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Short Communication

Sucrose synthase molecular marker associated with sugar content in elite sugarcane progeny

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Abstract

We describe the development and application of an expressed sequence tag (EST)-derived restriction fragment length polymorphism (RFLP) marker for sugarcane elite genotypes which can be used for quantitative trait loci (QTL) tagging for sugar content. EST-derived RFLP markers for proteins involved in sucrose metabolism have been used in Southern analysis for mapping and gene tagging in elite sugarcane clones. A single dose marker, obtained from a sucrose synthase EST associated with sugar content at the $\alpha = 0.01$ probability level, is presented for sugarcane breeding. Utilization of EST homologues to known genes for generation of molecular markers accelerated the identification of a QTL controlling an important trait-sugar content. Sugarcane bacterial artificial chromosome (BAC) clones hybridizing to the sucrose synthase EST were identified.

Key words: sucrose, synthase, expressed, sequence, tag.

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Sugarcane (*Saccharum* sp.) is probably the world's most valuable crop, valued at approximately US$143 billion per year. About 75% of world sucrose production comes from sugarcane which is grown in more than 100 countries (http://www.illovo.co.za/worldofsugar). Sugarcane breeding has become the cornerstone of all advanced sugarcane industries and it is now important that such breeding is integrated with biotechnology so that maximum advantage can be taken of new opportunities and that the current level of production is sustained (Hogarth *et al*., 1997). The high ploidy level and the cytogenetic complexity of sugarcane cultivars, involving varying chromosome sets and complex recombinational events, imposes difficulties in breeding this crop (Vettore *et al*., 2001). Two factors make sugarcane breeding difficult, one factor is that *Saccharum* species are some of the most genetically complex plants because they are polyploid and have relatively large genomes with a DNA content of about six pg (Arumuganathan and Earle, 1991), six times larger than the rice genome. The other factor is that commercial varieties are aneuploid because they have been developed from a series of inter-specific crosses and backcrosses to *Saccharum officinarum* and are polyploid with a high number of chromosomes, *S. officinarum* having a chromosome complement of 2n = 80 while the auto-octoploid *Saccharum spontaneum,* (da Silva *et al*., 1993) has a complement of 2n = 64 to 120.

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In conventional sugarcane variety improvement programs one cycle takes, on average, 12 years from hybridization to the release of cultivars. Molecular markers represent a valuable tool for the breeder because techniques that would increase the efficiency of indirect and early selection in sugarcane would exert a great impact on breeding for high sugar content and could increase the selection precision for early sugar content. In this study we describe an expressed sequence tag (EST)-derived restriction fragment length polymorphism (RFLP) marker associated with sugar content in the progeny of a cross between commercial sugarcane genotypes.

The highly technological agriculture practiced today is based on genetic breeding techniques which use statistics to detect the underlying genes that govern the inheritance of quantitative traits. Compared with traditional methods, mapping with molecular markers is more efficient in genetically dissecting complex traits and has more impact on breeding techniques. The importance of gene mapping was first shown in humans (Botstein *et al*., 1980), although the first genetic maps were actually made in plants (Tanksley *et al*., 1989).

Molecular markers have great potential for improving the efficiency of the breeding process, not only by targeting more traits to be selected in one generation but also by the precision with which genotypes can be selected. Informa-

tion obtained with these markers has contributed to a better understanding of evolution and genetics of both diploids and polyploids (Soltis and Soltis, 1993).

Marker assisted selection (MAS) may be of great importance for sugarcane breeding in that it could increase selection efficiency. The suitability of the genetic markers used in MAS depends on their repeatability, map positions and linkage to other economically important qualitative and quantitative traits (Staub *et al*., 1996). The EST technique has been used for the annotation of complete genome sequences in mammals (Vettore *et al*., 2001) and as a source of molecular markers for gene mapping in plants (Scott *et al*., 2000; da Silva and Ulian, 2001; da Silva *et al*., 2001; da Silva, 2001).

Restriction fragment length polymorphisms (RFLPs) are generated by the restriction digestion of genomic DNA which is immobilized onto nylon membranes and hybridized with genomic or cDNA probes (Southern, 1975). If the cDNA probe corresponds to the EST of a known gene it may increase the chance of tagging the quantitative trait loci (QTL) and allow the indirect but accurate selection of desirable genotypes. The development of EST-derived RFLP markers in sugarcane commercial genotypes for mapping and QTL tagging has been described by da Silva *et al*. (2001) and QTLs have been tagged in the progeny resulting from a wild sugarcane cross (Ming *et al*., 1998), but no QTL has been detected in the progenies of crosses involving commercial varieties. The present paper describes the tagging of a possible QTL for sugar content with an EST-derived RFLP marker using progenies of a cross between two elite commercial sugarcane genotypes.

In 1996 a cross was made at the Copersucar experiment station in Camamu (Bahia, Brazil) between the elite *Saccharum spp.* genotypes SP80-180 (the female line giving a high yield of cane with low sugar content) and SP80-4966 (the male line giving a low yield of cane with high sugar content). In September 1997 the 500 progenies resulting from the SP80-180 x SP80-4966 cross were planted in plots of 1 row, 3 m long, at the Copersucar experiment station in Piracicaba (Sao Paulo, Brazil) to provide a source of seed cane for a field trial which was planted at the same experiment station in April 1999 in 6-m long double-row plots. The statistical design was laid out as a complete block with two replications using the varieties RB72454, SP80-1816 and SP80-1842 as common checks. The trial was harvested in July 2000 and the sugar-content determined by calculating the apparent sucrose content by polarization (Pol value; grams of sucrose per kg per 100 g of fresh cane). In order to accelerate the identification of QTLs for sugar content, the results of this trial were used to identify 10 progenies with extremely high or low Pol values. These plants were then genotyped with EST-derived RFLP markers showing polymorphism between the SP80-180 and SP80-4966 parents.

For genomic DNA extraction and membrane preparation young leaves were collected from the SP80-180 and SP80-4966 clones, frozen in liquid nitrogen and ground with dry ice. Genomic DNA was extracted using the method utilized by Brasileiro and Carneiro, 1998. Good quality DNA samples were digested with restriction enzymes (*Eco*RI, *Eco*RV, *Dra*I and *Xba*I), separated by electrophoresis and transferred to nylon membranes as described by da Silva *et al*. (1993).

We obtained cDNA clones representing ESTs for sugar metabolism from the South African Sugar Association Experiment Station (SASEX; 10 clones) (Carson *et al*., 2001) and from the Sugarcane EST project (SUCEST; 19 clones) (Vettore *et al*., 2001). The clones were grown in liquid media for plasmid DNA extraction and the DNA inserts amplified using the polymerase chain reaction (PCR) and M13 primers, the PCR products being labeled with ^{32}P using the RediPrime (Amersham Pharmacia) according to the manufacturer's instructions. Hybridized membranes were laid onto X-ray films for one to seven days at -80 ºC, depending on the signal intensity.

The association of EST-derived RFLP markers with QTL was investigated using linear regression analysis of phenotype against genotype, which corresponds to Analysis of Variance (ANOVA) for a single factor model. This approach compares the phenotypic means of progeny presenting a marker with those of progeny without the same marker. For QTL tagging, the phenotypic means for those progeny containing the sugar associated marker and those where it was absent were compared, this method being equivalent to the linear regression of sugar content with respect to genotype (*i.e.* presence/absence of the marker) and is a special case of the maximum likelihood method (Lander and Botstein, 1989).

The cDNA clones carrying ESTs for proteins involved in carbohydrate metabolism (Table 1) used in combination with the four restriction enzymes yielded 176 polymorphic fragments in one or other of the parents (da Silva *et al*, 2001). Nine of the EST clones were used as DNA probes with the restricted digested DNA of a random sample of 108 F_1 progeny plants and generated 16 polymorphic fragments. The pA35 clone plus the *Eco*RV restriction enzyme yielded a restriction fragment of about 4 Kb (Figure 1) which was inversely associated ($R^2 = 0.24$, $\alpha = 0.01$) with Pol values (Table 2), indicating that plants without this marker would have a higher Pol value than plants with the marker and that this difference would be significant at the 99% probability level.

The sugar content of both parent and offspring clones ranged from 10.5 to 16.7 Pol units, with the low sugar parent (SP80-180) having a Pol value of 13.1 and the high sugar parent (SP80-4966) a Pol value of 14.5. Surprisingly, the polymorphic fragment generated by pA35-*Eco*RV is present in the high sugar parent (SP80-4966) but absent in the low sugar parent, which helps to explain the

Table 1 - List of cDNA clones, representing expresses sequence tags, used as restriction fragment length polymorphism (RFLP) marker probes.

Expressed sequence tag	Annotation
pA35	Sucrose synthase
pA47	Olygodendrocyte-specific proline-rich protein 2
pA65	Trypsin inhibitor
A84	Sucrose synthase
B63	Sucrose synthase
C17	Hypothetical protein similar to beta-1,3 glucanase
C48	Pyruvate dehydrogenase E1 component, alpha subunit
C81	Sucrose synthase
E15	Pyrophosphatase-fructose 6-phosphate 1-phosphotransferase (PFP)
E46	2-oxoglutarate/malate translocator
SCCCCL1001F02.g	Starch synthase homolog T9A21.90
SCCCCL2001A05.b	Malate dehydrogenase
SCCCCL3001E04.g	Malate oxidoreductase, chloroplast
SCCCCL3001F05.g	Rubb_pea rubisco subunit binding-protein beta subunit precursor
SCCCCL3001F10.g	Ubiquitin-carboxyl extension
SCCCCL3001H01.g	Aldehyde oxidase-2
SCCCCL3003D01.g	Aldehyde oxidase-2
SCCCCL3003F11.b	Citrate Synthase, glyoxysomal precursor
SCCCCL3120A08.b	NADPH dependent mannose 6-phosphate reductase
SCCCCL3120C01.b	Malate oxidoreductase
SCCCCL3120C10.g	Pgky_ wheat phosphoglycerate kinase, cytosolic
SCCCLR1001A05.g	Sucrose synthase-1
SCCCLR1024D02.g	Similar to ATP-citrate-lyase
SCCCLR1C01B09.g	Malate dehydrogenase
SCCCLR1C01G07.g	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic 1
SCCCRZ1004A10.g	Hypothetical protein T4L20.60
SCCCRZ2002B05.g	Putative hydroxymethylglutaryl-CoA lyase
SCCCRZ2C02E02.g	Wall-associated protein kinase
SCCCRZ2C03E11.g	Putative ripening-related protein

transgressive genotypes of the progeny. This suggests that SP80-4966 carries other alleles for high Pol, that compensate for the negative allele associated with the pA35 marker. The data are indicative of transgressive segregation (the occurrence of progeny with phenotypes outside the range determined by the parents) which is usually attributable to polygenic segregation (King and Stanfield, 1997).

The observed presence:absence segregation ratio (1:1, with $\chi^2 = 0.04$ and p 0.9) in a random sample of the mapping population suggests that the pA35 fragment is present in a single dose in the SP80-4966 genotype (da

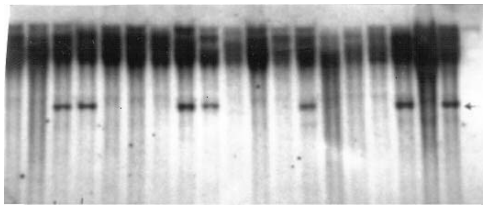

Figure 1 - Single dose restriction fragment generated by southern hybridization of marker pA35 with *Eco*RV digested genomic DNA of sugarcane progeny. From right to left, SP80-4966 parent (high apparent sucrose content by polarization (Pol value; grams of sucrose per kg per 100 g of fresh cane), SP80-180 parent (low Pol value), plants 1 to 17.

Silva and Sorrells, 1996). No association was found between the pA35 fragment and other traits, such as the tonnage of cane per hectare or fiber content (Table 2).

Results obtained with the EST pA35, which corresponds to the sucrose synthase gene, suggests that the polymorphic fragment is located near an allele coding for an isoform of the sucrose synthase enzyme. This transferase is part of the starch and sucrose metabolic pathway and takes part in the conversion of sucrose to starch (KEGG: Kyoto Encyclopedia of Genes and Genomes - http://www.genome.ad.jp/keg/). A putative allele coding for an active enzyme would explain the negative association found between the pA35 marker and the Pol value. The cDNA clone from which the pA35 EST was obtained was ^{32}P-hybridized to a set of nitrocellulose membranes representing the sugarcane bacterial artificial chromosome (BAC) Library (Tompkins *et al*., 1999) for the identification of the BAC clones carrying the sucrose synthase gene. The cDNA clone hybridized to the clones present in well P17 of library plate number (LPN) 30, and well C3 of LPN 35, information which may be used for the physical mapping and map-based cloning of this sucrose synthase allele.

The presence of a negative allele in the high sugar parent may be explained by the quantitative nature (controlled by numerous loci) of the sugar content trait. This fact also explains the occurrence of transgressive plants, resulting from genetic recombination between this major gene, controlling sugar content, and other favorable alleles. It also illustrates the importance of pyramiding favorable alleles in elite sugarcane genotypes. A marker-assisted selection (MAS) using this marker would have allowed the identification of 16 out of 22 transgressive progenies for low Pol values and 19 out of 42 transgressive progenies for high Pol values. The difference between the mean Pol values of plants without the marker and plants with the marker was -0.84, representing 5.9% of the progeny mean (14.05) or nearly 6 times greater than the 1% gain from selection for the same trait obtained in the last 10 years in the Copersucar breeding program without MAS. Because there was no association of the pA35 marker with other yield components (data not shown) we recommend the use of this marker for MAS aimed at increasing sugarcane Pol values.

Table 2 - F-test values from the linear regression analysis of the pA37-*Eco*RV marker as a binary independent variable for the apparent sucrose content by polarization (Pol value; grams of sucrose per kg per 100 g of fresh cane), plot biomass weight (tonnage of cane per hectare) and fiber content (%) of 108 progenies of the SP80-180 x SP80-4966 cross.

Source of variation	Degrees of freedom	Pol value	Plot biomass weight	Fiber content
Regression[1]	1	9.45*	0.07[ns]	0.02[ns]
Residual	106	-	-	-
Total	107			

[1]Pol regression coefficient = -0.59; *significant at the 5% probability level; [ns]not significant.

Lingle and Irvine (1994) correlated high levels of sucrose synthase activity with an increase in sucrose accumulation rate and plant ripening. However, results from other authors (Zhu *et al*., 1997; Botha, 2000) indicate no correlation between sucrose synthase activity and sucrose accumulation. These contrasting results could be due to an effect of genotypic differences between different varieties. An allele coding for an active enzyme reducing sucrose content would explain the negative association found between the pA35 marker and the Pol value.

The narrow range of Pol values existing between the two parent clones, appears to have been a consequence of using a cross between commercial parents as mapping population because varieties/clones with a low sugar content are normally discarded during the first selection stages of a breeding program. To be useful for MAS, this putative QTL needs to be validated in other crosses by investigating the same association with sugar content. Since sugarcane is the result of inter-specific hybridization, if this association is confirmed the origin of the QTL should be investigated, *i.e.* it should be ascertained whether the sucrose synthase allele causing the lower sucrose content is coming from *Saccharum officinarum* or *S. spontaneum*. Southern analysis performed using the sucrose synthase marker on *S. spontaneum* genotypes shows a high degree of polymorphism among the 11 genotypes investigated (data not shown). The mapping of this fragment on a commercial progeny would increase the efficiency of introgression programs, allowing the inclusion of new positive alleles from wild germplasm and reducing the normally associated genetic drag.

Another practical application of these findings in commercial sugarcane breeding would be the planning of crosses. The use of a single dose marker associated with a major QTL in a polymorphic cross for marker-assisted selection in sugarcane will allow more accurate selection because identification of high sugar genotypes would not involve environmental effects.

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