and Cluster Analysis by TRAP markers using salt related genes

The data of molecular markers as total allele scored (TA), polymorphic alleles (PA), polymorphic information content (PIC) and resolving power (Rp) obtained per TRAP markers were revealed as the number of bands amplified with each TRAP primer along with their details size range (bp) was given in the Table 3. Total 124 alleles, of which 81 (65%) were found polymorphic. The total numbers of alleles amplified by individual primer were ranged from 2 to 8 with an average of 4.13 alleles per primers. The PIC values were ranged from 0.10 (TRAP2R+Arb1, TRAP3R+Arb2, TRAP4R+Arb2) and to 0.41 (TRAP4F+Arb1) with an average of 0.30.

The Rp value varied from 0.33 to 4.33 for TRAP3R+Arb2, TRAP4R+Arb2, TRAP4R+Arb3 and TRAP1F+Arb3 respectively, with an average of 1.48. The PCR product was electrophoresed on agarose gels were presented in Fig.1. The range of % polymorphism was found about 25% to 100%. The Molecular markers derived from gene sequences commonly displays low level of polymorphism, which can limit their widespread use in genetic analysis (Varshney, 2005). The less polymorphism (42.5%) was obtained in 64 sugarcane genotypes by using 16 primer combinations (Suman, 2012). In the present study the mean PIC value found, near to result of PIC (0.28) were obtained by Suman et al. (Suman, 2012) and Creste et al. (2010), among 64 and 82 sugarcane genotypes respectively. Similarly, Devarumath et al. (2012), reported that the levels of polymorphism 73% and PIC value ranged from 0.17 to 0.31 with an average of 0.24 is obtained in 47 sugarcane genotypes by 23 pairs of TRAP markers. In the present investigation GS coefficients ranged from 0.53-0.91 with an average of 0.72 which revealed the existence of limited genetic variation among 18 sugarcane genotypes. A lowest similarly coefficient of 0.53% was present between genotypes Co 9805 and Co 419 while maximum similarly coefficient 0.91% was observed between genotypes Co ISH135 and Co 891010 Table 4. The dendrogram separated the eighteen sugarcane genotypes varieties into two main clusters Fig.2. The first cluster formed by two genotypes of Co 85004 and Co419 with 0.73% genetic similarity and cluster II showed the biggest cluster in the dendrogram with 14 genotypes that showed itself, two sub-cluster, (a and b) with five and nine genotypes, respectively. In the sub-cluster (a) the genotypes Co7704 and Co8014 showed common parents of Co 740 X Co 6304. And CoC671 is the male parents of Co86032 in the sub-group. In the sub-cluster b, ISH176 and Co89027 have a common male parent of Co6806 and also Co93074 with Co891010 has common female parent of Co6304.

Table 4. Mean genetic similarity among the 18 sugarcane genotypes generated by TRAP markers

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A	*																	
B	073	*																
C	061	069	*															
D	067	068	068	*														
E	064	068	064	082	*													
F	067	075	071	077	079	*												
G	070	070	067	071	075	083	*											
H	065	064	063	072	074	076	078	*										
I	058	053	058	058	056	066	062	063	*									
J	070	069	070	073	075	082	079	073	067	*								
K	068	070	070	070	073	078	079	073	065	086	*							
L	074	070	076	067	069	077	075	069	070	083	083	*						
M	067	064	068	060	062	072	070	065	066	073	076	081	*					
N	060	064	064	065	065	070	071	065	063	078	078	074	070	*				
O	068	067	070	068	063	078	074	071	067	081	084	082	083	075	*			
P	070	069	072	073	068	078	076	075	069	082	081	085	078	075	088	*		
Q	067	061	066	069	069	074	073	079	068	080	078	079	076	077	083	091	*	
R	070	067	070	073	070	078	077	071	067	084	081	080	076	076	086	089	087	*

Note: A-Co85004 B-Co419 C-Co7704 D-Co86032 E-Co8014 F-Co92008 G-Co775 H-CoC671 I-Co9805 J-Co94004 K-ISH176 L-Co89027 M-Co62175 N-Co87002 O-Co93017 P-ISH135 Q-Co891010 R-CoM265

*Single clone involved in genetic analysis study

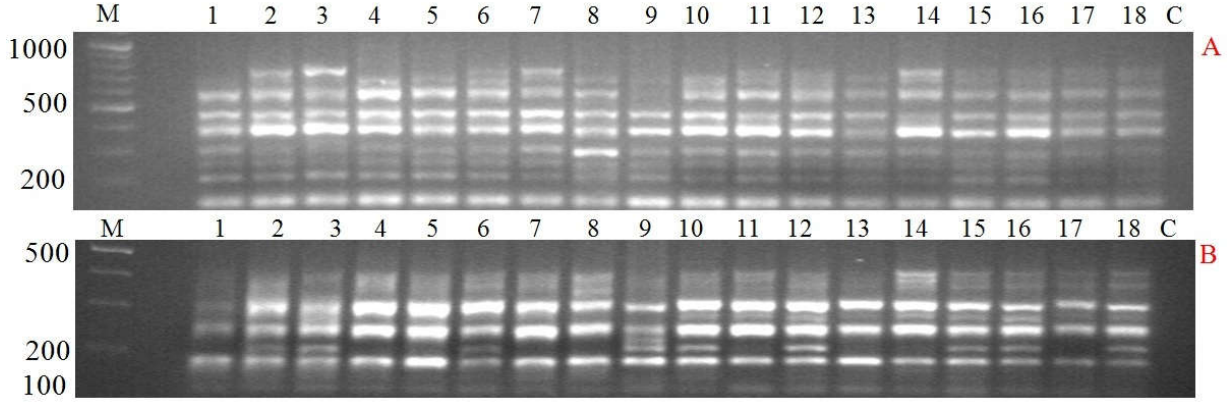

Figure 1. Molecular profiling of 18 sugarcane genotypes generated by TRAP markers A-TRAP1R+Arb3. B-TRAP2F+Arb1

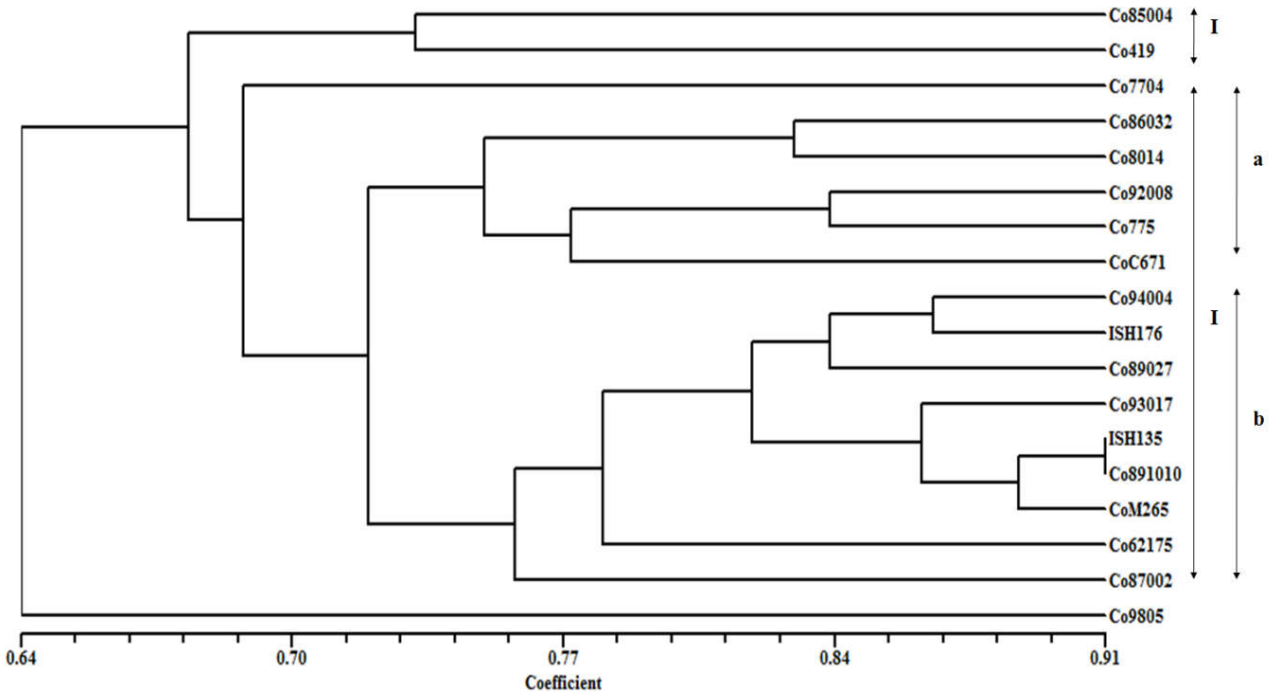

Figure 2. Dendrogram showing different distinct groups generated from TRAP marker of 18 sugarcane genotypes based on Jaccard's similarity coefficients and UPGMA method

The genotypes of Co7704 and Co9805 placed in the constructed dendrogram separately. The dendrogram, representing the association between eighteen sugarcane genotypes, indicated that progeny Co7704 and Co9805 had shown the most divergence among sugarcane varieties in the present study. Plant growth, productivity and distribution affected by environmental stress such as drought and salinity. Drought and salinity are the major abiotic stress and are the major limitations to food production, reducing average crop yield by more than 50% (Rodriguez, 2006) Mostly salinity is becoming increasingly significant in limiting growth of many crop plants, grasses and the cereals (Tester, 2005). In this study the candidate genes for screening the salt tolerant and susceptible sugarcane genotypes to identify potential candidate gene markers were identified. The candidate sequences were selected from the dataset generated in the study of cDNA-RAPD and SSH libraries. Study of salinity stress tolerance in sugarcane varieties is done by Saxena et al. (Tester, 2005). More research work of evaluating in sugarcane genotypes for their tolerance to salinity stress by protein markers is reported by Khaled et al. (Tester, 2005). Molecular markers are considered as genetic traits that are selectively neutral, free of epistatic interactions and unaffected by environment (Khaled,, 2010). Detection of molecular markers linked to salinity tolerance traits has provided plant breeders a new tool for selecting cultivars with improved salt-tolerance. Thus, understanding the molecular approaches on salinity stress will be helpful in developing selection strategies for salinity tolerance. However, molecular studies at DNA level may be of use to tag the specific gene responsible for salinity stress.

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Conflict of interest disclosure

The authors declare that there are no conflicts of interest.

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