IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN(P): 2347-4580; ISSN(E): 2321-8851
Vol. 5, Issue 1, Jan 2017, 99-104

DIFFERENTIAL GENE EXPRESSION IN FOXTAIL MILLET (*Setaria italica)* UNDER WATER STRESS

DIPTI GAWAI, M.P.MOHARIL, P.V.JADHAV & S.J.GAHUKAR

Biotechnology Centre, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

ABSTRACT

Foxtail millet (*Setaria italica* L.) is an important food and fodder grain crop in arid and semi-arid regions of Asia and Africa. Being an elite drought-tolerant crop, foxtail millet is thought to be an excellent experimental model in studying abiotic stress tolerance system. In recent years, semi-quantitative RT-PCR has emerged as a versatile technique in transcriptomics, as it can generate rapid measurement of mRNA levels in minimal tissue samples. The present study deals with semi-quantitative RT-PCR of gene specific markers for drought stress viz. DREB1 and DREB2 in two contrasting foxtail millet accessions IC97109 and IC97189. Based on the IDV values, the relative upregulation of DREB2 due to dehydration stress in tolerant accession was found to be 7.86 % whereas, DREB1 showed the relative upregulation of about 18.67% respectively. DREB1 and DREB2 showed higher expression in both unstressed and stressed condition in tolerant accession, IC97189. Analysis on the expression profiling of these genes revealed that the expression of genes was upregulated by water stress treatment, suggesting their role in drought tolerance of *Setaria italica* L.

KEYWORDS: Differential Expression Profiling, Semi-Quantitative RT-PCR, *Setaria italica,* Abiotic Stress

INDRODUCTION

Foxtail millet (*Setaria italica* L. Beauv) is one of the oldest cultivated millet crops serving as food grain in Asia and as forage/fodder in America, Australia and Africa ranking second in the world's total production of millets after pearl millet. India ranks second after China in the world in small millet production with Tamil Nadu and Andhra Pradesh as the leading producers. It is an important food and fodder grain crop in arid and semi-arid regions of Asia and Africa. Being an elite drought-tolerant crop and the attributes like small genome (1 C ~ 515 Mb; $2n = 2x = 18$), low amount of repetitive DNA, a highly conserved genome structure relative to the ancestral grass lineage, inbreeding nature and short life cycle, makes foxtail millet an excellent experimental model in studying abiotic stress tolerance (Devos et al., 1998; Jayaraman et al., 2008; Doust et al., 2009; Li and Brutnell, 2011; Zhang et al., 2012, Lata et al., 2013).

The adaptation of foxtail millet to low water conditions has been ascribed to its relatively small leaf area, the cell arrangement in its epidermis, its thick cell walls, and its ability to form a dense root system (Li, 1997). Also, the water use efficiency of foxtail millet has been shown to be higher than that of maize, wheat, and sorghum (Gu et al., 1987). However, the molecular mechanism underneath of its drought adaptation is still not clear. Understanding drought tolerance at molecular level particularly identification of relevant genes in foxtail millet is likely to pave the ways for mitigating the drought stress losses in major crops. An extensive germplasm collection of the crop is available, providing opportunities to study the various biological processes and to fetch out the molecular mechanism underneath its tolerance. The biological differences among the genotypes used, plant growth conditions, stress treatment conditions and their detection

methodologies may result in variation in extent of stress adaptive mechanism. The tolerant species may express some novel stress responsive genes. Hence, comparison of gene expression profiles between contrasting genotypes can provide much information in understanding the spatial and temporal patterns of gene expression required for abiotic stress tolerance**.** The identification, manipulation and comprehension of gene expression patterns would play an important part in unlocking the mysteries of drought responses and adaptation.

First report about analysis of differentially expressed transcripts (early- and late- induced) in foxtail millet cv. Prasad after dehydration stress was given by Lata et al. The previously reported as well as unknown genes suggests their function in possible regulation of dehydration adaptation in this crop (Lata et al., 2010). Various unknown genes in response to abiotic stress have been reported in foxtail millet. Zhang et al. (2012) identified 586 genes that were predicted to have roles in stress responses. Qi et al. (2013) analyzed the whole transcriptome of foxtail millet by using the next generation deep sequencing technology and identified a total of 2,824 genes with drought-responsive expression patterns (Qi et al., 2013). Differentially expressed signaling pathways of up-regulated genes in foxtail millet were studied and significantly up-regulated genes were identified in Yugu-1 in response to rust in foxtail millet (Li et al., 2015).

To gain a better understanding of the molecular responses of this crop to dehydration stress, various methods based on transcript profiling can be used for the analysis for differentially expressed genes. In the present study, semi-quantitative evaluation of the relative mRNA accumulation shows upregulation of genes involved in stress tolerance. The genes studied here for the tolerance mechanism showing higher expression can be explored in development of tolerant lines by MAS as a functional marker and can also be used as candidate genes for development of water stress tolerant transgenics in other related crops.

MATERIAL AND METHODS

Plant Material

The experimental material of the present investigation comprised of foxtail millet (*Setaria italica* L.) accessions procured from National Bureau of Plant Genetic Resources, Regional station, Akola. Two contrasting foxtail millet accession IC97189 (tolerant) and IC97109 (susceptible) (collectively based on the physiological and biochemical screening) (Gawai et al, 2013) were planted in triplicate in pots (20 litre capacity) separately. Pots were watered normally (once per day) until the plants attain 21day period. The 21-day-old seedlings were subjected to water with-holding experiments for a period of 9 days. Water treatment served as the control designated as unstressed (US). Seedlings were sampled by harvesting and freezing immediately in liquid nitrogen and stored at -80˚C for RNA isolation.

Total RNA Extraction

Total RNA was isolated using TRIzol (Invitrogen, USA) using a manufacturer's protocol with minor modifications and the concentration was determined using nanophotometer (IMPLEN, Germany). Following extraction, the isolated total RNA was electrophoresed in 1.5% denaturing agarose gel to check for RNA quality and integrity. cDNA was synthesized using First strand cDNA synthesis kit as per manufacturer's instructions and oligo-dT 18 mer primer and stored at -20˚C for further use.

Semi-Quantitative RT-PCR

Gene specific markers for drought stress studied earlier in various crops were utilized which consisted of DREB1

and DREB2. Reference genes for quantifying gene expression and to ensure proper normalization were utilised (Table 1). Semi-quantitative RT-PCR reactions were carried out with 20 µl of the reaction solutions using gene specific primers and α tubulin and EF-1α gene primers as internal controls. The reaction was performed by adding following components in order to sterile thin-walled PCR tubes for each PCR amplification reaction: 12.6µl of RNase-free water, 2µl of 10X PCR Buffer, 1.2µl of 50mM $MgSO_4$, 2.0µl of dNTP mix (2mM each dNTP), 0.5µl of upstream primer (0.1 µg/µl), 0.5µl of downstream primer (0.1 µg/µl), 1µl of experimental first-strand cDNA reaction, 0.2µl of *Taq polymerase* (5U/ul). The annealing temperature for each primer and amplified fragment size is given in Table 1. Amplifications were performed by a cycles of: 5.00 min at 94°C followed by 39 cycles each of 2 min at 94°C, 2 min at 50 -53°C, and 3 min at 72°C, and final extension of 15 min at 72°C .

Table 1: Drought Stress Specific Gene/TFs Primers Used During the Present Investigation

SN	Gene	Sequence		Annealing Temp	Reference
1.	DREB2	Forward	GCCTTGTAGTCATTTGGTGGTTT	51°C	Lata et al., 2010
		Reverse	CTCACAACTCCTTTTCTCAAGCT		
2.	DREB1	Forward	GGAGCAAGCAGAAACACACA	52°C	Nawaz et al., 2014
		Reverse	GCATCGGAAGCCAGAAAAGA		
		Reverse	GCTGGTTTGTCTGGTGGGAT		
3.	EF-1α	Forward	TGACTGTGCTGTCCTCATCA	50°C	Kumar et al., 2013
		Reverse	GTTGCAGCAGCAAATCATCT		
4.	TUB α	Forward	TACCAGCCACCATCTGTTGT	50°C	Kumar et al., 2013
		Reverse	GGTCGAACTTGTGGTCAATG		

Gel Electrophoretic Analysis and Gel Elution

Separation of amplified fragments was carried out using Bio-rad gel electrophoresis assembly. PCR amplification products were analyzed by agarose gel electrophoresis on 1.5 % agarose gel stained with ethidium bromide solution (0.5 µg/ml). The gel was run in 1X TBE buffer at 70-80 Volts for 45 minutes to 1.5 h. Standard ladders of 100bp and 1kb from (Fermentas^{TM}) sizes was used. Gel Doc system (Alpha innotech) was used for further analysis. The computer program AlphaEaseFC (From Alpha Innotech) was used to visualize, score and analyse the results.

Scoring Amplicons

Differential analysis on the basis of number of amplicons (present/absent) as well as differences in amplicon intensities to understand differential expression pattern in stressed and unstressed of tolerant and susceptible foxtail millet accessions was done. For TDFs those were expressed differentially with change in amplicons intensities were analyzed by densitometric analysis. This analysis based on pixel intensities of bands produced intensity derived values (IDVs) in AlphaEaseFC (Genetic technologies Inc.) image processing software. The relative intensity of amplified fragments provided basis for quantification of level of expression of gene as high, moderate, low and negligible. Generated values were used to compare intensity of gene expression in further studies.

Gel Elution, Sequence Characterization and Bioinformatics Studies

Gel elution of amplicons of interest was performed using Qiagen gel elution kit as per the manufactures protocol. Sequencing was done through GeneOmBiotech, Pune, India. The sequences were accessed using Bioedit software. The sequences synthesized using reverse primers were converted in reverse complement using Bioedit software. NCBI Blast tool was used for homology searches. Pair wise alignment was done using Clustalw sequence alignment tool

available at EBI (European Bioinformatics Institute) to align the recovered sequences with the sequences used for primer designing.

RESULT AND DISCUSSIONS

Even though the drought-tolerance capacity of foxtail millet is ascribed to its cellular and morphological characteristics (Li, 1997), there have been very less study on differentially expressed genes that impart drought tolerance to foxtail millet. Semi-quantitative RT-PCR has emerged as a versatile technique in transcriptomics, as it can generate rapid measurement of mRNA levels in minimal tissue samples. Differential gene expression pattern was studied by using candidate genes involved in drought tolerance reported earlier in other related crops. Strikingly, genes that were significantly upregulated under drought stress showed much higher expression in tolerant than in susceptible accession.

As the initial step in expression studies is isolation of good quality RNA, the RNA extracted in this study met the quality requirement for semi-quantitative RT-PCR analysis based on the agarose gel electrophoresis and alpha imager analysis. Amplification products presented in all reactions indicated that the reaction conditions adopted here were suitable for the analysis of differential expression in foxtail millet. There were neither primer-dimers nor the occurrence of non-specific amplifications. To confirm the normalisation of cDNA, appropriate internal control gene like α tubulin and EF1α were used which showed constant expression in unstressed and stressed condition and hence the normalization were found to be accurate.

In the present study, semi-quantitative evaluation of the relative mRNA accumulation of two genes namely, DREB2 and DREB1 induced by dehydration stress was performed. Tolerant accessions showed higher expression levels of drought responsive genes/TFs in stressed conditions. The gene studied here was DREB i.e. Dehydration Responsive Elements which have been reported to be involved in various types of abiotic stress responses via ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 1997). The DREB transcription factor family is one of the largest and is broadly divided into DREB1 and DREB2 sub families and each sub family contain several paralogs. In the present study, the DREB2 primer amplified a fragment of 215 bp, whereas, DREB1 amplified a fragment of 500 bp in the selected contrasting accessions in unstressed and stressed conditions. RT-PCR analysis showed an upregulation of transcript in stressed condition in both the accessions (Figure 1). Based on the IDV values, qRT-PCR analysis of DREB2 showed the relative upregulation of about 7.86 % due to dehydration stress in IC97189. Whereas, DREB1 showed the relative upregulation of about 11.80% (Figure 1). Both DREB1 and DREB2 showed higher expression in both unstressed and stressed condition in tolerant accession, IC97189. The up-regulation of these transcripts in the tolerant cultivar clearly suggests their role in providing dehydration stress tolerance to the selected accession.

Hence induction of these transcripts suggests that these genes might impart drought avoidance capacity to the tolerant accession in comparison to the sensitive one, as the expression analysis of up-regulated transcripts between drought tolerant and susceptible cultivars upon dehydration stress suggests their function in dehydration adaptation and tolerance in foxtail millet (Lata et al., 2010).

Sequence characterisation of the drought responsible genes was done to confirm that the results of amplification are not contaminated with foreign DNA. For DREB2, we were able to sequence a stretch of 215 bp with respect to 200 bp product length respectively. . This may be due to very minor modification in nucleotide sequence of this gene that has been

taken place during evolution process. Insertion of some nucleotides that occurred at 3' end may be of the sequences may be reason for the increase in length of PCR product. Database analysis of DREB2 with BLAST revealed that the deduced sequence has high homology to PREDICTED: Setaria italica dehydration-responsive element-binding protein 2A (LOC101780153) with an identity of 89% and query coverage of 68 %. However the sequencing results for DREB1 were difficult to reproduce regardless of repetitive experiments. This may be due the differences in genotype used for actual experiments and primer designing.

CONCLUSIONS

Water withholding experiments in plants can simulate a series of physiological and biochemical reactions leading to expression of genes related to drought-tolerance. Differential gene expression of genes involved in drought stress tolerance yielded valuable information about participation of these genes in providing tolerance ability to foxtail millet. These genes were found to be more active in growth phase. The high expression of these genes under stress condition confirms their role in drought tolerance. Here, both the genes showed considerable upregulation, hence, considering their importance in drought stress tolerance, more detailed study of their role in drought tolerance should be carried out. Further validation of these genes could help in identifying the potential candidate genes involved in drought tolerance. More emphasis should be given on analysis of the *cis*-regulatory elements in the promoter region of these genes showing high expression which could help to fetch the reason behind their tolerance.

REFERENCES

1. Devos KM, Wang ZM, Beales J, Sasaki T and Gale MD (1998) Comparative genetic maps of foxtail millet (*Setaria italica*) and rice (*Oryza sativa*). Theor Appl Genet 96:63–68
2. Doust AN, Kellogg EA, Devos KM, and Bennetzen JL (2009) Foxtail millet: a sequence-driven grass model system. Plant Physiol. 149:137–141
3. Gawai Dipti, Moharil MP, Gahukar SJ, Jadhav PV, Suprasanna P, Dani RG (2013) Global consultation on millets promotion for health and nutritional security, ISBN81-89335-47-2, pg 85-98
4. Gu SL, Liu J, Ren HR, et al. (1987) the relationship between foxtail millet and its environment. *In*: Shanxi Academy of Agricultural Sciences, eds, Foxtail millet cultivation in China, (In Chinese). Bejing: China Agricultural Press,pp 64–65
5. Jayaraman A, Puranik S, Rai NK, Vidapu S, Sahu PP, Lata C and Prasad M (2008) cDNA-AFLP analysis reveals differential gene expression in response to salt stress in foxtail millet (*Setaria italica* L.). Mol Biotechnol 40: 241–251
6. Kumar K, Muthamilarasan M, Prasad M (2013) Reference genes for quantitative Real-time PCR analysis in the model plant foxtail millet (*Setaria italica* L.) subjected to abiotic stress conditions.Plant Cell Tiss Organ Cult 115:13–22
7. Lata C, Gupta S, and Prasad M (2013) Foxtail millet: a model crop for genetic and genomic studies in bioenergy grasses. Crit Rev Biotechnol 33:328–343
8. Lata C, Sahu PP, Prasad M (2010) Comparative transcriptome analysis of differentially expressed genes in foxtail millet (*Setaria italica* L.) during dehydration stress. Biochem Biophy Res Commun 393:720–727

9. Li P, and Brutnell TP (2011) Setaria viridis and Setaria italica, model genetic systems for the Panicoid grasses. J Exp Bot 62: 3031–3037

10. Li YM (1997) Drought-resistant mechanism and genetic expression of foxtail millet. *In* Li YM (eds) Foxtail millet breeding. Beijing: China Agricultural Press, pp 433–434

11. Li ZY, Wang N, Dong L, Bai H, Quan JZ, Liu L, et al. (2015) Differential Gene Expression in Foxtail Millet during Incompatible Interaction with Uromyces setariae-italicae. PLoS ONE 10(4): e0123825

12. Nawaz M, Iqbal N, Idrees S, Ullah I (2014) DREB1A from Oryza sativa var. IR6: homology modelling and molecular docking. Turk J Bot 38: 1095-1102

13. Qi X, Xie S, Liu Y, Yi F, and Yu J (2013) Genome-wide annotation of genes and noncoding RNAs of foxtail millet in response to simulated drought stress by deep sequencing. Plant Mol Biol 83:459–473

14. Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response. Plant Physiol 115: 327-334

15. Zhang G, Liu X, Quan Z et al. (2012) Genome sequence of foxtail millet (Setaria italica) provides insights into grass evolution and biofuel potential. Nat Biotechnol 30:549–554

APPENDIX

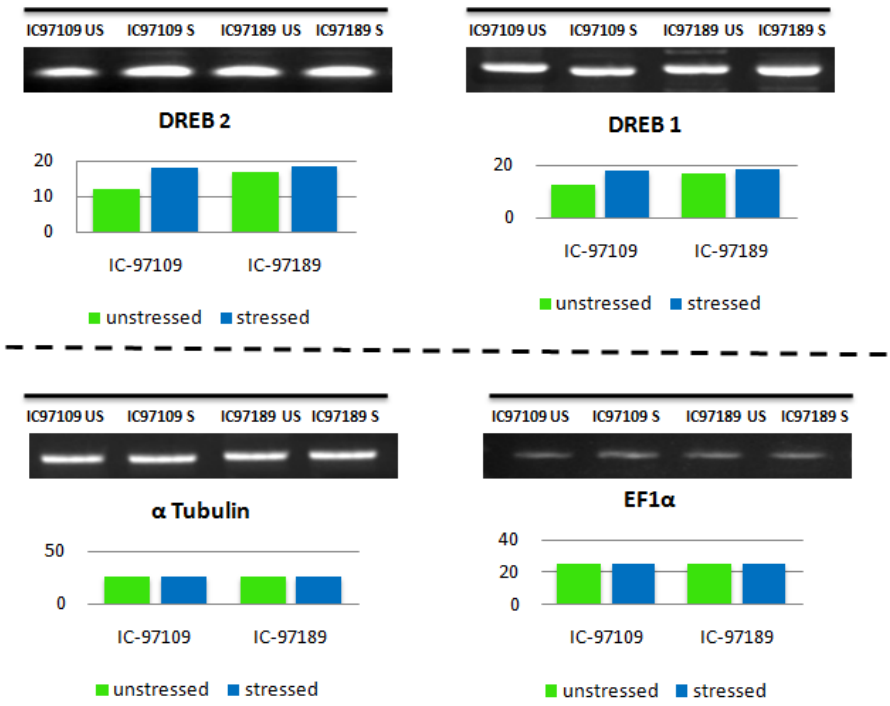

Figure 1: Semi-Quantitative RT-PCR Profile Of Drought Related Candidate Genes Samples from Two Contrasting Foxtail Millet Accessions, IC-97109, IC-97189 in Response to Water Stress Were Run on 1.5% Agarose Gel; Graphs Show Differential Expression of DREB2 and DREB1; A Tubulin and EF1 α Used as Internal Controls; US: Unstressed; S: Stress