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IDENTIFICATION OF ELITE SUGARCANE CLONES THROUGH TRAP

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Abstract

Sugarcane is being recognized as major source of sugar in the world. Identification of high sucrose clones endowed with other agronomic traits can be speedup with the help of specific molecular markers target region amplified polymorphism (TRAP). Results revealed that clone AEC81-0819 possesses high sucrose gene, tolerant to cold conditions and dehydrating conditions followed by L116. High activity of soluble acid invertase was observed in NIA98 and minimum was recorded in LRK-2001. Agronomic data showed that clone AEC81-0819 out yielded all clones in trial for commercial cane sucrose percent (CCS).

Introduction

Sugarcane is one of the most important economical crops in Pakistan. Most crop plants, including sugarcane as a typical glycophyte, are sensitive and grow poorly in cold and water stress condition (Taghian, 2002). Sugarcane also is used for ethanol and biomass production as well as alternative source of energy (Ming *et al.*, 2006; Khan *et al.*, 2009).

Sugarcane has long been recognized as one of the world's most efficient crops in converting solar energy into chemical energy. Sugarcane is a C_4 plant in which CO_2 is initially added to a 3 Carbon acid to form a 4 Carbon acid that is then transported to a region of the leaf where ribulosebiphosphate carboxylase is located. Reverse carboxylation enhances CO_2 concentration in the cell causing dramatically decrease in photorespiration (Wang *et al.*, 2008a). Photosynthetic efficiency can be translated to biomass yields. At maximum efficiency, the theoretical upper limit for sugarcane biomass production is estimated to be 281 t/ha-yr (Loomis & Williams, 1963).

Because of the urgent need to improve the efficiency of Pakistan sugar production it was decided to attempt to increase the levels of sucrose in breeding lines. Most of our current commercial varieties ranged in between 20.5-21.4 Brix value (Khan *et al.*, 2010). The work described here extends the Brix range of breeding lines significantly and increases the genetic variability for this character. The effect on selection of the use of the new clones will increase the sugar production of the country.

Genome sequences have a great potential in facilitating the crop improvement (Goff *et al.*, 2002; Yu *et al.*, 2002). The task of bridging this DNA sequence information with particular phenotypes relies on molecular markers. Express Sequence Tag Sites (ESTs) is a powerful bioinformatics tools for identification of putative functional genes. TRAP is a rapid and efficient PCR-based technique, which uses bioinformatics tools and the EST database information to generate polymorphic markers around targeted putative candidate gene sequences (Hu & Vick, 2003)

In this study several biochemical indexes including the content of sugar, activities of soluble acid invertase and response of cold/drought stress were assayed. The growth habit and cane yield contributing parameters were also studied in five sugarcane cultivars.

Materials and Methods

The experiment material comprising of 5 sugarcane clones viz., NIA-98, AEC81-0819, L116, NIA-2004 and LRK-2004 were planted in the field for three consecutive years from 2006-2008. The experimental layout was RCB design with 4 replications. The plot size was 8 x 10m, row to row distance one metre. Normal agronomic practices were followed through out the growth period. Field data was recorded for nine important agronomic characters viz., plant height (cm), plant girth (cm), number of stalks per stool, weight per stool (kg), sucrose %, commercial cane sugar (CCS) %, fiber %, cane yield (t/ha) and sugar yield (t/ha). Three stools were randomly taken from each plot to determine sugar contents according to sugarcane laboratory Manual for Queensland Sugar Mills (Anon., 1970) while three rows from each plot were harvested to record yield data. The mean and variance were computed from each treatment. Data on one plant crop and two ratoon crops was computed on above-mentioned parameters. Data was analysed by employing Duncan Multiple Range test (Steel & Torrie, 1980).

DNA Isolation: Fresh leaves of sugarcane clones viz., NIA-98, AEC81-0819, L116, NIA-2004 and LRK-2004 was collected from experimental field of NIA, Tandojam during September 2009. Genomic DNA was isolated through DNA isolation kit (Gentra system, Minnesota, USA) (Bibi, 2009) and DNA was quantified on spectrophotometer (Bio-mate 3), at absorbance of 260/280nm. The quality was further checked on 0.8% agarose gel.

Primers Designing: For this study, initially sequence of the three EST (express sequence tags) region has been selected by the use of bioinformatics tools to check the genomic polymorphism at the genes responsible for sucrose metabolism which has a major role in plant growth and development as it is a major product of photosynthesis and is the major carbohydrate form used as energy sources for growth or storage reserves and also selected genes responsible for cold tolerance. The first sequence is encoding the enzyme sucrose synthase (SuSy) NCBI accession No. AF263384. The other sequence encodes the enzyme soluble acid invertase (SAI) NCBI accession No. AF062735. The third sequence encodes pyrovate orthophosphate dikinase (PPDK). The fourth sequence encodes C-repeats/Dehydration Responsive Element (CRT/DRE) NCBI accession No. NM_118680. COR15a, Mischantus-PPDK and Calcium-dependent protein kinases (CDPK) were also sequenced for study the tolerant mechanism under cold stress with NCBI accession No. U01377, AY262272 and CF572977 respectively.

Primers: An 18-mer primer was used from the EST sequence of these enzymes paired with an arbitrary primer that targets the intronic and /or extronic region (AT- or GC-rich core).

Polymerase Chain Reaction: PCR was optimized for TRAP study in sugarcane with one fixed primer and two arbitrary primers. PCR reaction was carried out in 10µl reaction mixture containing 0.33mM dNTPs, 2.5mM Mg, 0.15 µM of each primer, 0.5u of Taq polymerase, 0.05% gelatin and 50ng of template DNA. The best amplification were obtained using following PCR programmed in Eppendorf Master Cycler: an initial denaturation for 4 min., at 94^{o}C, then 35 cycles: denaturation at 94^{o}C for 45 sec; 45 sec annealing at 45^{o}C; 1 min extension at 72^{o}C. Final extension was carried out at 72^{o}C for 7 min.

Amplified products were electrophoresed on 1.5% agarose gels containing 0.5 x TBE (Tris Borate EDTA) and 0.5µg/ml Ethidium bromide to stain the DNA. The PCR product was electrophoresing at 72 volts for 2 hours. Photograph was taken under UV light using gel documentation system.

Data Analysis: Data were scored as (1) presence of band and (0) absence of band from TRAP of amplification profile.

Results

CRT/DRE (Cold tolerance): Primer CRT/DRE amplified two segments of DNA, which were polymorphic. Gel analysis revealed that clone AEC81-0819 produce two bands (2.6kbp and 1.8kbp) whereas clones viz., L116 and LRK-2001 produce only one band (2.6kbp). Clones NIA-98 and NIA-2004 did not yield any band (Fig. 1). The extra band produce by the clone AEC81-0819 can be used as clone marker which proves the utility of TRAP for genome identification.

COR15A: Expression of cold-regulated genes (COR15A) is responsible for cold tolerance in sugarcane. Primer COR15A on amplification produces three bands, in which two were polymorphic and one monomorphic (segment of 765bp). Band size in clones AEC81-0819 and L116 was 2.34kbp and 1.78kbp, and clone LRK-2001 produced only one polymorphic band (2.34kbp) (Fig. 2).

Mischanthus-specific PPDK: Primer Mischanthus- PPDK produces single band in clones viz., AEC81-0819, L116, NIA-2004 and LRK-2001. The size of the band was 1.87kbp. Clone NIA-98 did not amplify the Mischanthus- PPDK sequences on the gel (Fig. 3).

Calcium-dependent protein kinases (CDPK): TRAP profile of CDPK revealed that AEC81-0819 and L116 contain four bands. Clones NIA-98 and LRK-2001 exhibit three bands and NIA-2004 produced only two bands (517bp and 344bp). The band size in case of clone AEC81-0819 was 2.6kbp, 1.4kbp, 618bp and 344bp and in case of L116 2.6kbp, 1.4kbp, 517bp and 344bp. Clone NIA-98 produces 2.6kbp, 618bp and 344bp while LRK-2001 yielded 2.6kbp, 1.4kbp and 618bp bands. The specific band produce by L116 and NIA-2004 (segment of 517bp) can be used for the identification of sugarcane clones through TRAP marker (Fig. 4).

Sucrose Synthase (**SUCSy):** Primer SucSy amplified three segments of DNA in which two were monomorphic (2.26kbp and 578bp) and one was polymorphic (segment of 120bp). Polymorphism was observed in clones NIA-98, AEC81-0819 and L116 (Fig. 5).

Soluble Acid Invertase (SAI): Primer SAI produced eight bands in which seven were polymorphic and only one was monomorphic. Gel analysis showed that maximum bands (five) were amplified with NIA-98 and minimum bands (two) were amplified with LRK-2001. Four bands were amplified with NIA-2004 whereas clones viz., AEC81-0819 and L116 produced three bands. DNA fragments ranged sized between 1.78kb- 199bp (Fig. 6).

Some specific bands were also identified which could be used as marker for clone identification. Gel analysis revealed that clone NIA-98 exhibit three specific segments of 1.78kbp, 956bp and 747bp. Clones NIA-2004 and LRK-2001 yielded a specific band which was of 1.26kbp and 1.06kbp in size respectively (Fig. 6).

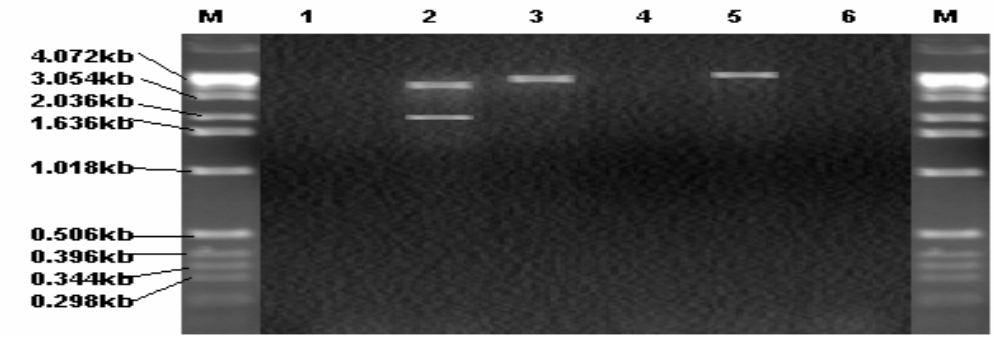

Fig. 1. TRAP profile of sugarcane clones using primer CRT/DRE, M=DNA marker, 1=NIA-98, 2=AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, 6=Blank.

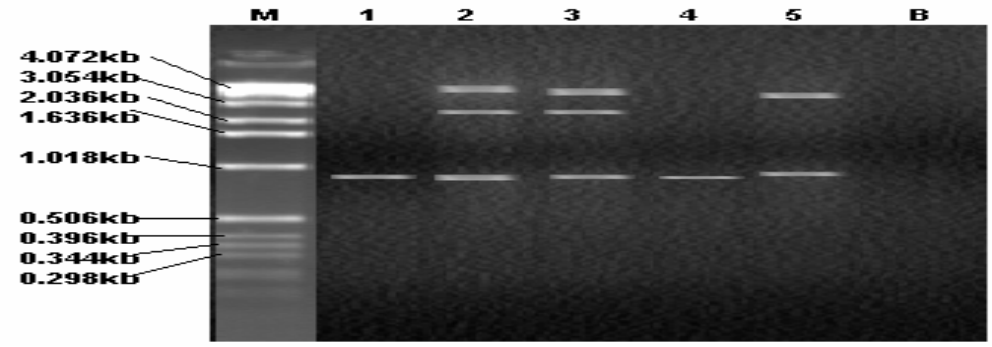

Fig. 2. TRAP profile of sugarcane clones using primer Core15A, M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, B=Blank.

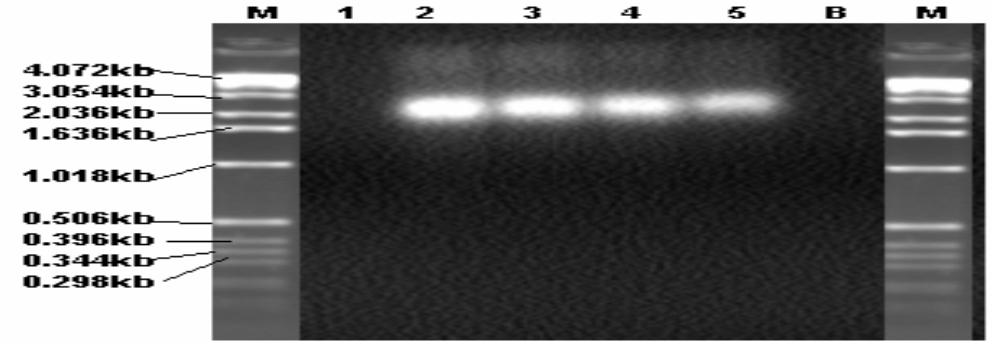

Fig. 3. TRAP profile of sugarcane clones using primer Mischanthus-PPDK M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, B=Blank.

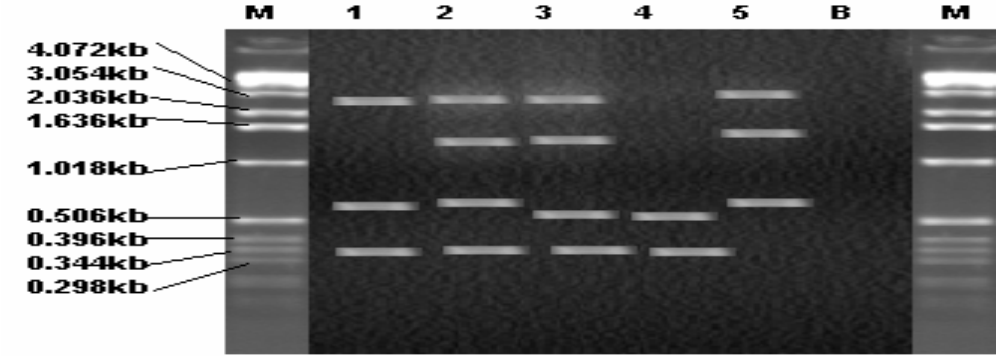

Fig. 4. TRAP profile of sugarcane clones using primer CDPK, M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, B=Blank.

Pyruvate Orthophosphate Dikinase (PPDK): Gel analysis revealed that clones viz., NIA-98, AEC81-0819, L116, NIA-2004 and LRK-2001 tagging the specific gene (PPDK) responsible for formation of PEP in mesophyll cell of the chloroplast which is primary acceptor of CO_2. DNA amplification with PPDK sequence yields 2.26kbp bands (Fig. 7).

Field data: The data regarding the average performance of all the clones is presented in Table 1. Stalks /stool (No), were significantly ($p \leq 0.05$) higher in AEC81-0819 (7.00), followed by NIA-98 (6.5) whereas lowest stalk per stool was observed in L116 (5.17). Non significant difference was observed for the trait cane girth among the tested clones. Weight per stool (kg) was significantly higher in AEC81-0819 (9.17) and minimum in L116 (6.5). Maximum CCS % (13.01) was noted in AEC81-0819 followed by L116 (12.13). Statistically significant higher cane yield (t/ha) was (81.0) recorded in AEC81-0819 and (80.33) NIA-98. Lowest cane yield was recorded in L116 (63.67). Highest sugar yield (t/ha) was (10.54) observed in AEC81-0819 and lowest (6.43) in Larkana-2001.

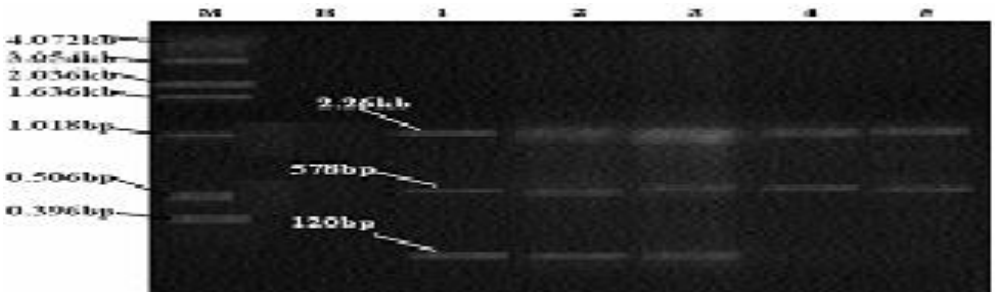

Fig. 5. TRAP profile of sugarcane clones using primer SucSy, M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4= NIA-2004, 5=LRK-2001.

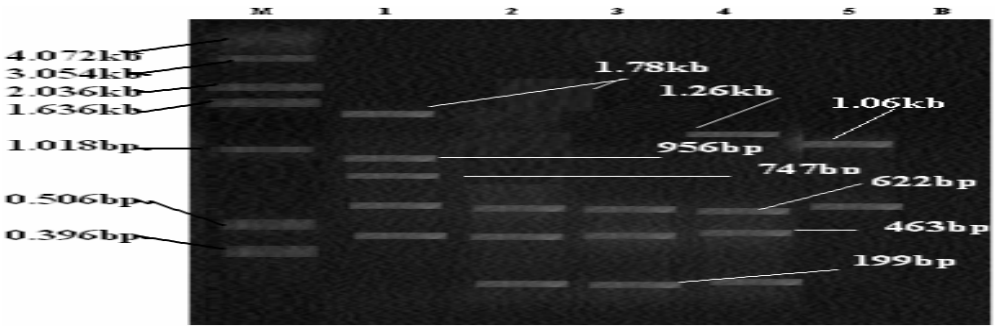

Fig. 6. TRAP profile of sugarcane clones using primer SAI, M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, B=Blank.

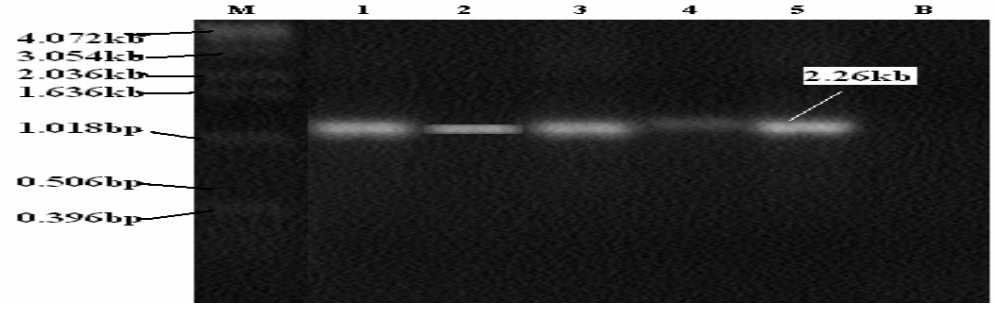

Fig. 7. TRAP profile of sugarcane clones using primer PPDK, M=DNA marker, 1=NIA-98, 2= AEC81-0819, 3=L116, 4=NIA-2004, 5=LRK-2001, B=Blank.

Table 1. Quantitative and qualitative performance of sugarcane clones.

Clone	Stalk per stool (Nos)	Cane girth (cm)	Weight per stool (kg)	Cane yield t/ha	CCS %	Sugar yield t/ha
AEC81-0819	7.00a	2.50ns	9.17a	81.00a	13.01a	10.54a
NIA-98	6.50ab	2.46ns	8.08b	80.33a	9.61c	7.72b
NIA-2004	5.50b	2.40ns	8.25b	73.67b	11.91b	8.77b
L116	5.17b	2.10ns	6.50c	63.67c	12.13ab	7.72b
Larkana-2001	5.40b	2.40ns	8.15b	70.67b	9.11c	6.43bc

DMR test (0.05): Means followed by the same letters are not significantly different from each other

Discussion

High molecular variation is common in clonally propagated, out-crossing polyploidy species and has been reported in (*Buchloe dactyloides)* buffalo grass (Huff *et al*., 1993); *(Bouteloua gracilis)* blue grama (Phan and Smith, 2000) and *Agropyron spp.)* crested wheatgrass (Mellish *et al*., 2002). Sugarcane is a tropical and subtropical crop. It faces two seasons' winter and summer so we need to identify such type of the genotypes which thrives well cold and drought condition. Water stress reduces sugarcane growth and invertase activity increases after 72 hours of stress (Alexander *et al*., 1972). Interestingly, water / cold stress is used in sugarcane to boost sucrose accumulation in the internodes (Stevenson, 1965). Therefore, the genotypes possessing cold tolerance may give high sucrose content (Ming *et al.*, 2006; Wang *et al.*, 2008b). In this study C-repeats/Dehydration Responsive Element (CRT/DRE), COR15a, Mischantus-PPDK and Calcium-dependent protein kinases (CDPK) were used to detect the cold tolerance gene.

CRT/DRE cis-element was found in AEC81-0819, L116 and LRK-2001 thus, ensuring the binding of cis-element with transcription factor CBFs and DREB1s, to activate the transcription of cold and dehydration responsive gene. The cold inducible gene also provides protection against cellular dehydration (Arro, 2005). In case of NIA-98 and NIA-2004 amplification of this element was not observed therefore it can be said that these varieties are not tolerant against cold conditions. The water potential of an internode is an important factor in determining import of assimilates in the plant (Alexander *et al*., 1972).

Primers CRT/DRE, COR15A, CDPK, SucSy and SAI revealed multiple bands per locus due to polyploidy nature of sugarcane. Janno *et al*., (2000) reported that the number of alleles generated per primer pair ranged from 9-20 using 5 SSR primer pairs on 96 sugarcane cultivars. Cordeiro *et al*., (2000) observed 3-12 alleles per primer across five sugarcane genotypes using 91 SSR primer pairs.

Primer Mischanthus-PPDK was amplified by all the genotypes except NIA-98. Like sugarcane, Mischanthus uses the NADP-malic enzyme pathway for photosynthesis (Naidu *et al*., 2003; Wang *et al*., 2008b). In saturating light and chilling temperature CO_2 absorption reduced in susceptible C4 species, thus reducing carbonoxylation efficiency *via* PEPc which lead to photoinhibition and photoxidation (Long *et al*., 1994; Chinthapalli *et al*., 2003). The presence of Mischanthus-PPDK gene facilitates large quantity of CO_2 uptake in cold / drought conditions to maintain adequate photosynthetic rate in the plant cells (Chinthapalli *et al*., 2003).

Calcium-dependent protein kinases (CDPK) is also involved in photosynthesis activity of the plant and more active during the cold tolerance (Arro, 2005). Results shows that AEC81-0819 and L116 showed 4 alleles of Calcium-dependent protein kinases, therefore, these clones perform well under cold stress due to efficient photosynthetic pathway. In contrast NIA-98 and LRK-2001 showed 3 alleles therefore

performance of these clones will be checked under cold conditions. In case of NIA-2004 only 2 alleles were observed thus making NIA-2004 more prone to cold conditions due to weak photosynthetic activity. Cheng *et al*., (2002) reported that it is not unexpected as CDPK are part of a larger super family that is involved in several biochemical pathways mostly in response to stress.

Sugarcane produce large amount of biomass and accumulate high concentration of sugar in the stem. Juice quality is depending on photosynthetic rate, utilization and net accumulation of sucrose. Sucrose metabolism in sugarcane is governed by several enzymes which were Sucrose synthase (SuSy), Soluble acid invertase (SAI) and Pyruvate orthophosphate dikinase (PPDK) (Zhu *et al.,* 1996). Sucrose synthase activity appeared to be related to total sugar (glucose1fructose1 sucrose) accumulation rate (Lingle, 1996). Zhu *et al.,* (1997) reported that high sucrose concentration in sugarcane was negatively correlated with soluble acid invertase activity.

Zhu *et al.,* (2000) also reported that SAI concentration is high in merismetic tissues but decrease rapidly during growth and development of internode. Therefore, sugarcane varieties that are of low sucrose level retain high levels of SAI. It was observed that SAI which is classified as part of the SUC gene family (sucrose accumulation), was actually closer to the cold-tolerant gene CDPK.

PPDK is an important rate limiting enzyme in plant photosynthesis and it is more active in C_4 plant (sugarcane) as compare to C_3 plants (Wang *et al.,* 2008b). Therefore, under stress conditions (drought and cold) the activity of PPDK does increase to combat the situation (Michalowski *et al.,* 1989; Moons *et al.,* 1998; Nogueira *et al.,* 2003). RNA profile of cold-responsive genes using filter arrays showed that PPDK transcripts significantly increased on transfer of a sugar cane hybrid (*Saccharum* sp. 'SP80-3280') from 26^{o}C to 4^{o}C (Nogueira *et al.,* 2003).

TRAP markers reveal mostly trait related polymorphisms, then it is plausible that selection, genetic drift and unequal contribution of parents through selection could alter genetic relationship among siblings for a particular trait-gene.

Results regarding the mean performance of genotypes for cane yield and its component showed significant differences expect cane girth. Whereas, mean values of different genotype for CCS% and sugar yield were significantly different. Khan *et al.,* (2009) reported that increase in cane yield is due to plant height, stalk per stool and weight per stool. Chaudary (1982) observed the increase in cane yield was directly influenced by combined effect of stalk per stool and weight per stool. According to Raman *et al.,* (1985) number of stalks per stool was a major yield contributing factor followed by height and girth. Singh & Sharma (1983) concluded that cane yield exhibited phenotypic association with stalk per stool. Our results are in agreement with these workers as far as stalks per stool and weight per stool is concerned. Sugar yield (t/ha) is mainly dependent on stalks per stool and CCS% (Khan *et al.,* 2002). Sangwan & Singh (1983) reported positive and significant association of sugar yield with CCS% and negative correlation with cane yield. The negative correlation of CCS% with cane yield and positive correlation with sugar yield is one of the major constraints in the improvement of sugarcane. It is concluded from the data that clone AEC81-0819 showed better agronomic performance than the other clones in the trial. Trap studies confirm the agronomic data.

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