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Sucrose specific TRAP markers as genus and species specific markers in *Saccharum* and *Erianthus* spp.

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Abstract

Sucrose specific candidate genes were used to identify species specific markers through Target Region Amplification Polymorphism (TRAP) based on a study involving nineteen *Erianthus* sp., *Saccharum officinarum* and *S. spontaneum* clones. In this study, 12 primer combinations comprising of the forward primers of four sequences of sucrose metabolizing genes and reverse primers of three arbitrary sequences were used to amplify the DNA samples of the species clones. A total of 296 bands within a size range of 38 bp to 1216 bp were detected. Out of these, 256 were polymorphic (86.49%) and 10 out of 12 combinations revealed more than 80% polymorphism. There were five *Erianthus* specific TRAP markers (SuSy + Arb3$_{108}$, SuSy + Arb3$_{260}$, SuPS(a) + Arb1$_{155}$, SuPS(a) + Arb2$_{370}$ and SAI + Arb3$_{107}$) that would help in identifying intergeneric hybrids in nobilization process. Eight *Saccharum* specific markers (SuSy+Arb2$_{144}$, SuSy+Arb3$_{135}$, SuPS(a)+Arb1$_{141}$, SuPS(a)+Arb1$_{518}$, SuPS(a)+Arb1$_{628}$, SuPS(a)+Arb2$_{212}$, SuPS(a)+Arb2$_{282}$ and SuPS(b)+Arb1$_{492}$ would enable detecting *Saccharum* genome during nobilization process especially when *Saccharum* is used as paternal parent. A marker specific to *S. officinarum* (SuSy+Arb1$_{960}$) owes promise as a marker linked to sucrose content, being present in all sucrose rich clones belonging to *S. officinarum* and absent in low sucrose forms of *Erianthus* and *S. spontaneum*.

Key words: TRAP markers, Sucrose specific candidate genes, *Erianthus* sp., *Saccharum* sp., nobilization

Introduction

A century of sugarcane varietal development activities have resulted in several improved commercial hybrids to yield 70% of total world sugar being produced from sugarcane. The commercial sugarcane cultivars are interspecific hybrids derived from the cultivated species *Saccharum officinarum* or noble cane with wild species *S. spontaneum* through nobilization and with *S. sinense* and *S. barberi* (Heinz 1986). After a spectacular yield and quality improvement initially, further gains through breeding were not appreciable. Breeders felt the need of incorporating hitherto unutilized *Saccharum* species clones and related genera including *Erianthus* and *Miscanthus*. *S. spontaneum* has played a significant role in the development of exotic and elite hybrids for commercial cultivation and is the repository of resistance sources to stresses and diseases. *Erianthus* is the closely related genus of sugarcane, readily crossable and presently attaining importance in breeding for improving ratoonability, biomass and tolerance to abiotic stresses, apart from contributing to the development of special varieties for biofuel. Hence, the practice of wide hybridization has been gaining momentum and has resulted in the development of several interspecific and intergeneric hybrids. The inherent problems associated with wide hybridization necessitate identification of true hybrids to further harness the new variability for sugarcane improvement. Molecular markers have been used in the characterization and analysis of hybridity (D'Hont et al. 1995 and Nair et al. 2006).

The Expressed sequence tag (EST) collections have added to our understanding of the sugarcane genome structure, number of alleles and the complex relationship of specific alleles and allele dosage to phenotypes. The availability of ESTs allows for large-scale gene expression analysis using a variety of tools that has made available several candidate genes of agronomic interest in sugarcane. Following sequence identification, a functional genomics project, the

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SUCEST-FUN Project (http:sucest-fun.org), was implemented to associate putative roles of sugarcane genes (Menossi et al. 2008). An application of this is the Target Region Amplification Polymorphism (TRAP) markers (Hu and Vick 2003). The usefulness of TRAP is tested to develop a new set of species and genus specific markers and is a polymerase chain reaction (PCR)-based marker system that takes advantage of the available EST database sequence information to generate polymorphic markers targeting candidate genes. The primer pair includes an anchored primer, which is generally a18-mer primer from the EST sequence of the candidate gene to pair with an arbitrary primer that targets the intronic and/or exonic region (AT- or GC- rich core) (Li and Quiros 2001). Using a pair of anchored and arbitrary primers to amplify coding regions in the genome, the resulting polymorphism should be reflective of diversity within functional genes (Arro 2005). This marker system has been proved to be of value in quantifying genetic diversity and was found advantageous as the genetic diversity was observed within the functional genes in sugarcane. Again, sequencing of TRAP amplicons from sugarcane and Blast X analysis has demonstrated that the TRAP primers could successfully amplify the anticipated candidate gene regions (Alwala et al. 2003). In the present study, anchored primers of three genes involved in sucrose metabolism *viz*., Sucrose Synthase (SuSy), Sucrose Phosphate Synthase (SuPS) and Soluble Acid Invertase (SAI) were paired with three arbitrary primers to identify species specific markers in *S. officinarum*, *S. spontaneum* and *Erianthus* species to aid in identifying true intergeneric and interspecific hybrids among the progeny derived through crossing these species.

Materials and methods

A total of 19 clones comprising eight of *S. officinarum*, six of *S. spontaneum*, three of *Erianthus arundinaceus* and one each of *E. procerus* and *E. bengalense* were taken for the study as indicated in Table 1.

Of the two primers used in TRAP system, the forward was the fixed or anchored primer designed from the EST sequences of three major genes involved in the metabolic pathway in regulation of sucrose accumulation *viz*., SuSy, SuPs and SAI. The sequences of these genes were obtained from Genbank databases and specific primers were designed using the Primer3 software (Rozen and Skaletsky 2000). Two primers were designed for Sucrose Phosphate Synthase, *viz*., SuPSa and SuPSb. The optimum size, maximum size and minimum size of the primers were set as 18-20 nucleotides. Details of the primers are given in Table 2.

The second primer was the arbitrary primer which was basically a AT- and GC-rich primer to supposedly target introns and exons, respectively (Li and Quiros 2001). These primers comprised of three selective nucleotides at the 3‘ end, four nucleotides of AT- or GC-rich content in the core region and 11 nucleotides as filler sequences at the 5‘ end (Alwala et al. 2003). The basic rules of primer design such as self-complementarity and maintenance of 40 to 60% GC content were followed. These four fixed primers were paired with three arbitrary primers resulting in a total of twelve TRAP primer combinations for the study (Table 1).

PCR amplification and electrophoresis

DNA from the 19 species clones (Table 2) was isolated using CTAB method (Murray and Thompson 1980) and

Table 1. List of fixed and arbitrary primer sequences used for identifying TRAP markers

Primers	Gene	Source sequence	Primer sequence
Forward			
Fixed	Sucrose Synthase (SuSy)	AY670698	AGCCATTGGTCTGGTAGTGG
	Sucrose Phosphate Synthase (SuPS)a	AB001338	ATTCTGGTGAAACGCCAAAC
	Sucrose Phosphate Synthase (SuPS)b	AB001338	ACCCCAAGCATCATAAGCAC
	Soluble Acid Invertase (SAI)	AY302083	GTGGGAGTGCATCGACTTCT
Reverse			
Arbitrary	Arbitrary 1		GACTGCGTACGAATTAAT
	Arbitrary 2		GACTGCGTACGAATTGAC
	Arbitrary 3		GACTGCGTACGAATTTGA

quantified in Nanodrop 100 DNA quantifier. PCR reactions were performed in Eppendorf master cycler with a total reaction volume of 10 µl reaction mixture containing 0.33mM dNTPs, 2.5mM Mg, 0.15 µM of each primer, 0.5U of Taq polymerase and 50ng of template DNA. PCR cycling conditions included a single cycle of 4 minutes at 94^{o}C, followed by 35 cycles of 94^{o}C for 45 seconds, 53^{o}C of annealing temperature for 45 seconds and 72^{o} C for one minute, with a final extension of 72^{o}C for seven minutes. PCR products were resolved on a seven percent non-denaturing polyacrylamide gel using 1X TBE buffer and silver stained adopting standard protocols. The gels were visualized in UV in a gel documentation system (Alpha Innotech).

Results and discussion

The markers used in sugarcane genomic research are mostly random markers like SSR and AFLP derived from polymorphic region randomly dispersed in the genome. Gene specific markers like TRAP would aid in identifying genes of agronomic importance along with analyzing the genetic architecture of species clones and hybrid derivatives. The fixed primers are anchored in the same region on the gene and the arbitrary primers would provide sampling variation for a trait through appropriate combination of fixed and arbitrary primers (Arro 2005). This being the advantage, our study was used to distinguish species clones with TRAP markers specific to genes governing sucrose content as sucrose is a major trait that exhibits variation between the wild and cultivated species. Sucrose specific candidate genes were also tested for the same purpose.

Table 2. List of Sugarcane clones used for the study

Erianthus species	*S. spontaneum*	*S. officinarum*
IK 76-62 (*E.arundinaceus)*	SES 168	57 NG 136
IK 76-99 (*E. arundinaceus)*	Iritty 2	Penang
IK 76-91 (*E. arundinaceus)*	SES 600	Laukona
IND 84-394 (*E. procerus)*	SES 106B	Awela 68
SES 2401 *(E. bengalense)*	S. spontaneum Coimbatore	57 NG 215
	SES 106A	28 NG 224 Keong Mia Moi

A total of 296 amplified products were scored from 12 primer pairs in the PAGE gels, accounting to 24.66 bands per primer, which ranged from 18 bands with SuPSy +Arb1 to 45 in SuSy +Arb2 combinations. The details of amplifications detected are provided in Table 3. Bands of size ranging from 40 bp to 1216 bp were observed, revealing the efficiency of TRAP markers to amplify small to large band sizes and the observations were consistent with the earlier reports (Hu and Vick 2003; Li and Quiros 2001; Arro 2005). Majority of the bands were polymorphic with a high mean polymorphism of 86.49 %. The high level of polymorphism revealed the efficiency of TRAP markers similar to the commonly used random markers like SSR and AFLP in analysing *Saccharum* complex. This also revealed polymorphism in the investigated material with regard to the sucrose genes. Percent polymorphism being an index of marker informativeness and a qualitative measure of the usefulness of a marker for large scale use in marker based studies, TRAP marker system emerged as a favourable option in molecular studies in sugarcane. Again, the highly polymorphic banding profile could be attributed to the complex polyploid genetic nature of the species with homologous and homoeologous chromosomes (D'Hont et al. 1994; D'Hont et al. 1996 and Ming et al. 2001).

The extent of polymorphisms among the four gene specific primers of SuSy, SuPS(a), SuPS(b) and SAI were 84.04%, 88.46%, 85.94% and 88.33%, respectively. Similarly, among the three arbitrary primers, Arb 1 exhibited 89.25% polymorphism compared to 87.83 % with Arb 2 and 81.82 % with Arb3 primer. The high polymorphism with the individual fixed and arbitrary primers indicated polymorphism within the gene-targeted region for sucrose content. Such markers would be more promising and meaningful than random DNA markers in terms of characterizing genetic diversity and for marker-trait association studies facilitating genetical studies in the polyploid genome of sugarcane. The primer pairs SuPS(b) and Arb 3 and SuPS(a) and Arb 1 detected high polymorphism of 95.83 % and 92.59 % respectively and are suggested for use in DNA fingerprinting of sugarcane hybrids.

The amplified markers were surveyed for species and genus specificity. The purpose of this exercise was mainly due to large scale utilization of *Erianthus* and *S. spontaneum* as vital genetic resources in the recent years to breed for climate resilient varieties with drought, salinity and high temperature tolerance.

Table 3. Twelve TRAP primer combinations and the amplified products generated in *Saccharum* and *Erianthus* species clones

Primer combinations	Band size (bp)	Mono-morphic bands	Poly-morphic bands	Total no. of bands	Percent poly-morphism
SuSy+Arb1	50-960	7	16	23	69.57
SuSy+Arb2	50-1056	5	40	45	88.89
SuSy+Arb3	45-1067	3	23	26	88.46
SuPS(a)+Arb1	59-1216	2	25	27	92.59
SuPS(a)+Arb2	58-831	3	25	28	89.29
SuPS(a)+Arb3	71-818	4	19	23	82.61
SuPS(b)+Arb1	40-1020	5	13	18	72.22
SuPS(b)+Arb2	76-1075	3	19	22	86.36
SuPS(b)+Arb3	36-907	1	23	24	95.83
SAI+Arb1	66-903	2	18	20	90.00
SAI+Arb2	67-904	3	17	20	85.00
SAI+Arb3	76-997	2	18	20	90.00

Mean no. of bands / primer = 24.66 Mean = 86.49%

Also sugarcane is emerging as a multi-utility crop for biomass, fibre and energy. Though readily crossable with sugarcane, intergeneric hybrids are difficult to obtain and identification of genuine hybrids among the progeny is difficult and time consuming based on gross morphology. The polymorphic TRAP markers revealed 14 to be species specific between *Saccharum* and *Erianthus* (Table 4). The five *Erianthus* specific markers (present in *Erianthus* but absent in *Saccharum*) were SuSy + $Arb3_{108}$ SuSy + $Arb3_{260}$ (Fig. 1a), SuPS(a) + $Arb1_{155}$, SuPS(a) + $Arb2_{370}$ and SAI + $Arb3_{107}$ (Fig. 1b). Similarly, there were eight *Saccharum* specific markers (absent in *Erianthus*) *viz*., SuSy+$Arb2_{152}$ (Fig. 1c), SuSy+$Arb3_{135,}$ SuPS(a)+$Arb1_{141,}$ SuPS(a)+$Arb1_{518,}$ SuPS(a)+$Arb1_{628,}$ SuPS(a)+$Arb2_{212,}$ SuPS(a)+$Arb2_{282}$ and SuPS(b)+$Arb1_{492}$ which would enable detecting *Saccharum* genome during nobilization process especially when *Saccharum* is used as paternal parent. Thus the results of the present study provided practical application in introgression breeding to identify true hybrids involving these two genera and to differentiate *Erianthus* clones from other members of the *Saccharum* complex. Additionally,

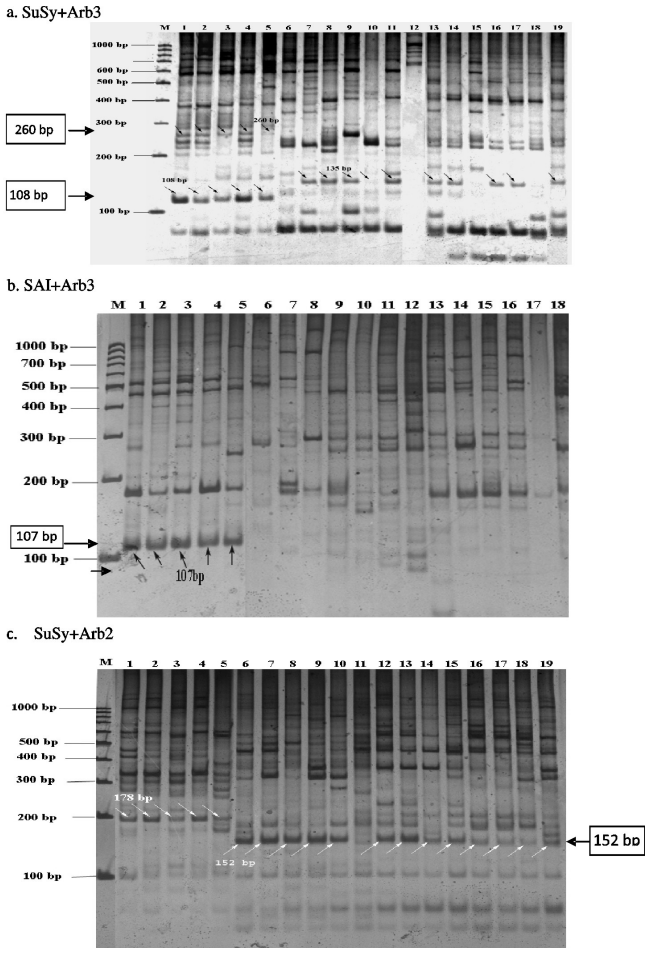
Table 4. Polymorphic bands present and absent among the species clones of *Erianthus* sp., *S. spontaneum* and *S. officinarum*

S.No.	Primer combinations	Band (bp)	*Erianthus* sp.					*S. spontaneum*						*S. officinarum*							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Erianthus specific markers																					
1	SuSy + Arb 3	108	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A
2		260	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A
3	SuPS(a)+Arb1	155	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A
4	SuPS(a)+Arb2	370	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A
5	SAI + Arb 3	107	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Saccharum specific markers (present in *S. spontaneum* and *S. officinarum*)																					
1	SuSy+Arb2	152	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
2	SuSy+Arb 3	135	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
3	SuPS(a)+Arb 1	141	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
4		518	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
5		628	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
6	SuPS(a)+Arb2	212	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
7	SuPS(b)+Arb1	492	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
8	SAI + Arb 2	121	A	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P
S. officinarum specific markers																					
1	SuSy+Arb 1	960	A	A	A	A	A	A	A	A	A	A	A	P	P	P	P	P	P	P	P

A= absent; P= present

SuSy+Arb1$_{960}$ was a useful TRAP marker specific to *S. officinarum*.

Other markers like SuPS (b)+Arb2$_{282}$ and SAI+Arb2$_{121, 450}$ were absent in *Erianthus*, but did not show *Saccharum* specificity, being present in most but all *Saccharum* clones studied and needed further analysis to establish their use in introgression breeding. Species demarcation is not very distinct in *Saccharum* genus with several overlapping accessions, being evolved through natural hybridization in places of origin and diversity where the different species grew together (Heinz 1987). The high polymorphim information content of the TRAP markers as revealed from the study and their unique composition involving a pair of specific gene and arbitrary primers point towards the application of this marker system in sugarcane systematics to identify true to the species clones in the world germplasm collection.



Figs. 1. (a-c): Polymorphism in *Saccharum* and *Erianthus* species generated by different TRAP primer combinations. Lanes 1-5: *Erianthus* 1-IK 76-62, 2-IK76-99, 3-IND 84-394, 4-IK 76-91, 5-SES 2401; 6-11: *S. spontaneum* 6-SES 168, 7-Iritty-2, 8-SES 600, 9-SES 106B, 10-*S. spontaneum* Coimbatore, 11- SES 106A; 12-19: *S. officinarum* 12-57NG 136, 13-Penang, 14-Leukona, 15-Awela 68, 16-57NG215, 17-28NG224, 18-Keong, 19-Mia Moi M-100 bp ladder

Although these markers portray the level of diversity in sugarcane and the related genus, there is a need to translate this information into a working system useful for sugarcane breeders. Expression profiling and sequence analysis of these four sucrose synthase genes failed to show variation between high and low sucrose forms of sugarcane that limited the application of these genes to be used as candidate gene markers in identifying clones with high sucrose (Lavanga and Hemaprabha 2012). This finding prompted us to explore tha use of TRAP markers with the combination of sucrose gene specific and arbitrary marker specific primers for identifying a reliable marker for sucrose content. Among the twelve markers that showed genic specificity, a marker *viz*., SuSy+Arb1$_{960}$ owes promise as a marker linked to sucrose content, being present in all sucrose rich clones belonging to *S. officinarum* and absent in low sucrose forms of *Erianthus* and *S. spontaneum* and owes promise as a marker linked to sucrose content. Further studies are being pursued in backcross breeding programmes to validate this marker so as to explore its use in marker assisted selection for sugar content. By increasing the fixed primers associated with the expression of sucrose trait, maximum extent of the trait polymorphism could be revealed for every gene in the metabolic pathway of the trait. TRAP markers have advantage over random DNA markers which are severely limited in linkage and QTL analysis studies because such linkages can be broken by genetic recombination in the absence of closely linked marker and the gene of interest. Moreover, gene targeted molecular markers like TRAP are more promising and meaningful than random DNA markers in terms of characterizing genetic diversity. Whereas random DNA markers are derived from polymorphic sites spread widely in the genome, gene targeted markers are derived from polymorphisms within genes and thus reflect functional polymorphism (Andersen and Lubberstedt 2003). The TRAP technique, taking advantage of the availability of sequence information, should therefore be useful in sugarcane genomics research involved in marker-trait association.

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