

Transcriptional insights into sugarcane aquaporin genes under water deficit conditions

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Research Article

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Abstract

Water deficit in soil during formative growth stage adversely hinders the crop productivity. Plant develop a key chain of mechanisms to cope these strains. Characterization of genotypes under water deficit will provide the basis for breeding new germplasm for efficient utilization of water and nutrients and adaptation to water stress. To achieve this, two tolerant (Co 98014 and Co 0118) and two sensitive (CoJ 85 and Co 89003) sugarcane genotypes were assessed for antioxidant response followed by differential expression of three aquaporin genes (ShPIP2-1, ShPIP-5 and ShPIP2-6) under two water deficit conditions. The MDA and H₂O₂ contents were significantly higher ($p < 0.05$) in sensitive genotypes as compared to tolerant ones, whereas SOD activity was higher ($p < 0.05$) in tolerant than sensitive genotypes. The transcript analysis of AQPs reveals upregulation of ShPIP2;5, whereas down-regulation of ShPIP2;1 and ShPIP2;6 when plants were imposed to water deficit conditions. The findings under study suggested the role of PIP2 AQPs in regulation of plant water status under water deficit conditions.

Key Message

Substantial alterations in aquaporin (PIP2) gene transcripts as well as higher antioxidant activity of tolerant genotypes witnessed the role of AQPs in sustaining plant water status during stress conditions.

Introduction

Sugarcane (*Saccharum officinarum* L.), an economically important and the largest area occupying Indian crop suffers due to various biotic and abiotic constraints. Among various abiotic constraints, drought or water deficit is the most drastic factor in regions suffering with water scarcity, that adversely affects this crop from initial growth to development phases (Kumar et al. 2019). Production of sugarcane is worsening with the rapid expansion of drought affected areas of the world. As a major part of sugarcane growing area in India is directly affected by drought that alters plant functioning at very early stages. Sugarcane production can still be improved further if we can put a check on a major agricultural problem *i.e.* water stress. Thus, the varieties having tolerance towards drought is prior need to sustain sugarcane productivity during this changing climate scenario.

Drought stress is known to decreased chlorophyll and relative water contents, water potential and affects other various metabolic and physiological processes (Singh et al. 2016, Kumar et al. 2019). Water deficit induce reactive oxygen species (ROS) accumulation and lipid peroxidation in plants that could have an effect on membrane lipids, proteins and nucleic acids, resulting in increased membrane leakage of solutes under drought stress (Liu 2011). Thus, cellular damage is caused in plants due to oxidative stress (Miller et al. 2010). To reduce such stress, plants have developed a complex multitude of antioxidant defense systems to limit ROS and maintain redox homeostasis such as superoxide dismutase, catalase and peroxidase etc. (Kumar et al. 2019).

In apart to the physio-biochemical changes, a coordinated set of signalling networks are involved to control stress consequences by regulating numerous of genes that encode protein for plant survival (Chaves et al. 2003). The genes having role in stress acclimation encode for chaperones (HSPs), aquaporins (water channel proteins), proteases, detoxifying enzymes and free radical scavengers. Aquaporins (AQPs) are water facilitating proteins that transport water, gases (NH₃ and CO₂) and small solutes (urea, H₃BO₄ and H₄SiO₄) through the membrane (Maurel et al. 2008, Zelazny et al. 2009). Importance of AQPs in water transport suggest role of these proteins in various physiological responses to abiotic stresses like drought (Heinen et al. 2009). Amino acid identity of plant AQPs classify aquaporins into five subfamilies: plasma membrane intrinsic protein (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like proteins (NIPs), small basic intrinsic proteins (SIPs) and X intrinsic proteins (XIPs) (Sakurai et al. 2005). Similarly, phylogenetic study based on nucleotides sequence revealed 33 isoforms of AQPs consisting four subfamilies *i.e.* 13 PIPs, 11 TIPs, 6 NIPs and 3 SIPs (de-Andrade et al. 2016). The subfamily PIP can be further subdivided into PIP1 and PIP2 (Chaumont et al. 2000), consists five and eight proteins, respectively. Three PIP2 isoforms involved in drought stress in higher plants. The transcript study of three PIPs (ShPIP2;1, ShPIP2;5 and ShPIP2;6) evaluated in two sugarcane genotypes (IACSP94-2094 and IACSP97-7065) under water deficit through qPCR showed these isoforms were responsive to drought and their expression pattern were dependent of genotype, experimental conditions (de-Andrade et al. 2016). The isoform PIP2 exhibit more efficient water channel activity than PIP1 (Chaumont et al. 2000, Kaldenhoff and Fischer 2006). The abundance of PIP2 transcripts has been established by qPCR studies suggest their presence in different tissues and organs under water stress conditions (Zhang et al. 2008, de Andrade et al.2016).

Drought tolerance in sugarcane is not same in all species, besides in certain species, mechanisms can operate concomitantly producing tolerance through their amalgamated effects. No particular studies of tolerance mechanisms in response to water stress has been done in the sugarcane genotypes of semi-arid region of India. Even the type of tolerance mechanism for drought tolerance are not fully understood. Thereby, breeding for drought tolerance in sugarcane require a depth understanding of divergent resistance mechanisms. Molecular techniques, particularly gene expression profiles has been proved as a reliable tool to identify transcript involved in drought tolerance (Rodrigues et al. 2009). Therefore, present study was designed on evaluation of sugarcane genotypes of semi-arid region of India through antioxidant activity and differential expression pattern analysis of aquaporin genes under different water deficit conditions.

Materials And Methods

Plant materials and experimental conditions

Two tolerant (Co 98014, Co 0118), and two sensitive (CoJ 85, Co 89003) sugarcane genotypes of semi-arid region of India that have contradictory response to water stress (Kumar et al. 2019) were chosen under this study. Experimental trial was conducted under rain out shelter, field laboratory, Department of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P., India and

qRT-PCR was performed at DUVASU, Mathura, India. Each genotype was planted in plastic pots (39 × 33 cm²) containing 25.0 kg of soil and 5.0 kg farm yard manure (FYM) in completely randomized design. Healthy and infection free sugarcane setts (2 budded) were prepared and treated prior to planting with 0.2 percent mixture of mancozeb + carbendazim for 10 min followed by chlorpyrifos 25 EC for 5 min. The soil pH, electrical conductivity and organic carbon were 7.2, 1.3 dSm⁻¹ and 4.3 g/kg. Particle size distribution (%) was 58 (sand), 25 (silt) and 22 (clay). Three setts were planted in each pot with three biological replications. After 60 days of planting, thinning was done by leaving only three plants in each pot. Moisture of soil contents was determined as per gravimetric method. Soil moisture content obtained was 23.9% (0–20 cm soil depth) at the time of sowing and it was 18.1%, 5.9% (0-20 cm soil depth) on 135th and 150th day, respectively. The similar management inputs like fertilizer and insecticides for proper growth and disease control were provided. On 120th day, samples were collected and marked as control, after that plants were imposed to water withholding. Next sampling was done on 135th and 150th day after imposing water deficit. The third expanded leaf from top was collected as sample in aseptic manner by minimizing activity of ribonucleases at 09:00 h, frozen in LN₂ and immediately stored at -80 °C. The samples were collected in two replications, one for analysis of antioxidant activity and another one for AQPs expression.

Climatic variables

The meteorological observations during experimental period were recorded by an automatic weather station of Indian Institute of Farming System and Research (IIFSR), Modipuram, Meerut, India. The observation recorded were min and max temp, percent relative humidity in morning and evening, average rainfall and bright sunshine for Jan-2018 to June-2018 (Fig. 1).

Antioxidant analysis

Malondialdehyde (MDA) assay was done by the measuring thio-barbituric acid reactive substance (Heath and Packer 1968) and expressed as μM MDA/g FW using extinction coefficient of 155 mM⁻¹cm⁻¹. H₂O₂ content was measured by the peroxidase coupled assay according to Veljovic-Jovanovic et al. (2002). Superoxide dismutase (SOD) measurement was performed by nitroblue tetrazolium (NBT) method of Beyer and Fridovich (1987) and expressed as units/mg protein.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIZOL reagent (Invitrogen, USA) with some modifications. The 200 mg of leaf sample was crushed in LN₂ and 1.5 ml of TRIZOL was added and transferred to RNase free tube. Then, 30 μl β-mercaptoethanol (BME) and 40 μl dithiothreitol (DTT) was added and mixed vigorously by pipetting by passing several times through tip of pipette. Samples were incubated for 5 min at room temp by adding chloroform (300 μl) and centrifuged (12000g, 15 min at 4C). Upper aqueous phase was separated carefully without disturbing interphase and precipitated by adding chilled isopropanol (250 μl), 1.5 M NaCl (125 μl), 0.8 M potassium acetate (125 μl) and incubated for 10 min. The samples were

centrifuged (12000g, 10 min, 4°C) and supernatant was decanted. RNA pellet was washed twice with 1 ml of 75% ethanol and pellet was air dried and dissolved in 75 µl nuclease free water (NFW). Residual genomic DNA contamination was removed using DNase I (GeNei, Bangalore) as per the manufacturer instructions. The concentration of RNA was determined using Biophotometer (Eppendorf, Germany). The integrity of RNA was analyzed using 0.08% agarose gel electrophoresis. A total of 36 samples of RNA were isolated and reverse transcribed to cDNA in a final volume of 20 µl using m-mulv RT-PCR kit (GeNei, Bangalore) by taking 1µl oligo dT, 1µl random Hexamer primers and 2 µg of total RNA. The volume was finalized to 12 µl by NFW and incubated at 65 °C for 10min. The tubes were ice chilled and master mix was prepared by taking 10 µl of 5X reaction buffer, 1 µl m-mulv RT, 0.5µl each dNTPs and 1 µl RNase inhibitor. To minimize pipetting error, the master mix was prepared in one PCR tube (0.2 ml) by taking multiple values of required reagents as per the number of samples and mixed. The 8 µl of master mix was mixed to each tube and incubated with 1 µl oligo dT and 1 µl of random hexamer primers. Reaction was carried out in thermal cycler at 37 °C for 1 h followed by 95 °C for 5 minutes. The cDNA was stored at -20 °C.

Primer sequences

Primer for sugarcane AQPs used under study were taken from already published primer sequences (de-Andrade et al. 2016). These sequences were further aligned using PRIMER-BLAST at NCBI for specificity. β -tubulin (Andrade et al. 2015) was amplified as internal reference gene (Table 1).

Quantitative real time PCR (qRT-PCR)

The qRT-PCR was performed with Hi-SYBr Master Mix (MolBio-HIMEDIA) using CFX-96 real-time thermal cycler C-1000 (BIO-RAD) for gene expression in a final volume of 20 µl containing 10 µl of Hi-SYBr Mix (MolBio-HIMEDIA), 1 µl each forward and reverse primer and cDNA template (≤ 100 ng). A total of three biological samples with three technical replicates were used for each gene. The thermal profiles were set with an initial temp 95 °C for 3min followed by 36 cycles of PCR (95 °C for 30 sec, 56.5 °C for ShPIP2;1, 51 °C for ShPIP2;5, 57 °C for ShPIP2;6 and 58.5 °C for b-tubulin for 30 sec with plate read) and 72 °C for 30 sec. The melt curve was obtained between 65 °C to 95 °C with an increment of 0.5 °C per 5 sec and plate read. No template control (NTC) also included. For normalization, b-tubulin was taken as housekeeping gene. The differential expression was calculated by obtaining cycle threshold (Ct) value using the equation $2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen 2001).

Data analysis

Data analysis was estimated using software SPSS (SPSS 20.0 for windows, Chicago, USA). Analysis of Variance (ANOVA) was performed following Tukey's-b multiple range test and significant difference was determined at probability level of 5%.

Results

Meteorological variables

The meteorological observations during experimental period are presented in Fig. 1. The weekly min temperature was 5.9°C whereas max temp was 44.6°C. Total rainfall received was 62.3 mm, relative humidity varies from 61.4 to 96.0% in morning wherein evening time it varied from 17.6 to 68.7% in evening and 167.9 h of bright sunshine (BSS) during entire experimental period.

Lipid peroxidation

Thio-barbituric acid reactive substance (MDA content) underwent an increase under water deficit in case of all genotypes. However, the proportion of its contents was significantly increased in Co 89003 (1.9 fold) and CoJ 85 (1.84 fold) than Co 98014 and Co 0118 (Table, Fig 2a).

H₂O₂ content

A significantly higher ($p < 0.05$) H₂O₂ content was observed in sensitive genotypes (CoJ 85 and Co 89003) during both stress treatments whereas tolerant ones (Co 98014 and Co0118) didn't showed any remarkable changes (Table 2, Fig. 2b).

Superoxide dismutase (SOD)

During water deficit conditions, a dramatic increase in SOD activity was showed in tolerant genotypes as compared to sensitive ones. The genotype Co 98014 was shown with the highest and Co 89003 with the lowest SOD activity (Table 2, Fig 2c).

Expression pattern of AQPs

The relative mRNA transcript of ShPIP2;1, ShPIP2;5 and ShPIP2;6 was increased under water deficit in all genotypes (Fig 3a-3c). ShPIP2;1 exhibited significant change under water deficit conditions in genotype Co 98014, Co 0118 and CoJ 85 whereas genotype Co 89003 didn't show any remarkable change in ShPIP2;1 transcript level. On 135th day, the genotype Co 98014 exhibited a dramatic increase in ShPIP2;1 transcript but it was decreased significantly on 150th day as compared to control (120th day). The genotype Co 0118 showed significantly decreased in ShPIP2;5 expression in both water deficit conditions. A significantly decrease on 135th day and increase on 150th day in transcript abundance of ShPIP2;1 was shown by genotype CoJ 85 when compared to 120th day (Fig. 3a).

A dramatic increase in ShPIP2;5 transcript was observed in water deficit level-II (150th day) in comparison to both normal (120th day) and water deficit level-I (135th day) (Fig. 3b). The transcript level of ShPIP2;5 under water deficit was significantly higher in tolerant genotypes (Co 98014 and Co 0118) than sensitive ones (CoJ 85 and Co 89003). In genotype Co 89003, ShPIP;5 expression didn't show any remarkable changes however, genotype CoJ 85 was observed with significant difference in ShPIP2;5 transcript on 150th day when compared to controlled conditions (120th day).

The ShPIP2;6 expression profile was significantly ($p>0.05$) similar in tolerant genotypes, however Co 98014 showed similar expression on 120th and 135th day whereas, Co 0118 on 120th and 150 day. A significant increase ($p<0.05$) in ShPIP2;6 expression was observed on 135th day as compared to control in genotype Co 0118. However, it was significantly decreased in tolerant (Co 98014 and Co 0118) and increased in sensitive genotypes (CoJ 85 and Co 89003) on 150th day. The sensitive genotypes showed significantly decreased ShPIP2;6 expression on 135th day.

Discussion

Unfavorable circumstances like water deficit induce ROS production that causes oxidative injuries to plant cell. In response to oxidative stress, plant develop its antioxidant defense system to maintain cellular homeostasis (Liu et al. 2011, Kumar et al. 2019). It is known that water deficit is detected early in roots, and subsequently its effects trigger modifications in leaves (Locy et al. 2002).

Malondialdehyde (MDA) is a by-product generated by cell under oxidative stress (Huang et al. 2012). The oxidative degradation of lipids by ROS generated oxidative stress leads to lipid peroxidation and increase in H₂O₂ content (Skorzynska-Polit 2007). Increase in MDA and H₂O₂ content production is the indicator of oxidative damage (Abbas et al. 2014). This suggests a correlation between drought and oxidative stress (Munné-Bosch et al. 2001). Malondialdehyde contents and H₂O₂ content of leaves increased for all the genotypes under water deficit conditions. However, sensitive genotypes (Co 89003 and CoJ 85) showed higher increase in MDA and H₂O₂ contents than tolerant genotypes (Co 98014 and Co 0118), indicating the oxidative stress under water deficit condition (Fig. 2a). Increased MDA content has also been seen in stressed sugarcane (Pagariya et al. 2011), wheat and fababean plants under drought stress conditions (Sultan et al. 2012, Siddiqui et al. 2015). The sugarcane plants under water stress accompanied high level of H₂O₂ content with the increase in lipid peroxidation (Boaretto et al. 2014). Manimekalai et al. (2018) reported 50.5% lesser lipid peroxidation in tolerant varieties as compared to susceptible varieties in sugarcane. To scavenge and detoxify MDA content and H₂O₂ production at intracellular level, antioxidant enzymes are induced to protect the plants under drought stress conditions (Patade et al. 2011, Kumar et al. 2019). Under current study, SOD was significantly increased under water deficit conditions (Fig. 2c). The SOD activity was higher in tolerant genotypes than sensitive ones responding to water deficit stress. It indicated that the genotypes induced oxidative stress and their antioxidant enzymes caused to detoxify cells. The results under study are similar to previous reports in sugarcane (dos-Santos and Silva 2015), wheat (Ekmekci and Terzioglu 2005).

Aquaporins are integral membrane proteins, present in all plant organs/tissues and participate in transfer of water and solutes. Plasma membrane integral proteins (PIPs) are highly influenced by environmental constraints like water deficit or drought (Maurel et al. 2008). Gene expression study through real-time quantitative PCR had been successfully used to identify stress-induced genes in barley (Maraschin et al. 2006), transposable elements in sugarcane and regulatory pathways of stress tolerance in *M. truncatula* (Merchan et al. 2007). The response to water deficit is detected early in roots and leaves (Locy et al.

2002). Drought stress affected the expression pattern of PIP2 proteins in plants leaves of rice (Li et al. 2008, Sakurai et al. 2005) and maize (Zelazny et al. 2009). Difference in AQPs expression was also showed in tolerant and sensitive rice (Lian et al. 2006). Expression pattern of PIP Aquaporins vary with the level of stress, species and isoform (Galmes et al. 2007). The AQPs expression pattern under study revealed upregulation of ShPIP2;5, and down-regulation of ShPIP2;1 and ShPIP2;6 when plants were imposed to water deficit conditions. Findings of study are in harmony with earlier researchers reports (Silva et al. 2013, de Andrade et al. 2016). HT-SuperSAGE libraries analysis of two bulks under drought showed divergent AQPs expression in tolerant and sensitive sugarcane genotypes indicated AQPs expression is genotype specific (Silva et al. 2013). Cultivars of same species also respond differently to drought as per their tolerance ability (Heinen et al. 2009). Jang et al. (2004) also reported AtPIP2;1 and AtPIP2;5 were up-regulated whereas AtPIP2;6 was down-regulated in leaves of *Arabidopsis thaliana* under 250 mM mannitol stress. The tolerant genotypes showed higher antioxidant enzyme activity were comparatively more upregulated the ShPIP2;5 expression during water deficit genotypes than sensitive ones suggesting ShPIP2;5 gene may possess a possible mechanism for plant to direct the water flux for specific tissues, organs or cells that are vital for plant survival during water deficit. On the other hand, down regulation of some AQPs might be necessary to stop the aquaporin synthesis at low moisture availability in soil to minimize plant water loss as well as maintain turgor pressure in leaves (Almeida-Rodriguez et al. 2010, Afzal et al. 2016).

Conclusion

The study discriminated wide variations in antioxidant responses. Differential expression pattern of AQPs reveals that PIP2 was responsive to water deficit conditions. The ShPIP2;5 upregulation suggested its role in maintenance of cell water status under water deficit as these proteins affects water transport. However, further protein profiling-based studies need to be employed in other crops to explore the role of these proteins.

Declarations

Author contribution

Conceptualization and Data curation, D.K.; Formal analysis and Investigation, D.K. and N.M.; Writing – original draft, D.K. All authors read and approved the manuscript.

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Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Tables

Table 1: Gene transcripts and their primer sequences

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ShPIP2;1	TCGGTCGCTCTTGTTCAG	GCAGAGAAGCAGCAGGAGAC
ShPIP2;5	CTGACCAAGTGGTCGCTGTA	GTGTCCGTCTGGTGCTTGTA
ShPIP2;6	TGAACGGAGAAGGAGACCAC	CAACACACGCACACCATAACA
b-tubulin	GGAGGAGTACCCTGACAGAATGA	CAGTATCGGAAACCTTTGGTGAT

Table 2. Changes in SOD activity, MDA and H₂O₂ content under water deficit in comparison to control condition (120th day).

Sugarcane genotypes	SOD		Lipid Peroxidation (MDA)		H ₂ O ₂ Content	
	% Changes		Fold changes		% Changes	
	135 th day	150 th day	135 th day	150 th day	135 th day	150 th day
Co 98014	136.9	187.7	0.4	0.7	39.4	61.4
Co 0118	196.5	233.5	0.6	0.7	42.3	61.2
CoJ 85	80.3	137.2	1.4	1.8	100.2	145.9
Co 89003	66.3	114.7	1.5	1.9	117.5	160.1

Figures

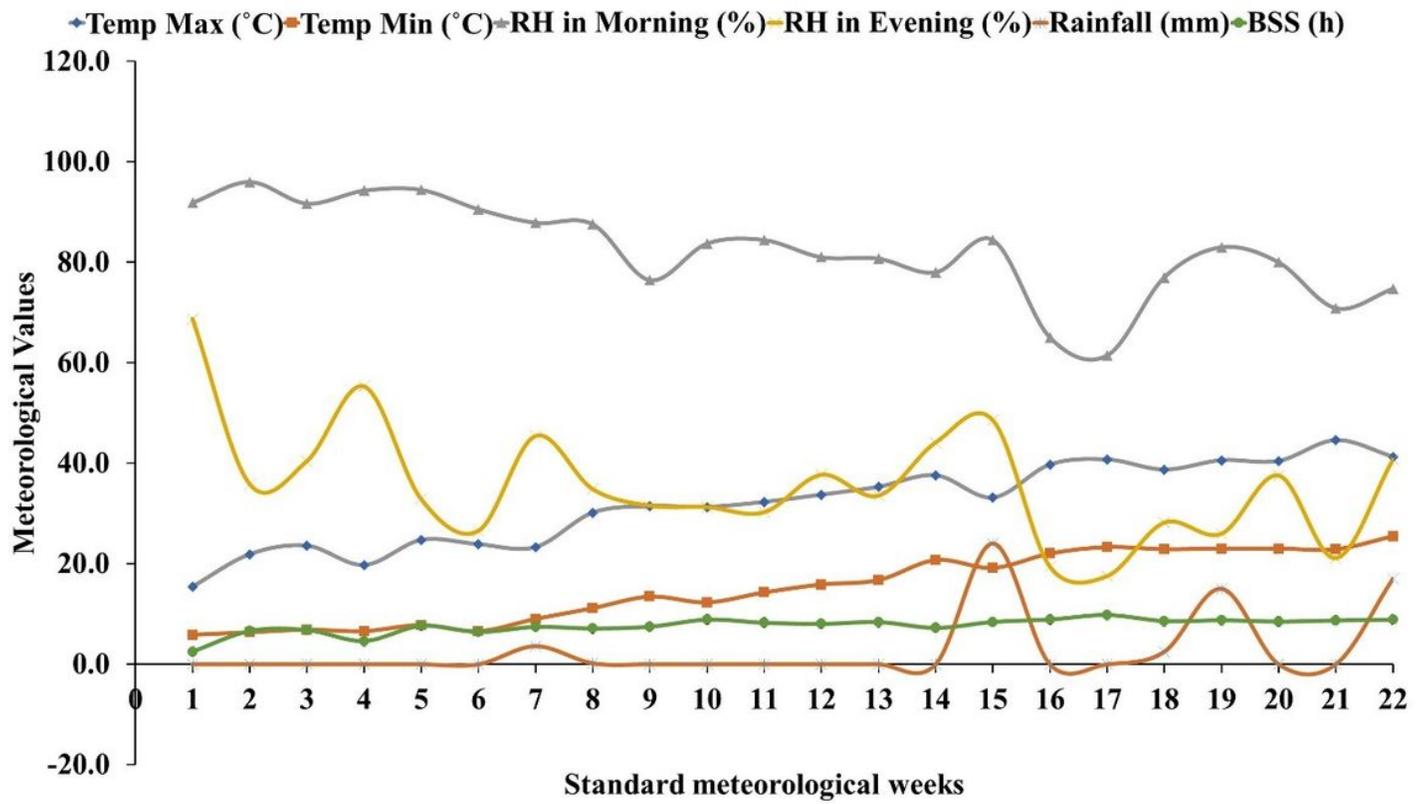


Figure 1

Meteorological observations recorded during experimental period. Note- Temp: Temperature, Max: Maximum, Min: Minimum, RH: Relative Humidity, BSS: Bright Sun Shine

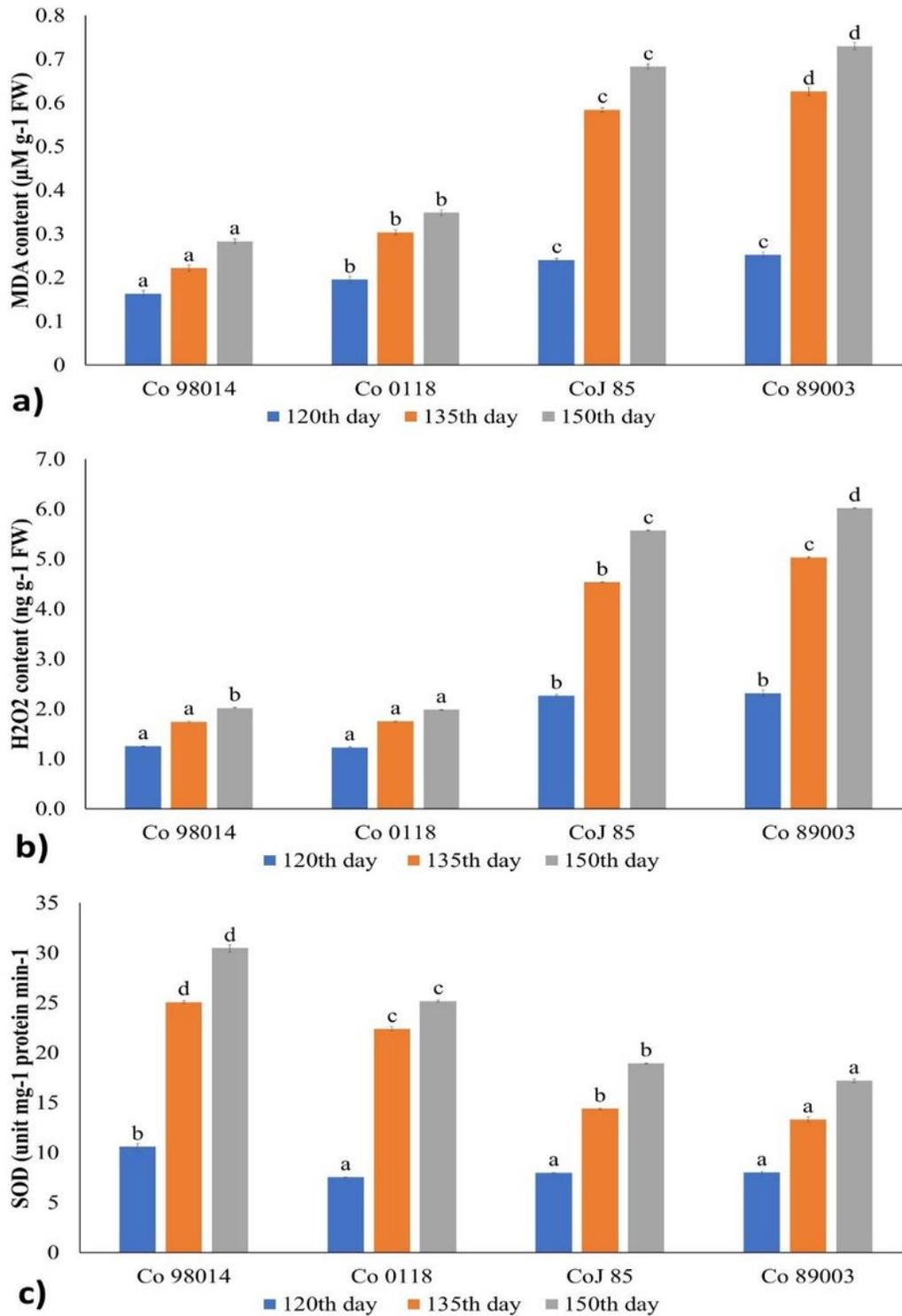


Figure 2

Observations for Lipid peroxidation (MDA content) a), H₂O₂ content b), and Superoxide dismutase (SOD) c) in sugarcane genotypes. Bars represent mean value and error bars indicated the standard error ($\pm\text{SE}$). The different alphabetical letters upon error bars differ significantly ($p < 0.05$).

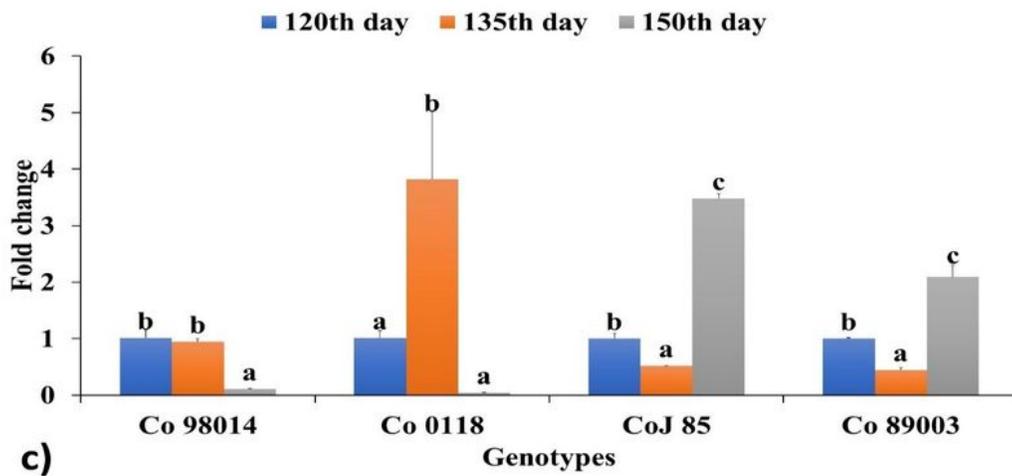
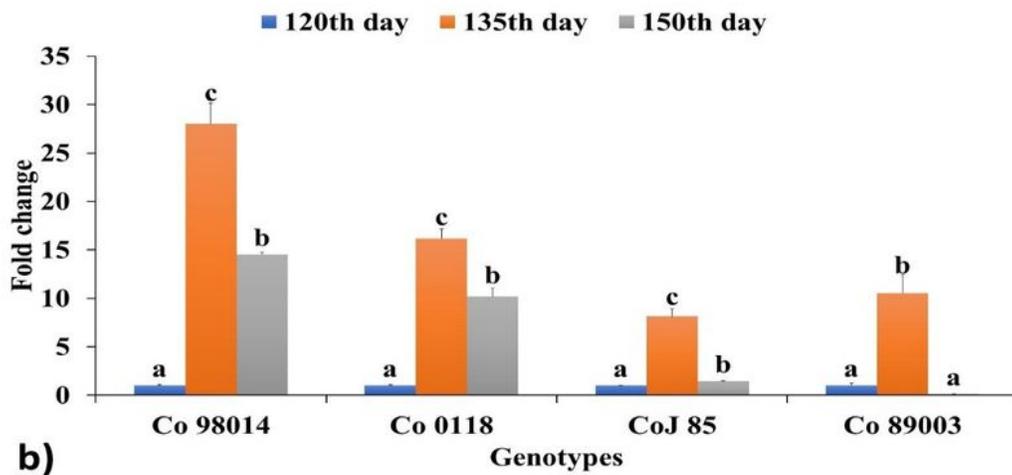
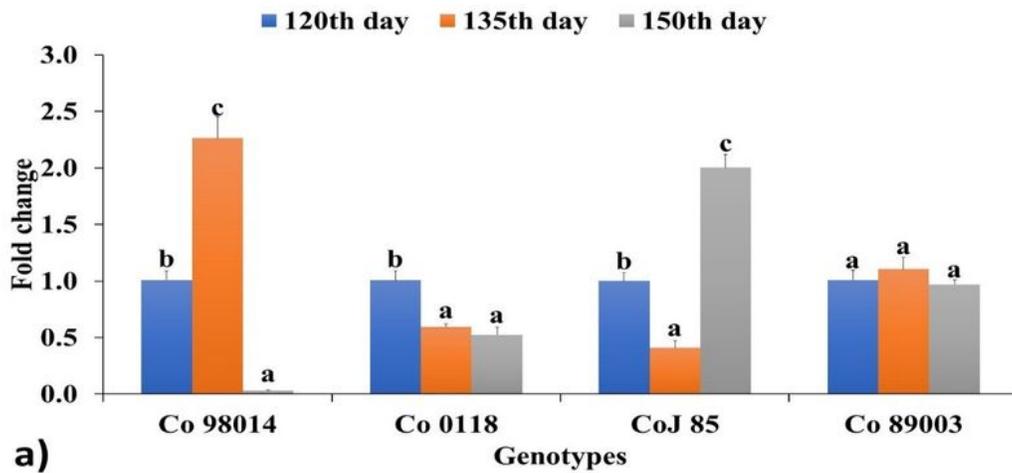


Figure 3

Fold change in expression pattern of ShPIP2;1 a), ShPIP2;5 b), ShPIP2;6 c) gene. Bars represent mean transcript value and error bars indicated the standard error (\pm SE). The different alphabetical letters upon error bars differ significantly ($p < 0.05$).