Short Communication

PCR Primers for identification of high sucrose *Saccharum* genotypes

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ABSTRACT

The progeny of a cross between high sucrose sugarcane clone *S. officinarum* 'Gungera' and a low sucrose clone *S. spontaneum* 'SES 603' resulted in interspecific hybrids that were named as ISH-1 to ISH-29 and graded on the basis of sucrose content. Hybrids ISH-1, ISH-5, ISH-17 and ISH-23 were selected as very high sucrose (65 to 100 mg/g tissue) genotypes, whereas ISH-10, ISH-11, ISH-12 and ISH-25 were very low sucrose (2 to 25 mg/g tissue) genotypes. DNA from leaves of both the parent clones, as also the progeny hybrids, was amplified using selected primers, in order to identify markers for sucrose content. Ten specific primers were examined: primers 'A' and 'B' that detect polymorphism in promoter region of sucrose synthase-2 gene; primers AI, SS and SPS that were designed on the basis of nucleotide sequences of genes for acid invertase, sucrose synthase and sucrose phosphate synthase enzymes, respectively and primers MSSCIR43, MSSCIRI, SMC226CG, SMC1039CG and SCB07 selected for relation to sucrose accumulation process. DNA products specific to low or high sucrose clones were identified. Primer 'A' and AI amplified DNA products of size 230 and 500 bp, respectively only in high sucrose genotypes ('Gungera', ISH-1, ISH-5, ISH-17 and ISH-23), while primer SMC226CG generated a DNA product of size 920 bp only in low sucrose genotypes ('SES 603', ISH-10, ISH-11, ISH-12 and ISH-25). Ten random decamer primers were also examined, but their products did not show relationship to sucrose content of genotypes. **[Physiol. Mol. Biol. Plants 2010; 16(1) : 107-111]** *E-mail : kapilvinayak@gmail.com*

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INTRODUCTION

Identification of high sucrose genotypes at early stages of growth is of immense importance in sugarcane breeding work. Efforts have, therefore, been made to identify and characterize genes related to sucrose content (Papini-Terzi *et al*., 2009) and develop markers for these (Ming *et al*., 2001; Lingle *et al*., 2001; Lingle and Dyer 2001; 2004). Using a maize shrunken-1 (sh 1) probe, a restriction fragment length polymorphism (RFLP) marker linked to brix values in sugarcane has been identified (Ming *et al*., 2001). A c-DNA for sucrose synthase gene (*Sus2*) in sugarcane, homologous to maize gene that produces shrunken-1 phenotype, was subsequently cloned (Lingle and Dyer, 2001). A comparison of sequences of promoter region of this gene in high and low sucrose types revealed that promoter sequences of the gene were polymorphic between genotypes and therefore sugarcane species that are known to be polyploids would seem to contain multiple forms of the *Sus2* gene. The sequence differences in the promoter region were primarily due to different indels (insertions – deletions) ranging from 233 to 247 bp and the possibility of using these variable indels as PCR markers for identifying different alleles of *Sus2* gene in high and low sucrose genotypes was explored. On the basis of these observations Lingle and Dyer (2004) designed two sets of primers named 'A' and 'B' based on two different indels in promoter region of sugarcane *Sus2* genes.

Identification of PCR primers that could form an amplification product indicating sucrose content will have immense practical value. The first requirement for a study of this type however, is the availability of genotypes with a wide range of sucrose content. The variability in existing sugarcane germplasm is not large and hence crosses were made between *S. officinarum*, Gungera; a high sucrose clone and *S. spontaneum,*

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SES603; a low sucrose clone to obtain progeny showing wide variation for sucrose content. The parents and progenies labeled ISH-1 to ISH-29 were used as experimental materials in the present study. Results indicate the possibility of using PCR based markers for identification of high sucrose genotypes at early stages of growth.

MATERIALS AND METHODS

Raising inter-specific hybrids

S. officinarum clone 'Gungera' (high sugar, female parent) was crossed with *S. spontaneum* clone 'SES603' (low sugar, male parent) at Sugarcane Breeding Institute, Coimbatore. Setts from parent clones and seed fluff obtained as a result of this cross was transported to CCS Haryana Agricultural University, Regional Research Station, Karnal. Seed fluff was raised on brick beds and germinated seedlings were transferred to the field. Twenty-nine inter-specific hybrids raised from this fluff were tested for sucrose content by anthrone method as described by Van Handel (1968).

Primer design

Three primers AI, SS and SPS were specifically designed for this work. For designing of these primers, nucleotide sequences for genes related to acid invertase, sucrose synthase and sucrose phosphate synthase respectively were downloaded in FASTA format from NCBI database (http:// www.ncbi.nlm.nih.gov). These sequences were aligned using CLUSTALW multiple alignment tool (http://www.ebi.ac.uk/Tools/clustalw) and primers AI, SS and SPS designed. Primers 'A' and 'B' have been reported to generate PCR product related to *Sus2* gene in *Saccharum* (Lingle and Dyer, 2004) species. Primers MSSCIR43, MSSCIRI, SMC226CG, SMC1039CG and SCB07 were selected on the basis of their reported relationship to sucrose metabolism. Of these five, SMC226CG and SMC1039CG have been specifically designed for *Saccharum* species (Cordeiro *et al*., 2000) and SCB07 was taken from SUCEST database (http:// sucest.cna.unicamp.br/en/) for expected EST homology with pyrophosphate dependent phosphofructo-1-kinase like protein (Pinto *et al*., 2004)**;** gene bank acc no. AB013392). The sequences of each of the forward and reverse primers are given in Table 1. In addition, the following ten random primers obtained Integrated DNA Technologies, Coralville, USA were also tested: KJ 1 (5' GAA ACG GGT G 3'); KJ 2 (5' GTG ATC GCA G 3'); KJ 3 (5' TCG GCG ATA G 3'); KJ 4 (5' GTC CAC ACG G 3'); KJ 5 (5' CTG CTG GGA C 3'); KJ 6 (5' GTA GAC CCG T 3'); KJ 7 (5' GGG CCG TTT A 3'); KJ 8 (5' CCA CCA ACA G 3'); KJ 9 (5' TTG AGA CAG G 3'); KJ 10 (5' TTC GAC CAT C 3').

DNA extraction and amplification

Freshly collected tissue (100 mg) from top visible dewlap leaf of sugarcane plants was crushed using liquid nitrogen and genomic DNA was extracted using a kit specifically designed for sugarcane (Bangalore Genei, India).

Table 1. Forward and reverse sequences of ten micro satellite primers used for PCR amplification.

Primer Name	Forward Sequence	Reverse Sequence
A	5' TCG GGA CGA ATC TGT TGA G 3'	5' GCA TAC AAA GGA CAA TAA TAA AAG A 3'
B	5' GAT TCG ATG TGA TGG CAA GCA C 3'	5' GCA TAC AAA GGA CAA TAA TAA AAG A 3'
MSSCIR43	5' ATT CAA CGA TTT TCA CGA G 3'	5' AAC CTA GCA ATT TAC AAG AG 3'
MSSCIRI	5' CTT GTG GAT TGG ATT GGA T 3'	5' AGG AAA TGG ATT GCT CAG G 3'
SMC226CG	5' GAG GCT CAG AAG CTG GCA T 3'	5' ACC CTC TAT TTC CGA GTT GGT 3'
SMC1039CG	5' AGG TGA GAG TTC CTG GCT TTC CA 3'	5' TGT GC TGGC AAG CCC CTA CTT 3'
SCB07	5' ACG AGA ACC ACA GCC ACC AG 3'	5' GGA GGT AGT CGG TGA AGT GC 3'
AI	5' CAA GTT CTA CGC GTC CAA GAC 3'	5' CAG ATG TCC GTG ACC ATT AGT 3'
SS	5' TTG GGT ATG CTC GCT CTT CT 3'	5' TAC TGA CTC CGC ACA AGC AC 3'
SPS	5' TGA GAA GAG CTC GCT GAA CA 3'	5' GCT AGC AGA GGG ACA ACC TG 3'

Genomic DNA was amplified following the protocol described by Dhawan *et al.* (2006). The PCR reaction mixture (25 µl) contained 13.0 µl double distilled water, 2.5 µl 10x assay buffer with 15 mM $MgCl_2$, 1.0 µl primer (0.5 µl forward and 0.5 µl reverse primer; 50 to 60 ng/µl), 10 µl mixture of dNTPs (2.5 mM each) and 1.0 µl sample DNA (50 ng/µl). The mixture was spinned for 5 sec and placed in the thermocycler (Biometra) programmed to initial denaturation step of 6 min followed by addition of 0.5 µl *Taq* polymerase (1.5 U *Taq* polymerase) and spinning for 5 sec. The following programme with forty cycles was used for amplification: denaturation at 94°C for 1 min; annealing at 52°C for 2 min; extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were separated on 1.4% (w/v) agarose gels and visualized by ethidium bromide staining under photodyne UV Trans illuminator (ULTRA-LUM, New Delhi) and photographed with red filter.

RESULTS AND DISCUSSION

Crossing high sucrose parent *S. officinarum* 'Gungera' with a low sucrose parent *S. spontaneum* 'SES 603' resulted in twenty-nine interspecific hybrids (ISH-1 to ISH-29). The very high sucrose types selected for this study (ISH-1, ISH-5, ISH-17 and ISH-23) showed sucrose content between 65 to 100 mg/g of tissue, whereas very low sucrose types (ISH-10, ISH-11, ISH-12 and ISH-25) had sucrose content between 2 to 25 mg/g of tissue (Table 2). The material thus developed was ideal for a study of this kind, as it had a wide range for sucrose content that was not available in commercial germplasm and also had the advantage of similarity in parentage.

Primer 'A' and 'B' described by Lingle and Dyer (2004) based on specific sequences in promoter region of *Sus2* alleles, produced amplification products of 230 and 120 bp, respectively. The 230 bp product was observed in clone 'Gungera' and hybrids ISH-1, ISH-5, ISH-17, and ISH-23 all high sucrose genotypes and was absent in clone 'SES 603' and other low sucrose progenies. Primer set B produced 120 bp product in all genotypes except 'SES 603' and ISH 5 (Table 3). Thus, of these two primers, 'A' seems to be useful in identifying *Sus2* allele related to high sucrose and may be used for identification of high sucrose genotypes.

Three primer pairs were specifically designed for this study on the basis of available nucleotide sequences of three most important enzymes of sucrose synthesis, i.e., acid invertase (primer AI), sucrose synthase (primer SS) and sucrose phosphate synthase (primer SPS). Of these, primer AI generated six amplification products, while primers SS and SPS generated one product each (Table 3). One of these products of size 500 bp produced by primer AI was found to be present in high sugar genotypes only and absent in low sugar genotypes.

Primer SCB07 did not amplify in any of the sugarcane genotypes. Amplification using other primer pairs namely MSSCIR43, MSSCIRI and SMC1039CG showed products with no specificity to high or low sucrose genotypes. However, primer SMC226CG generated a product of size 920 bp which was observed to be present only in low sucrose genotypes of sugarcane, viz., parent clone 'SES 603', ISH-10, ISH-11, ISH-12 and ISH-25 but totally absent in high sucrose genotypes, viz., parent clone 'Gungera', ISH-1, ISH-5, ISH-17 and ISH-23.

Some workers have employed PCR-RAPD to analyze genetic diversity amongst the sugarcane varieties. Harvey *et al*. (1994) and Harvey and Botha (1996) used a total of 41 random decamer primers and 20 inter-specific sugarcane hybrids and *S. spontaneum* clones for this purpose. Nair *et al*. (1999, 2002) used 25 RAPD primers and resolved 262 bands in 28 sugarcane varieties, of which 167 were polymorphic.

Table 2. Sucrose content (mg/g tissue) in 6th internode from bottom in sugarcane clones *S. officinarum* 'Gungera', *S. spontaneum* 'SES 603'and their inter-specific hybrids. Values in the table are mean ± S.E.

Genotypes	Sucrose (mg/g tissue)
'SES 603'	25.5 ± 6.79
'Gungera'	65.1 ± 0.55
ISH-1	78.9 ± 2.16
ISH-5	91.8 ± 2.56
ISH-10	24.8 ± 0.57
ISH-11	4.4 ± 1.60
ISH-12	12.9 ± 0.76
ISH-17	74.4 ± 3.23
ISH-23	71.4 ± 1.72
ISH-25	17.9 ± 0.78

Table 3. Amplification products formed in PCR reaction using ten micro satellite primers in sugarcane parents 'SES 603', 'Gungera and their selected high and low sucrose progenies. "+" indicates presence of a product; "–" indicates absence of a product.

Primer	Product size (bp)	SES 603	Gun-gera	ISH -1	ISH -5	ISH -10	ISH -11	ISH -12	ISH -17	ISH -23	ISH -25
A	230	–	+	+	+	–	–	–	+	+	–
B	120	–	+	+	–	+	+	+	+	+	+
AI	500	–	+	+	+	–	–	–	+	+	–
	390	–	–	–	–	–	+	–	+	–	+
	350	–	–	+	–	+	+	–	+	+	+
	210	–	–	–	–	+	–	–	–	–	–
	180	–	–	+	–	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	+	+
SS	90	+	+	+	+	+	+	+	+	+	+
SPS	180	+	+	+	+	+	+	+	+	+	+
MSSCIR43	650	–	–	+	–	–	+	–	+	–	+
	520	–	–	–	–	–	+	–	+	–	–
	480	–	–	–	–	–	+	–	+	–	–
	290	–	+	+	–	+	+	–	+	+	+
	190	+	+	+	–	–	+	–	+	+	+
	100	+	+	+	–	–	+	–	+	+	+
MSCIRRI	820	–	–	+	–	+	+	+	+	+	+
	550	–	–	+	–	+	+	+	+	+	+
	430	–	–	+	–	+	+	+	+	+	+
	150	–	+	+	+	+	+	+	+	+	+
	50	+	+	+	+	–	–	–	–	–	–
SMC226CG	920	+	–	–	–	+	+	+	–	–	+
	430	+	+	+	+	+	+	+	+	+	+
	320	+	+	+	+	+	+	+	+		+
	180	+	+	+	+	+	+	+	+	+	+
	90	+	+	+	+	+	+	+	+	+	+
SMC1039CG	1000	–	+	+	–	–	+	+	+	–	+
	80	+	+	+	+	+	+	+	+	+	+

SCBO7 – No amplification

Identification of genes associated with sucrose content is important for marker assisted selection in sugarcane breeding (da Silva *et al.*, 2001) and for developing transgenic varieties (Menossi *et al.,* 2008). Microsatellites have been used to detect gene for sucrose synthase an important enzyme of sucrose metabolism in sugarcane (Botha and Black, 2000; Lingle, 1999; Lingle and Dyer, 2001). Also, sucrose synthase markers have been used for quantitative trait loci tagging for sugar content (da Silva and Bressiani 2005). Earlier, Lingle and Irvine (1994) correlated high levels of sucrose synthase activity with an increase in sucrose accumulation rate. Lingle and Dyer (2004) cloned and compared sequences of the promoter region of *Sus2* from 'Muntok Java', a *S. officinarum* x *S. spontaneum* hybrid and 'PIN 84-1', *S. spontaneum*. Identified PCR markers for *Sus2* may be useful molecular markers for identifying different *Sus2* alleles.

Use of sequence differences in the promoter region of *Sus2* gene for designing PCR markers for identifying different alleles of *Sus2* gene in high and low sucrose genotypes is an interesting approach. Two sets of primers (A and B) designed on the basis of such variable indels in the promoter region produced specific PCR products of 230 and 120 bp, respectively. In the present study, the 230 bp product was specifically produced in high sucrose genotypes. Thus, of the ten primers studied, primer 'AI', SMC226CG and primer 'A' seem to be useful in determining genotypes for sucrose content.

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