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THE siRNA EFFICACY OF SOLUBLE ACID INVERTASE DOWN-REGULATION IN SUGARCANE (*SACCHARUM SPP.*)

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ABSTRACT

Sugarcane (*Saccharum* sp. hybrids) is a C4 grass used as a major source of sucrose. Invertase enzymes hydrolyse sucrose into hexose sugars reducing the production markedly. Soluble acid invertase role is always a case of discussion for having a major or minor role in the breakdown process in sink tissues. Bio-deterioration is another serious problem accomplishes the sucrose production, the delay between harvest and milling of sugarcane cause enormous depreciation in cane tonnage as well as sugar recovery. Beside another many factors, it was improved that both neutral and acid invertase present in cane stalk and both have tendency to increase after harvest. In the present study, sugarcane cultivar G.99/103, *Saccharum officinarium*, was used to establish *In-planta* transformation experiment for down-regulation of soluble acid invertase gene using siRNA application. The transgenic plants were examined chemically and genetically to estimate the percentage of silencing and its impact on the sucrose content. The enzyme activity showed reduction compared to control in most transgenic plants and consequently the decrease in expression level of soluble acid invertase increase the Brix value significantly in some of the transgenic plants.

INTRODUCTION

Photosynthesis process in mature leaves produces sucrose which is exported according to the demands of the plant to the sink tissues. Plant growth and development are determined by photosynthesis product and its partition way (**McCormick et al 2006**). Enzymes influence the complicated process of sucrose accumulation beside other factors like the genetic makeup of cultivar, the storage compartment and enviromental factors. The sucrose accumulation biochemistry still needs further studies (**Moore, 2005**).

Sucrose content is correlated with the difference between sucrose phosphate synthase and invertases activities during intermodal developmental stages (**Zhu et al 1997**). Providing growing tissues with hexoses as a source of energy is one of the proposed physiological functions of invertases (**ApRees, 1974**). Generating sucrose concentration gradient between different tissues and the plant response to enviromental condition was also another estimated function of invertases (**Eschrich, 1980; Benhamou et al 1991**).

A negative correlation between soluble acid invertase(*SAI)* activity and sucrose content in mature and immature internodes was estimated and on the other hand, a positive correlation between SAI activity and hexoses sugar content was present (**Verma *et al* 2011**).

Sugarcane is a crop with great potential for metabolic engineering, but progress has been limited by highly efficient transgene silencing. The potential exists to utilize efficient gene silencing in molecular improvement through down-regulation of sugarcane genes. However, sugarcane is highly polyploid and heterozygous, which might complicate efforts to employ transgene-mediated silencing of endogenous genes. Hairpin mediated gene silencing could be one of the powerful tool for the molecular improvement of this important crop. (**Osabe et al 2009**).

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Small interfering RNA (siRNA) and microRNA (miRNA) has emerged as one of most powerful approaches for crop improvement. RNAi based on siRNA is one of the widely used tools of reverse genetics which aid in revealing gene functions in many species. This technology has been extensively applied to alter the gene expression in plants with an aim to achieve desirable traits. As per the FDA guidelines, small RNA (sRNA) based transgenics are much safer for consumption than those over-expressing proteins. (**Kamthan et al 2015**)

Differences in sucrose metabolism may contribute to differences in sucrose accumulation among *Saccharum* genotypes. Gene expression and sucrose metabolism were studied in a high sucrose genotype, and a low sucrose genotype. Quantitative PCR is undoubtedly a popular method used for identification of microRNAs. Selection primarily for sucrose content has modified physiological characters, and impacted sucrose content and yield. Through studies, sugarcane maturation can be evaluated by technological indices such as Pol, Brix, Purity and Reducing Sugars. Sugarcane quality cannot be analyzed only by its sucrose content, although it is the most important indicator. (**Lingle et al 2001; Lingle et al 2010; Silva and Segato 2011; Yang et al 2016**).

Development of genotype independent gene transformation method is of great interest on many plants. *In-planta* transformation methods are free from somaclonal variation and easier, simpler and quicker than tissue culture -based methods. One of the gene transformation methods is the meristem based regeneration methods (shoot apex method) which proved its effectiveness in different plants. (**Niazian et al 2017**)

MATERIALS AND METHODS

Plant material

The middle portion of stem at the age of 12 month sugarcane cultivar G.99/103 (US.74-3XCP.76-1055) was used for preparation of nodal cutting setts (a small portion of stem with one axillary bud). The setts were washed and sterilized with 1% mercuric chloride solution for one hour, then washed several times with sterilized distilled water.

Construct

The sequence of *SAI1* gene with accession number JQ406875.1 was downloaded from NCBI and used to design siRNA complementary to *SAI* gene in order to down-regulate it. The website PSSRNAit was used and from the candidate results, the siRNA sequences was tested for efficiency, RISC binding antisense score, target accessibility, off-targeting and secondary structure prediction. According to the results of those parameters (unpublished data) siRNA with efficiency value of 10.31 and sequence (5\-UUGUUGAAGAGGA-ACACGCCG-3\) was used to design short hairpin RNA (shRNA) molecule with loop sequence (5\-TTGGATCCAA-3\). The shRNA molecule was synthesized and purchased from Metabion Company. The vector pFGC5941 was purchased from ARBC stocks, Ohio State University. To get rid of CHSA intron from the vector, it was digested with both the restriction enzymes used in the borders of shRNA molecule; XbaI and NcoI. The synthesized siRNA molecule was ligated in the vector pFGC5941, and then introduced in *Agrobacterium tumeficiens*strain LB4404. The transformation of Agrobacterium was confirmed by PCR using primers designed for the vector. The sequence of the forward primer was 5'-GGATGACGCACAATCCCACT-3' and reverse primer sequence was 5'-ATCATGCGATCATA-GGCGTC-3'.

In-Planta genetic transformation

The sugarcane cultivar was transformed according to the modified method of **Mayavan et al (2015)**. Agrobacterium was suspended in MS medium containing 2% tween20, 5% sucrose and 100µM acetosyringone. The sugarcane setts were pricked several times randomly and gently using sterile hypodermic needle. The setts were injected and immersed on Agrobacterium suspension at room temperature for five hours. After the incubation, the setts were removed from the suspension and air dried on sterilized paper. The infected sugarcane sets were co-cultivated at 25+2˚C overnight in desiccators under complete darkness. After co-cultivation; the sugarcane setts were washed with sterile distilled water containing 500mg/L cefotaxime to kill Agrobacterium. Each sugarcane sett was transferred to 500ml tissue culture bottle containing 100ml of sterile water supplemented with 30mg/L BASTA to be partially immersed and let it to grow. The water with BASTA was replaced on alternate days to avoid bacterial growth. The primary shoots from BASTA resistant sets were trimmed, sown in plastic pots containing patmos, sand and clay. The setts in plastic pots were irrigated once in two days with water containing

BASTA. The putatively transformed sugarcane plants were transferred to the field and tested to confirm transformation.

Transgenic plants analysis

The produced transformed plants from *In-planta* transformation experiment of G.99/103 cultivar were selected using BASTA then, were tested carefully in three different steps to avoid chimerism.

Invertase assay

The invertase enzyme activity was detected as an indicator for the silencing of soluble acid invertase gene. This experiment calculated the activity of the three isoforms of invertase; soluble acid invertase, neutral invertase and cell wall invertase. The three month leaves of the transformed plants were crushed using liquid nitrogen then the invertase activity was detected according to **Miller (1959)**. Extraction by phosphate buffer pH 7.5 (50 mM) containing 1 % PVP with 1 to 4 ratios (1 g to 4 ml chilled buffer) was conducted. The mixture was incubated for 30 min at 37ºC.The reaction was terminated by addition of one ml Dinitrosalicylic Acid (DNS) reagent to one ml reaction mixture, and then the test tubes were placed in a boiling water bath for 5 min and cooled in an ice bath. One ml of distilled water was added and measured at $O.D_{540}$. A standard curve using glucose solution (0 – 2mg/ml) was established using the same volumes of the mixture (1 ml glucose + 1 ml DNS + 1 ml distilled water). The buffer solution was used as a blank.

Gene expression analysis

RNA was extracted from transgenic and control plants according to Trizol modified method described by **Sah et al (2014)**. A fresh tissue snap was frozen in liquid nitrogen and homogenized in sterile pestle and mortar. One ml Trizol was added to the tissue. The samples were vortexed for six seconds then incubated in water bath for ten minutes at 65˚C.500µl chloroform was added, then vortex for 15 seconds. The samples were incubated again but at room temperature for ten minutes, centrifuged at 12,000xg for 15 minutes at 4˚C. The supernatant was mixed with of 3x volume of ice-cold 100% (v/v) ethanol and incubated at −20˚C for one hour, then collected by centrifugation at 12,000 xg for 15 minutes at 4˚C for precipitation, air-dried briefly, and dissolved in 50 µL of nuclease-free water. A second round of precipitation was performed, using an equal volume of 4M LiCl (100µL) and kept at −20˚C overnight. The RNA pellet was recovered by centrifugation at 12,000 g for 15 minutes at 4˚C, washed with 70% (v/v) ethanol, air-dried, and dissolved in 30-50µL of nuclease-free water.

The RNA samples concentration was measured using Bio-Rad spectrophotometer system. The promega DNanse system was used to get rid from any residual DNA in the samples. The reaction was conducted as manufacturer recommendations. The DNase treated RNA was used to synthesize cDNA. After adding one µl of oligodT to RNA the reaction was incubated at 65˚C for 5 min. The samples were thaw on ice for two minutes, and then reverse transcriptase one µl 5x buffer, two µl of dNTPase and one µl of reverse transcriptase enzyme were added. The samples were incubated for one cycle in PCR thermocycler at 42˚C for one hour and other termination cycle at 70˚C for 5 min carried out. cDNA concentration was measured using Fluoromter. To conduct the reaction, 100ng of cDNA was used for all the samples.
Primer pair for soluble acid invertase and actin as a house keeping gene (normalizer) was used for comparative quantitation analysis (QRT-PCR) in three replicates. The soluble acid invertase primer sequence was used by **Prathima et al (2011)** with the sequence F5\-TCCTTGCTTGCCTCTCAAAT-3\ and R5\-ACAAATGTAGCCCTGCCTTG-3\ while actin was designed from sugarcane actin gene with accession number AY742219 using Genescript tool. The sequence of forward actin primer was 5\-GCTGATCGTATGAGCAAGGA-3\ and the reverse was 5\- GATCGACCCTCCTATCCAGA-3\.

Total soluble solids analysis

Seven and eight months transformed sugarcane plants were tested for the total soluble solids as an indicator for sucrose content to determine how far could silencing effect on sucrose accumulation. For Brix value a few drops of juice were used by refractmeter. The reads were statically analyzed using JASP 0.8.0.1 to identify the significance of the difference between control and transgenic plants.

RESULTS AND DISCUSSION

The transgenic cultivar G.99/103 sprouted after one week of incubation on BASTA herbicide while positive control sprouted after three days of incubation in water only. In the first stage of selection, primary shoots developed normally showing significant resistance to BASTA and negative control failed to sprout. On the other hand, the positive control which was grown on water only grew normally and fast. In the second stage of selection, the transgenic shoots were trimmed and transferred into plastic pots and irrigated with water containing BASTA at 30 mg/L. Twenty two shoots from transgenic cultivar G.99-103 completed to the pots stage. From 50 sets, only seven transformed plants were selected to be transferred to the field at four months of age. It was noticed that no tillers were obtained in the pots stage for both transgenic and the control plants. While the four month transformed plants of G.99/103 planted in the field propagated tillers which varied in their numbers.

The invertase activity

A small portion of leaves from two months old plants in the pots stage were ground using liquid nitrogen and the invertase enzymes activity measured as an early indicator of silencing taking into consideration the protein concentration. It was noticed that the lowest values were for T5and T4with values 9.15 and 9.34U/mg protein, respectively. On the other hand, T1 showed a remarkable value of 35.7U/mg protein which is higher than control 32.84U/mg protein, raising a question mark. This higher value may be a result of environmental effect or a response to the treatment as dosage compensate of soluble acid invertase gene loss by the other forms **(Fig. 1)**. Two enzymes in sugarcane leaves, invertase and sucrose synthase produce hexoses from sucrose. It is known that high vacuolar invertase decrease the amount of sucrose available for both storage and export, while low acid invertase activity directs sucrose accumulation (**Winter et al 1994; Burge and Bieleski, 1962**). It is obvious that the data for T3(21) was not collected as the leaves were so exhausted and have low mass, so any sample collected from it could cause death to the plant so it has been subjected for the further analysis after its complete cure.

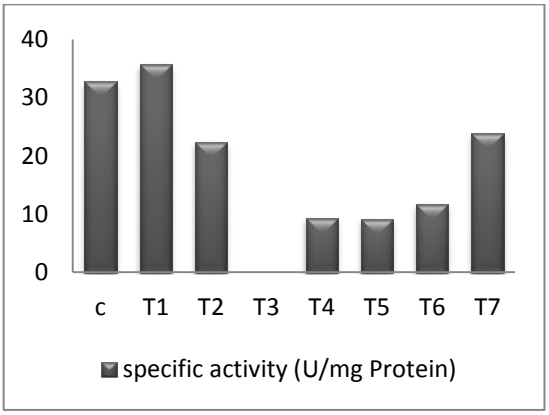

Fig. 1. A diagram shows the specific activity values SAI of transgenic plants in contrast to control

Comparative Quantitation Analysis

The gene expression level of the soluble acid invertase for the transgenic plants was measured by QRT-PCR using actin as a normalizer. Despite the differences in the enzyme activity between the transgenic plants, yet all of them showed a decrease in the expression of SAI enzyme than the control by approximately less than ten folds **(Fig. 2)**. The decrease in invertase activity and its transcript level was confirmed with both high and low sucrose cultivars. The positive control between SAI activity and hexose sugar content reflect its role in sucrose partitioning in various intermodal tissues (**Verma et al 2011**).

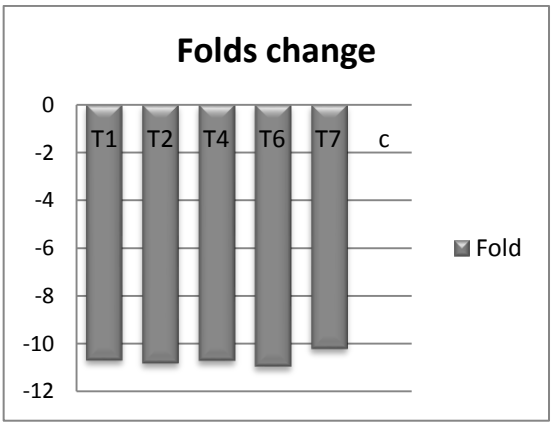

Fig. 2. The gene expression fold change of soluble acid invertase in transgenic plants compared to the control

Total soluble solids analysis

The Brix value was measured as an indicator of sucrose content in the transgenic plants. Seven and eight month plants were used for the reads as the sucrose accumulation starts from the 7th month. The differences in Brix values between transgenic lines and control are shown in the descriptive plot **(Fig. 3 and 4)**. As its obvious T1 line has less sucrose content than control one, while the other lines have superior brix values than control.

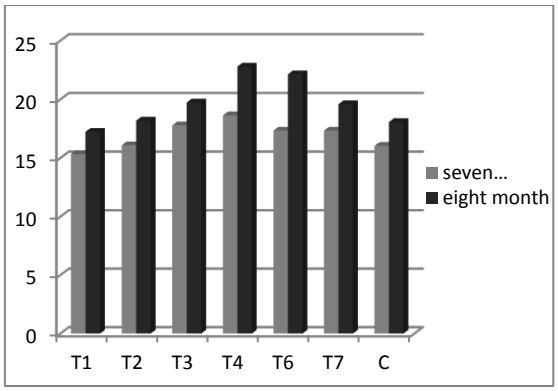

Fig. 3. The change in mean values of Brix for seven and eight month transgenic plants and control

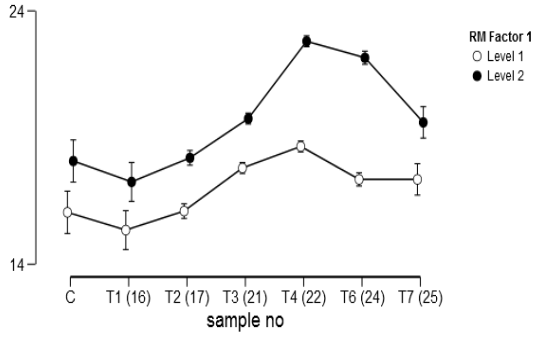

Fig. 4. Descriptive Plot for the sucrose content in seven and eight month transgenic plants compared to control

A statistical analysis using JASP 0.8.0.1 was used **(Table 1)**. Significant differences between all the transgenic plants and the control appeared except for T2 where there was non-significant difference with the control (P= 0.991). However, there was a significant difference between T1 and the control in a negative manner where the sucrose value decreased (P < 0.001). The T1 manner is explained as the enzyme activity for it was higher than control revealing the need for further analysis to determine the physiological performance of this plant. It might be a dosage compensation response of the plant by other invertase isoform. The rest transgenic lines have a significant difference on the expected road with the same value (P< 0.001). The highest sucrose content values obtained from T4 and T6 reflects a promising expectation for these plants.

Table 1. The statistical comparison of Brix values between control and transgenic plants

Post Hoc Comparisons - sample no					
		Mean Difference	SE	t	p tukey
C	T1	0.762	0.123	6.180	< .001
	T2	-0.087	0.123	-0.709	0.991
	T3	-1.712	0.123	-13.880	< .001
	T4	-3.663	0.123	-29.684	< .001
	T6	-2.688	0.123	-21.782	< .001
	T7	-1.413	0.123	-11.448	< .001

Brix value was one of the most common used indexes to evaluate sugarcane. Through studies, sugarcane maturation can be evaluated by technological indexes such as Pol, Brix, Purity and Reducing Sugars. Sugarcane quality cannot be analyzed only by its sucrose content, although it is the most important indicator. (**Silva and Segato, 2011**). According the Brix results T4 and T6 could be promising lines for further evaluation.

Although sucrose content as a trait is of relatively high heritability, progress in its improvement in the varieties has been slow. The increases in sucrose yield have been accomplished through convential breeding programmes; however, these were attained mainly through improvement in cane yield, not in sucrose content. Understanding differences in the expression of genes related directly or indirectly to sucrose accumulation in different *Saccharum* spp. is an important step for improvement of sucrose content **(Jackson, 2005)**.

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كفاءة تقنية siRNA فى تخفيض التعبير الجينى لانزيم الاتفرتيز فى قصب السكر

[149]

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الكلمات الدالة: جزيئات siRNA، انزيم الانفرتيز، محتوى السكروز، قصب السكر

الموجـــــز

نبات قصب السكر من اهم النباتات الاقتصادية فى انتاج السكروز،الا ان انزيمات الانفرتيز لديها قدرة هائلة على تحويل السكروز الى سكريات احادية مختزلة. ويوجد جدل واسع حول ما اذا كان انزيم Soluble acid invertase ذو دور واسع النطاق ام دور محدود فى كل من عمليتى هدم السكروز فى الأنسجة التخزينية و عملية التدهور التى تحدث بعد عملية كسر المحصول نتيجة تأخر عمليات التصنيع. فى هذه الدراسة تم استخدام الصنف التجارى G.99/103 بهدف خفض التعبير الجينى لانزيم Soluble acid invertase باستخدام تقنية siRNA. تم إحـداث عمليـة النقل الوراثى باستخـدام تقنيـة In-Planta transformation. وقد تم تصميم جزئ shRNA على تتابع جين Soluble acid invertase واستخدام الناقل الوراثى التعبيرى (pFGC5941) فى عملية الأستنقال الوراثى. تم الحصول على سبع نباتات مهندسة وراثيا من الصنف G.99/103 وقد تم انتخابهم بعناية وتقييمهم كيميائيا ووراثيا. وقد لوحظ انخفاض النشاط الانزيمى عند تقديره كيميائيا فى معظم النباتات المهندسة وراثيا مقارنة بالنبات غير المهندس وراثيا وقد تباينت قيم الإنخفاض بين النباتات. أما على مستوى التعبير الجينى كان الانخفاض فى كل النباتات بدون استثناء بنسب اقل من عشرة اضعاف. وعند دراسة مردود هذا الانخفاض على نسبة المواد الصلبة الذائبة، وجد ارتفاع معنوى فى بعض النباتات وبخاصة النبات T4 الذى كان له أعلى نسبة سكر بين النباتات الناتجه باستخدام هذه التقنيه.

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