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Assessment of the new Next Generation Sequencing (NGS) tools to identify genes associated with tolerance to water deficit in sugarcane

Evaluación de las herramientas de secuenciación masiva (NGS) para identificar genes asociados con tolerancia al estrés hídrico en caña de azúcar

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Abstract

The aim of this research was to evaluate the performance of the new Next Generation Sequencing (NGS) technologies in comparative transcriptomics experiments, aiming to identify genes associated with tolerance mechanisms to abiotic stress such water deficit or flooding in sugarcane. Despite being widely used in most of current comparative transcriptomics studies, it is important to test the utility of NGS technologies in species such as sugarcane considering its genome complexity and the fact that there is no reference genome, which could be of use in this type of studies. For this purpose, in this investigation, tolerant and susceptible varieties to drought or flooding were selected and independently subjected to stress (medium or severe levels) due to drought or flooding, in order to induce the production of mRNAs of interest. For each of these, leaves were collected and cDNA libraries were produced (for a total of 12). Each library was sequenced using NGS methodologies (Illumina-RNA-Seq) and data were analyzed using specialized bioinformatics software. Among the genes that were observed as differentially expressed it was possible to identify orthologs of those previously associated with tolerance for the traits of interest. Also, it was possible to detect differences in expression levels of highly similar transcripts. Our results provide evidence that support the use of NGS technologies in transcriptomics studies in genetically complex species such as sugarcane.

Key words: Sugarcane, drought, flooding, cDNA, next generation sequencing, transcriptome.

Resumen

En la investigación se evaluó la utilidad de las nuevas tecnologías de secuenciación masiva NGS en la identificación de genes expresados en variedades de caña, bajo condiciones de estrés por déficit hídrico o por anegamiento. No obstante que en la actualidad la secuenciación masiva NGS es la metodología preferida en estudios de transcriptómica comparativa, en el caso de la caña de azúcar (*Saccharum* spp.) es necesario verificar su utilidad, si se tiene en cuenta la complejidad de su genoma y que herramientas útiles, como un genoma de referencia, no se encuentran disponibles. Para el estudio se seleccionaron dos variedades de caña para cada tipo de estrés (una tolerante y una susceptible) tanto en el caso del déficit hídrico como en el caso del anegamiento. Cada una de estas variedades se mantuvo bajo condiciones de estrés (niveles medio o severo) por déficit de agua o por anegamiento para inducir la expresión de los ARNm de interés. Para cada una se crearon tres bibliotecas de ADNc a partir de tejido foliar (para un total de 12 bibliotecas), las cuales se secuenciaron usando la metodología Illumina-RNA-Seq. Los resultados de expresión diferencial obtenidos a partir de estos análisis mostraron ortólogos de genes previamente identificados como contribuyentes a la tolerancia causada por el déficit hídrico o el anegamiento. También fue posible diferenciar entre los niveles de expresión de transcritos altamente similares. Los resultados aquí presentados permiten concluir la utilidad de las metodologías NGS en estudios de transcriptómica comparativa de caña de azúcar.

Palabras clave: Caña de azúcar, déficit hídrico, anegamiento, ADNc, secuenciación masiva NGS, transcriptoma.

Introduction

The Cauca River Valley has 230.303 ha of planted sugar cane (*Saccharum* spp.), of which 121.830 ha (52.9%) are dry areas with water deficit (WD); while 26.370 ha (11.45%) correspond to areas with higher water levels (flooded areas). The remaining area corresponds to semi-dry areas (68.630 ha) representing 29.8% and foothill areas (13,818 ha) representing 6%. The water shortage in sugarcane has led to an estimated production loss of 42%, while those generated by excessively wet soils reach 54% (Cruz *et al.,* 2000, 2009).

So far, stress due to WD or water logging has been handled successfully by using appropriate systems of irrigation and drainage. Those systems have been implemented in more than 80% of the cultivated area in the region, reducing the amount of used water in the areas that need irrigation and therefore maintaining high levels of production throughout the whole cultivated area. However, the costs of those systems are high and represent approximately 45% of total production costs (Campos *et al.*, 2009).

Currently, it is necessary to have genotypes of sugarcane with the ability to maintain good levels of production under abiotic stress conditions, especially water deficit or water logging. To address these conditions, the Department of Breeding in the Research Center of Sugarcane Colombia (Cenicaña) works in evaluating a gene-bank. Besides, the center seeks to implement biotechnological tools to accelerate the process of genetic improvement of sugarcane varieties.

Nowadays advances in methodologies for massive sequencing NGS (Next Generation Sequencing), specifically the RNA-Seq sequencing, have allowed numerous studies of comparative transcriptomics seeking to identify responsible genes that contribute to the development of a feature specific. Such studies start from short sequences ('short reads') of cDNA (complementary deoxyribonucleic acid), generated from cellular RNA (ribonucleic acid) of the organism and usually compares the gene expression of the same individual or a group of individuals under different treatments (Trapnell *et al.,* 2012). By methodologies that require considerable computing power calculation, it is possible to quantify the number of short sequences corresponding to genes or transcripts of a condition or an individual. Through the comparisons, the identification of genes with significant differential expression is possible. In this type of analysis is often used a genome or transcriptome known as a reference point, however, it is also possible to do so without having known information of the species under study ('non-model species') thanks to the Novo assembly techniques applied to NGS data.

Although the concept of comparative transcriptomics, supported by the sequencing RNA-Seq and used in non-model species such as common bean (*Phaseolus vulgaris* L.) (Wu *et al.*, 2014), the cabernet sauvignon grape variety (*Vitis vinifera*) (Li *et al.,* 2014) and the cultivated species of diploid wheat (einkorn wheat) (Fox *et al.*, 2014), the sugar cane is an unique case because of its polyploid genome (octadecaploid), aneuploid (2n = 100-130), large size > 10 GB (Revised in D'Hondt, 2005), which could hamper the analysis of genomic data. Therefore, the aim in this research was to test the usefulness of the new sequencing tools to identify genes associated to stress tolerance WD and/or waterlogging in varieties of sugarcane, in order to confirm its usefulness in varietal breeding strategies supported in biotechnology. Whereas in other crop species have been identified genes that contribute to tolerance of stress caused by the WD and waterlogging, it was decided to use this information to evaluate the usefulness of results obtained in the analysis of this study.

Materials and methods

Plant material and stress induction

Varieties SP 71-6949 and 74-275 MZC, previously characterized (Viveros, 2011) as tolerant and susceptible to water stress (WD) were used respectively. The varieties used for waterlogging stress were CC 01-1940 and CC 93-4418, listed by the Variety Program of Cenicaña as tolerant and susceptible to this feature, respectively.

Stress induction by water deficit

The water deficit (WD) stress induction was performed under greenhouse conditions on 5 months old plants, which were planted in tanks of 0.8 m deep and 2 m diameter. The varieties were planted in a completely randomized model with three replicates per treatment. Samples of leaf tissue, about 5 g, were collected from plants that grown under normal irrigation conditions (control) or stress conditions. Stress

condition was defined as when the moisture content of the soil is depleted approximately 75% from the available water (average stress), and when the moisture level had reached a level close to the wilting point (severe stress). To monitoring moisture levels in the soil, a probe Diviner-2000 was used (Sentek, Australia). Before collecting the leaf tissue to confirm the induction of stress in each of the plants, the leaf temperature, stomatal conductance, fluorescence of photosystem II and chlorophyll content were measured. The instruments used were High Temperature Thermometer IR (Extech Instruments, USA), porometer SC-1 (Decagon Devices, USA), FluorPenFP 100 model Z990 (Qubit Systems, Canada) and Chlorophyll Meter Konica Minolta SPAD 502 (Sensing Americas, USA), respectively.

Stress induction by waterlogging

To induce the waterlogging stress condition, plants of 4 months old were planted in lysimeters (1.2 m long x 1.5 m wide x 2 m deep) and then flooded for a period of 2 to 14 days, or maintained under normal moisture conditions. The experiment was set up using a model of complete randomized block with three replicates per treatment. Tissue samples were collected from the leaves of plants grown under normal irrigation (control) or stress conditions, specifically after 2 days (average stress) and 14 days under waterlogging (severe stress). To monitor the phenotypic changes indicating stress, the presence of surface roots at harvest time was evaluated.

RNA Extraction

Total RNA from each of the varieties was extracted from 100 mg of leaf tissue using Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Once the RNA was extracted, it was dissolved in RNAse free ultrapure water to determine its concentration in a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Additionally, 5 µg RNA were treated with DNAse I (Ambion, cat#: AM 2222) dissolved in ultrapure water, which were used for construction of the cDNA libraries.

Construction of cDNA libraries and massive sequencing Illumina

The construction and sequencing process of cDNA libraries required total RNA samples treated with DNAse I, which were sent to the University of Illinois at Urbana-Champaign (http://www.igb.illinois.edu/biotech/htdna).
Twelve libraries were sent in total, corresponding to two varieties under three treatments (control, medium stress and severe stress) in the case of WD, and two varieties under three treatments (control, medium stress and severe stress) in the case of waterlogging. Each library was sequenced at each end (paired-end sequenced), using the sequencing kit TruSeq SBS (version 3) and Casava1.8 package (pipeline 1.9). The sequencing process was performed in a sequencing platform HiSeq1000.

Bioinformatic and statistical analysis

Once the sequences were obtained, different software packages were used to identify genes that showed differential expression between treatments in each experiment (EH or WD). Among them, the CLC Genomics Workbench package, version 4.6.1 (CLC bio, Aarhus, Denmark) and EST database of sugarcane, http://sucest-fun.org/ (Vettore *et al.,* 2003), were used for the alignment process and the estimation of RPKM values (readings per kilobase transcript reads assigned per million). Estimated values of RPKM for each of the sequences were used in the DEGseq program (Wang *et al.*, 2010) to identify genes that showing differential expression ($P < 0.001$) between treatments (Control vs. Middle stress and Control vs Severe stress) within each experiment (WD and waterlogging). Finally, the annotation of the sequences of interest was conducted with the Blast2GO program (Conesa *et al.*, 2005). Statistical analyses were performed with SAS package, version 9.3.

Results and discussion

Abiotic factors such as water shortage or waterlogging promote biochemical and physiological changes in plants, for example, changes in the opening or closing of the stomata and uptake of CO2, changes in the rate of cell division and photosynthesis, among others. These changes occur as an adaptation to new environmental conditions and are the consequence of activation or inactivation of a number of genes. These genes could be considered as responsible for tolerance to drought stress (Shinozaki and Yamaguchi-Shinozaki, 2007) due to their function during periods of stress. Identifying the genes that contribute to tolerance by water stress represents a clear benefit in agriculture, as it would provide the ability to generate new varieties with lower water consumption in areas where water is scarce, reducing losses production due to abiotic stress and lower cost

of crop production.

The usefulness of NGS sequencing methodologies in identifying genes associated with tolerance to water stress, such as WD and waterlogging in sugarcane, was evaluated in this project. Given the large size of the genome of sugar cane (about 10GB) and ploidy level, among other features, it could hamper the reliable identification of differentially expressed genes (GED). It is noteworthy that in the analysis process of NGS-RNA-Seq data, the first step requires an alignment of the sequences produced to a transcriptome reference. If not, it should be produced from the set of short sequences novo. Since the first reconstruction of the genes found in the sequences of NGS, it is possible to quantify the number of fragments that form part of a gene and thereby determine whether the reconstructed genes have changed their expression between the study treatments (Trapnell *et al.,* 2012). In this study the hypothesis that very similar genes can be differentiate, expressed under the same condition of interest, was also evaluated due to the large genome size or transcriptome that sugarcane has.

In order to promote changes in the expression in the genes of interest, sugarcane varieties, previously classified as tolerant or susceptible to WD or waterlogging stress, were independently maintained under control or stress conditions. Therefore, gene expression of both treatments could be compare, as well as the identification of the differentially expressed genes. Induction of the stress condition in each of the experiments was confirmed by monitoring physiological variables such as leaf temperature, stomatal conductance, fluorescence of photosystem II and leaf chlorophyll content, in the case of WD; and develop of shallow roots in the case of waterlogging. Figure 1 shows the increase of leaf temperature as the stress also increased for the WD treatment, while stomatal conductance, fluorescence of photosystem II and chlorophyll content of leaves decreased. These results were expected since the typical changes of plants under WD stress have already been documented. As consequence, there is stomatal closure and reduction in gas exchange (Barbosa *et al.*, 2013; De Almeida Silva *et al.*, 2011; Liu *et al.,* 2011). In the case of waterlogging, stress induction was observed in root development, given that stress waterlogging does not necessarily generate alterations in leaf temperature, gas exchange or photosynthesis (Glaz *et al.,* 2004). By contrast, when oxygen concentration in the soil decreases as a consequence of excess water, changes like more aerenchyma in the surface roots was observed, facilitating the capture of oxygen in the roots (Eavis, 1972) (Figure 2).

Leaf RNA is used as a starting point to evaluate the usefulness of the tools of mass sequencing for gene expression studies in sugarcane, especially due to its simplicity when the leaf RNA is extracted, and the availability of data from other studies (Tougou 2012;. Vettore *et al.,* 2003). They assessed the gene expression in leaves, allowing comparisons with the results of this study. However, gene expression is influenced by the environment and considered specific for a tissue. In other words, the genes expressed in a given moment in the leaves may become different from those expressed in the root, suggesting that the evaluation of gene expression in roots may provide additional in-

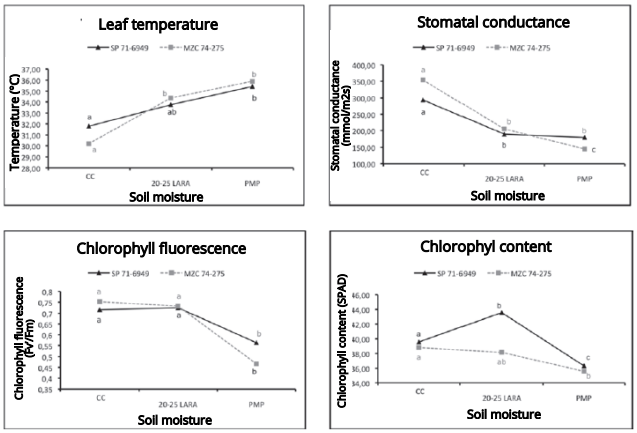

Figura 1. Physiological parameters measured in cane varieties used under normal irrigation or under stress conditions WD.

CC: field capacity, 20-25 LARA: water layer rapidly absorbed between 20-25%, PMP: wilting point. The different letters (a, b, and c) between each of the points for the same variety indicate statistically significant differences (P < 95%).

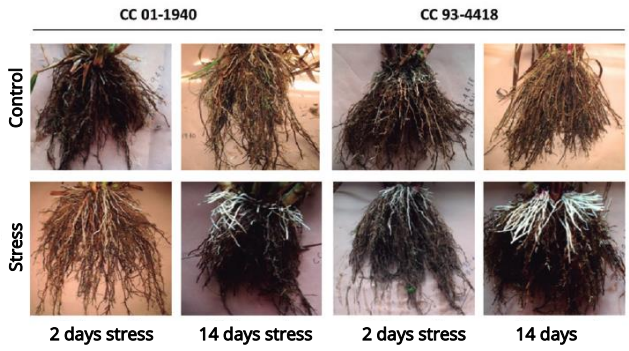

Figure 2. Roots of sugarcane from 4 months old plants under normal irrigation (control) or waterlogging (stress).

formation for both WD and waterlogging treatments. Figure 3 shows that each of the samples has solid ribosomal bands. The quality of RNA extracted from leaves, which was used in the construction and sequencing of RNA-Seq libraries, was assessed by absorbance ranges 260/280, in addition to visual evaluation of 28S and 18S ribosomal bands obtained for all samples. Figure 3 shows that each of the samples has strong ribosomal bands. Together with the obtained ranges, which remained between 1.8 - 2.0 (data not shown), it can be conclude the RNA used for library construction was of high quality.

In this study a minimum of 9,461,897 sequences for each of the 12 libraries constructed (Table 1) was sequenced. Additionally, public transcriptome sugarcane was used, called SUCEST as reference. Although SUCEST does not faithfully represent the transcriptome of sugarcane, because currently there is no sequenced genome, it is an excellent tool that gathers 43,143 transcripts (which in this case could be interpreted as genes) and their possible variants, which indeed are not far from what we would expect to see in a genome such as sugarcane. The total possible stored transcripts in SUCEST could be reconstructed for each library between 30,219 to 33,419, which were used to identify differentially expressed genes. In other words, genes of a variety that were over or under-expressed under stress conditions were identified. The results of the quantification of the gene expression showed a total of 3193 GED for WD stress and 1481 for waterlogging stress. Figures 4 and 5 show the summary of this information in addition to the differences between the number of GED present for both, tolerant and susceptible for each of the two groups.

The possible function in the different cell biological processes of genes was evaluated through comparisons with stored genes from

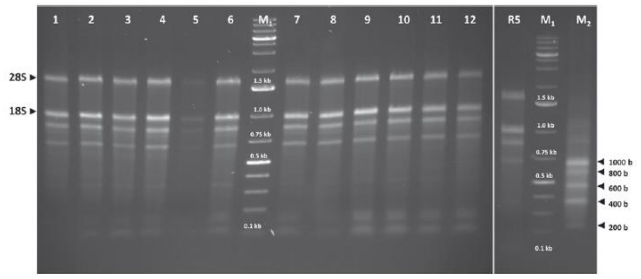

Figure 3. Total RNA extracted from leaf tissue of sugarcane varieties under normal or water stress conditions.

Samples 1-3 correspond to the tolerant genotype (SP 71-6949) for the WD condition. Samples 4-6 correspond to the susceptible genotype (CZM 74-275) for the WH condition. Samples 7-9 correspond to genotype tolerant to waterlogging condition (CC 01-1940). Samples 10-12 correspond to the condition susceptible to waterlogging (CC93-4418) genotype. Samples 1, 4, 7 and 10 correspond to the control treatment for each of the experiments and varieties. Samples 2, 5, 8 and 11 correspond to the middle stress for each of the experiments and varieties. Samples 3, 6, 9 and 12 correspond to severe stress treatment for each of the experiments and varieties. R5 sample: repetition of sample 5. M1: DNA molecular weight marker of 100 base pairs (Fermentas), M2: RNA molecular weight marker (Fermentas).

Table 1. General characteristics of the sequencing process.

Total constructed libraries	12
Size of generated sequencing fragments	75 - 150 pb
Number of sequencing fragments in the 12 libraries	9'461,897 – 22'911,803
Number of genes ('contigs') identified in the 12 libraries	30,219 – 33,419

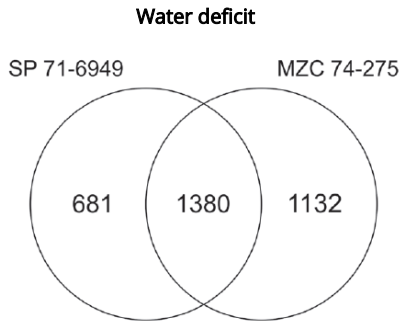

Figure 4. Number of GED identified in the variety of tolerant (SP 71-6949) and susceptible (CZM -74 to 275) sugarcane in the two (medium and severe) levels of water deficit stress.

Tolerant genotype with 2061 GED (681 exclusive) and susceptible genotype with 2512 GED (exclusive 1132) were identified. 1380 GED were common between the two varieties.

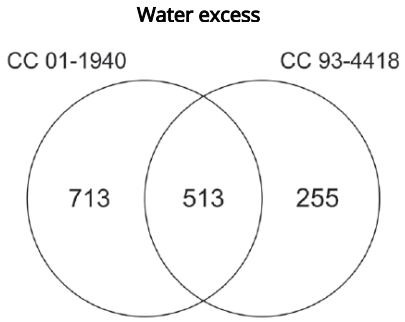

Figure 5. Number of GED identified in the variety of tolerant (CC 01-1940) and susceptible (CC 93-4418) sugarcane in two (medium and severe) stress levels of water excess (flooding).

Tolerant genotype with 1226 GED (713 exclusive) and susceptible genotype with 768 GED (255 Exclusive) were identified. 513 GED were common between the two varieties.

the database of non-redundant protein (nr: Non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF) at NCBI (www.ncbi.nlm.nih.gov) using the Blat2GO program. Figures 6 and 7 indicate this type of information for GED tolerant genotypes (SP 71-6949 and CC 01-1940) used in this study. The activation genes associated with stress response and abiotic stimulus are related with the metabolic pathways, among others, which confirm the results of similar studies (Aparecida-Rodrigues *et al.*, 2009; Vettore *et al.*, 2003).

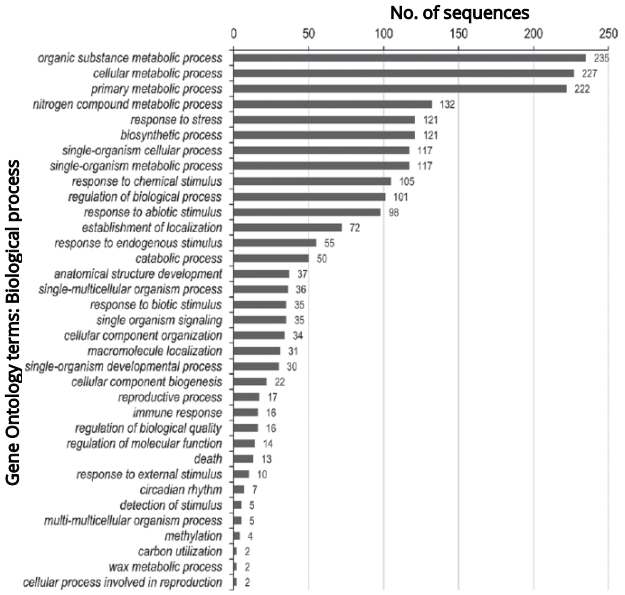

Figure 6. Biological processes associated with the differentially expressed genes (n = 681) exclusively on the variety SP 71-6949 of sugarcane during the period of water stress (WS), in the levels of medium and severe stress levels.

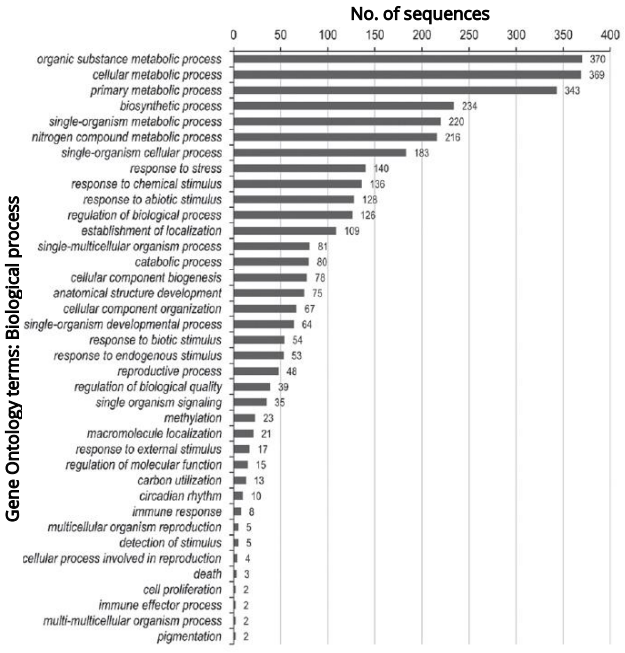

Figure 7. Biological processes associated with the differentially expressed genes (n = 713) exclusively on the variety CC 01-1940 of sugarcane during the period of waterlogging stress levels of medium and severe stress.

Finally, to assess the possibility of identifying GED with high similarity expression it was necessary to monitoring the DREB gene (dehydration response element binding) and the FER gene (Ethylene sensitive to factors) in case of WD stress, and the ADH family (alcohol dehydrogenase) in the case of stress waterlogging. Within the GED transcripts identified, seven transcripts were categorized as DREF, 29 as the FER and 3 as ADH. Each of these gene families have been extensively characterized as contributing to waterlogging tolerance and WD in several monocotyledonous and dicotyledonous (Tougou *et al.*, 2012;. Quan *et al.*, 2010; Shinozaki and Yamaguchi-Shinozaki, 2007; Li *et al.*, 2005). Additionally, similarity between the seven GED varied between 43.9% and 90.5% for the DREB family. In the case of ERF the range of similarity for the 29 GED varied between 31.2% to 96.4%. The three identified GED for waterlogging treatment, showed a similarity between them from 39% to 57%. Table 2 provides a partial list of GED (WD treatment) belonging to the families ERF and DREF. Table 3 shows the list of the three GED belonging to the family ADH from the waterlogging experiments. These results confirm the possibility of differentiating between highly similar GED which have been previously associated with the characteristics of interest in other crops.

Conclusions

Achieving varieties of sugarcane with capacity for more efficient use of water resources would have a significant impact on the sugar industry in Colombian, as it would reduce costs and production losses in areas where stress occurs due to deficit or over supply of water resources. In this sense, Cenicaña works on identifying genes of interest so that they can implement breeding strategies based on genetic transformation or varietal selection using molecular markers.

The preliminary results shown in this document are the first step in identifying these genes and confirming the usefulness of the

Table 2. Partial list of GED belonging to the DREF and ERF families, identified under the levels of medium and severe water deficit stress in the SP 71-6949 (tolerant) and MZC 74-275 (susceptible) varieties. Expression levels (log2 (Fold-Change)) and values of significance (P-value) of the GED are also displayed during the two stress levels in each variety. The empty cells denote that no differential expression of the GED was presented.

Id. Sequence*	Family	Description of the sequence	SP 71-6949				MZC 74-275			
			Medium Stress		Severe stress		Medium Stress		Severe stress	
			log_2(FC)	p-value	log_2(FC)	p-value	log_2(FC)	p-value	log_2(FC)	p-value
SCACLR1129E10.g	DREB	dehydration-responsive element-binding protein 1a-like	3.58	1.4E-06	—	—	—	—	—	—
SCRFHR1009G06.g	DREB	dehydration-responsive contains burp pf03181	—	—	—	—	1.58	6.5E-04	—	—
SCSFLR2031C10.g	DREB	dehydration-responsive element-binding protein 1a-like	2.81	2.6E-09	—	—	-2.13	3.5E-08	-1.84	4.0E-07
SCVPRZ3026C06.g	DREB	dre binding factor	5.45	5.9E-18	—	—	—	—	—	—
SCACLR2022D03.g	ERF	ethylene-responsive transcription factor 4	1.99	1.1E-08	—	—	-2.16	8.5E-15	-3.77	2.7E-25
SCCCLR1077H02.g	ERF	ethylene-responsive transcription factor 1-like	-0.97	1.9E-08	-0.92	7.3E-08	—	—	—	—
SCJFRZ2025A06.g	ERF	ethylene-responsive transcription factor 4	2.18	4.0E-14	—	—	-1.52	1.1E-11	-3.46	1.5E-29
SCJLFL4183C08.g	ERF	ethylene-responsive transcription factor erf025-like	3.95	2.4E-05	—	—	—	—	-1.87	2.3E-04
SCSGLR1025F06.g	ERF	ethylene-responsive transcription factor 1-like	—	—	—	—	-0.89	2.9E-06	-0.87	2.3E-06

* Id according to the data base SUCEST (www.sucest-fun.org)

Table 3. List of GED belonging to the ADH family, identified under the levels of medium and severe waterlogging stress in DC 01-1940 (tolerant) and DC 93-4418 (susceptible) varieties. Expression levels (log_2(Fold-Change)) and values of significance (p-value) of the GED are also displayed during the two stress levels in each variety. The empty cells denote that no differential expression of the GED was presented.

Id. Sequence*	Family	Description of the sequence	CC 01-1940				CC 93-4418			
			Medium Stress		Severe stress		Medium Stress		Severe stress	
			log_2(FC)	p-value	log_2(FC)	p-value	log_2(FC)	p-value	log_2(FC)	p-value
SCBFLR1026G03.g	ADH	aryl-alcohol dehydrogenase - like	—	—	-0.81	4.2E-04	—	—	—	—
SCEPRZ1011A02.g	ADH	cinnamyl alcohol dehydrogenase 2	—	—	1.83	1.1E-09	—	—	—	—
SCEQLR1029E05.g	ADH	tpa: cinnamyl-alcohol dehydrogenase family protein	-0.55	9.4E-09	—	—	-0.67	1.4E-11	—	—

* Id según especificado en la base de datos SUCEST (www.sucest-fun.org)

NGS methodologies, in a genetically complex species such as sugar cane.

Although the observed results are so far promising, these require biological replicates to confirm the expression of the identified genes in this study, differentially expressed and that could serve as candidate in molecular breeding strategies.

References

Aparecida-Rodrigues, F.; de Laia, M.L.; and Zingaretti, S.M. 2009. Analysis of gene expression profiles under water stress in tolerant and sensitive sugarcane plants. Plant Sci. 176:286 - 302.

Barbosa, D.; Ciríaco E.; Custódio, R.; Menossi, M.; and Silveira, M. 2013. Physiological limitations in two sugarcane varieties under water suppression and after recovering. Theoretical and Experimental Plant Phys. 25(3):213 - 222.

Campos, R. A.; Cruz, D. M.; and Torres, J. S. 2009. Riego con caudal reducido. Cenicaña. Cali, Colombia. Work document No. 694. 14 p.

Conesa, A.; Götz, S.; García-Gomez, J. M.; Terol, J.; Talón, M.; and Robles, M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21:3674 - 3676.

Cruz, J.R.; Silva, M.L.; and Torres, J. 2000. Nuevas opciones de drenaje. p 13. In: Memorias del V Congreso de la Asociación Colombiana de Técnicos de la Caña de Azúcar (Tecnicaña). Cali. Co-

lombia. 4-6 October, 2000. Tenicaña. Cali, Colombia. p. 13.

Cruz, J. R.; Torres, J. S.; Besosa, R.; and Rojas, R. 2009. Función de respuesta de la caña al agua. In: Memorias del VIII Congreso de la Asociación Colombiana de Técnicos de la Caña de Azúcar. Cali.; Colombia. 16-18 September, 2009. Tenicaña. Cali, Colombia. p. 256 – 262.

D´Hont, A. 2005. Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and bananas. Cytog. Genome Res.109:27 - 33.

De Almeida Silva, M.; Jifon, J. L.; Sharma, V. Da Silva J. A.; Caputo, M. M.; Damaj, M. B.; Guimarães E. R.; and Ferro, M. I. 2013. Use of physiological parameters in screening drought tolerance in sugarcane genotypes. Sugar Tech. 13(3):191 - 197.

Eavis, B. W. 1972. Effects of flooding on sugarcane growth 2. Benefits during subsequent drought. Proc. Int. Soc. Sugarcane Techn. 14:715 - 721.

Fox, S. E.; Geniza, M.; Hanumappa, M.; Naithani, S. *et al.* 2014. De novo transcriptome assembly and analyses of gene expression during photomorphogenesis in diploid wheat *Triticum monococcum*. PloS One 9(5): e96855.

Glaz, B.; Morris, D. R.; and Daroub, S. H. 2004. Sugarcane photosynthesis, transpiration, and stomatal conductance due to flooding and water table. Crop Sci. 44:1633 - 1641.

Quan, R.; Hu, S.; Zhang, Z.; Zhang, H.; Zhang, Z.; and Huang, R. 2010. Overexpression of an ERF transcription factor TRSF1 improves rice drought tolerance. Plant Biot. J. 8:476 - 488.

Li, Q.; He, F.; Zhu, B. Q.; Liu, B.; Sun, R. Z.; Duan, C.Q.; Reeves, M. J.; and Wang, J. 2014. Comparison of distinct transcriptional expression patterns of flavonoid biosynthesis in Cabernet Sauvignon grapes from east and west China. Plant Phys. Bioch. 84:45 - 56.

Li, X. P.; Tian, A. G.; Luo, G. Z.; Gong, Z. Z.; Zhang, J. S.; and Chen, S. Y. 2005. Soybean DRE-binding transcription factors that are responsive to abiotic stresses. Theor. Appl. Gen. 110:1355 - 1362.

Liu, Y.; Subhash, C.; Yang, J.; Song, C.; Zhao, J.; and Li, J. 2011. Maize leaf temperature responses to drought: Thermal imaging and quantitative trait loci (QTL) mapping. Envir. Exp. Bot. 71(2):158 - 165.

SAS Institute Inc. 1991. SAS System for Linear Models (3rd Edition). Cary, NC. SAS Institute Inc. p 329.

Shinozaki, K.; Yamaguchi-Shinozaki, K. 2007. Gene networks involved in drought stress response and tolerance. J. Exp. Bot. 58(2):221 - 227.

Tougou, M.; Hashiguchi, A.; Yukawa, K.; Nanjo, Y. et al. 2012. Responses to flooding stress in soybean seedlings with the alcohol dehydrogenase transgene. Plant Biot. 29:301 - 305.

Trapnell, C.; Roberts, A.; Goff, L.; Pertea, G. et al. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7(3):562 - 578.

Vettore, A.L.; da Silva, F.R.; Kemper, E.L.; Souza, G.M. et al. 2003. Analysis and functional annotation of an expressed sequence tag collection for the tropical crop sugarcane. Genome Res. 13:2725 - 2735.

Viveros, C A. 2011. Identificación de características asociadas con la mayor eficiencia en el uso de agua para la producción de caña de azúcar. PhD thesis. Universidad Nacional de Colombia - Palmira. Palmira, Valle, Colombia. p. 120.

Wang, L.; Feng, Z.; Wang, X.; Wang, X.; and Zhang, X. 2010. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26 136 - 138.

Wu, J.; Wang, L.; Li, L.; and Wang, S. 2014. De novo assembly of the common bean transcriptome using short reads for the discovery of drought-responsive genes. PloS One. 9(10):e109262.