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Quantitation of differential expression of transcription factors under moisture deficit stress in sugarcane

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Moisture deficit stress, one of the abiotic stresses, affects sugarcane growth and development and reduces cane and sugar yields. Transcription Factors (TFs) are master regulatory proteins in all living cells which have the capability of activating or repressing transcription of stress responsive genes in order to activate the stress tolerance mechanism. Study of expression profiles of TF genes which regulate the expression of stress responsive genes help to elucidate the regulatory biology of stress tolerance. Expression of 17 sugarcane TF genes in moisture deficit stress sensitive and tolerant varieties under different moisture deficit stress conditions were quantified in quantitative real-time PCR. Expression of seven TF genes namely, WRKY, NAC, bZIP, DREB, G2 like, Homeobox and TUB showed significant difference between the stress tolerant and susceptible varieties under both moderate and severe moisture deficit stress conditions. In stress tolerant variety, of these seven TF genes, bZIP showed highest expression both under moderate (22.39 fold) and severe stress (13.45 fold) conditions than other TF genes. Expression of bZIP gene in moisture deficit stress susceptible variety was significantly low under moderate (1.09 fold) and severe (3.63 fold) moisture deficit stress condition. GRAS TF gene under moderate stress condition (4 fold) and Homeobox gene under severe stress condition (6.06 fold) showed highest expressions than other TF genes in moisture stress susceptible variety. These differentially expressed TFs among the moisture stress tolerant and sensitive varieties hold promise for improving abiotic stress tolerance in sugarcane through their use as the potential candidate genes in marker assisted selection and in genetic transformation.

Key words: Transcription factors, moisture deficit stress, qRT- PCR, sugarcane.

INTRODUCTION

Sugarcane is, an important industrial crop, used primarily for production of sugar and ethanol. It is being cultivated in more than 100 tropical and subtropical countries (Waclawovsky et al., 2010). Sugarcane is a highly water demanding crop and significant extent of existing growing areas cannot meet the water requirement during the most

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critical water demanding formative growth stage of the plant (Naidu, 1978; Ramesh, 2000). Supplementary irrigation is not a favorable option due to the added cost component which adversely affects the profit margin of the farmers. Hence, development of moisture deficit stress tolerant varieties either through conventional breeding or genetic manipulation is the most sustainable solution (Moore, 1987; Jain and Chattopadhyay, 2010) to mitigate the adverse effects comes from abiotic stresses.

Breeding for moisture deficit stress tolerance in sugarcane is complicated due to both the polyploid nature of the crop and the complexity of the trait (Swapna and Hemaprabha, 2010). Recent advancements in molecular tools help in identification of genetic factors involved in the responses of plants to moisture deficit stress. The available data indicate that moisture deficit stress tolerance is a complex physio-chemical process, in which many biological macro and micro molecules such as nucleic acids (DNA, RNA, microRNA), proteins, carbohydrates, lipids, hormones, ions, free radicals and mineral elements are involved (Bayoumi et al., 2008). Identification of genes which can regulate multiple biochemical and development pathways related to moisture deficit stress tolerance could be the best candidates to improve the performances of crops during moistures deficit stress. Among the stress responsive genes, TF genes are master regulatory proteins in all living cells. They often exhibit sequence specific DNA binding and are capable of activating or repressing transcription of multiple genes (Latchman, 2003). Interactions of TFs and *cis* elements in the promoter regions of stress responsive genes up-regulate the expression of many downstream genes and activate the stress tolerance mechanism (Agarwal and Jha, 2010). Hence, the ability of TFs to regulate the multiple biochemical and development pathways related to moisture deficit stress tolerance can be exploited to alter the performances of sugarcane during moisture deficit stress conditions. Information on the TFs which show differential expression under different moisture deficit stress conditions and quantification of the expression of TFs in susceptible and tolerant varieties are the prerequisites for understanding the regulatory biology of stress perception and modulation. This would help in identification of candidate genes which can be considered to target breeding for improved moisture deficit stress tolerance in sugarcane.

Hence, the present experiment was designed to identify the differentially expressed TFs and study the differences in their levels of expression under different moisture deficit stress conditions in sugarcane genotypes reported as moisture stress tolerant and sensitive.

MATERIALS AND METHODS

Two sugarcane varieties reported as moisture stress tolerant (Co 94008) (Gomathi and Vasantha, 2010) and moisture stress sensitive (Co 775) (Hemaprabha and Swapna, 2012; Manel and Sumangala, 2017) were grown in pots filled with sterilized sand (single bud setts per pot) under greenhouse condition without light or temperature control. The experiment was arranged in a completely randomized design, with six biological replications for unstressed and stressed plants. All plantlets were maintained under same growth condition and equally watered (250 ml per pot) in alternate days with 25% of Hoagland solution per pot (Hoagland and Arnon, 1950). After 2 months of growth, four replications of each variety were subjected to moisture deficit stress by withholding the irrigation (stressed) and rest of the plants were regularly irrigated (unstressed). The water content of the sand was measured by gravimetric methods to observe the moisture reduction during the experimental treatment (Black, 1965). Root samples of two biological replicates of stressed and unstressed plants were collected separately 8 (moderate stress) and 10 days (severe stress) after withholding the irrigation (Rodrigues et al., 2009) and stored at -80°C.

Preparation of cDNA

Total RNA was isolated from stressed and unstressed root samples of each biological replicates separately using TRIzol reagent according to the instructions given by manufacture (SIGMA-ALDRICH PVT. LTD.USA). The quantity and quality of total RNA were checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and 1% formaldehyde Agarose gel electrophoresis. Single stranded cDNA was prepared by using High Capacity cDNA Reverse Transcription kit (cat#4374966, Ambion, USA) as per the protocol given by manufacturer.

The sequences of available forty-one sugarcane transcription factor families (http://grassius.org/browse family.html/species=Sugarcane) were selected for quantification of expression. The similarity portion of the sequence of the members of TF families were identified and targeted for designing the primer pairs. The Primer3plus software (http://frodo.wi.mit.edu/cgi-bin/primer3plus/primer3plus_www.cgi) was used for designing the primer pairs and they were synthesized at Sigma-Aldrich Pvt. Ltd. (Germany) after confirming the specificity by BLAST searching them against SUEST database.

qRT-PCR and data analysis

Genomic DNA of sugarcane varieties, Co 94008 and Co 775 were used as the template and Polymerase Chain Reactions (PCR) were performed to confirm the applicability of the primers.

The Eppendrof master cycler realplex machine and its default program (95°C for 10 min, followed by 40 amplification cycles, 95°C for 15 s, 59-62°C for 20 s and 68°C for 20 s) were employed in 10 µl reaction mixture consisting of 1.0 ng cDNA, 200nM of each gene-specific primer and 5 µl of 2x SYBR green reagents (Cat.#4368706, Ambion, USA). Expression levels of TFs were quantified using ΔΔCt method (Livak and Schmittgen, 2001). Sugarcane glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GAPDH F: 5' CACGGCCACTGGAAGCA 3' and GAPDH R: 5`TCCTCAGGGTTCCTGATGCC 3') reported as a stable gene in moisture stress studies (Ling et al., 2014) was used as the reference gene for normalization. Two each of technical and biological replications from each treatment were used to avoid the handling errors and to confirm the reproducibility of the results. Significant differences in gene quantitation between the stress and unstressed conditions and genotypes were analyzed on the basis of T-test sat $\alpha = 0.05$ using Microsoft Excel program.

RESULTS AND DISCUSSION

Phenotypic changes such as leaf rolling, wilting etc.

Table 1. Details of specific primers used for detection of transcription factors and amplicon size.

TF name	Forward primer sequence 5` - 3`	Reverse primer sequence 5` - 3`	Tm (°C)	Expected amplicon size (bp)	Observed amplicon size (bp)
ALFIn like	GCCTCTGGTTGTCATATGTCTT	AAGGCCAAGAGGTTCCATTT	51.4	120	100 - 200
AP2 EREBD	TCAAGCAGCAGCAGAGGTAA	TCGGTGCTCATCTCCTTCTT	51.8	121	100 - 200
ARF	TGGGGAGTTACGTGTTGGAG	TGCAAGAACACCAAGATGCA	51.7	110	100
ARR-B	CCCAGCTTTGACCTCCCTG	CTCCTCGACGGTCATCTCC	55.4	191	100 - 200
BBR- BPC	CACTGGTGTTGGTATGGTGG	ACCAAAGCCTAAGAAGCCTAAG	53.0	159	100 - 200
bZip	CAATGACCCTAGCAGACCCT	GTGGATTTGCAGCAACGGTA	52.8	153	100 – 200
E2F-DP	TCAAAATTCCGCCACACACA	GATGTCCCATACGCCTAGCT	51.7	197	200
G2 –like	ATTCAGAAGTACCGGCTGCA	TGCTCATCCATTTCCGCTTG	51.8	202	200
GRAS	ACTGTTCTGATGGCACCTGA	TCCCTTAGCCGTTTCTCTGG	52.8	209	200
GRF	ATCTCCCCTCCTCCCCTG	CAGGAAGGAGGATTGGGGAC	50.4	172	200
Homeobox	ACATGATCTGGGGCAACTGA	TACAGGCACAATTGGACCCT	51.8	165	100 – 200
MYB	GTTCCCTGCATGCTGAAACA	GGAACATTCACGGACACACC	52.8	233	200 – 300
TCP	GCTCATCCGTAACGCCAAG	CTGCTCGGACGGCTCAGT	54.1	116	100 - 200
TUB	AGATGTCTCGGCATGCTG	ACCTCTCCTCCTGATCCTCC	53.1	108	100
DREB	CACACAATCCAAGGGGCTTC	TGCCTCGTCTCCTTGAACTT	66.6	207	200
NAC	AAGTGAAAAGCTCCCCTCGA	TTTTCCCTCCTCTGGCTCTG	65.0	173	100 – 200
WRKY	GCGGGACCCCAGCTTCAAG	CCACGCCATGTCAAGCCGC	71.4	209	200

undergone by the moisture stress tolerant and sensitive varieties and reduction of the moisture content of sand potting medium during the experimental period were recorded (data not shown). A total of 17 primer pairs mentioned in Table 1 were designed to quantify the expression of TFs and their feasibility was checked in PCR with genomic DNA of the selected sugarcane varieties (Co 94008 and Co 775). Since all the primer pairs showed amplifications with genomic DNA (Plate 1a), their expressions were further quantified in qRT-PCR. Melting curve analyses for each primer pair were performed and confirmed the occurrence of specific amplification peaks and the absence of primer-dimer formation. Further, specificity of amplicons were confirmed by loading the qRT-PCR products on 4% Agarose gel and all the selected TF genes showed the expected size of the amplicons (Plate 1b).

Differential expression of TFs in moisture stress tolerant and susceptible varieties

Differential expressions of TFs probably govern expression of stress-inducible genes either cooperatively or independently, and may constitute gene network in various responses for abiotic stresses (Joshi et al., 2016). Therefore, the TFs might play a role in the differential responses of moisture stress tolerant and susceptible varieties to moisture deficit stress. In this study, significant difference in expression of seven TF genes namely WRKY, NAC, bZIP, DREB, G2 like, Homeobox and TUB were found between tolerant (Co 94008) and sensitive (Co 775) varieties under moderate and severe stress conditions (Table 2).

Among the differentially expressed TFs, bZIP showed the highest expression under moderate (22.39 fold expression) and severe (13.45 fold expression) stress condition. To date, several bZIP TFs have been functionally characterized, including those shown to be responsive to abiotic stress in rice (Lu et al., 2009) and *Arabidopsis* (Uno et al., 2000). For example, over-expression of OsbZIP23 or OsbZIP72 enhances the drought tolerance of rice and these two TFs are involved in ABA-dependent drought signal transduction (Lu et al., 2009). In this study also, higher expression of bZIP were recorded in moisture stress tolerant genotype than the sensitive genotype. The

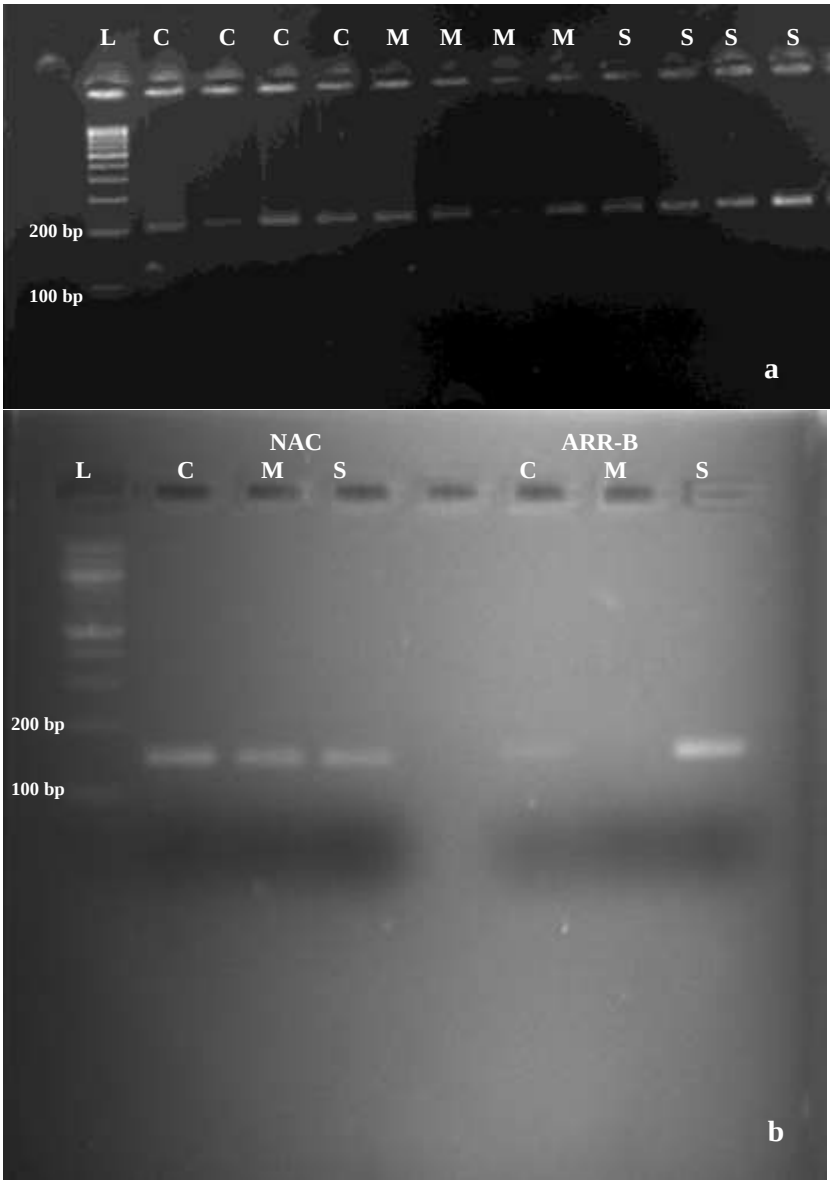

Plate 1. (A) Fractionation of PCR products generated from amplification of genomic DNA with the primers targeted the DREB gene, (B) Fractionation of qRT-PCR products generated from NAC and ARR-B primers on 4% agarose gel. L: Ladder, C: unstress condition, M: moderate stress condition and S: severe stress condition.

expression pattern of miRNA of bZIP factor in sugarcane indicated that tolerant plants adjust their transcriptome to increase the bZIP factor, which may activate the transcription of drought-related genes (Agustina et al., 2013).

Over-expression of the NAC transcription factor family members in *Arabidopsis* showed up regulation of several stress inducible genes in the transgenic plants and significant increase in drought tolerance (Tran et al., 2004). In the present study also, NAC TF gene showed significant up regulation than the unstressed condition in both the sugarcane varieties (Figures 1 and 2). Further, expression of NAC gene was significantly high in moisture stress tolerant variety than susceptible variety.

DREB is a known ABA independent abiotic stress responsive TF that is expressed predominantly in moisture stressed root (Liu et al., 1998; Sakuma et al., 2002). Over expression of DREB gene and enhancement of moisture stress tolerance were reported in *Arabidopsis* (Liu et al., 1998), tobacco (Kasuga et al., 2004), rice (Oh et al., 2005) and potato (Behnam et al., 2006). Up-regulation of DREB TF in moisture stress tolerant

Table 2. Relative changes in the expression of transcription factors under stressed condition in Co 94008 and Co 775 sugarcane varieties

TF Family	Co 94008			Co 775			Calculated T value	
	Fold expression			Fold expression				
	C	M	S	C	M	S	M	S
GRAS	1	4	2	1	4	4	1.14	14.91**
GRF	1	0	0.06	1	0	0.03	UD	3.38*
G2 –like	1	0.19	0.08	1	1.41	1.18	19.58**	12.09**
Homeobox	1	4	5.66	1	3.03	6.06	134.35**	6.44**
TUB	1	1	0.86	1	0.38	0.47	8.05**	6.97**
WRKY	1	1.4	0.68	1	3.4	1.01	135.19**	14.95**
NAC	1	4.12	1.94	1	1.43	1.33	66.03**	32.06**
bZIP	1	22.39	13.45	1	1.09	3.63	81.80**	211.31**
DREB	1	3.35	1.68	1	1.39	0.39	55.96**	56.08**

Table t value (5%, df: 3) = 3.18*, Table t value (1%, df: 3) = 5.84**
Where; UD: Transcripts undetectable, C: Unstress condition, M: Moderate stress condition and S: Severe stress condition.

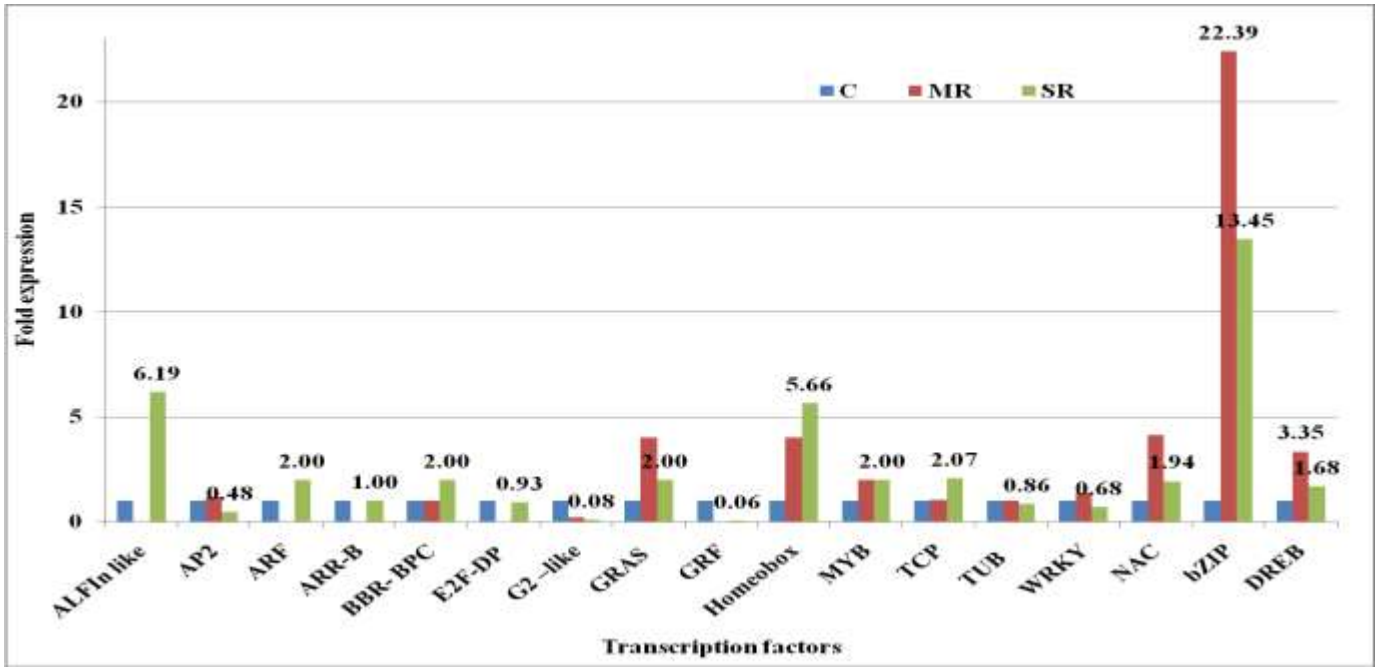

Figure 1. Real time PCR data for expression of transcription factors under stressed and control condition in sugarcane variety Co 94008, where: C: unstress condition, MR: moderate stress condition and SR: severe stress condition.

sugarcane varieties than sensitive was observed by Agustina et al. (2013) and the expression patterns of DREB in this experiment were also in accordance with the earlier reports. In this study also, DREB TF gene showed differential expression between sensitive and tolerant varieties under moisture deficit stress condition. Under moderate stress condition, it showed 3.35 fold up regulation in tolerant variety and 1.39 in sensitive variety. Though, this gene was down regulated under severe stress condition compared to moderate stress condition in both varieties, expression in moisture stress tolerant variety Co 94008 showed higher expression than the sensitive variety. Hence, this gene may also contribute to moisture stress tolerance in Co 94008 variety.

Homeobox, a family of TFs are found only in plants and its over-expression increases tolerance to water stress (Dezar et al., 2005). Higher levels of expression of Homeobox were observed in tolerant genotype than sensitive genotype under moisture stress (Agustina et al., 2013). In the present study, up regulation of expressions

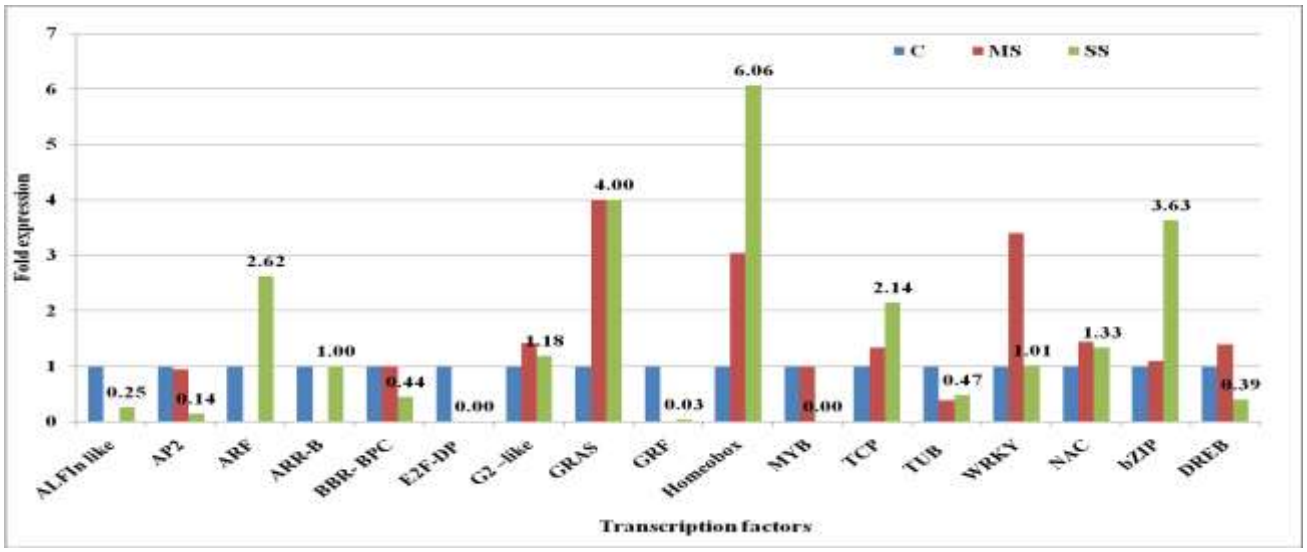

Figure 2. Real time PCR data for expression of transcription factors under stressed and control condition in sugarcane variety Co775, where: C: unstress condition, MS: moderate stress condition and SS: severe stress condition

of Homeobox TF was recorded for both the varieties under stress conditions and its expression were significantly different between the tolerant and sensitive variety (Table 2). Hence, Homeobox may also play a role in enhancing moisture stress tolerance in tolerant variety.

Several WRKY proteins were shown to be involved in plant drought and salinity stress responses in various species such as rice, tobacco and *Arabidopsis* (Golldack et al., 2011; Wu et al., 2009; Qiu and Yu 2009; Song et al., 2010). As an example, over-expression of OsWRKY11 under the control of HSP101 promoter led to enhanced drought tolerance, as shown by the slower leaf-wilting and increased survival rate of green plant parts (Wu et al., 2009). Further, previous research had demonstrated that WRKY proteins may act as activators or repressors of ABA stress hormone which plays essential role in plant responses to abiotic stress signaling (Chen et al., 2011). Ren et al. (2010) reported that over-expression of some WRKY proteins do not result in drought tolerance, thus they may need either co-factors or some posttranslational modifications to activate the downstream genes for stress tolerance. Recently, two research groups (Shang et al., 2010; Chen et al., 2010) reported the function of a group of structurally related WRKY proteins, in ABA signaling. Shang et al. (2010) showed that some WRKY proteins (WRKY40 in *Arabidopsis*) act as a central negative regulator among the WRKY proteins and could directly inhibit the expression of several important ABA responsive genes such as ABF, ABI, DREB, MYB and RAB, by directly binding to the W-Box sequences upstream of their promoters. Some WRKY genes (WRKY18 and WRKY60 in *Arabidopsis*) have a positive effect on plant ABA sensitivity and increase plant sensitivity to abiotic stresses (Chen et al., 2010). In this experiment, WRKY gene showed the lower expression in tolerant variety (1.40 fold under moderate and 0.68 fold under severe stress) and higher in sensitive variety (3.40 fold under moderate and 1.01 fold under severe stress). Hence, it may enhance the sensitivity to moisture stress by playing the main role in ABA signaling.

Over-expression of the members of MYB TF family in different plant species showed increased tolerance to different abiotic stresses such as drought, chilling and freezing (Vannini et al., 2004; 2007; Pasquali et al., 2008). In the present study, MYB gene showed higher expression in tolerant genotype than sensitive genotype. MYB may act as enhancer of moisture stress tolerance.

Further, expression of GRAS and GRF TF genes significantly differed between susceptible and tolerant varieties only under severe stress condition. The GRAS gene showed same level of expression in both varieties under moderate stress conditions (4 fold) and it was significantly low in Co 94008 (2 fold expression) than Co 775 (4 fold expression). These GRAS TFs are known to play a crucial role in diverse plant growth and development, ranging from gibberellic acid signaling, root radial patterning, light signal transduction and axillary shoot meristem formation (Hirsch and Oldroyd, 2009). Despite their important regulatory roles in *Arabidopsis*, the biological properties of GRAS members are largely unknown. One of GRAS TFs namely, OsGRAS23, has been identified in rice that is involved in drought stress response through regulating expression of stress-responsive genes (Xu et al., 2015). The functions of a number of identified GRAS genes and their role in moisture stress tolerance have not been characterized.

Expression of GRF TF gene under severe stress

condition was significantly low in Co 775 (0.03) than moisture stress tolerant Co 94008 (0.06) variety. The GRF transcription factors are involved in cell proliferation and strongly expressed in actively growing and developing tissues, such as shoot tips, flower buds, and roots, but weakly in mature stem and leaf tissues (Kim et al., 2003). Shunwu et al. (2012) reported that GRF TF was expressed under drought condition in rice. However, the functions of GRF genes related to drought resistance are unknown. Further studies on the functions of GRF genes will make obvious the role of this transcription factor in moisture stress tolerance.

Of the TFs which show significant differential expression between drought susceptible and tolerant varieties, expression of Homeobox showed increase with prolonged moisture deficit stress. Five TFs namely, GRAS, WRKY, NAC, bZIP and DREB showed up regulation of expression under moderate moisture deficit stress and down regulation under severe stress condition in tolerant variety Co 94008. It is presumed that the genes expressed during the course of gradual stress in tolerant species are responsible for altering the cellular metabolism, leading to adaptation under severe stress (Govind et al., 2009). These TFs genes may also provide the necessary induction to the plant to adapt and survive under severe stress.

Conclusion

Roots are the primary site of perception and injury, for several types of water limiting stresses including salinity and drought, in many circumstances; it is the stress sensitivity of the root that limits the productivity of the entire plant (Atkin et al., 1973; Steppuhn and Raney, 2005). Further, TFs are regulators of transcription and have the potential for coordinated regulation of genes relevant to stress tolerance (Xiong and Zhu, 2002). In this study, the bZIP TF gene out of the identified TFs: WRKY, NAC, bZIP, DREB, G2 like, Homeobox and TUB, reported from the analysis of Co 94008 and Co 775 varieties, may play an important role in moisture deficit stress tolerance in sugarcane. This information will be a valuable starting point for further research on these genes to check their potential as candidate genes to use as the targeted genes in moisture stress tolerance breeding programs. Further, the information generated may aid in isolation of most specific regulatory TFs and their promoters in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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