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Saccharum spontaneum Gene Tagging by Markers Developed From Sugarcane Expressed Sequence Tags

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ABSTRACT

Sugarcane (*Saccharum* spp.) cultivars are considered to be genetically constrained because the limited gene pool from which they are derived originated from a few inter-specific hybrids among *Saccharum officinarum* and its wild relative *S. spontaneum*. As a result of this and other limitations, a basic breeding program was established at the USDA ARS Sugarcane Research Laboratory in Houma, Louisiana, with the goal of identifying and introgressing exotic genes in order to overcome yield and other physiological barriers that breeders may encounter. A plateau appears to have been reached in sugarcane cultivars for sugar content, as suggested by limited genetic gains from breeding for this trait in different sugarcane programs worldwide. The objective of this study was to develop molecular markers to characterize the genetic diversity for sugar composition within a collection of *S. spontaneum*. During the 2004 harvest season, 50 *S. spontaneum* accessions were evaluated for sugar and cane yield components. We developed TRAP (Target Region Amplification Polymorphism) markers from sugarcane genes involved in carbohydrate metabolism and detected marker-trait associations in these genomic regions from *S. spontaneum*. Identification of *S. spontaneum* alleles with positive or negative effects for sugar and fiber content will aid in the development of parental lines for improved sugar and biomass composition through the introgression of genes from this exotic source.

Additional Index Words: sugar content, gene, genetic gain, breeding

Progress in sugarcane genetic studies has been slow because of its genomic complexity, which includes high ploidy levels of inter-specific hybrids involving *Saccharum officinarum* and *S. spontaneum* (Sreenivasan et al., 1987). Difficulties in applying conventional plant breeding techniques to sugarcane result from the fact that commercial varieties possess different proportions of chromosomes and aneuploid chromosome sets. Genetic breeding efforts have been successful in increasing biomass production despite limited success in increasing sugar content. Legendre (1995) found that the average sucrose content of new candidate varieties decreased 3.5% on the fifth cycle of recurrent selection, while Bonnett et al. (2004), noted there has been no increase in sugar content over the last 40 years. These observations suggest that a limit has been reached for this trait, which is supported by a quantitative trait loci (QTL) analysis on interspecific F_1 populations (Ming et al., 2001) which suggests that improved varieties of sugarcane may have a limited (biased subset) population of genes controlling sugar content, resulting from the narrow genetic basis characteristic of these varieties (Hogarth, 1987).

One way to overcome obstacles in breeding for sugar content is to identify and introduce alternative alleles controlling sugar metabolism into commercial germplasm from alternative *Saccharum* species. Natural sources of biodiversity for sucrose synthesis might enrich the narrow genetic basis of sugarcane by means of novel alleles. This process, facilitated by molecular techniques, presents the potential to increase sugar productivity of commercial varieties.

Wild populations of *S. spontaneum* have played an important role in the development of modern sugarcane cultivars (Berding and Roach, 1987). This is the species with the widest distribution among those from the so called *Saccharum* complex (Tai and Miller, 2002) and is the one that probably presents the greatest potential source of genetic variation. However, a limited number of *S. spontaneum* genotypes have been used in the production of modern

sugarcane cultivars (Berding and Roach, 1987). Only two genotypes of *S. spontaneum* were used in the initial crosses made in the late 19th and early 20th centuries in India and Java (Martin, 1996). It has been estimated that an average of 15-25% of chromatin in commercial sugarcane is derived from *S. spontaneum* (d'Hont et al., 1996).

Natural sources of biodiversity for sucrose synthesis, together with genotyping, may enrich the narrow genetic basis of sugarcane with novel alleles to increase sugar productivity. The exploitation of the genetic variation present within *S. spontaneum* will help sugarcane breeders meet the challenge in the use of genetic resources for the benefit of sugarcane breeding.

The first step to identify alleles controlling agronomic traits in wild species of *Sacharum* requires the characterization and evaluation of the group's germplasm. One recommendation of the 1996 Sugarcane Crop Germplasm Committee report is to conduct research to fine tune molecular techniques for use in the characterization and evaluation of *Saccharum* species (http://www.ars-grin.gov/npgs/cgc_reports/sugar.html). Since the introgression of useful alleles, once identified, through traditional breeding methods would be expensive and lengthy on account of "genetic drag" or accidental introduction of alleles with undesirable effects in other traits (Lander and Boltstein, 1989), identifying molecular markers associated with desirable traits would allow for a more efficient development of new cultivars.

TRAP (Target Region Amplification Polymorphism) is a recently developed PCR-based marker technique that uses DNA sequence information from expressed sequence tags (ESTs). This procedure has been proposed to identify polymorphism in plants (Hu and Vick, 2003) and applied in sugarcane studies with success (Alwala et al., 2005). An important application of TRAP markers for introgression breeding results from their ability to associate alleles with either negative or positive effects on traits of interest (Alwala et al., 2005). This technology is more efficient for gene tagging than using random markers, i.e., amplified fragment length polymorphisms (AFLPs) or simple sequence repeats (SSRs).

ESTs have proven useful in identifying genes that are expressed preferentially in certain tissues or cell types of multicellular organisms (Adams et al., 1991). Another useful aspect of ESTs lies in their ability to access genetic information from species with complex genomes, such as sugarcane (Vettore et al., 2001). The Sugarcane EST Project (SUCEST - http://sucest.lad.ic.unicamp.br/en) has built a database containing 238,000 ESTs from 26 sugarcane cDNA libraries, each being constructed from several organs and tissues sampled at different developmental stages of the plant growth. Breeding for sugar content may benefit from this project since many genes potentially involved in sucrose biosynthesis, catalysis and transport have been identified in the sugarcane EST collection. This database represents a source of DNA sequences that can be used to develop molecular markers to assist the genetic breeding, which is considered the basis for the highly technological agriculture of modern times.

The TRAP technique allows plant breeders to explore DNA sequence information available in the Genebank and thereby target genes of interest. When targeting genes involved in sucrose accumulation, EST's would be used to investigate the enzymes participating in metabolic pathways involved in sucrose metabolism. One such pathway, is the metabolism of complex carbohydrates. The *Saccharum officinarum* Gene Index (SOGI), as part of the Institute for Genomic Research (TIGR), contains a total of 254,635 such ESTs. From these, 24,711 are tentative consensus sequences (TCs), 276 of which belong to the metabolism of complex carbohydrates pathway and represent a potential source of TRAP markers for tagging sucrose content QTLs.

To test if alleles with positive effects for sucrose content could be found in *S. spontaneum* and introgressed into commercial sugarcane genotypes, we used ESTs involved in sucrose accumulation from the metabolism of complex carbohydrates pathway to develop TRAP markers. These markers were then applied to a set of 50 *S. spontaneum* genotypes showing variation in sugar content.

MATERIALS AND METHODS

Germplasm Characterization and Juice Analysis. Fifty *S. spontaneum* genotypes currently maintained vegetatively at the USDA ARS Sugarcane Research Laboratory (SRL), Houma, Louisiana were transplanted to 76-l cans for performance evaluation of quantitative traits and data were collected for juice analysis (fiber, brix, sucrose, and purity). On December 2, 2002, the *S. spontaneum* collection was cut and stalks were evaluated for juice quality characteristics in the SRL's juice quality laboratory (Table 1).

Data mining. We searched the TIGR-SOGI database (http://www.tigr.org) for the EST sequences of genes involved in sucrose metabolism and designed forward Polymerase Chain Reaction (PCR) primers from the EST sequence of 4 of those genes using the software Primer 3 (Table 2) developed by the Whitehead Institute for Biological Research and available at http://www.genome.wi.mit.edu/. The

Table 1. *Saccharum spontaneum* genotypes maintained and at the USDA ARS Sugarcane Research Laboratory, Houma, Louisiana. Brix = percentage of soluble solids; Purity = percentage of sucrose over total sugars.

Variety	Brix	Fiber (%)	Purity (%)	Sucrose (%)
SES 84/58	9.1	31.3	67.5	6.1
SES 234A	7.3	38.3	53.1	3.9
SES 147B	12.0	29.8	72.7	8.7
Tainan	8.8	33.9	57.6	5.1
MPTH97-216	17.1	23.1	77.3	13.2
US 56-13-7	11.2	30.3	75.1	8.4
US 56-15-8	10.9	31.8	72.2	7.9
MPTH97-3	10.1	26.8	60.8	6.1
MPTH97-107	14.7	27.2	73.3	10.8
MPTH97-200	10.5	30.0	57.4	6.0
MPTH97-204	12.6	27.5	72.1	9.1
MPTH97-209	8.3	23.7	55.4	4.6
MPTH97-218	11.4	21.6	70.0	8.0
MPTH97-233	10.4	26.3	55.8	5.8
MPTH98-388	14.6	26.1	75.3	11.0
Guangxi86-5	11.9	25.4	75.7	9.0
Guangxi87-21	13.0	29.9	66.3	8.6
Coimbatore	9.1	37.6	55.0	5.0
Djatiroto	8.9	31.3	51.5	4.6
Holes	4.4	33.5	25.1	1.1
IMP 9068	13.3	46.4	63.8	8.5
IMP 9089	8.8	33.2	59.3	5.2
IND 81-80	6.3	36.4	27.5	1.7
IND 81-142	5.2	54.9	21.2	1.1
IND 82-144	7.2	42.9	39.5	2.8
IND 81-161	7.2	31.6	41.6	3.0
IND 81-165	4.9	36.7	22.5	1.1
IND 82-257A	4.0	34.7	21.9	0.9
IND 82-311	6.1	37.1	31.3	1.9
MOL 1032B	7.6	27.3	48.6	3.7
MOL 1032A	11.3	36.8	66.9	7.6
PCA-NOR 84-2A	10.0	31.8	65.4	6.5
PCAV 84-12A	9.7	35.2	60.6	5.9

Table 1 (Continued). *Saccharum spontaneum* genotypes maintained and at the USDA – Agricultural Research Unit's Sugarcane Research Laboratory, Houma, LA. Brix = percentage of soluble solids; Purity = percentage of sucrose over total sugars.

Variety	Brix	Fiber (%)	Purity (%)	Sucrose (%)
PCAV 84-12B	9.7	36.7	57.2	5.6
PCAV 84-12C	9.6	33.0	62.8	6.0
SH 249	14.9	28.9	68.1	10.2
SES 323A	4.6	35.5	31.8	1.5
SES 234B	8.4	32.9	59.5	5.0
SES 231	8.2	30.0	60.7	5.0
SES 205A	10.4	28.7	69.1	7.2
SES 189	8.8	28.6	59.0	5.2
SES 114	7.3	10.7	38.6	2.8
SES 006	10.3	23.3	63.9	6.6
S 66-121-A	4.6	30.4	32.9	1.5
S 66-084B	10.6	30.8	61.3	6.5
S 66-084A	7.1	29.8	52.9	3.8
Guangxi87-22	11.9	27.4	72.3	8.6
Longchuan	12.9	22.6	78.7	10.2
PIN 84-1-B	11.5	31.8	65.4	7.5
PQ 84-3	7.6	33.8	47.0	3.6

parameters we used for the design of the primers were the same as those suggested by Hu and Vick (2003).

Molecular Analysis. We used the CTAB method for plant DNA extraction following the procedures described by da Silva (2001). We then synthesized arbitrary primers with an infrared modification (either IRDye700 or IRDye800) to allow the visualization of the PCR products. We performed PCR reactions in a MJ PTC-100 thermocycler under the same conditions as described in Hu and Vick (2003), and visualized the PCR amplifications in a Li-Cor 4300 DNA Analysis System (Li-Cor, Lincoln, NE) according with the manufacturer's recommendations. Negative controls consisted of PCR primers and all components of the PCR reaction mix, but mili Q water was used in place of sugarcane genomic DNA.

Statistical Analysis. Associations between markers and phenotypes were measured using the analysis of variance model, which is a regression model containing only qualitative independent variables (Neter, 1990). Regression analysis was performed using the SAS PROC REG (SAS Institute), using the genotype information generated by each marker as the indicator variable which received the value 1 or 0 for presence or absence, respectively. Each indicator variable was regressed against the results obtained from the juice analysis, which was considered the response variable. This method is also known as association mapping (Risch and Menkangas,

Table 2 - Sugar metabolism ESTs used to develop TRAP markers.

Name	EST	Protein	Forward
TRAP14	SCQGFL8014B03.g	Sucrose-phosphate synthase	GATTCCTGTTTTGGCTTC
TRAP17	SCRUFL1019B03.g	Beta-fructofuranosidase - Neutral invertase like protein	CTCTGCTCTGCTCTGCTT
TRAP18	SCRFRZ3055B06.g	Hexokinase	CATAGCGCACAACTCAAT
TRAP19	SCSFSB1068H04.g	Glucose-6-phosphate isomerase	ATACAAAATCCGGAGGAA

1996) and is a special application of the Method of Maximum Likelihood (Lander and Botstein, 1989).

RESULTS

Juice analysis of *S. spontaneum* genotypes. On December 2, 2002, we evaluated the *S. spontaneum* collection for juice quality characteristics in the laboratory at the Ardoyne farm. Clones Spont #17, Spont #24 and Spont #37 had a bunchy grass appearance and were too thin to be evaluated for juice quality characteristics. The results of this analysis showed a large amount of variation (Table 1), which justified the investigation of the level of polymorphism in genomic regions involved on sucrose metabolism. For this, we targeted the genes coding for the following enzymes [see Alexander (1973); also the Kegg web site at http://www.genome.ad.jp/kegg/metabolism.html] to develop the TRAP markers:

1. Sucrose-phosphate synthase (Gene Bank Accession #: CA291601). Sucrose-phosphate synthase is among the several enzymes present to assure adequate amounts of carbohydrates necessary for sucrose synthesis.

2. Neutral Invertase (Gene Bank Accession #: CA202913). In mature stalk tissue, where growth processes have practically ceased, there is a predominance of a neutral invertase which governs the active accumulation of sucrose in the vacuole.

3. Hexokinase (Gene Bank Accession #: CA077511). An enzyme that takes part in 6 different metabolic pathways: a) glycolysis and gluconeogenesis, b) fructose and mannose metabolism, c) galactose metabolism, d) starch and sucrose metabolism, e) Streptomycin biosynthesis and f) aminosugars metabolism.

4. Phosphohexose isomerase (Gene Bank Accession #: CA171931). An enzyme that takes part on 3 different metabolic pathways: a) glycolysis and gluconeogenesis, b) pentose phosphate pathway and c) starch and sucrose metabolism

We developed TRAP primers from these carbohydrate-related ESTs to assess the genetic variability present in a group of *Saccharum spontaneum* genotypes. Markers produced by these primers were given a sequential number not related to the EST from which they were developed.

Primers developed from these ESTs (Table 2) were utilized in PCR reactions with the same arbitrary primers presented by Hu and Vick (2003), which were designed with either an AT- or GC-rich motif to anneal with an intron or exon, respectively (Li and Quiros, 2001), to explore the genetic variability present in each locus. Our data showed substantial genetic variation at these loci, as indicated by the presence of polymorphic markers.

This strategy proved to be successful for detecting polymorphism among *S. spontaneum* genotypes. Marker TRAP 19, for example, developed from the SUCEST EST SCSFSB1068H04.g, which corresponds to the gene for Glucose-6-phosphate isomerase (EC 5.3.1.9) when used in combination with the arbitrary primer GACTGCGTACGAATTTGA (Arbi 3 from Li and Quiros, 2001) produced 37 different fragments, 9 being polymorphic.

The marker TRAP 18, developed from the gene coding for enzymes EC 2.7.1.1 and TRAP 19 presented fragments showing statistically significant association with sugar and fiber content (Table 3).

To test whether or not the polymorphic markers were already present in commercial genotypes, we applied them to a group of 50 commercial-type hybrids (Table 4). Some of those markers proved to be absent in all of those genotypes as can be seen in Fig. 1, which suggests that they are specific to *S. spontaneum* and associated with new QTLs controlling sucrose content not yet present in the modern commercial germplasm.

Table 3. – TRAP Markers showing association with carbohydrate related traits, and the statistical parameters obtained from the simple linear regression analysis. Probability level refers to the probability of the type I error for the observed t test.

Marker	Trait	Parameter Estimate	t-test	Probability Level
Trap 18.2-380	Brix	-4.08	-3.65	0.0009
Trap 18.2-380	Purity	-23.05	-4.03	0.0003
Trap 18.2-380	Sucrose	-3.9	-3.4	0.0018
Trap 19.1	Fiber	-4.68	-2.32	0.0261
Trap 19.4	Sucrose	2.37	2.02	0.0511
Trap 19.6	Brix	2.65	2.25	0.0300
Trap 19.6	Fiber	-6.05	-2.25	0.0149

Table 4 – Sugarcane commercial genotypes used to assess *S. spontaneum* specificity of TRAP markers.

Bulk	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5
1	CP96-1252	CP95-1913	CP97-1362	CP97-2068	CP95-1712
2	CP80-1827	CP89-2377	CP81-1405	CP70-1133	BADILA
3	L62-96	L65-69	L60-25	SP79-1117	SP79-93
4	US99-2	US93-15	CP70-321	HOCP93-776	HOCP00-960
5	HOCP96-540	US02-98	HOCP91-555	LCP85-384	US01-39
6	HOCP95-988	US02-99	L97-128	US02-96	US93-17
7	US01-40	US90-18	US02-95	HOCP85-845	US02-97
8	CP72-1210	TCP87-3388	TCP89-3505	TCP93-4245	TCP02-4579
9	H99-295	HB99-1	RSB99-36	CP83-606	LCP80-10
10	RSB99-32	H96-133	RSB99-27	H99-966	CP92-675

DISCUSSION

Tai and Miller (2002) characterized the morphology and sugar composition of the World Collection of *Saccharum* in Miami, Florida, which included *S. spontaneum* genotypes. This species has played a major role in the production of sugarcane varieties adapted to different climate conditions and for disease and insect resistance. The agronomic habits of 32 apparent mosaic-resistant *S. spontaneum* genotypes were studied and reported by Dunckelman and Breaux (1972) to ascertain their potential utilization as breeding material. *S. spontaneum* genotype US 56-15-8 is particularly sweet with a juice Brix reading of 11.5% (and with similar juice Brix reading of 10.9% in our evaluation). Genotypes US 56-15-8 and MPTH 97-216, from Thailand, had a juice Brix reading of 10.9% and 17%, respectively in our evaluation (Table 1). The collections found in Southeast Asia (East Zone Region III including Thailand and Myanmar), which have wider leaves and a common number of chromosomes (2n = 80), tend to

be larger-stalked compared to the low chromosome genotypes from India. MPTH and GUANGXI genotypes of *S. spontaneum* are extremely important in sugarcane breeding because of the higher sugar accumulation and the vigor they impart in the development of novel elite germplasm (breeding parents and varieties).

Because we were interested in sugar content, we targeted enzymes involved in sucrose metabolism. The primary enzymes of interest are invertases (EC 3.2.1.26), sucrose synthase (EC 2.4.1.13) and sucrose-phosphate synthase (EC 2.4.1.14) (Lingle, 1997). The entire gene encoding one of the forms of sucrose synthase, *Sus*2, in sugarcane has been isolated and sequenced (Lingle and Dyer, 2004). When comparing the sequences of the promoter region of *Sus*2 from 'Muntok Java,' a high sucrose *S. officinarum* x *S. spontaneum* hybrid, and 'PIN 84-1,' a low sucrose *S. spontaneum,* these authors found that the sequences were polymorphic within and between the genotypes, primarily due to the presence of different large insertion-deletions (indels). These indels have been suggested as useful molecular markers for identifying different *Sus*2 alleles. Since the arbitrary primers used to generate TRAP markers are designed to explore polymorphic regions inside gene sequences, the polymorphism detected in our work may have resulted from indels present in the genes studied.

Partial sequencing of cDNAs to produce expressed sequence tags and comparing the resulting sequences to public databases provides researchers with a means to gather large amounts of genetic information and potentially identify new genes. A growing number of bio-informatic approaches permits one to explore ESTs for the identification of important genes (Yang et al. 2000). Since partial DNA sequence of key genes in the carbohydrate metabolism pathway is available in a public database (TIGRE-SOGI), one can now directly investigate how much of the genetic variation relates directly to sugar content (Ming et al., 2001).

With the goal of finding direct associations between molecular markers and genes involved in sucrose metabolism, we investigated the DNA sequences of functionally characterized candidate genes as targets for region amplification polymorphism analysis. TRAPs may not be as effective for detecting polymorphism in the *S. officinarum* genome of commercial hybrids, given the narrow genetic basis of commercial varieties (Hogarth, 1987). A similar situation has been observed on the homogeneous D genomes of wheat, where TRAPs were less effective than SSRs for detecting polymorphism (Liu et al., 2005). However, TRAPs were effective in detecting genetic variation among S. spontaneum genotypes and may be useful when applied to other *Sacccharum* species.

We plan to use the association mapping approach to identify genomic regions containing loci involved in sugar related traits. Because of its detection power, this approach has been used frequently for the mapping of complex-trait loci (Pritchard and Rosenberg, 1999). However, if population subdivision resulting from nonrandom mating (Pritchard and Rosenberg, 1999) is present, statistical associations between the arbitrary marker and the quantitative trait is likely to occur without a physical linkage between marker and trait. One efficient way to detect stratification is to type additional unlinked markers. In the case of stratification, the unlinked markers should also show associations with the phenotype (Pritchard and Rosenberg, 1999).

In order to test whether or not population stratification was present, we applied a TRAP primer obtained from an EST that is unrelated to sucrose metabolism. In this case we chose to use the EST SCCCLR1022B11.g which codes for a cysteine protease component of protease-inhibitor complex [*Zea mays*] and has been annotated as "Stress response; Cell rescue activities" (http://www.tigr.org). Because this unlinked marker did not show any association to the traits investigated (Table 5), indicating no stratification, the associations of the TRAPs developed from sugar-related ESTs with the sugar-related traits were considered valid (Pritchard and Rosenberg, 1999).

Table 5 – Results of unlinked marker developed form an EST for insect resistance showing no association with sugar-related traits.

Trait	*F value*	*Probability of F*
Brix	0.14	0.71
Purity	0.07	0.80
Sucrose	0.00	0.96

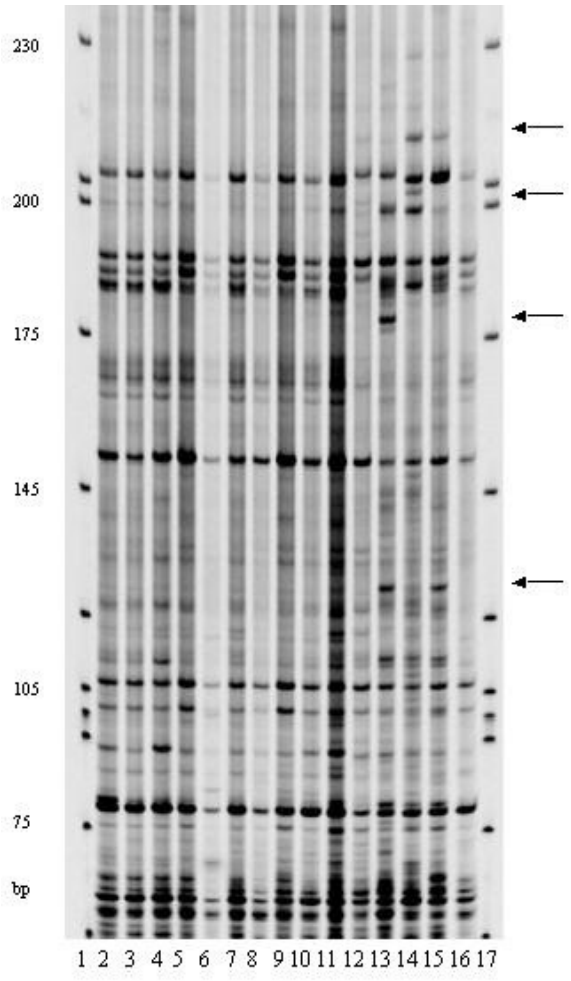

Fig. 1. Results of marker TRAP19, Arbi3 on DNA of commercial sugarcane genotypes , *S. spontaneum* and *S. officinarum*. Lanes 1 and 17 correspond to size markers; lanes 2 to 11 correspond to commercial varieties DNA bulks 1 through 10, each comprised of five genotypes; lanes 12 – 15 correspond to the following *S. spontaneum* genotypes: SES147B; MPTH97-3; MPTH97-216 and S. spontaneum unknown, in this order; lane 16 corresponds to the *S. officinarum* genotype Badila. Arrows indicate *S. spontaneum*-specific markers.

The identification of *S. spontaneum* specific markers (Fig. 1) indicates that new and unknown alleles not yet present in the modern commercial genotypes can be tagged. The alleles conferring positive effects, once identified, can be more efficiently introgressed with the assistance of the sucrose-related TRAP markers that we developed.

To create F1 populations for genetic mapping, we have used the high sucrose *S. spontaneum* genotype MPTH97-216 identified herein in hybridization crosses with commercial varieties. We will apply the TRAP markers being reported to study the segregation of sugar content in these populations to tag *S. spontaneum* positive alleles to be introgressed into commercial varieties.

In conclusion, the molecular markers developed from genes involved in sucrose metabolism were useful for genotyping *S. spontaneum* acessions. We targeted four functionally characterized candidate genes involved in sugar metabolism and detected *S. spontaneum* specific polymorphic markers that are not present in commercial genotypes. The identification of *S. spontaneum* specific markers (Fig. 1) indicates that new and unknown alleles not yet present in the modern commercial genotypes can be tagged. The alleles conferring positive effects, once identified, can be more efficiently introgressed with the assistance of the sucrose-related TRAP markers that we developed.

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