Ur Rehman S *et al.* (2022)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 50, Issue 3, Article number 12765
DOI:10.15835/nbha50312765
Research Article

A view of transcriptome during cold stress in sugarcane using *Saccharum spontaneum* genome

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Abstract

Transcriptomic data of two sugarcane cultivars 'ROC22' and 'GT08-1108' were investigated for the expression analysis of cold responsive genes. The raw RNA Seq data of the sugarcane cultivars were downloaded from the SRA NCBI database and were reanalyzed and mapped by using *Saccharum spontaneum* genome. In the *Saccharum spontaneum* reference genome, 83826 unigenes were annotated and, among these, 46,159 (55%) were functionally annotated with Gene Ontology (GO) categories. In the transcriptome-based analysis, 183,515 unigenes were assembled and, among these, 110,021 (60%) were functionally annotated with GO categories. For the cultivar GT08-1108, using the reference genome pipeline, 11,652 (13.9%) unigenes were differentially expressed (7,238 upregulated; 4,414 downregulated), while 16,145 (8.8%) were differentially expressed (8,965 upregulated; 7,180 downregulated) using transcriptome-based pipeline. In the cultivar ROC22, 11,516 (13.7%) genes were differentially expressed (7,174 upregulated; 4,342 downregulated) and 20,317 (11.1%) (10,898 upregulated; 9,419 downregulated) for the genome and transcriptome-based analysis, respectively. In the genome analyses, among downregulated genes, 3,248 were coincident between the two cultivars, the remaining 1,166 differentially expressed only in 'GT-1180' and 1,094 only in 'ROC22'. With the transcriptome assembly, 13,113 genes were deferentially expressed in both cultivars, the remaining 3,032 unique to 'GT08-1108' and 7,204 in 'ROC22'. We concluded that sugarcane in response to cold stress expresses many genes, although the transcriptome assembly overestimated the number of unigenes and, consequently, a higher number of differentially expressed genes. This may be due to difficulties in separating homeologues from paralogue genes. When a reference genome is available, we recommend its use since genes predicted on a reference genome tend to be more accurate.

Keywords: bioinformatics pipeline; RNA-Seq; *Saccharum* spp. L.

Received: 01 Jul 2022. Received in revised form: 20 Aug 2022. Accepted: 22 Aug 2022. Published online: 29 Sep 2022.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Sugarcane (*Saccharum* hybrid) stores a high concentration of sugar in its stem and is cultivated in both tropical and subtropical regions of the globe about 35° N and 35° S from the equator (Cheavegatti-Gianotto *et al*., 2011). Due to sugar production property, it has been known to mankind since early in the history, now it is an important commercial crop cultivated in several countries that gives essential by-products such as sugar, ethanol, and bagasse (James, 2008; Xu *et al*., 2018; Figueroa-Rodriguez *et al*., 2019). According to the UNFAO, the world's top 10 sugarcane producing countries includes Brazil ranked top, India second, followed by China, Thailand, Pakistan, Mexico, Colombia, Indonesia, Philippines, and United State (Saleh and Halim, 2018). Pakistan ranked fifth with average sugarcane production of 73.40 million tons in an area of 1.2 million hectares (Khan *et al*., 2019). Plants may expose to various abiotic and biotic stresses which can adversely affect the plant yield, growth, and development (Agarwal *et al*., 2006).

Both biotic and abiotic stresses affect the Sugarcane plants during their life cycle, biotic stresses include several pathogens like bacteria, fungi, and viruses which cause many diseases (OECD Consensus Documents, 2016). Water stress, drought/salinity stress, light/heat stress, and cold/freezing stress all affect sugarcane growth, yield, and metabolism (Hussain *et al*., 2004; Rehman *et al*., 2020). The ideal temperature for sugarcane crop germination is 26-32 °C and the optimum temperature for crop growth is 30-33 °C. Owing to climatic vagaries many stresses such as high-temperature, salinity, cold temperature, flooding, and toxicity of elements during the crop growth period in the subtropical regions, restrict the active growth period of the crop up to 8-9 months. During the peak winter period (December and January), the temperature falls below 5 °C, whereas, during the month of May-June, the average temperature reaches more than 43 °C. Temperature more than 38 °C affects photosynthesis in sugarcane and increases the respiration rate, which ultimately affects the cane productivity and juice quality in the region (Moore, 1987; Que *et al*., 2014).

In the genome of sugarcane, the complexity in copy number of genes, high repetitive contents, and heterozygosity, due to this it is limited to understanding the transcriptome of sugarcane (Grivet and Arruda, 2002; Hotta *et al*., 2010; Manimekalai *et al*., 2022). From the alternative splicing in this potentially complex transcriptome, the result is not clear how many transcript isoforms (Hoang *et al*., 2017). Each sugarcane cultivar has its own unique set of chromosomes ranging from 80 to 130; the modern sugarcane is polyploid and a hybrid between *Saccharum officinarum* and *Saccharum spontaneum*. Sugarcane genome has approximately 12 copies of each gene, with a total predicted gene count of around 35,000 (Vettore *et al*., 2003; Souza *et al*., 2011; Serba *et al*., 2021) Despite the economic importance of sugarcane in the sugar and biofuel industry, a reference genome is not yet available.t Transcriptome data of sugarcane have been based on reference transcript assembly or Saccharum officinarum gene indices. Therefore, the data of sugarcane Transcriptome in limited in relation to transcript isoforms and transcript length (Hoang *et al*., 2017).

Aim of the study

For the analysis of transcriptome sequence data analysis based on a reference genome or the assembled transcripts (reference-free) recently the genome of sugarcane was sequenced (Zhang *et al*., 2018), so in this study, we analysed the transcriptome data of two Chinese sugarcane 'ROC22' and 'GT081108' by using a reference genome. We also assembled the transcriptome and aligned the RNA-Seq reads in the transcripts. Therefore, the objective of our work was to compare two commonly used bioinformatics pipelines in the polyploid genome of *Saccharum* hybrid. As the genome sequence has been recently published, we tested whether a reference genome-based pipeline is better than the one from Trinity that uses assembled transcripts as a reference for reading mapping.

Materials and Methods

Experimental materials and cold stress treatment

Two sugarcane cultivars namely 'ROC22' and 'GT08-1108' were selected (Shi-yun *et al*., 2018). Cold stress experiment was performed at the Guangxi Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, China. RNA sequenced data (57.41 GB) of the selected sugarcane cultivars were downloaded from the SRA, NCBI database, where the samples were arranged with three biological replicates with a cold stress treatment time point (4 °C) and control (28 °C) (Table 1). Total RNA was extracted from the selected sugarcane cultivars (cold treated as well as normal sugarcane plants) by using RNA extraction kit (Tiangen). The extracted RNA was further confirmed by agarose gel electrophoresis and for RNA degradation and contamination detection, Nanodrop was used, while Qubit Fluorometer was used for RNA concentration. Furthermore, Agilent 210 was used to detect RNA integrity. For mRNA enrichment, magnetic beads with oligo (dT) were used, and a fragmentation solution was added to interrupt mRNA. cDNA with a six-base random primer was synthesize from the mRNA as a template. Buffer, dNTPS, and DNA polymerase were used to synthesize double stranded cDNA library. After cDNA library construction, the samples were sent for the Illumina High throughput sequencing to Beijing Nuohe Zhiyuan Biological Company.

Table 1. The details of samples of sugarcane used for analysis

Sample (NCBI code)	Cultivar	Temperature
SRX4505112	'GT08-1108'	4 °C
SRX4504973	'GT08-1108'	4 °C
SRX4504931	'GT08-1108'	28 °C
SRX4504686	'GT08-1108'	28 °C
SRX4495660	'ROC2'	4°C
SRX4494234	'ROC2'	4 °C
SRX4494233	'ROC2'	28 °C
SRX4494227	'ROC2'	28 °C

Quality control and cleaning of transcriptome raw data

The transcriptomic raw data of the selected sugarcane cultivars were analysed for quality control with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and low-quality sequences, as well as Illumina adaptors, were removed with Trimmomatic (Bolger *et al*., 2014). The sugarcane reference genome was used to align the reads by using STAR software and HTSeq-count was used for counting the reads aligned in each gene (Zhang *et al*., 2018). The number of reads from each library aligned in each gene was used as the measurement of gene expression levels.

Differential expression analysis and normalization of data and transcription factor

For differential expression analysis between treatments and normalization of RNA sequence data, R library edgeR was used (Robinson *et al*., 2010). Initially, the samples were filtered for normalization, and the expression of genes with 1 count per million (CPM) for at least two samples were excluded. TMM (Trimmed Mean) (Robinson and Oshlack, 2010) method was used for estimating the normalization factors of each sample, and to equalize and minimize the volume and high expression of genes and differences in sample size.

During differential expression analysis, the package edgeR assumes the negative binomial (NBD) distribution of the sequences count digital discrete data related to each sample. Therefore, Conditional Maximum Likelihood (CML) estimation was used for sequential analysis of gene to gene (tag-wise) dispersions, and Bayes empirical strategy assumed the shrinkage in tag-wise (gene-wise) distribution of residual variation.

Differential gene expression analysis (DGE) and over dispersed data were estimated by Fisher Exact test (Robinson and Smyth, 2008). The likelihood ratio test (LRT) was carried out for both low temperature and control experimental conditions. Moreover, the False Discovery Rate (FDR) (P-value) gave us the significance of the differential expression with a threshold of an FDR<0.05. Log fold change ratio (LogFC) measured the magnitude of differential expression (Quackenbush, 2002). The upregulated and downregulated genes under cold stress were defined as the genes expressed with LogFC ≥ 2 and LogFC value ≤-2 value respectively. Further, the DEGs were further grouped with cluster analysis, annotated, and visualized within biochemical pathways.

Gene ontology and gene ontology enrichment analysis

Agrigo (Tian *et al*., 2017) gene enrichment analysis was performed for the differentially expressed genes in sugarcane cultivars during cold stress. Besides, Agrigo is web-based online database and kit of tools to provide deep support to the agriculture community for gene enrichment analysis (Zhou *et al*., 2010; Tian *et al*., 2017). The Gene Ontology and enrichment analysis were categorized into three that are: 1) Biological process, 2) Molecular function, 3) Cellular components.

Results

RNA sequence data analysis

In the *Saccharum spontaneum* reference genome, 83826 unigenes were annotated and, among these, 46,159 (55%) were functionally annotated with Gene Ontology (GO) categories. In the Transcriptome-based analysis, 183,515 unigenes were assembled and, among these, 110,021 (60%) were functionally annotated with GO categories. For the cultivar 'GT08-1108', using the reference genome pipeline, 11,652 (13.9%) unigenes were differentially expressed (7,238 upregulated; 4,414 downregulated), while 16,145 (8.8%) were differentially expressed (8,965 upregulated; 7,180 downregulated) using Transcriptome-based pipeline. In the cultivar 'ROC22', 11,516 (13.7%) genes were differentially expressed (7,174 upregulated; 4,342 downregulated) and 20,317 (11.1%) (10,898 upregulated; 9,419 downregulated) for the genome and Transcriptome based analysis, respectively (Table 2). In the genome analyses, among downregulated genes, 3,248 were coincident between the two cultivars, the remaining 1,166 differentially expressed only in 'GT-1180' and 1,094 only in 'ROC22'. With the Transcriptome assembly, 13,113 genes were deferentially expressed in both cultivars, the remaining 3,032 unique to 'GT08-1108' and 7,204 in 'ROC22'.

Table 2. The total number of genes and transcripts and the number of differentially expressed elements for 'GT1108' and 'ROC22' sugarcane cultivars under cold stress treatment

Unigenes	Genome-based		Assembled Transcriptome	
	'GT08-1108'	'ROC22'	'GT08-1108'	'ROC22'
Total	83,826 (46,159 annotated)		183,515 (110,021 annotated)	
Differentially expressed	11,652 (13.9%)	11,516 (13.7%)	16,145 (8.8%)	20,317 (11.1%)

Analysis of differential gene expression

Cold tolerance gene expression analysis

The differential expression analyses of cold regulated genes were analyzed by edgeR: and almost 11,652 cold genes differentially expressed were identified in 'GT081108'. Among these genes, nearly 7,238 upregulated cold tolerance genes were expressed, while 4,414 genes were downregulated. While 11,516 differentially expressed genes were identified in 'ROC22' and among these cold regulated genes, 7,174 were upregulated while 4,342 were down-regulated. Further, the PCA analysis was performed to view the variation among expressed genes under control and cold conditions. Where the first component explains the variation (92%) as a function of the control condition and under cold stress, and the second component explains the variation

among the cultivars (Figure 1). Thus, by this preliminary analysis, it is possible that the gross difference in the number of reads per gene is much more related to the temperature conditions in which the plants were submitted than to the differential response of the sugarcane cultivars under the cold stress.

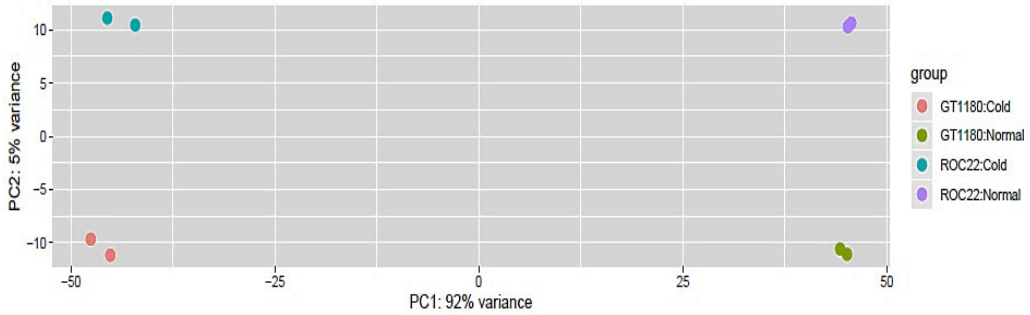

Figure 1. Principal Component Analysis (PCA) Scatter plot of the cold tolerance gene expression, each point represents one experiment for gene expression during cold stress treatment and normal samples

The clustering of transcripts in sugarcane during cold and normal conditions was investigated using Pearson dissimilarity (Figure 2) revealed high confusability in the correct identification of the samples. The cluster analysis showed two large clusters of control and cold stress samples were formed, being identified within these two groups with the greater similarity between the biological replicates Thus, by this preliminary analysis, it is possible that the gross difference in the number of reads per gene is much more related to the temperature conditions in which the plants were submitted than to the differential response of the sugarcane cultivars under the cold stress (Figure 5 and 6).

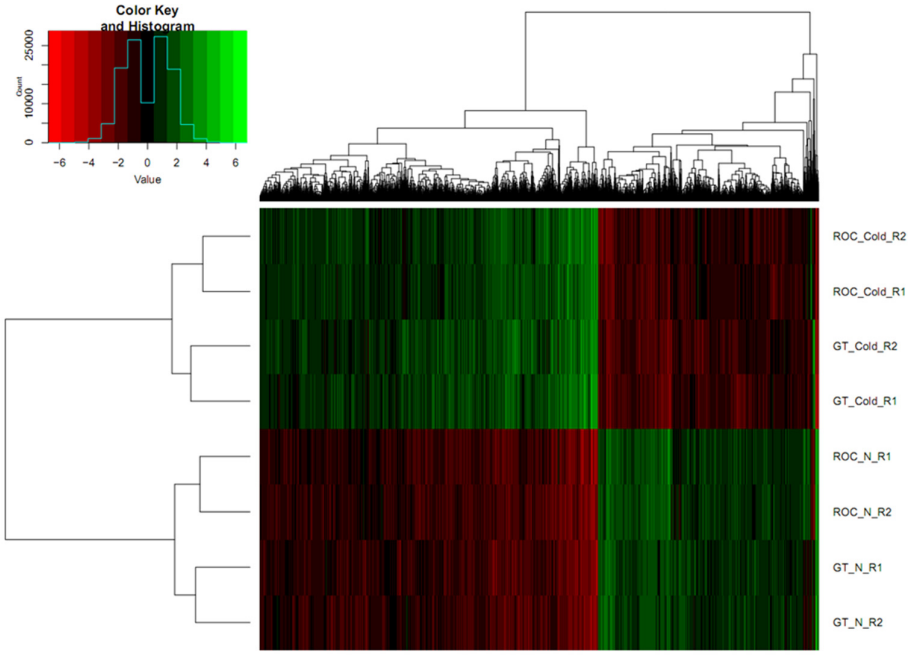

Figure 2. Heatmap and hierarchical cluster of RNA-Seq transcriptome analysis for 23167 differentially expressed cold tolerance genes during cold treatment and control samples

Green or red colors showed differentially upregulated or downregulated genes, correspondingly. The mean points were corrected background and transformed to the log2 scale. Genes with at least a 2-fold change with FDR< 0.05 were considered significant.

The Venn diagram shows that there are 7, 238 genes out of 23,167 differentially expressed genes that were significantly upregulated in 'GT08-1108', while 7,174 genes were significantly upregulated in 'ROC22' (Figure 3). While the two cultivars share 5,652 genes combined. 1,586 genes were specific to 'GT08-1108' and 15, 22 genes were specific to 'ROC22'. Analyzing down regulated genes, 4,414 genes were differentially down regulated in 'GT08-1108' out of 23,167 differentially expressed genes, while in 'ROC22' 4,342 genes were downregulated respectively (Figure 4.). The two cultivars shared 3,248 combines, while 1,166 genes were specific to 'GT08-1108' and 1,094 genes were specific to 'ROC22'.

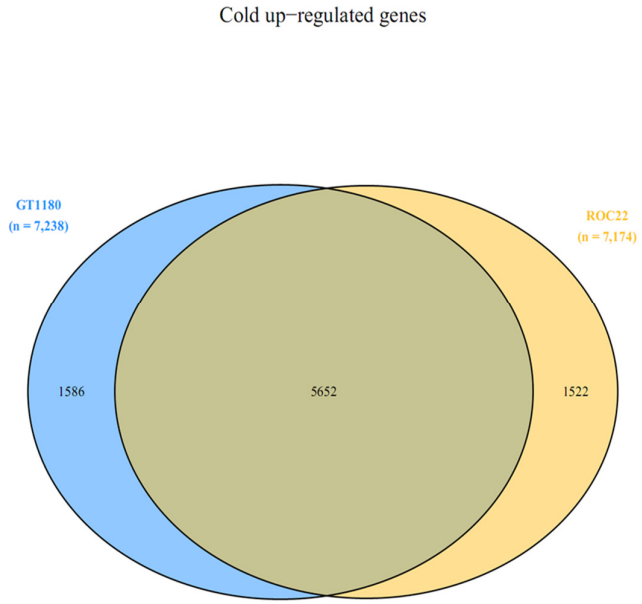

Figure 3. Venn diagram of the 'GT1108' and 'ROC22' cultivars showing the differentially expression of upregulated genes during cold stress treatment only genes with LogFC values ≥ 2 and FDR < 0.05 were included

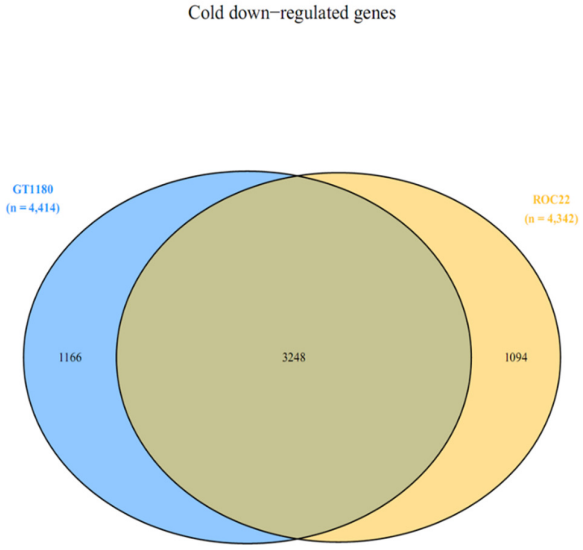

Figure 4. The Venn diagram of GT1108 and ROC22 cultivar shown the differentially expression of down regulated genes during cold stress treatment, only genes with Log FC values ≥ -2 and FDR < 0.05 were included

Visualizing the MA plot, it is also possible to visualize the similar shape of genes distribution in the two cultivars and close number cold regulated genes (Figures 5 and 6).

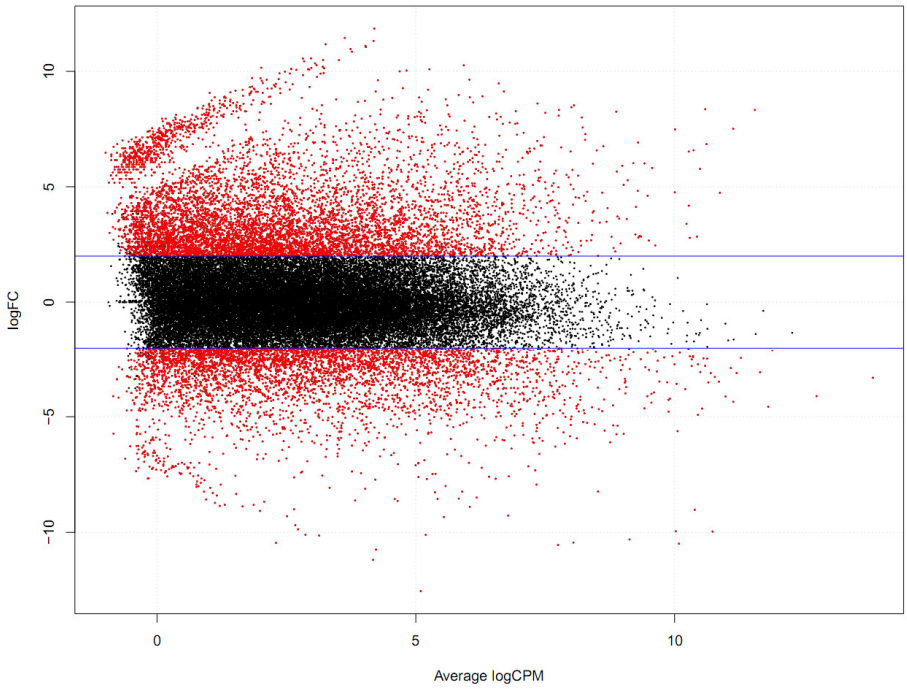

Figure 5 MA plot shown the differential expression of cold regulated genes in the 'GT08-1108' cultivar The red color represents the significant genes differentially expressed at most FDR < 0.05 and Log FC values ≥ 2 and ≤ -2

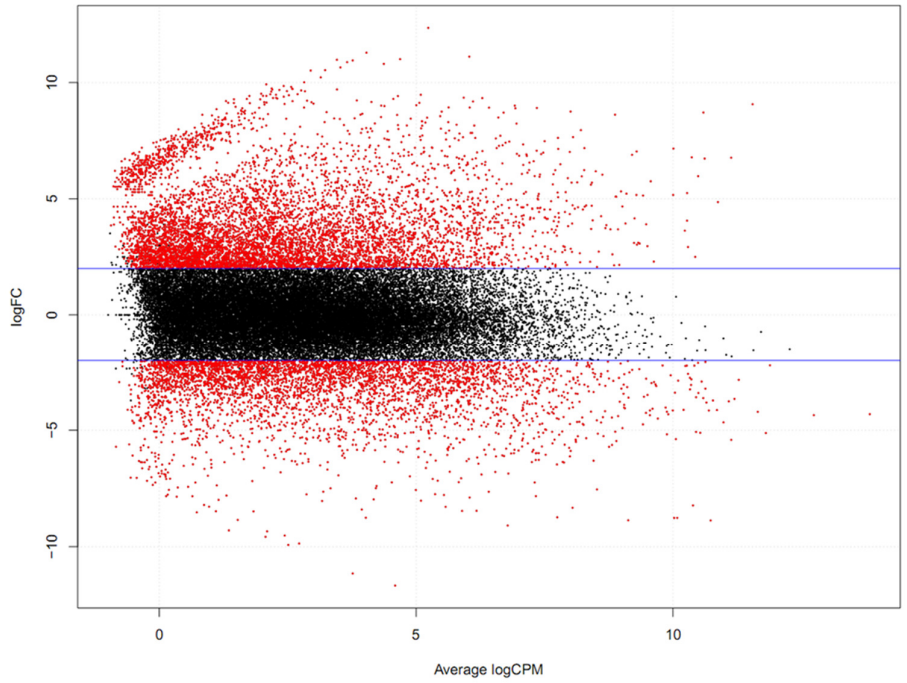

Figure 6. MA plot shown the differential expression of cold regulated genes in the 'ROC22' cultivar

Gene annotation and enrichment analysis of differentially expressed cold regulated genes

The gene enrichment analysis of these data showed that the two cultivars showed the same mechanism in response to cold stress. Many genes were recorded significantly expressed in both cultivars during cold stress. Gene ontology annotation of differentially expressed genes was performed by using Agrigo gene enrichment analysis. Among down regulated genes, there was enrichment for those involved in photosynthesis, water transport, glucose transport, respiration, etc., in both cultivars. Among up-regulated genes, there was enrichment for those involved in rRNA and ribosome biogenesis, protein refolding, regulation of transcription, and the obvious response to cold, among other categories, for both cultivars (Figure S1, S2, S3, S4, S5, S6, S7 and S8).

The differentially expressed annotated genes of cultivar 'GT08-1108' during cold stress were considered into three categories, i.e., Biological Process, Molecular Function, and Cellular Components. The 'GT08-1108' in the cold stress treatment had shown differential expression of cold tolerance genes in response to various physiological mechanisms (Table 3 and Table S1, S2, and S3).

Table 3. Detail of gene annotation of 'GT08-1108' differentially expressed genes during cold stress

Gene	logFC	logCPM	LR	Pvalue	FDR	GO_ID	GO definition	Category
Sspon.06G0010960-1A	-9.0261	10.38908	759.1406	4.13E-167	2.72E-163	GO:0006355	regulation of transcription, DNA-templated	biological process
Sspon.06G0010960-2B	-10.3088	9.128431	729.5461	1.12E-160	6.49E-157	GO:0006355	regulation of transcription, DNA-templated	biological process
Sspon.03G0013100-1A	8.362379	10.59189	727.2723	3.51E-160	1.80E-156	GO:0006950	response to stress	biological process
Sspon.03G0013100-1A	8.362379	10.59189	727.2723	3.51E-160	1.80E-156	GO:0009228	thiamine biosynthetic process	biological_process
Sspon.03G0013100-1A	8.362379	10.59189	727.2723	3.51E-160	1.80E-156	GO:0052837	thiazole biosynthetic process	biological_process
Sspon.03G0013100-1A	8.362379	10.59189	727.2723	3.51E-160	1.80E-156	GO:0055114	oxidation-reduction process	biological_process
Sspon.06G0025360-3D	9.132777	6.702637	673.9798	1.36E-148	5.71E-145	GO:0007165	signal transduction	biological_process
Sspon.06G0025360-3D	9.132777	6.702637	673.9798	1.36E-148	5.71E-145	GO:0016567	protein ubiquitination	biological_process
Sspon.06G0025360-3D	9.132777	6.702637	673.9798	1.36E-148	5.71E-145	GO:0018298	protein-chromophore linkage	biological_process
Sspon.06G0025360-3D	9.132777	6.702637	673.9798	1.36E-148	5.71E-145	GO:0048511	rhythmic process	biological_process
Sspon.02G0030140-2C	6.918423	9.292616	664.4327	1.62E-146	6.24E-143	GO:0090305	nucleic acid phosphodiester bond hydrolysis	biological_process
Sspon.01G0051480-1C	-7.93272	7.330759	656.6325	8.07E-145	2.86E-141	GO:0003333	amino acid transmembrane transport	biological_process
Sspon.04G0011170-3C	8.205048	7.502473	647.2206	8.99E-143	2.96E-139	GO:0006355	regulation of transcription, DNA-templated	biological_process
Sspon.02G0017380-2B	8.611494	7.350816	645.0294	2.69E-142	8.29E-139	GO:0006000	fructose metabolic process	biological_process
Sspon.02G0017380-2B	8.611494	7.350816	645.0294	2.69E-142	8.29E-139	GO:0006013	mannose metabolic process	biological_process
Sspon.03G0035130-2C	7.511996	11.13164	632.9289	1.15E-139	3.33E-136	GO:0006950	response to stress	biological_process
Sspon.07G0013280-2B	2.538011	5.605842	115.6486	5.67E-27	1.11E-25	GO:0009941	chloroplast envelope	cellular_component
Sspon.05G0013620-1A	3.944825	3.40033	115.6333	5.72E-27	1.12E-25	GO:0005737	Cytoplasm	cellular_component
Sspon.06G0013240-3C	-2.43437	5.832789	115.5199	6.05E-27	1.18E-25	GO:0009570	chloroplast stroma	cellular_component
Sspon.01G0008760-3D	5.365915	2.367819	115.4332	6.33E-27	1.24E-25	GO:0005789	endoplasmic reticulum membrane	cellular_component
Sspon.01G0008760-3D	5.365915	2.367819	115.4332	6.33E-27	1.24E-25	GO:0016021	integral component of membrane	cellular_component
Sspon.03G0036680-2P	-2.81856	4.493954	115.3867	6.48E-27	1.27E-25	GO:0000123	histone acetyltransferase complex	cellular_component

Sspon.03G0036680-2P	-2.81856	4.493954	115.3867	6.48E-27	1.27E-25	GO:0000785	Chromatin	cellular_component
Sspon.03G0036680-2P	-2.81856	4.493954	115.3867	6.48E-27	1.27E-25	GO:0005667	transcription factor complex	cellular_component
Sspon.04G0002510-3C	5.636285	3.045118	115.3401	6.63E-27	1.29E-25	GO:0016021	integral component of membrane	cellular_component
Sspon.01G0002030-2C	2.463409	4.702138	115.3033	6.75E-27	1.32E-25	GO:0009507	Chloroplast	cellular_component
Sspon.01G0012480-1A	2.841966	5.403219	115.2203	7.04E-27	1.37E-25	GO:0005622	Intracellular	cellular_component
Sspon.01G0048730-2D	-1.98317	4.662433	40.37435	2.10E-10	1.20E-09	GO:0005524	ATP binding	molecular_function
Sspon.02G0023800-4D	-1.27937	5.357711	40.38826	2.08E-10	1.20E-09	GO:0003677	DNA binding	molecular_function
Sspon.02G0023800-4D	-1.27937	5.357711	40.38826	2.08E-10	1.20E-09	GO:0008270	zinc ion binding	molecular_function
Sspon.04G0015320-2B	7.428988	0.594033	40.38531	2.09E-10	1.20E-09	GO:0005471	ATP:ADP antiporter activity	molecular_function
Sspon.04G0015320-2B	7.428988	0.594033	40.38531	2.09E-10	1.20E-09	GO:0005524	ATP binding	molecular_function
Sspon.04G0015340-2B	3.671573	1.006586	40.37079	2.10E-10	1.21E-09	GO:0004497	monooxygenase activity	molecular_function
Sspon.04G0015340-2B	3.671573	1.006586	40.37079	2.10E-10	1.21E-09	GO:0005506	iron ion binding	molecular_function
Sspon.04G0015340-2B	3.671573	1.006586	40.37079	2.10E-10	1.21E-09	GO:0020037	heme binding	molecular function
Sspon.02G0015920-1A	-1.59245	4.219714	40.35518	2.12E-10	1.22E-09	GO:0016787	hydrolase activity	molecular_function
Sspon.02G0042300-1P	2.40227	1.731929	40.34295	2.13E-10	1.22E-09	GO:0016787	hydrolase activity	molecular function
Sspon.03G0027670-1B	-1.42982	4.135042	40.34818	2.13E-10	1.22E-09	GO:0004758	serine C-palmitoyltransferase activity	molecular function
Sspon.03G0027670-1B	-1.42982	4.135042	40.34818	2.13E-10	1.22E-09	GO:0030170	pyridoxal phosphate binding	molecular function
Sspon.05G0018830-4D	-2.65959	2.885706	40.349	2.12E-10	1.22E-09	GO:0005524	ATP binding	molecular function
Sspon.07G0027630-1B	-1.90024	3.003928	40.35562	2.12E-10	1.22E-09	GO:0020037	heme binding	molecular function
Sspon.07G0027630-1B	-1.90024	3.003928	40.35562	2.12E-10	1.22E-09	GO:0046872	metal ion binding	molecular function
Sspon.08G0018910-3D	7.314548	0.111064	40.34528	2.13E-10	1.22E-09	GO:0005215	transporter activity	molecular function

During the gene annotation of sugarcane cultivar 'ROC22', the differentially expressed genes during cold stress were considered in three categories, namely biological process, molecular function, and cellular components (Table 4, Table S4, S5 and S6).

Table 4. Detail of gene annotation of 'ROC22' differentially expressed genes during cold stress

Gene	logFC	logCPM	LR	P Value	FDR	GO_ID	GO definition	Category
Sspon.06G0010960-1T	-8.8709	10.73379	987.0747	1.16E-216	5.35E-212	GO:0006355	regulation of transcription, DNA-templated	Biological process
Sspon.03G0013100-1A	8.714428	10.59189	751.3594	2.03E-165	2.34E-161	GO:0006950	response to stress	Biological process
Sspon.03G0013100-1A	8.714428	10.59189	751.3594	2.03E-165	2.34E-161	GO:0009228	thiamine biosynthetic process	Biological process
Sspon.03G0013100-1A	8.714428	10.59189	751.3594	2.03E-165	2.34E-161	GO:0052837	thiazole biosynthetic process	Biological process
Sspon.03G0013100-1A	8.714428	10.59189	751.3594	2.03E-165	2.34E-161	GO:0055114	oxidation-reduction process	Biological process
Sspon.06G0010960-1A	-8.22469	10.38908	701.1425	1.69E-154	1.56E-150	GO:0006355	regulation of transcription, DNA-templated	Biological process
Sspon.06G0034300-1D	-8.75868	10.08356	700.0457	2.92E-154	2.25E-150	GO:0006355	regulation of transcription, DNA-templated	Biological process
Sspon.01G0051480-1C	-7.82504	7.330759	697.6376	9.76E-154	6.44E-150	GO:0003333	amino acid transmembrane transport	Biological process
Sspon.06G0010960-2B	-8.8649	9.128431	690.0034	4.46E-152	2.57E-148	GO:0006355	regulation of transcription, DNA-templated	Biological process
Sspon.06G0010960-3C	-8.73441	7.747341	685.2255	4.88E-151	2.50E-147	GO:0006355	regulation of transcription, DNA-templated	Biological process

Sspon.02G0030140-2C	7.038342	9.292616	677.7472	2.06E-149	9.53E-146	GO:0090305	nucleic acid phosphodiester bond hydrolysis	Biological process
Sspon.02G0017380-2B	8.911113	7.350816	644.1668	4.15E-142	1.47E-138	GO:0006000	fructose metabolic process	Biological process
Sspon.02G0017380-2B	8.911113	7.350816	644.1668	4.15E-142	1.47E-138	GO:0006013	mannose metabolic process	Biological process
Sspon.01G0028840-1A	7.415706	6.741887	632.1765	1.68E-139	5.17E-136	GO:0009793	embryo development ending in seed dormancy	Biological process
Sspon.04G0011170-2B	7.657201	8.055123	624.2963	8.70E-138	2.51E-134	GO:0006355	regulation of transcription, DNA-templated	Biological process
Sspon.02G0003080-2C	8.623846	8.872335	622.0002	2.75E-137	7.46E-134	GO:0006950	response to stress	Biological process
Sspon.02G0003080-2C	8.623846	8.872335	622.0002	2.75E-137	7.46E-134	GO:0009228	thiamine biosynthetic process	Biological process
Sspon.02G0005540-3C	9.192582	6.108551	138.551	5.52E-32	1.35E-30	GO:0000139	Golgi membrane	Cellular component
Sspon.02G0005540-3C	9.192582	6.108551	138.551	5.52E-32	1.35E-30	GO:0016021	integral component of membrane	Cellular component
Sspon.07G0015480-4D	-4.38823	8.931823	138.4778	5.73E-32	1.40E-30	GO:0005737	Cytoplasm	Cellular component
Sspon.01G0004310-1A	-2.21858	6.327873	138.4758	5.73E-32	1.40E-30	GO:0016021	integral component of membrane	Cellular component
Sspon.05G0010980-2B	-2.55609	5.21341	138.3306	6.17E-32	1.51E-30	GO:0005737	Cytoplasm	Cellular component
Sspon.01G0046530-2C	-3.4984	5.075343	138.3253	6.19E-32	1.51E-30	GO:0009570	chloroplast stroma	Cellular component
Sspon.03G0033420-1B	-2.4762	7.198554	137.9278	7.56E-32	1.84E-30	GO:0008287	protein serine/threonine phosphatase complex	Cellular component
Sspon.03G0033420-1B	-2.4762	7.198554	137.9278	7.56E-32	1.84E-30	GO:0016021	integral component of membrane	Cellular component
Sspon.03G0007130-3C	3.137238	5.676721	137.7818	8.13E-32	1.98E-30	GO:0005739	Mitochondrion	Cellular component
Sspon.08G0019390-2C	2.93887	6.552519	137.7679	8.19E-32	1.99E-30	GO:0005634	Nucleus	Cellular component
Sspon.08G0019390-2C	2.93887	6.552519	137.7679	8.19E-32	1.99E-30	GO:0005667	transcription factor complex	Cellular component
Sspon.02G0030420-1A	-2.87695	4.233017	137.2127	1.08E-31	2.62E-30	GO:0016021	integral component of membrane	Cellular component
Sspon.08G0000430-1P	5.032969	1.976474	73.67273	9.22E-18	9.63E-17	GO:0050660	flavin adenine dinucleotide binding	Molecular function
Sspon.06G0013110-2B	2.177385	5.156148	73.66786	9.24E-18	9.66E-17	GO:0008375	acetylglucosaminyltransferase activity	Molecular function
Sspon.01G0021790-1P	-2.04753	4.029061	73.63027	9.42E-18	9.84E-17	GO:0015194	L-serine transmembrane transporter activity	Molecular function
Sspon.07G0008360-1P	-2.03741	4.400111	73.61691	9.48E-18	9.90E-17	GO:0003993	acid phosphatase activity	Molecular function
Sspon.06G0021210-1B	-1.58444	6.465062	73.61443	9.50E-18	9.91E-17	GO:0016853	isomerase activity	Molecular function
Sspon.03G0030700-1B	3.471103	2.850102	73.6072	9.53E-18	9.95E-17	GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	Molecular function
Sspon.03G0030700-1B	3.471103	2.850102	73.6072	9.53E-18	9.95E-17	GO:0019139	cytokinin dehydrogenase activity	Molecular function
Sspon.03G0030700-1B	3.471103	2.850102	73.6072	9.53E-18	9.95E-17	GO:0050660	flavin adenine dinucleotide binding	Molecular function
Sspon.04G0008130-1A	4.226656	2.264577	73.60521	9.54E-18	9.95E-17	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	Molecular function
Sspon.04G0008130-1A	4.226656	2.264577	73.60521	9.54E-18	9.95E-17	GO:0016762	xyloglucan:xyloglucosyl transferase activity	Molecular function
Sspon.01G0055390-1C	-2.11588	5.059533	73.55097	9.81E-18	1.02E-16	GO:0052692	raffinose alpha-galactosidase activity	Molecular function
Sspon.05G0015300-4D	1.913092	5.573124	73.49816	1.01E-17	1.05E-16	GO:0008270	zinc ion binding	Molecular function
Sspon.05G0021970-1B	-1.6491	4.874546	73.47903	1.02E-17	1.06E-16	GO:0004722	protein serine/threonine phosphatase activity	Molecular function

Discussion

RNA sequencing also called transcriptome sequencing is a method using next-generation sequencing to analyse the total transcriptome of biological samples (Morin *et al*., 2008; Wang *et al*., 2009; Chu *et al*., 2012). In the present study, the RNA sequence data of two sugarcane cultivars namely 'ROC22' and 'GT-081108' (Shi-Yun *et al.,* 2018) were analysed and mapped the reads with reference sugarcane genome recently published (Jisen *et al.,* 2018), to study the expression analysis of cold tolerance genes and the mechanism of sugarcane in response to cold stress. The early response of the plant to cold is inhibition of photosynthesis by changing the pigment composition, decreased quantum efficiency (Fryer *et al*., 1995), also modified thylakoid membranes, and impaired chloroplast development (Nie *et al*., 1995). Most of the cold tolerance genes during cold stress play a key role in defense and cell wall composition. Arabinoxylan arabinofuranohydrolase (plasticizing of the cell), these are expressed during stress and is a structural component of the cell, and serves as phenolic reservoir, maintain membrane fluidity by rehydration and production of antioxidant activates. (Moore *et al*., 2013). In our data metabolic process of xyloglucan was upregulated by 10.0 logFc2 while many genes were upregulated in both cultivars like genes for cell wall biogenesis, and cell wall composition.

During low temperature plants also express pectin and lignin for strengthening and decreasing the pore size of the cell wall. In our data analysis, the cell wall pectin metabolic process, pectin, and other organic and inorganic substances, were upregulated by 1.0 and 4.0 logFc2, while the lignin biosynthetic process was also upregulated by 4.2 logFc2, activation of phospholipase C activity, phospholipase A2 activity, acyl-[acyl-carrier-protein] desaturase activity was also upregulated by 4.0, 4.8, 4.3 logFc2, They help in double bond insertion to hydrocarbon chain of fatty acid to produced unsaturated fatty acid (Pang *et al.,* 2013). The phospholipase is enhanced which anchors the microtubules to the plasma membrane and causes rearrangement in cytoskeleton confirmation, therefore activating the calcium channels (Fowler and Thomashow, 2002).

During low temperature the Na+ ions are increasing and these excessive Na+ ions must be organized in the vacuole to maintain the cellular hemostasis. Plant cells have H+ pyro-phosphatases and H+ ATPase produced proton motive force and transport ions like Na+ (Conde *et al*., 2011). In our data ATPase activity, coupled with the transmembrane movement of substances, ATPase inhibitor activity, ATPase activity was upregulated by 1.2, 1.5, 1.6 logFc2. In membrane fluidity, during cold temperature, the cold sensor detects the change and initiates the signal transduction machinery. During low temperatures in s calcium channels and histidine kinase, these two components work as sensors (Xiong *et al*., 2002). The plant cell membrane in response to low temperature raise the level of calcium cytosolic levels and transmit the signal through Ca^{2+} regulated protein-like protein kinase act as a responder and calcineurin and calmodulin act as a sensor. The sensor transmits changes in different protein phosphorylation status and normalized the gene expression of cold responsive genes, calcium dependent protein kinase and phosphatase act as responder have effector domains that recognized the message to downstream targets (Reddy and Reddy, 2004). During low temperature the multifunctional protein like calmodulin, Ca^{2+} dependent protein kinase increases the level of calcium (Nogueira *et al.,* 2003). The calcium dependent protein kinase and Ca^{2+} ATPase also act as pumps of calcium to increase the level of calcium as a result of transmitting calcium signal the cell hemostasis are restored (Sze *et al*., 2000). In our data analysis, the calcium dependent kinase protein kinase activity and calmodulin dependent kinase activity were highly Upregulated by 3.8 logFc2.

Inositol and calcium, abscisic acid (ABA), and reactive oxygen (ROS) are secondary messengers and play a major role in cold signal transduction. During cold stress in plants, the MAPK pathways play key importance in the production of compatible antioxidants and osmolytes. In our data protein, tyrosine kinase activity was highly upregulated by 4.8 logFc2, G-protein coupled receptor activity was also Upregulated by 2.3 logFc2, these proteins working as the cold sensor (Xiong *et al.,* 2002).

During seed germination, stomal closure, and ABA inducible gene expression the phosphatase protein also acts as MAPK phosphatase involved in positive regulation. In our analysis, the protein phosphatase activity

was upregulated by 2.3 logFc2. In cold-inducible gene expression, the Ras-Gene small GTPase bind with a cascade of Ras-MAPK and also acts as a positive regulator, in our data analysis activity of these small Ras-Gene (GTPase) was upregulated by 1.6 logFc2. Kinase diphosphate nucleoside also acts as a positive regulator of MAPK cascade and enhances expression during cold stress (Kovtun *et al*., 2000). Kinase diphosphate nucleoside activity in our data analysis was upregulated by 1.8 logFc2. ABA dependent gene expression in our analysis was also upregulated genes involved in ABA catabolic activity, ABA 8-hydroxylase activity, ABA activated signal pathways were highly upregulated by 6.2, 6.8 logFc2, these genes also work as a secondary messenger during low temperature.

Enzymes such as glucosidases, beta-1, 3 glucan hydrolases, peroxidases, alcohol dehydrogenases, glutathione, S-transferases, acetyltransferases and phosphatases, ascorbate peroxidase APX, catalase CAT, Superoxide dismutase SOD, glutathione peroxidases GPX, monodehydroascorbate reductase MDHAR, dehydroascorbate reductase DHAR, glutathione S-transferase GST, glutathione reductase GR, and peroxiredoxin PRX, there were more than 13 types of enzymes which are located in different organs of cell and help for searching oxygen species ROS during various abiotic stress condition in plants (Gill and Tuteja, 2010; You and Chan, 2015). In our data analysis enzymes like peroxisome, cellobiose glucosidase activity, peroxidase activity, hydrolase activity, alcohol dehydrogenase (NAD) activity, glutathione transferase activity, glutathione peroxidase activity, oxidoreductase activity was differentially upregulated by 3.2, 3.6, 2.5, 1.3, 5.2 logFc2.

Simple sugar is also accumulated during cold stress and plays a key role in preventing the cell membranes from cold damage (Strauss and Hauser, 1986). In plants, the soluble sugars remove hydroxyl radicals, induced protein synthesis indirectly, and provide improvement in the cold resistance ability of plants. Reports showed that soluble sugars and protein contents showed a positive correlation with the hardiness of plants to cold (Luo *et al*., 2002). In our data sucrose transmembrane transporter activity, glutamine-fructose-6-phosphate transaminase (isomerizing) activity was upregulated by 1.1, 1.2 logFc2, 140 genes were upregulated for sucrose, 50 genes for fructose, while 200 genes were upregulated for sucrose in both cultivars 'GT08-1108' and 'ROC 22'. Sugars alcohols are also a key characteristic and accumulation during cold stress, in our data analysis 560 genes were upregulated for sugar alcohols.

In plants, proline is a major osmolyte, which is involved in response to various abiotic stresses, and excessive production of proline in plants enhanced osmo-tolerance (Gubis *et al.,* 2007). In sugarcane cultivars, the free amino acid content is varied but some cold tolerant cultivars have a high content of free amino acid (Huang *et al*., 2015). In proline synthesis, the key enzyme is delta-1-pyrroline-5-carboxylate to synthesize P5CS and these enzymes also play important role in cold accumulation (Szekely *et al.,* 2008). In our data analysis, peptidyl-prolyl cis-trans isomerase activity was upregulated by 2.3 logFc2, for proline metabolic process and peptidyl-proline modification 33 genes were upregulated in both cultivars.

During cold stress, photosynthesis is extremely affected, like light reaction, photorespiration, and the Calvin cycle (Yan *et al*., 2004). Research work has shown that in sugarcane plants during cold stress photosynthesis is affected, therefore delaying the supply of carbon for synthesis, storage, and transport of carbohydrates (Machado *et al*., 2013; Sales *et al*., 2012). In our data analysis genes for photosynthesis were down regulated in both cultivars, in down regulated differentially expressed genes in 'GT08-1108', 68 genes for photosynthesis, 85 genes for photosynthesis light harvesting, while 40 genes for regulation of photosynthesis, 15 genes for light reaction, 5 genes for dark reaction were upregulated. In 'ROC22' 52 genes were Upregulated for photosynthesis, while 205 genes were down regulated for photosynthesis.

Recently ubiquitin conjugation has been found and identified as an important regulator of stress responsive transcription factors and many other regulatory proteins. During abiotic stress, ubiquitination plays important role in regulating the transcription process which is essential for adaptation (Lyzenga and Stone, 2012). Our data showed protein ubiquitination was highly upregulated by 8.0 logFc2, while 479 genes in 'GT08-1108' and 450 in 'ROC22' were upregulated. Heat shock proteins or chaperones (HSPs) in cell response to adverse environment are produced, heat shock proteins have been found produced during cold

stress like HSP90, HSP70, and Small HSPs (Timperio *et al*., 2008) heat shock proteins (HSPs) are also found in Maize (Kollipara *et al*., 2002) rice, chicory (Degand *et al*., 2009) and poplar (Renaut *et al*., 2004). In our analysis, the protein refolding was highly upregulated by 8.0 logFc2. While 53 genes were upregulated in 'GT08-1108', and 48 in 'ROC22'.

Chilling or cold stress in plants induces oxidative stress (Sato, 2001) and collects reactive oxygen species upon cold exposure (ROS). During cold stress, there is a significant increase in the antioxidant systems (Baek and Skinner, 2003). In our analysis, 1200 genes were differentially upregulated for oxidoreductase activity in 'GT08-1108' while 1987 genes were upregulated in 'ROC22'. Plants expressed hormones and secondary metabolites in response to cold stress. In our analysis of secondary metabolite, biosynthetic processes were upregulated by 7.0 logFc2, auxin-activated signaling pathway, cytokinesis, ethylene biosynthetic process was Upregulated by 1.0, 3.5 logFc2. While 470 genes were upregulated for the hormonal metabolic process in 'GT08-1108', and 460 genes in 'ROC22'.

Furthermore, the data analysis of both pipelines was compared for the differential expression analysis of cold responsive genes in sugarcane. Shi-yun *et al*. (2018) mapped the reads with reference transcriptome assembly, the analysis showed that 183,515 unigenes were assembled and, among these, 110,021 (60%) were functionally annotated with GO categories. In our analysis using the reference genome of *Saccharum* spontaneum 83826 unigenes were annotated and, among these, 46,159 (55%) were functionally annotated with Gene Ontology (GO) categories. For the cultivar GT08-1108, using the reference genome pipeline, 11,652 (13.9%) unigenes were differentially expressed (7,238 upregulated; 4,414 downregulated), while 16,145 (8.8%) were differentially expressed (8,965 upregulated; 7,180 downregulated) using Transcriptome-based pipeline. In the cultivar ROC22, 11,516 (13.7%) genes were differentially expressed (7,174 upregulated; 4,342 downregulated) and 20,317 (11.1%) (10,898 upregulated; 9,419 downregulated) for the genome and Transcriptome based analysis, respectively (Table 1). In the genome analyses, among downregulated genes, 3,248 were coincident between the two cultivars, the remaining 1,166 differentially expressed only in 'GT-1180' and 1,094 only in 'ROC22'. With the Transcriptome assembly, 13,113 genes were deferentially expressed in both cultivars, the remaining 3,032 unique to 'GT08-1108' and 7,204 in 'ROC22'.

Conclusions

The sugarcane genome is a complex and polyploidy, very few studies have been conducted on the sugarcane transcriptomic and genomics. In this study the transcriptomic analysis of cold responsive genes was studied in sugarcane, from the results we concluded that sugarcane plants during cold stress showed diverse mechanisms, these mechanisms, including regulation of transcriptional factors activity, gene expression to cold stress, kinase activity, cold signaling and many more. During cold stress, many genes were significantly upregulated, and downregulated, among downregulated mostly the genes were responsible for fluid transport, photosynthesis, etc. Furthermore, the Transcriptome data was mostly analyzed by using two main bioinformatics pipelines, the reads were mapped either using a reference genome and if a reference genome is not available then reads were mapped with reference transcripts (de novo assembly). Therefore, in this study, we mapped the RNA reads with the reference genome, based on the results of both pipelines we conclude that the Transcriptome assembly overestimated the number of unigenes and, consequently, a higher number of differentially expressed genes. This may be due to difficulties in separating homologues from paralogue genes. The RNA-Seq approach will provide a powerful tool to investigate the genes that contribute to abiotic stress tolerance in many economically important crop species with a lack of genomic information.

Authors' Contributions

SUR conceptualized and prepared the experiment manuscript draft, KM, ACP, YQ, and EN designed, reviewed, and improved the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors are thankful to the National Key Research and Development Program of China (2018YFD1000503), Higher Education Commission Islamabad, Pakistan, and the Brazilian National Council for Scientific and Technological Development (CNPq), for supporting and funding this project.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Report 25:1263-1274. *https://doi.org/10.1007/s00299-006-0204-8*

Bolger AM, Lohse M, Usadel B (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114-2120. *https://doi.org/10.1093/bioinformatics/btu170*

Cheavegatti-Gianotto A, De-Abreu HMC, Arruda P, Bespalhok Filho JC, Burnquist WL, Creste DE, Fatima Grossi-De-Sa SM (2011). Sugarcane (*Saccharum* X *officinarum*): a reference study for the regulation of genetically modified cultivars in Brazil. Tropical Plant Biology 4:62-89. *https://doi.org/10.1007/s12042-011-9068-3*

Chu Y, Corey DR (2012). RNA sequencing: platform selection, experimental design, and data interpretation. Nucleic Acid Therapeutics 22:271-274. *https://doi.org/10.1089/nat.2012.0367*

Conde A, Chaves MM, Geros H (2011). Membrane transport, sensing and signaling in plant adaptation to environmental stress. Plant and Cell Physiology 52:1583-1602. *https://doi.org/10.1093/pcp/pcr107*

Degand H, Faber AM, Dauchot N, Mingeot D, Watillon B, Cutsem PV, Morsomme P (2009). Proteomic analysis of chicory root identifies proteins typically involved in cold acclimation. Proteomics 9:2903-2907. *https://doi.org/10.1002/pmic.200800744*

Figueroa-Rodríguez KA, Hernández-Rosas F, Figueroa-Sandoval B, Velasco-Velasco J, Aguilar Rivera N (2019). What has been the focus of sugarcane research? A bibliometric overview. International Journal of Environmental Research and Public Health 16:3326. *https://doi.org/10.3390/ijerph16183326*

Fryer MJ, Oxborough K, Martin B, Ort DR, Baker NR (1995). Factors associated with depression of photosynthetic quantum efficiency in maize at low growth temperature. Plant Physiology 108:761-767. *https://doi.org/10.1104/pp.108.2.761*

Fowler S, Thomashow MF (2002). Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell 14:1675-1690. *https://doi.org/10.1105/tpc.003483*

Gill SS, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48:909-930. *https://doi.org/10.1016/j.plaphy.2010.08.016*

Grivet L, Arruda P (2002). Sugarcane genomics: depicting the complex genome of an important tropical crop. Current Opinion in Plant Biology 5:122-127. *https://doi.org/10.1016/S1369-5266(02)00234-0*

Gubis J, Vankova R, Cervena V, Dragunova M, Hudcovicova M, Lichtnerova H, Dokupil T Jurekova Z (2007). Transformed tobacco plants with increased tolerance to drought. South African Journal of Botany 73:505-511. *https://doi.org/10.1016/j.sajb.2007.03.011*

Hotta CT, Lembke CG, Domingues DS, Ochoa EA, Cruz GM, Melotto Passarin DM, ... Crivellari AC (2010). The biotechnology roadmap for sugarcane improvement. Tropical Plant Biology 3:75-87. *https://doi.org/10.1007/s12042-010-9050-5*

Hoang NV, Furtado A, Mason PJ, Marquardt A, Kasirajan L, Thirugnanasambandam PP, ... Henry R J (2017). A survey of the complex transcriptome from the highly polyploid sugarcane genome using full-length isoform sequencing and de novo assembly from short read sequencing. BMC Genomics 18(1):1-22. *https://doi.org/10.1186/s12864-017-3757-8*

Hoang NV, Furtado A, Mcqualter RB, Henry RJ (2015). Next generation sequencing of total DNA from sugarcane provides no evidence for chloroplast heteroplasmy. New Negatives in Plant Science 1(2):33-45. *https://doi.org/10.1016/j.neps. 2015.10.001*

Hussain A, Khan ZI, Ghafoor MY, Ashraf M (2004). Review sugarcane, sugar metabolism and some abiotic stresses. *https://doi.org/10.3920/QAS2015.0824*

James G (2008). Sugarcane.1st ed. John Wiely and Sons.

Khan MT, Khan IA, Yasmeen S, Nizamani GS, Afghan S (2019). Sugarcane Biofuels and Bioenergy Production in Pakistan. In: Current Scenario, Potential, and Future Avenues Sugarcane Biofuels. Springer, Cham pp 175-202.

Kollipara KP, Saab IN, Wych RD, Lauer MJ, Singletary GW (2002). Expression profiling of reciprocal maize hybrids divergent for cold germination and desiccation tolerance. Plant Physiology 129:974-992. *https://doi.org/10.1104/pp.000729*

Kovtun Y, Chiu WL, Tena G, Sheen J (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proceedings of the National Academy of Sciences 97:2940-2945. *https://doi.org/10.1073/pnas.97.6.2940*

Luo HL, Weili X Jianzhong L (2002). Study on the acceptation of *Acacia mangium* to low temperature stress. Journal of South China Agriculture University 23:51-53.

Lyzenga WJ, Stone SL (2012). Abiotic stress tolerance mediated by protein ubiquitination. Journal of Experimental Botany 63:599-616. *https://doi.org/10.1093/jxb/err310*

Lyzenga WJ, Stone SL (2012). Abiotic stress tolerance mediated by protein ubiquitination. Journal of Experimental Botany 63(2):599-616. *https://doi.org/10.1093/jxb/err310*

Machado DFSP, Ribeiro RV, Silveira JAG, da Filho JRM, Machado EC (2013). Rootstocks induce contrasting photosynthetic responses of orange plants to low night temperature without affecting the antioxidant metabolism. Theoretical and Experimental Plant Physiology 25:26-35.

Moore PH (1987). Breeding for stress resistance. In: Heinz DJ (Ed). Sugarcane Improvement through Breeding. pp 503-542.

Morin RM, Bainbridge A, Fejes M, Hirst M, Krzywinski M, Pugh T, McDonald H, Varhol R, Jones S, Marra M (2008). Profiling the HeLa S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing. Biotechniques 45(1):81-94. *https://doi.org/10.2144/000112900*

Moore JP, Nguema-Ona EE, Vicre-Gibouin M, Sorensen I, Willats WGT, Driouich A, Farrant JM (2013). Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. Planta 237:739-754. *https://doi.org/10.1007/s00425-012-1785-9*

Manimekalai R, Suresh G, Singaravelu B (2022). Sugarcane transcriptomics in response to abiotic and biotic stresses: a review. Sugar Tech 15:1-24. *https://doi.org/10.1007/s12355-021-01098-9*

Nie GY, Robertson EJ, Fryer MJ, Leech RM, Baker NR (1995). Response of the photosynthetic apparatus in maize leaves grown at low temperature on transfer to normal growth temperature. Plant, Cell and Environment 18:1-12. *https://doi.org/10.1111/j.1365-3040.1995.tb00538.x*

Nogueira FT, De Rosa VE, Menossi M, Ulian EC, Arruda P (2003). RNA expression profiles and data mining of sugarcane response to low temperature. Plant Physiology 132:1811-1824. *https://doi.org/10.1104/pp.102.017483*

OECD (2016). Safety Assessment of Transgenic Organisms in the environment. Vol: 6. *OECD Consensus Documents*, Harmonisation of Regulatory Oversight in Biotechnology, OECD Publishing, Paris. *https://www.oecd.org/chemicalsafety/biotrack/safety-assessment-of-transgenic-organisms-in-the-environment-volume-6-9789264253421-en.htm*

Pang T, Ye CY, Xia X, Yin W (2013). *De novo* sequencing and transcriptome analysis of the desert shrub, *Ammopiptanthus mongolicus*, during cold acclimation using Illumina/Solexa. BMC Genomics 14:488. *https://doi.org/10.1186/1471-2164-14-488*

Quackenbush J (2002). Microarray data normalization and transformation. Nature Genetics 32:496-501. *https://doi.org/10.1038/ng1032*

Que Y, Su Y, Guo J, Wu Q, Xu L (2014). A global view of transcriptome dynamics during *Sporisorium scitamineum* Challenge in sugarcane by RNAseq. PLoS One Journal pone 9:106476. *http://dx.doi.org/10.1186/1471-2164-15-996*.

Rehman SU, Que Y, Khan S, Novaes E, Muhammad K (2020). Molecular study of CBF and WCOR14 genes in selected sugarcane cultivars and its wild relative *Saccharum spontaneum* L. Journal of Animal and Plant Sciences 30:913-922. *https://doi.org/10.36899/JAPS.2020.4.0107*

Reddy VS, Reddy AS (2004). Proteomics of calcium-signaling components in plants. Photochemistry 65:1745-1776. *https://doi.org/10.1016/j.phytochem.2004.04.033*

Robinson MD, Mccarthy DJ, Smyth GK (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139-40. *https://doi.org/10.1093/bioinformatics/btp616*

Robinson MD, Oshlack A (2010). A scaling normalization method for differential expression analysis of RNA-Seq data. Genome Biology 11:1-9. *https://doi.org/10.1186/gb-2010-11-3-r25*

Robinson MD, Smyth GK (2008). Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics 9:321-332. *https://doi.org/10.1093/biostatistics/kxm030*

Renaut J, Lutts S, Hofmann L, Hausman JF (2004). Responses of poplar to chilling temperatures: proteomic and physiological aspects. Plant Biology 7:81-90. *https://doi.org/10.1055/s-2004-815733*

Sabiha-Hanim S, Halim NAA (2018). Sugarcane bagasse pretreatment methods for ethanol production. In: Fuel Ethanol Production from Sugarcane. Intechopen. *http://dx.doi.org/10.5772/intechopen.81656*

Sales CRG, Ribeiro RV, Machado DFSP, Machado RS, Dovis VL, Lagôa AMMA (2012). Gas exchange and carbohydrate balance in sugarcane plants under root stressful conditions. Bragantia 71:319-327.

Serba DD, Meng X, Schnable J, Bashir E, Michaud JP, Vara Prasad PV, Perumal R (2021). Comparative transcriptome analysis reveals genetic mechanisms of sugarcane aphid resistance in grain *Sorghum*. International Journal of Molecular Sciences 22(13):7129. *https://doi.org/10.3390/ijms22137129*

Shi-Yun T, Li-Tao Y, Yang-Rui L (2018). Comparative analysis on transcriptome among different sugarcane cultivars under low temperature stress. Biotechnology Bulletin 34:116-124. *http://dx.doi.org/10.13560/j.cnki.biotech.bull.1985.2018-0522*

Souza GM, Berges H, Bocs S, Casu R, D'Hont A, Ferreira JE, Henry R, Ming R, Potier B, Sluys MV, Vincentz M, Paterson AH (2011). The sugarcane genome challenge: strategies for sequencing a highly complex genome. Tropical Plant Biology 4:145. *https://doi.org/10.1007/s12042-011-9079-0*

Strauss G, Hauser H (1986). Stabilization of lipid bilayer vesicles by sucrose during freezing. Proceedings of the National Academy of Sciences 83:2422-2426. *https://doi.org/10.1073/pnas.83.8.2422*

Sze H, Liang F, Hwang I, Curran AC, Harper JF (2000). Diversity and regulation of plant Ca2+ pumps: insights from expression in yeast. Annual Review of Plant Biology 51:433-462. *https://doi.org/10.1146/annurev.arplant.51.1.433*

Sato Y (2001). Heat shock-mediated APX gene expression and protection against chilling injury in rice seedlings. Journal of Experimental Botany 52:145-151. *https://doi.org/10.1093/jexbot/52.354.145*

Strauss G, Hauser H (1986). Stabilization of lipid bilayer vesicles by sucrose during freezing. Proceedings of the National Academy of Sciences 83(8):2422-2426. *https://doi.org/10.1073/pnas.83.8.2422*

Szekely G, Abraham E, Cseplo A, Rigo G, Zsigmond L, Csiszar J, ... Koncz C (2008). Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant Journal 53:11-28.

Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, Xu W, Su Z (2017). Agrigo v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Research 45:122-129. *https://doi.org/10.1093/nar/gkx382*

Timperio AM, Egidi MG, Zolla L (2008). Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). Journal of Proteomics 71:391-411. *https://doi.org/10.1016/j.jprot.2008.07.005*

Vettore AL, da Silva FR, Kemper EL, Souza GM, da Silva AM, Ferro MI, ... Nobrega MP (2003). Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane. Genome Research 13(12):2725-2735. *http://www.genome.org/cgi/doi/10.1101/gr.1532103*

Wang Z, Gerstein M, Snyder M (2009). RNA-Seq: A Revolutionary Tool for Transcriptomics. Nature Review Genetics 10:57-63. *https://doi.org/10.1038/nrg2484*

Xiong L, Schumaker KS, Zhu JK (2002). Cell signaling during cold, drought, and salt stress. The Plant Cell 14:165-183. *https://doi.org/10.1105/tpc.000596*

Xu S, Wang J, Shang H, Huang Y, Yao W, Chen B, Zhang M (2018). Transcriptomic characterization and potential marker development of contrasting sugarcane cultivars. Scientific Reports 8(1):1-1. *https://doi.org/10.1038/s41598-018-19832-x*

Yan J, He C, Wang J, Mao Z, Holaday SA, Allen RD, Zhang H (2004). Overexpression of the Arabidopsis 14-3-3 protein GF14 lambda in cotton leads to a "stay-green" phenotype and improves stress tolerance under moderate drought conditions. Plant Cell Physiology 45:1007-1014.

You J, Chan Z (2015). ROS regulation during abiotic stress responses in crop plants. Frontiers in Plant Science 6:1092. *https://doi.org/10.3389/fpls.2015.01092*

Zhang J, Zhang X, Tang H, Zhang Q, Hua X, Ma X, ... Ming R (2018). Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L. Nature Genetics 50(11):1565-1573. *https://doi.org/10.1038/s41588-018-0237-2*

Zhou D, Zhou X, Ling Y, Zhang Z, Su Z (2010). Agrigo: a GO analysis toolkit for the agricultural community. Nucleic Acids Research. *https://doi.org/10.1093/nar/gkq31*.