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STMS markers for fingerprinting of varieties and genotypes of sugarcane (*Saccharum* spp.)

G. Hemaprabha, P. Govindaraj and N. K. Singh[1]

Sugarcane Breeding Institute, Coimbatore 641 007
[1]National Center for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012

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Abstract

Forty five Genbank derived STMS primers were tested on 36 sugarcane (*Saccharum* spp.) varieties and genetic stocks to identify the most suitable primers for clonal identification in sugarcane. Initially, molecular profiles of 25 elite hybrids of diverse origin were generated with these primers to identify those with high polymorphism information content. Each hybrid could be distinguished individually by twenty primers, while the remaining primers were effective in combination of two or three. The extent of genetic differences brought about by somaclonal variation and induced mutation in sugarcane was significantly less compared with that brought about by conventional breeding. Based on PIC values, 25 most polymorphic STMS primers were identified for testing on genetically more similar genotypes including six somaclones and five induced mutants derived from a sugar-rich variety *viz.*, CoC 671. Highly similar molecular profiles of these clones reflected their similar genetic architecture. Seventeen primers detected genetic differences among the mutants, in contrast to only three, among the somaclones indicating that induced mutations created more genetic changes than somaclonal variations. Thirteen most suitable primers that could differentiate commercial hybrids as well as the mutants and somaclones were identified for routine fingerprinting of sugarcane hybrids.

Key words: Sugarcane, STMS primers, molecular fingerprinting, hybrids, somaclones, induced mutants

Introduction

The present day commercial sugarcane varieties are the products of intercrossing and selection of hybrids of interspecific origin. Identification of varieties for maintaining varietal purity is done by a set of morphological descriptors based on gross morphology of cane [1, 2]. Though characterization based on morphological characters is easy and economical, availability of a large number of varieties suited to specific agro-ecological conditions of India invite constant attention in assuring varietal purity. Continuous variation of these traits and the influence of environment that modifies their expression are problems to the unambiguous identification of varieties. A more satisfactory understanding of the genotype can be realized by anatomical, cytological, biochemical and molecular characterization. During the past two decades, several molecular marker systems have been developed with a range of applications to aid breeding, selection and identification of plant breeder's materials.

Among the molecular markers, microsatellites or simple sequence repeats consisting of short tandem repeats of di- to penta-nucleotide sequence motifs [3] are widely used as genetic markers for their abundance, codominant, multi-allelic and highly polymorphic nature, ease of scoring with PCR and high reproducibility. The sequences flanking specific microsatellite loci are used for designing primers to amplify individual microsatellite loci and the technique is described as sequence tagged microsatellite loci (STMS) [4]. Results of microsatellite analysis in sugarcane have indicated the power of this marker system in quantifying genetic diversity in sugarcane [5] and identifying cane varieties in several countries [6, 7]. STMS is found to be a very useful molecular marker system for plant variety characterization [8, 9].

In this study, STMS primers developed from sugarcane sequences in the Genbank database were used on a representative sample of 36 sugarcane germplasm comprising of varieties with varied geographical adaptation in tropical and subtropical India and mutants and somaclones derived from a high sucrose commercial clone CoC 671. The aim was to assess the utility and efficiency of these markers in generating clear and unambiguous fingerprints for their continued use in molecular fingerprinting of sugarcane varieties and genetic stocks. This will help the process of DUS (distinctiveness, uniformity and stability) testing for varietal identification and protection.

Materials and methods

Plant material and STMS primers: Twenty five elite hybrids of commercial status and genetic stocks *viz.*,

Co 7314, Co 87270, CoLk 8102, BO 91, Co 94005, Co 94008, Co 6304, Co 86032, Co 740, Co 8347, Co 89003, Co 1148, CoC 671, Co 85004, Co 6806, Co 7201, Co 91002, Co 7704, Co 775, Co 62198, Co 86002, Co 96002, ISH 35, ISH 41 and ISH 69, six somaclones of a sugar rich Indian commercial hybrid *viz.*, CoC 671 (Co 88006, Co 88025, Co 89005, Co 89034, Co 94003 and Co 94012) and five induced mutants developed through gamma irradiation of the calli of the same parent *viz.*, CoC 671 (Co 91017, Co 94007, Co 99012, Co 200002 and Co 200003) were taken for the study. A total of 45 polymorphic STMS primers developed from the Genbank sequences containing microsatellite repeats were used for detecting the genetic polymorphism among the commercial hybrids. These primers are NKSCSSR 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 34, 38, 40, 42, 43, 45, 46, 49, 50, 51, 52, 53, 56, 57, 61, 63, 65, 69. Details regarding the primers are available at www.nrcpb.org/STMS.html. Among these 25 primers were tested on the somaclones and mutants (Table 1).

PCR Amplification and electrophoresis: DNA from young leaves of the above parental clones was isolated using CTAB method [10] and quantified by ethidium bromide staining after agarose gel electrophoresis using known concentration of λ DNA. The samples were diluted to get a final concentration of 20ng/µl for PCR amplification. The PCR was performed in a thermal cycler (Gene Amp PCR System 9700, ABI) using a 10. µl reaction mix consisting of final concentration of 20ng template DNA, 1pmol each of forward and reverse primers, 0.5 units Tag polymerase, 1 × Taq DNA polymerase buffer, 2mM dNTPs and 1.75mM $MgCl_2$. The basic cycling profile was 5 min at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature standardized for each primer (ranging from 51°C to 59°C) and 50 seconds extension at 72°C and a final extension of 5 minutes at 72°C. PCR products were resolved through polyacrylamide gel electrophoresis (PAGE) on 7.5% non-denaturing polyacrylamide gels with 0.8% cross linker using 0.5 × TBE buffer in a vertical electrophoresis apparatus (Hoefer S.E 600 Ruby) and stained with ethidium bromide. The gels were visualized under UV light in a gel documentation system (Flurochem^{TM} 5500, Alpha Innotech) to detect polymorphism.

Band scoring and analysis: Bands visualized on PAGE gels were sized using 50bp ladder, and scored for their presence as 1 and absence as 0. Binary matrices consisting of Os and 1s were analyzed to obtain Jaccard's similarity coefficients among the parental clones using NTSYS-pc software (Version 2.0), Exeter Biological Software, Setauket (NY). Genetic similarity matrix generated using Jaccard coefficients was used for clustering using the unweighted pair group method with arithmetic average (UPGMA) algorithm in NTSYS-pc [11]. Polymorphism information content (PIC) was calculated using the formula $PIC = 1 - \sum P_{ij}^2$, where P_{ij} is the frequency of the jth allele for the ith locus summed across all alleles for the locus [12].

Results and discussion

As the traits in sugarcane are fixed by vegetative propagation through stem cuttings, there is uniformity and stability of the DNA fingerprints once generated. This has made DNA fingerprinting a reliable method to varietal identification in this crop.

Twenty five elite hybrids of diverse origin were analyzed with 45 polymorphic sugarcane STMS markers. There was wide range of variability among them (Fig. 1). The number of bands detected with each primer ranged from 3 (with NKSCSSR 1) to 23 (with NKSCSSR 27) with a total of 552 bands on ethidium bromide stained PAGE gels. Average number of bands observed on PAGE gels was 12.27 bands per primer compared to 2-12 bands on agarose gels as reported by Cordeiro *et al.* [5] on a similar set of sugarcane clones. Twenty STMS primers among 45 tested could differentiate all the 25 commercial hybrids. These primers were NKSCSSR 14, 15, 16, 17, 21, 23, 24, 25, 27, 28, 30, 34, 42, 45, 46, 52, 53, 63, 65 and 69. While the STMS primer NKSCSSR 52 was found to be useful in combination with three STMS primers, combinations of two of the remaining 24 primers were able to distinguish all the commercial clones studied (in separate reactions). Thus use of PAGE gels improved the efficiency of STMS analysis by providing more information from each primer and for developing clear fingerprints. PIC values of these primers ranged from 0.44 for NKSCSSR 69 to 0.73 for NKSCSSR 1, 17, 21 and 63 (Table 1). Microsatellites were found to be valuable not only for their rapidity to generate markers, but also for their ability to discriminate 96 cultivars of Mauritius with just two primer pairs [7].

Based on the results on elite hybrids, 25 primers with more PIC values were identified for generating the molecular profiles of genetically more similar clones comprising of somaclones and mutants. PIC values and nucleotide sequences of these primers are provided in Table 1. A total of 297different bands were detected from 25 STMS primers from six somaclones and five induced mutants of CoC 671, of which 47 bands (17.94%) were polymorphic. The high level of molecular uniformity of these lines reflected extremely similar genetic nature of these clones. An example of their relative uniformity is shown in Fig. 2 where only a

Table 1. STMS primers selected based on their discriminatory power on commercial hybrids and genetic stocks and tested on CoC 671 derived somaclones and mutants

Sl. No.	STMS Primers	Primer sequence		PIC values
		Forward (F)	Reverse (R)	
1	NKSCSSR 1	tggcatgtgtcatagccaat	coccaactgggacttttaca	0.73
2	NKSCSSR 2	gctgtcccgttccaagttac	gcgaccggattatgatgatt	0.70
3	NKSCSSR 3	cgtgttcctcttcaacaacg	tgcttcgctatatatgggttca	0.55
4	NKSCSSR 5	atagctcccacaccaaatgc	ttggcaaaattgacccaaat	0.67
5	NKSCSSR 12	cagccacgtgatgcttttct	ccgatccatcagtttcaggt	0.55
6	NKSCSSR 16	gacagaatatgccatggataacaa	cgttctctggtcctattgagc	0.67
7	NKSCSSR 17	gctcgccatgaatagaaagg	accgaggtaggagggagtgt	0.73
8	NKSCSSR 20	cagccaagggtgagaaaaag	tttactatgcaccaagatacacg	0.69
9	NKSCSSR 21	taagccattgggaagaggtg	ctgatgcctgggaatctttc	0.73
10	NKSCSSR 22	gctaagttgccggatgagaa	gtgatggcgtgaacaatgac	0.67
11	NKSCSSR 23	taaacccccgaaaaagaacc	tccggaggtagatccatttg	0.69
12	NKSCSSR 25	tccatgcatgcgtgtagttt	agtgcacaacgttcttgctg	0.71
13	NKSCSSR 26	gttctcgacatgggcctact	ctgcactttcggtccttttt	0.64
14	NKSCSSR 27	tggatttgggtaaggatgga	taatgcctctgggctcaaat	0.67
15	NKSCSSR 30	ctccttctccttcgcatcct	caccttctggagcacgtta	0.69
16	NKSCSSR 40	gatggaggctttgcaatgat	gcatgtcccactgaactgaa	0.67
17	NKSCSSR 45	gtoggtcgtgagaaggaaag	cacgtataaaggcctgtgg	0.50
18	NKSCSSR 51	tttggagagcaaggagcaat	ccaccgtatgcatgaaagaa	0.60
19	NKSCSSR 52	ggcctatggaacgaagttca	cagccttttcttcgcaaaac	0.57
20	NKSCSSR 56	ctatacggcaaacgcaacct	tatacgtcgcatgcaccatc	0.71
21	NKSCSSR 57	cgagcctcoctccatagatt	accaccaccaacctcatctc	0.58
22	NKSCSSR 61	ttggacatggcaagtctttg	aggaacctccaagaacaca	0.63
23	NKSCSSR 63	gattggaaacatgggattgg	ggagaccctccttccttcagt	0.73
24	NKSCSSR 65	aacatgccaccattccata	ttgttgccacacacacacac	0.55
25	NKSCSSR 69	atgggagtgggactgaagag	aaattaaaaccccggagcag	0.55

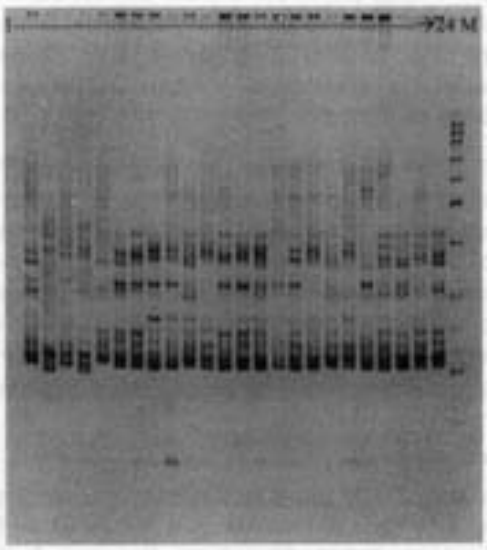

Fig. 1a. NKSCSSR 17

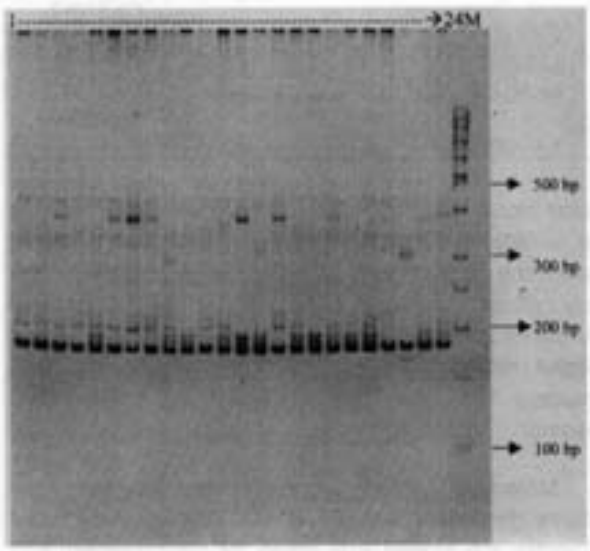

Fig. 1b. NKSCSSR 20

Fig. 1. Fingerprints of elite hybrids and genetic stocks generated by two STMS primers

Lane 1. Co 7314, 2. Co 87270, 3. CoLk 8102, 4. BO 91, 5. Co 94005, 6. Co 94008, 7. Co 6304, 8. Co 86032, 9. Co 740, 10. Co 8347, 11. Co 89003, 12. CoC 671, 13. Co 85004, 14. Co 6806, 15. Co 7201, 16. Co 91002, 17. Co 7704, 18. Co 775, 19. Co 62198, 20. Co 86002, 21. Co 96002, 22. ISH 35, 23. ISH 41, 24. ISH 69, M-50 pb ladder

Table 2. Polymorphism detected in the somaclones and mutants of a sugar rich Indian hybrid CoC 671 using 25 STMS primers

Sl. No.	Primer No.	Number of bands	Percentage of polymorphic bands	Genotypes distinguished
1	NKSCSSR 1	8	25.00	Co 200002
2	NKSCSSR 2	10	40.00	Co 200003
3	NKSCSSR 3	8	37.50	Co 200002
4	NKSCSSR 5	8	37.50	Co 200002
5	NKSCSSR 12	10	20.00	Co 200002
6	NKSCSSR 16	12	0.00	-
7	NKSCSSR 17	16	12.50	Co 94003, Co 88025
8	NKSCSSR 20	15	20.00	Co 200002, Co 200003
9	NKSCSSR 21	15	6.67	Co 200002
10	NKSCSSR 23	16	25.00	Co 200002, Co 99012
11	NKSCSSR 25	14	14.30	Co 200002
12	NKSCSSR 26	10	0.00	-
13	NKSCSSR 27	15	0.00	-
14	NKSCSSR 30	10	20.00	Co 89005, Co 94012, Co 91017
15	NKSCSSR 40	11	18.20	Co 200002
16	NKSCSSR 45	16	0.00	-
17	NKSCSSR 46	16	0.00	-
18	NKSCSSR 51	10	0.00	-
19	NKSCSSR 52	18	0.00	-
20	NKSCSSR 56	6	50.00	Co 200002
21	NKSCSSR 57	12	8.30	Co 200002
22	NKSCSSR 61	12	41.70	Co 89005, Co 88025, Co 99012, Co 91017, Co 94007
23	NKSCSSR 63	11	45.50	Co 200002
24	NKSCSSR 65	11	27.30	Co 91017, Co 200002
25	NKSCSSR 69	7	0.00	-

mutant clone (Co 200002) showed polymorphism. This was in accordance with their morphological similarity among one another and with their original parent. The study clearly showed that the extent of genetic differences brought about by somaclonal variation and induced mutation in sugarcane is significantly less compared with that brought about by conventional breeding.

Molecular profiles of six somaclones and five mutants generated with 25 STMS primers were used to identify the clones that could be distinguished with individual primers. The clones Co 89034 and Co 94007 exhibited exactly similar molecular profiles with all the primers thereby making their distinction difficult. This observation was in accordance with their high level of morphological uniformity. The STMS primers as given in Table 2 could distinguish other clones. Seventeen

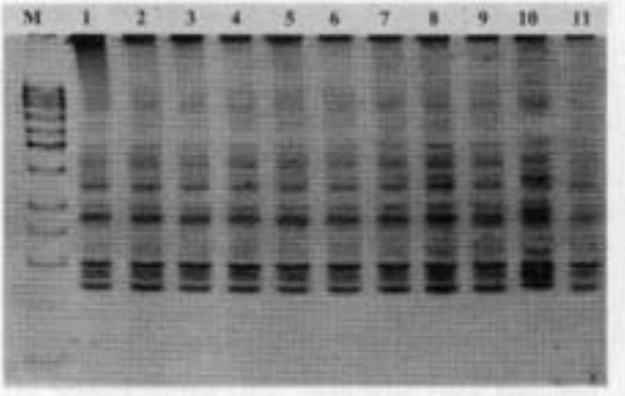

Fig. 2. Fingerprints of eleven CoC 671 derived somaclones and mutants generated by the STMS primer NKSCSSR 2 Lane M: 50 bp ladder, Lane 1. Co 89005, 2. Co 88006, 3. Co 89034, 4. Co 94012, 5. Co 94003, 6. Co 88025, 7. Co 91017, 8. Co 94007, 9. Co 99012, 10. Co 20002, 11. Co 200003

STMS primers *viz.*, NKSCSSR 1, 2, 3, 5, 12, 14, 17, 20, 21, 23, 25, 30, 56, 57, 61, 63 and 65 with very high discriminatory power could differentiate closely related clones of CoC 671. The mutant clone, Co 200002, was the most diverse and could be distinguished by eleven STMS primers *viz.*, NKSCSSR 1, 2, 3, 12, 14, 20, 21, 25, 56, 57and 63. Seventeen primers could detect genetic differences among the mutant clones, in contrast to three primers that showed polymorphism among the somaclones indicating that induced mutation created higher frequency of genetic differences over somaclonal variations. Thirteen STMS primers *viz.*, NKSCSSR 1, 2, 5, 17, 20, 21, 23, 25, 30, 56, 61, 63 and 65 were best suited for fingerprinting sugarcane varieties due to their high discriminatory power to distinguish the commercial hybrids as well as the derivatives of CoC 671.

The study highlighted the efficiency of STMS markers for fingerprinting sugarcane hybrids by providing sufficient number of new STMS markers for fingerprinting sugarcane varieties. The study also showed that somaclonal variation brought about less number of genetic alterations compared to induced mutations, while outcrossing led to larger genetic rearrangements in the heterozygous and polyploid genome of sugarcane.

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