Journal of Sugarcane Research (2019) 9 (1) : 71 - 85

RESEARCH ARTICLE

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN THE TRANSCRIPTOME OF SUGARCANE VARIETY Co 86032 EXPOSED TO OXIDATIVE STRESS

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Abstract

Sugarcane is an economically substantial crop used for sugar and latest for ethanol production. NGS technologies have started to resolve the complex nature of sugarcane genome and leads to the large-scale discovery of high through put markers like single nucleotide polymorphisms (SNPs). Transcriptome sequencing is preferred over genome sequencing as it finds the SNP on the genes. In this study, transcriptome sequencing of the sugarcane variety Co 86032 under oxidative stress was performed to identity SNPs related to differentially expressed genes. Nearly, 55.4 million high quality clean reads were generated out of 57.6 million unfiltered raw reads. The *de novo* assembly of the clean reads using Trinity resulted in an average of 61,671 transcripts. Functional annotation of these transcripts with Uniprot database showed that 71% of transcripts had significant match with Viridiplantae protein database. Around 87,834 single nucleotide variants were identified in the differentially expressed (535 up-regulated and 593 down-regulated) transcripts. Thirteen SNPs were predicted in up regulated transcripts from control and 21 SNPs from upregulated transcripts from stressed samples with reference to pooled transcripts. From the down regulated transcripts between control and stressed sample, we identified 80 SNPs and 92 SNPs respectively. The data in this study forms an important resource for future sugarcane improvement programs.

Key words: Sugarcane, Oxidative stress, Transcriptome analysis, Single nucleotide polymorphisms, *de novo* assembly.

Introduction

Sugarcane belongs to the genus *Saccharum* and to one of the largest grass family *Poaceae* that includes other economically important cereal crops like sorghum, maize, wheat, rice, and many other forage crops. Sugarcane (*Saccharum spp.* hybrids) has been serving as the major source of world's raw sugar for centuries. Despite being used for sugar, sugarcane has recently gained attention for the production of ethanol, which is an important renewable biofuel that can relieve the current energy crisis such as dependency on fossil fuels and the emission of greenhouse gases (Savage 2011).

Plants undergo number of abiotic and biotic stresses throughout their life cycle including extreme temperatures, drought, salinity, metal toxicity, nutrient deficiencies and various diseases that can decline their productivity. Virtually all environmental and biotic stresses accompany oxidative stress which can cause damage to the cell components and may lead to their dysfunction. Oxidative stress is induced as a result of overproduction and accumulation of reactive oxygen species (ROS), especially the production of hydrogen peroxide (H_2O_2) within the plant cell (De Azevedo et al. 2005). A basal level of H_2O_2 is essential to maintain regular metabolic

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activities (Halliwell 2006) but at higher levels can cause oxidative damage and finally lead to cell death (Gadjev et al. 2008). H_2O_2 is involved in the control of the senescence process (Peng et al. 2005), protection against pathogen attack (Kumar et al. 2011), the decline of stress intensity at low light (Zhang et al. 2011) and the alleviation of drought stress (Ishibashi et al. 2011), and it can affect the expression of hundreds of genes (Yun et al. 2010). Many of the sugarcane cultivars are vulnerable to abiotic and biotic stresses that results in drastic reduction in production potential.

The complex repetitive content and heterozygosity of sugarcane makes the interpretation of genome architecture as a challenging one. The monoploid genome size of sugarcane ranges from 800 to 900 Mb (D'Hont and Glaszman 2001), nearly similar to the size of sorghum genome (730 Mb) (Paterson et al. 2009) and twice the size of rice genome (450 Mb) (Song, et al. 2018, International Rice Genome Sequencing Project, 2005). Mosaic monoploid genome of sugarcane variety R 570 (Garsmeur et al. 2018), genome of *S. Spontaneum* genotype AP85-441 (Zhang et al. 2018) and genome sequences of the hybrid cultivar SP80-3280 (Riaño-Pachón and Mattiello 2017) are available at present.

Transcriptome sequencing has become more fascinating than genome sequencing while considering relevant traits. This may be due to the fact that in complex organisms, genome sequences vary randomly as a result of repeated contents in the non-coding regions of DNA whereas the transcriptome sequences indicate the genes and the levels of expression. Strategies in RNA-Sequencing technologies (RNA-Seq) have made sugarcane transcriptome analysis easier. Vargas et al. (2014) has reported the transcriptome analysis on drought tolerance of sugarcane associated with diazotrophs. Six sugarcane varieties that showed polymorphisms have been studied using Illumina based transcriptome analysis (Cardoso-Silva et al. 2014). RNA-Seq analysis is extensively used for the identification of SNPs (D'Hont 2005). Recently, transcriptome of six sugarcane genotypes involved in response to pokkah boeng, drought and leaf abscission were characterized, and SNPs were identified by Xu et al. (2018).

The objective of the study is to identify SNPs present in the transcripts from the control and oxidative stressed samples. Though it is the same genotype is taken for control and stress treatment, but the changes in the expression pattern of genes and its isoforms are expected. Hence the SNPs can also be different between transcripts from control and treated samples. The present study involved sequencing of the transcriptome of the sugarcane variety Co 86032 subjected to oxidative stress and analysing the transcriptome for single nucleotide variants. The differentially expressed genes in response to stress are analysed from the assembled transcripts for the detection of SNPs in those genes. Further identified SNPs can be used to develop markers for oxidative stress.

Materials and Methods

Oxidative stress treatment and collection of tissues

The sugarcane variety Co 86032 was used in the present experiment and the plants were raised in pots. The experiment was a randomized complete design with four treatments and two replications, consisting of 0 ppm (control), 300, 500 and 1000 ppm of hydrogen peroxide. The plants were maintained in a glass house in pots containing red soil and FYM (3:1) as potting mix. Water was sprayed on the plants for the control treatment. For the stress treatment, the plants (60 days-old) were sprayed with hydrogen peroxide until all leaves were completely wet at 8 am consecutively for 3 days. All the completely opened leaves and young leaves above the ground part including the

meristem were collected on second (48 h) and third (72 h) day of spraying for RNA isolation.

Total RNA isolation, RNA-Seq library preparation and sequencing

About 2 g of tissues were ground to fine powder with liquid nitrogen, and total RNA was isolated using TRIzol reagent (Invitrogen). The RNA was treated with DNase (Promega, USA) to remove residual DNA. The RNA concentration and quality were checked with Nanodrop spectrophotometer and an aliquot of the samples was run on Agilent RNA Bioanalyzer chip to check for integrity prior to Illumina sequencing at Genotypic Technologies Pvt Ltd. (Bangalore, India). The RNA isolated from 300, 500 and 1000 ppm hydrogen peroxide treated plants were pooled before library preparation and sequencing.

RNA-Seq library preparation was performed at Genotypic Technology's Genomics facility as per NEXTFlex RNA library as per the recommended protocol of NEXTFlex RNA sample preparation guide (Cat # 5138-08). 2 μg of Qubit quantified total RNA was used for sequencing library preparation according to the manufacture's (NEXTFlex) instructions. The library was amplified using 10 cycles of PCR for enrichment of adapter ligated fragments. The prepared library was quantified using Qubit and validated for quality by running an aliquot on Tapestation (Agilent High Sensitivity D10000). The library was sequenced using Illumina NextSeq500 sequencer (Illumina Inc., San Diego, CA, USA).

The collected paired end raw reads were filtered and cleaned based on quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) software. Adapters and low quality bases towards 3'-end were processed by GT proprietary script (Genotypic ABLT Script). The high-quality clean reads were further assembled *de novo*.

***De-novo* transcriptome assembly and clustering**

The reads were *de novo* assembled using Trinity (default k-mers i.e 25Trinity; Grabherr et al. 2011) software which includes three independent modules: Inchworm, Chrysalis, and Butterfly, applied sequentially to process large volumes of RNA-seq reads. *Inchworm* assembles the RNA-seq data into the unique sequences of transcripts. *Chrysalis* clusters the Inchworm contigs into clusters and constructs complete *de Bruijn* graphs for each cluster. *Butterfly* then processes the individual graphs in parallel, tracing the paths that reads and pairs of reads take within the graph.

Trinity generated assembly were further clustered into unigenes based on the similarity between the sequences using CD-HIT version 4.5.4 (Cluster Database at High Identity with tolerance; http://weizhongli-lab.org/cd-hit/) to reduce the repetition without elimination of sequence diversity that is used for further transcript annotation for the differential gene expression.

Functional annotation of transcripts

The clustered transcripts with >=300 bps in length were annotated with Viridiplantae protein sequences from Uniprot database. GO terms were annotated to the transcripts and pathway analysis was done using KAAS Server (http://www.genome.jp/tools/kaas/; Moriya et al. 2007). *Arabidopsis lyrate*, *Cucumis sativus*, *Arabidopsis thaliana, Citrus sinensis, Solanum lycopersicum, Fragaria vesca, Theobroma cacao, Vitis vinifera, Glycine max* were considered as references for pathway analysis.

Analysis of differentially expressed genes (DEGs)

DEGs between the assembled transcripts from control and stressed samples were analysed using DESeq R package (Anders and Huber, 2010). Variance for each gene was estimated and DGEs

were identified by negative binomial distribution. Once the DGEs were calculated, based on the log2 fold change, the results were separated as up regulated, down regulated and neutral. The annotations for the DEGs were separated from the previously annotated transcripts.

Identification of SNPs

To identify the SNPs, the reads of control and stress samples were pooled together. The pooled reads were then aligned to the unigenes of the transcriptome which was taken as the reference, with bowtie 2 (Langmead and Salzberg, 2012) program and the consensus were generated using Samtools and VarScan (Koboldt et al. 2012). About 95 % of the reads were mapped to the reference. The positions that had different allele between the samples in comparison were further filtered to get only the homozygous markers with a depth threshold of 40x as it is polyploid.

Results and Discussion

Transcriptome analysis: sequencing and assembly

The sequencing using the Illumina platform resulted in 30.07 and 27.62 million raw reads for control and stressed samples respectively. After quality checking and pre-processing , 28.85 (control) and 26.64 (stressed) million trimmed high-quality clean reads were obtained. *De novo* assembly using the Trinity program (resulted in 59,963 transcripts with an average length of 932 base pairs (bp) and an N50 of 1223 bp for control sample and 63,379 transcripts with an average length of 956 bp and an N50 of 1286 bp for treated sample. The N50 was smaller than those obtained from sugarcane in other studies (Cardoso-Silva et al. 2014; Hoang et al. 2017). A total of 52,118 clustered transcripts with an average length of 898 bp and an N50 of 1172 bp were obtained for control and 55,483 reads with an average length of 918 base pairs (bp) and an N50 of 1235 bp were obtained for treated sample after clustering by CD-HIT. The blast annotation results of transcripts showing > 30% identity as cut off was presented in Table 1.

Table 1. Summary of the transcript annotations using Uniprot Protein Database

Transcripts	Control	Stressed
Total Transcripts (Cluster)	52,118	55,483
Total Annotated Transcripts	39,696	36,707
Total Unannotated Transcripts	12,422	18,776

Identification of differentially expressed genes (DEGs)

Differential gene expression between the oxidative stress and control samples were analysed using DESeq package. Total of 4,828 transcripts were found to be differentially expressed based on two fold change for up regulation and down regulation. Around 535 transcripts were up regulated (> two fold change for up regulation) and 593 transcripts were down- regulated (> two fold change for down regulation). Around 3,700 transcripts with fold change +/- 1 were considered as neutrally regulated. Forty one transcripts were found to be expressed only under stress and 43 transcripts were expressed only under control conditions. Further filtering the DEGs with P-value <=0.05 resulted in 43 up regulated and 166 down regulated transcripts. Gene ontology classification of the DEGs showed that, out of 535 up regulated transcripts, 492 were assigned with GO terms and out of 593 down-regulated transcripts, 522 were assigned with GO terms.

Filtering of DEGs and identification of SNPs

A total of 1,32,709 and 1,41,582 unfiltered SNPs were predicted from 52,118 transcripts (control) and 55,483 transcripts (stress) respectively, Cardoso-Silva et al. (2014) identified 7,08,125 putative SNPs in sugarcane. Most of the SNPs

identified were in transcripts of uncharacterised proteins. For identification of significant SNPs in the differentially expressed transcripts, 4,828 DE transcripts were further filtered based on the GO terms consisting of stress, which resulted in 114 DEGs. Out of which 10 transcripts were upregulated, 14 were down-regulated and 90 were found to be neutrally regulated. Five transcripts were filtered by P value <=0.05 which includes 1 up-regulated and 4 down-regulated transcripts.

From the 10 up-regulated transcripts (four isoforms of peroxidase gene, an isoform of ACSL; long-chain acyl-CoA synthetase gene, two isoforms of ATCYSC1; L-3-cyanoalanine synthase/ cysteine synthase gene, one isoform of DNA excision repair protein ERCC-4, an isoform of PIK3C3; phosphatidyl inositol 3-kinase and an isoform of PARN; poly(A)-specific ribonuclease), 26 SNPs from transcripts of stressed sample and 66 SNPs from transcripts of control samples were identified. No SNPs were identified in PIK3C3 and PARN. Further the SNPs were filtered based on quality score > 40 which resulted in 13 SNPs (control transcripts) and 21 (stress transcripts) (Table 2). The annotation results revealed that 12 SNPs (control sample transcripts) and 14 SNPs (stressed sample transcripts) were identified in ACSL (long-chain acyl-CoA synthetase) gene encoding long-chain acyl-CoA synthetase involved in fatty acid metabolism pathway also involved in response to endoplasmic reticulum stress. From the stressed transcripts, three SNPs were identified in the highly expressed transcript that corresponded to ATCYSC1 (L-3-cyanoalanine synthase/ cysteine synthase) gene encoding L-3-cyanoalanine synthase/ cysteine synthase involved in the biosynthesis of cysteine from serine. Two SNPs were found to be located in the ERCC4 gene encoding DNA excision repair protein ERCC-4 that plays a significant role in nucleotide excision repair and was involved in response to oxidative stress. Also one SNPs (control transcripts) and two SNP (stressed) were found in the peroxidase gene involved in phenylpropanoid biosynthesis. According to Mittler et al. (2004), peroxidase is an antioxidant enzyme that plays an important role in the antioxidative defense system. Both control and stressed samples has a common SNP which is a guanine (G) to thymine (T) transversion at the 146th position, whereas the transcripts under stress consisted of T to Cytosine (C) transition at the 563rd position on the gene. Leu et al. (2018) has identified a novel peroxidase gene from sugarcane variety ROC22 which was found to play an important role during abiotic and biotic stress.

While considering the 14 down-regulated transcripts (four isoforms of Lipoxygenase gene, an isoform of AGXT; alanine-glyoxylate transaminase / serine-glyoxylate transaminase / serine-pyruvate transaminase, eight isoforms of Peroxidase gene and an isoform of PARN), 106 SNPs in control and 117 SNPs in treated samples were identified. No SNPs were identified in PARN gene. For getting high quality SNPs, the SNPs were further filtered based on quality score. The filtering resulted in 80 SNPs and 92 SNPs (Table 3) respectively in control and treated samples. In the control sample, 3 SNPs were found in the Lipoxygenase gene that plays a key role in the lipid metabolism pathway and is also involved in response to cold and salt stress. Lipoxygenase gene was also found to be differentially expressed in sugarcane under low potassium stress conditions (Zeng et al. 2015). Thirty eight SNPs in control and 44 SNPs in stressed samples were identified in which 21 SNPs were common in the AGXT gene encoding alanine glyoxylate and serine pyruvate amino-transferase gene involved in glyoxylate: dicarboxylate metabolism and amino acid metabolism respectively. The peroxidase gene was found to have 39 SNPs in control sample 48 SNPs in stressed samples. Of which, 21 SNPs were

Table 2. SNPs in the differentially up regulated transcripts in stressed condition

Sample	Gene name	Expression	Pathway	SNP positions	Reference	SNV
Stressed	Peroxidase isoform 1	2984	Phenyl propanoid biosynthesis	146	G	T
				219	G	A
	ACSL; long-chain acyl-CoA synthetase	107	Fatty acid degradation, Peroxisome, Fatty acid metabolism,	229	G	T
				336	C	T
				353	C	G
				449	G	A
				457	A	G
				503	A	C
				552	G	A
				563	C	T
				774	C	A
				777	C	T
				1368	C	T
				1449	C	T
				1758	T	C
				2342	A	G
	DNA excision repair protein ERCC-4	382	Nucleotide excision repair	345	C	T
				2646	G	A
	ATCYSC1; L-3-cyanoalanine synthase/ cysteine synthase	964	Biosynthesis of amino acids, Sulfur metabolism	25	A	G
				32	C	G
				57	T	G
Control	Peroxidase isoform 1	912	Phenyl propanoid biosynthesis	146	G	T
	ACSL; long-chain acyl-CoA synthetase	22	Fatty acid degradation, Peroxisome, Fatty acid metabolism,	229	G	T
				336	C	T
				353	C	G
				449	G	A
				457	A	G
				503	A	C
				563	C	T
				774	C	A
				777	C	T
				1368	C	T
				1758	T	C
				2342	A	G

Table 3. SNPs in the differentially down regulated genes in stressed condition

Sample	Gene name	Pathway	SNP positions	Reference	SNV
Stressed	Peroxidase isoform 2	Phenylpropanoid biosynthesis	156	A	T
			342	T	C,G
			345	A	G
			382	C	T
			384	T	A
			389	T	C
			390	C	G
			392	G	T
			393	A	G
			483	T	G
			500	C	T
			511	C	G
			513	C	G
			516	G	A
			525	C	G
			612	G	A
			615	A	G
			633	G	C
			636	G	C
			642	G	C
			699	G	C
			702	C	G
			715	G	T
			720	T	C
			729	T	C
			735	A	G
			736	A	T
			741	C	T
			749	G	C
			850	C	T
			859	G	A
			862	A	G
			874	G	C
			881	T	C
			916	G	T
			985	C	T
			994	C	A
			1006	C	T
			1079	A	G
			1088	T	C
			1173	T	C
			1184	A	C
			1339	G	C
			1374	C	T
			1376	C	G
			1377	C	T
			1488	C	T
			1523	G	C

Stressed	AGXT	Peroxisome; Glyoxylate and dicarboxylate metabolism	84	A	C
			111	G	T
			173	C	T
			191	G	C
			209	T	C
			227	T	C
			239	C	A
			245	T	G
			332	A	G
			374	G	C
			422	C	T
			447	G	A
			512	C	T
			515	C	G
			516	C	A
			517	A	T
			566	C	G
			590	C	G
			593	G	C
			608	G	C
			704	C	G
			746	A	G
			752	G	C
			764	T	C
			780	A	G
			782	G	A
			866	A	C
			977	T	G
			983	C	T
			1034	T	C
			1037	T	C
			1043	G	C
			1049	C	G
			1052	T	C
			1082	G	C
			1088	C	T
			1089	G	A
			1180	A	G
			1265	C	T
			1274	G	T
			1292	G	C
			1319	T	C
			1337	A	C
			1339	A	T

Control	Lipoxygenase	Lipid metabolism, oxylipin biosynthesis	489	T	C
			490	T	G
			1023	G	C
Control	Peroxidase isoform 2	Phenylpropanoid biosynthesis	77	T	C
			80	A	C
			108	T	C
			140	C	T
			156	A	T
			197	C	T
			209	G	C
			297	G	C
			303	G	C
			342	T	G
			382	C	T
			384	T	A
			442	A	G
			447	C	G
			461	A	G
			500	C	T
			518	T	C
			525	C	G
			537	G	C
			607	C	T
			612	G	A
			615	A	G
			629	T	C
			633	G	C
			699	G	C
			702	C	G
			715	G	T
			723	C	T
			881	T	C
			916	G	T
			945	A	T
			1079	A	G
			1088	T	C
			1339	G	C
			1374	C	T
			1376	C	G
			1377	C	T
			1477	G	A
			1523	G	C

			209	T	C
			227	T	C
			239	C	A
			245	T	G
			332	A	G
			374	G	C
			422	C	T
			447	G	A
			512	C	T
			516	C	A
			517	A	T
			566	C	G
			590	C	G
			593	G	C
			608	G	C
			622	G	C
			623	A	G
			704	C	G
Control	AGXT	Peroxisome; Glyoxylate and dicarboxylate metabolism	746	A	G
			752	G	C
			764	T	C
			780	A	G
			782	G	A
			866	A	C
			977	T	G
			983	C	T
			1034	T	C
			1037	T	C
			1043	G	C
			1049	C	G
			1052	T	C
			1180	A	G
			1265	C	T
			1274	G	T
			1292	G	C
			1319	T	C
			1337	A	C
			1339	A	T

Table 4. Unique SNPs present in the Co 86032 control condition

Gene	Regulation	SNP positions	Reference	SNV
Peroxidase isoform 1	Up	563	T	C
AGXT	Down	622	G	C
		623	A	G
Peroxidase isoform 2	Down	77	T	C
		80	A	C
		108	T	C
		140	C	T
		197	C	T
		209	G	C
		297	G	C
		303	G	C
		442	A	G
		447	C	G
		461	A	G
		518	T	C
		537	G	C
		607	C	T
		629	T	C
		723	C	T
		945	A	T
		1477	G	A

commonly found in both control and stressed sample. Mostly the transition type of variation was observed in the expressed transcripts in sugarcane. In the present study, the highest quality SNPs in the control was at 442nd position which corresponds to an adenine (A) to G, and in the stressed sample it is in the 382nd position which was a C to T transition. The unique SNPs present in the control and stressed sample transcripts are listed in table 4 and 5 respectively. These identified SNPs has to be further validated using different validation methods (Clevenger et al. 2015) to find whether they are true so that it can be further used for the development of markers and further genomic studies in sugarcane. Such developed functional markers can be widely used for genomic selection and gene editing (Muhammad et al. 2018).

Acknowledgements

Authors thank Director, ICAR- Sugarcane Breeding Institute for the support in carrying out the work.

Table 5. Unique SNPs present in the Co 86032 stressed condition

Gene	Regulation	SNP positions	Reference	SNV
Peroxidase isoform 1	Up	219	G	A
AGXT	Down	84	A	C
		111	G	T
		173	C	T
		191	G	C
		515	C	G
		1082	G	C
		1088	C	T
		1089	G	A
Peroxidase isoform 2	Down	345	A	G
		389	T	C
		390	C	G
		392	G	T
		393	A	G
		483	T	G
		511	C	G
		513	C	G
		516	G	A
		636	G	C
		642	G	C
		720	T	C
		729	T	C
		735	A	G
		736	A	T
		741	C	T
		749	G	C
		850	C	T
		859	G	A
		862	A	G
		874	G	C
		985	C	T
		994	C	A
		1006	C	T
		1173	T	C
		1184	A	C
		1488	C	T

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Received: July, 2019; Revised & Accepted: September, 2019