The Pharma Innovation Journal 2023; 12(8): 2625-2630

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(8): 2625-2630
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www.thepharmajournal.com
Received: 20-06-2023
Accepted: 27-07-2023

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Expression study of WRKY transcription factors during vegetative stages in pearl millet (*Pennisetum glaucum* L. R. Br)

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Abstract
WRKY is a member of an important plant specific major transcription factor family showing expression in majority of plant species. Little is known about expression of WRKY transcription factors in pearl millet. So the objective of present study is to analyse expression level of WRKY transcription factors during different developmental stages. Leaves and roots sample of two genotypes were studied. Out of four reference genes namely Actin, PP2A, TIP-41 and UBQ 5, Tip-41 was found most stable reference gene. Relative quantification of WRKY transcription factors was carried out for four WRKY transcription factor genes which revealed an alteration in expression level at different developmental stages and tissue. Present study is helpful to understand the importance of WRKY transcription factors during developmental stages. Detailed expression study of WRKY genes specific for particular gene or specific conditions may provide an important insight to unravel unknown mechanisms of plant growth and development.

Keywords: Pearl millet, c-DNA, qRT-PCR, WRKY transcription factor, normalization

Introduction
Pearl millet (*Pennisetum glaucum* L. R. Br.), an important multipurpose coarse grain cereal, has been grown primarily in arid and semi-arid regions of India and Africa for food, feed, fodder and fuel purpose (FAO, 2000; Khairwal *et al*., 2007) [8, 14]. To survive under changing environmental condition plants directs temporal and spatial gene expressions necessary for normal development and proper response to the physiological or environmental stimulus. Although pearl millet is considered as stress model plant, the genome sequence information of pearl millet is limited which restricts our knowledge regarding expression pattern of different genes during entire life cycle of plant, discovery of novel genes and hence crop improvement (Shivhare *et al*., 2016) [25]. Analysing expression of different genes from diverse biological samples provides the basis to study molecular mechanism of different genes.
Transcription factors are the proteins which regulates gene expressions through binding to cis-regulatory specific sequences in promoters of their target genes. It can activate downstream genes, involving physiological change and response to biotic and abiotic stress by binding cis-acting elements (Song *et al*., 2018) [27]. The identification of transcription factors helps to construct regulatory network for intrinsic development processes. Large numbers of transcription factors are identified in plants, amongst them WRKY transcription factors is one of the 10th largest families of transcriptional regulators (Ruthson *et al*., 2010) [20]. It has wide role in regulating plant process including plant growth and development like seed development (Luo *et al*., 2005) [18], seed dormancy and germination (Zou *et al*., 2008) [33], flowering (Chen *et al*., 2018) [6], metabolic pathways (Sun *et al*., 2003), morphogenesis (Johnson *et al*., 2002) [13], plant growth (Chen *et al*., 2002) [4] and senescence (Chen *et al*., 2017) [5]. The information generated here would be useful to understand the biological role of WRKY transcription factors in pearl millet. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) is reported as one of the very useful method for gene expression studies in plants (Higuchi *et al.,* 1993) [13]. It is one of the most reliable methods for gene expression study and can be applied for even plants having very low amount of RNA concentration (Fraga *et al*., 2008) [10].

Materials and Methods
Seeds of two pearl millet genotypes 7042 S and P-7-4 used in our study were grown in pots filled with sterilized soil under greenhouse condition with 28 °C/20 °C day/night temperature.

Leaves and roots tissues were sampled at seven and fourteen day after sowing condition and immediately snap frozen with liquid nitrogen and stored in -80 °C for further use, total eight samples were taken and subjected to RNA isolation for our study.

Table 1: List of genotype and nomenclature given to each sample

Sr. No.	Name	Characteristics (genotype, tissue, stage of sample harvest)	Sr. No.	Name	Characteristics (genotype, tissue, stage of sample harvest)
1	G1L1	P-7-4; leaf; 7 DAS	5	G2L1	7042S; leaf; 7 DAS
2	G1L2	P-7-4; leaf; 14 DAS	6	G2L2	7042S; leaf; 14 DAS
3	G1R1	P-7-4; root; 7 DAS	7	G2R1	7042S; root; 7 DAS
4	G1R2	P-7-4; root; 14 DAS	8	G2R2	7042S; root; 14 DAS

Total RNA isolation and its qualitative and quantitative assessment

Total RNA isolation from respective samples were carried out using standard TRIZOL (Invitrogen, Gmbh, Karlshuae, Germany) method with minor modifications in the protocol. Necessary conditions were maintained during isolation and isolated RNA was immediately snap frozen and stored at -80°C for downstream processing. RNA concentration and purity were determined through Nanodrop spectrophotometer ND 1000 (V.3.3.0, Thermoscientific, USA) at a wavelength ratio A260/280 and A260/230. Also RNA quality was verified through electrophoresis in 1.5% agarose gel. Total RNA (2.0 µg) was taken from all the samples individually and c-DNA synthesis was carried out using first strand cDNA synthesis kit (Fermentas) following the manufacturer's instructions. Total 20 µl reaction was set up and after incubation it was diluted to 100 µl by adding nuclease free water and stored at -20 °C for further use.

qRT-PCR primer design

Primer sequences for reference genes study were selected from reference (Saha *et al*., 2012) [21]. Total four primers were selected to study the endogenous gene showing stable expression and for normalization of relative quantity values of WRKY transcription factors.

While pearl millet complete genome sequence has not been yet released, the sequence information related to WRKY transcription factors were taken from BLAST results of our previous study of transcriptome of pearl millet (Kulkarni *et al*., 2016) [15]. The fasta sequence of WRKY transcription factor related transcripts were retrieved from obtained transcriptomic data and inputted to the batch primer 3 and oligodT software tool and primers were designed by keeping following criteria: Tm (melting temperature) of 55-65 °C, amplicon length of 100-200 bp, primer length of 19-25 nucleotide with optimum of 20 nucleotide, GC content of 45-60% and rest of the parameters were kept by default.

Quantitative real-time PCR: The amplification specificity of all the reference genes as well as candidate WRKY transcription factor related primers were checked by performing end point PCR and PCR product were analysed through 1.5% agarose gel electrophoresis. Primers showing bands of respective size were selected for further real time PCR. First qRT-PCR for candidate reference genes were carried out using SYBR green detection chemistry (Wong *et al.,* 2005) [31] on Bio-Rad CFX manager real time PCR machine. Total 10 µl reactions volume was set up by adding 1 µl c-DNA, 0.3 µl each forward and reverse primer, 5µl SYBR green master mix (Takara, Japan) and 3.4 µl nuclease free water. The cycling conditions were: 95 °C for 3 minutes, denaturation at 95 °C for 10 seconds, annealing and extension at 60° for 30 seconds. The melt curve was performed sequentially after real time PCR with temperature gradient 55-95 °C for each primer. It is a quality control step which revealed primers amplified were related to specific gene product and not the spurious result. Melt curve analysis provides dissociation curve of primer, so presence of single peak represents specificity of primer as well as absence of primer dimer. Total four reference genes namely, Actin, PP2A, TIP-41 and UBQ 5 were selected for identification of most stable reference genes amongst all tissue samples. The specificity of amplification reaction was verified using melt curve analysis (Codori *et al.,* 2001) [7].

Data analysis: Selection of stable reference gene through geNorm (Vandesompele *et al*., 2002) [29] and Normfinder (Anderson *et al*., 2004) software tools: Biorad CFX manager has inbuilt geNorm software programme which calculates average expression stability value (M) of each particular gene (http://medgen.ugent. be/genorm) (Livak *et al*., 2001) [17]. Larger the M value lower the stability and vice versa. The M value should not be greater than 1.0 otherwise it represents that respective gene is not stable to be considered as suitable reference gene. Normfinder ranks the stability of reference gene independent from other reference gene. Ct value of all the samples were imported to geNorm software and M value was calculated for each reference gene.

Real time PCR of four WRKY transcription factor was carried out following the protocol used for candidate reference gene. The reaction volume is also kept 10 µl and also melt curve was performed after completion of 40 cycles. Gene expression analysis was carried out through $2^{-\Delta\Delta Ct}$ method (Livak *et al*., 2001; Schmittgen *et al*., 2000) [17, 23]. Primers of WRKY transcription factors named PM_WRKY 3, PM_WRKY 17, PM_WRKY 28 and PM_WRKY 30 were taken for expression analysis. Information regarding primers is given in Table 1.

Table 1: List of primer sequences used for amplification of transcription factor gene in qRT-PCR analysis.

Primer name		Sequence (5' to 3')	Amplicon size
PM_WRKY 3	F	TGCTCGATCTCCTTCCACTC	139
	R	TCTCGTCCGCTAAGACCTTTC	
PM_WRKY 17	F	AGGTGTCCTCTCCCTCTTTTTC	140
	R	CAACGGCCATGTCTAAATAAATC	
PM_WRKY 28	F	CCACACCAAGTTTCTACATGACC	147
	R	GAAGGCGAGCACAACCATAC	
PM_WRKY 30	F	AGTATGTGATCTGGGTCGGTTT	126
	R	ATGAACAGCAGCAGCACACTAT	

Results and Discussion

RNA isolation at critical stages is the most important consideration for our research criteria. RNA isolation was carried out from eight different sample tissues individually at seven and fourteen day after sowing, these are two initial stages of vegetative cycle of pearl millet where fast level of growth and development seems to occur. Total RNA isolation was straightforward. All isolated RNA samples were checked for quality as well as quantity and had satisfied the necessary requirements (Table 2). Absorbance ratio A260/280 of all samples was ranged from 1.9 to 2.16, which is in agreement with normal purity standards of RNA. All the samples not having genomic DNA contamination were only taken for c-DNA synthesis.

Table 2: Qualitative and quantitative assessment of total RNA of pearl millet leaves and root tissue

Sr. No.	Samples	Total RNA (ng/µl)	A260/280	A260/230
1	G1L1	671.92	2.06	1.99
2	G1L2	939.3	2.16	2.04
3	G1R2	626.5	2.03	2.04
4	G1R2	810.1	1.99	2.11
5	G2L1	1966.2	2.05	2.02
6	G2L2	615.51	1.97	2.03
7	G2R1	1461.3	2.01	1.97
8	G2R2	680.4	1.98	2.05

Expression profiling of candidate reference gene

Expression level of four candidate reference genes namely actin, PP2A, TIP-41, ubiquitin was checked through performing qRT-PCR. Reference genes were analysed through end point PCR before performing qRT-PCR for primer specificity, amplification and for amplicon size determination (Reddy *et al*., 2015; Gimeno *et al*., 2014; Wang *et al*., 2015; and Chandana *et al*., 2012) [19, 11, 30, 3]. Reference genes confirmed obtained amplification of primers of respective size in all the samples through end point PCR were selected for qRT-PCR. Cycling threshold values were obtained for selected four candidate reference genes during real time PCR. Melt curve analysis shown absence of multiple peaks in all samples and non -template control (NTC), which indicated absence of primer dimer or non-specific products. qRT-PCR result of candidate reference genes revealed variations in cycling threshold values ranged from 18.88 (UBQ 5) to 33.7 (Actin). Generally Ct value less than 29 are strong positive reactions indicates abundant target nucleic acid in the respective samples. Hence our experimental results revealed presence of abundant to moderate level of target nucleic acid in different samples.

Selection of stable reference gene through geNorm and Normfinder softwares: M value indicates stability of selected candidate reference genes in geNorm analysis (Andersen *et al*., 2004) [1]. M value of reference genes was higher as compared to cut off value 1.0. So stepwise exclusion of candidate reference genes with highest M values (Silveira *et al*., 2009; Fan *et al*., 2013) [26, 9], which allowed samples to be ranked according to the stable expression pattern. Experimental result revealed Actin had higher M value. So after exclusion of Actin and ubiquitin, M value of other reference genes was decreased to 0.6 and was in range of cut off value that is 1.0. Hence, from among all reference genes, TIP-41 was finally selected as most stable reference gene.

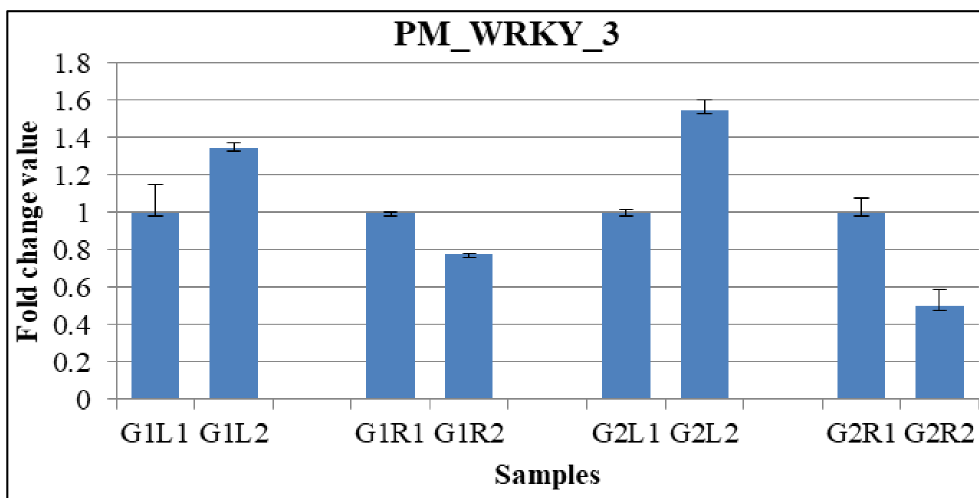
Expression analysis of WRKY primers

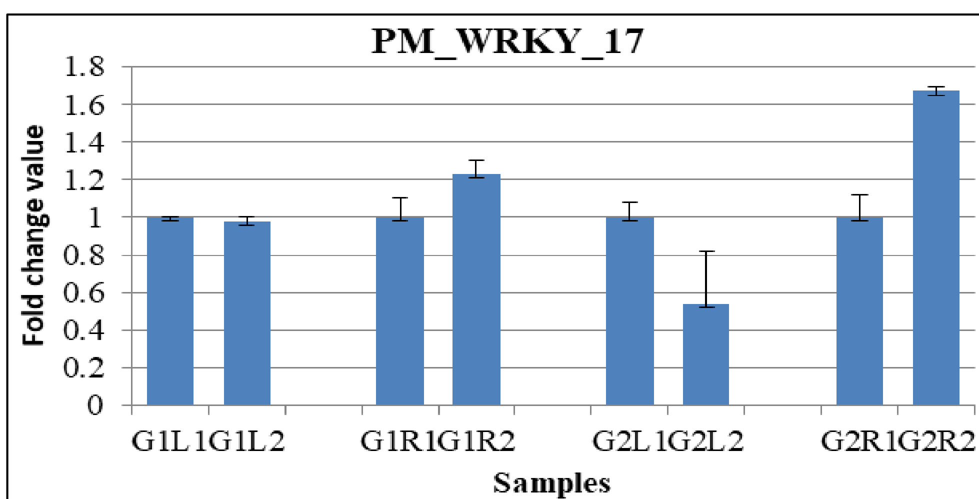
Expression study of four WRKY transcription factors was carried out through manual method by $2^{-\Delta CtCt}$ method. The result indicated expression of all WRKY transcription factors at both the developmental stages in all tissue samples. The specific amplification was obtained for all tissues along with strong to moderate amplification as well as the melt curve analysis of all tissue showed specific amplification as single peak was observed. This indicated the presence of specific PCR product and absence of primer dimer among all the samples.

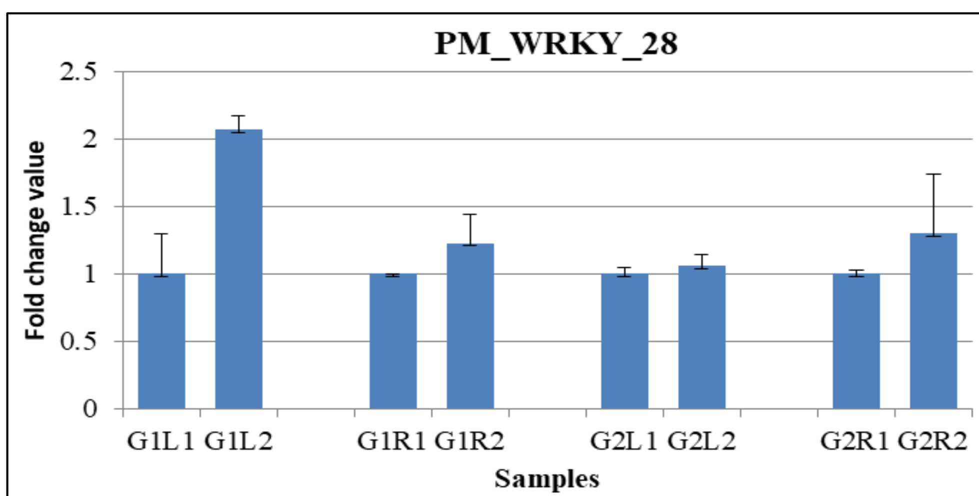
Primer PM_WRKY 3 was found as probable WRKY transcription factor 40 like sequence in our transcriptome BLAST result which was carried out by Kulkarni *et al*., 2016 [15]. Moderate level of amplification was found among all root and leaf tissues during qRT-PCR. Both vegetative stages had almost similar gene expression pattern of respective transcription factor showing upregulation in leaf while downregulation in root tissue when compared with seven to fourteen day stage (Fig. 1). The highest fold change was found in 14 day stage P-7-4 leaf tissue with 1.55 fold change value and it showed lowest 0.5 fold change in 14 day old root tissue in same genotype. WRKY transcription factor 40 is found in number of plant species showing its important role during different developmental stages viz. embryogenesis, germination as well as hormone controlled process to different biotic and abiotic stress response (Bakshi *et al*., 2014) [2]. WRKY40 related protein also regulates ABA signalling (Yan et al., 2012) [16, 32]. ABA receptor spans chloroplast envelope and its cytosolic c-terminal interacts with WRKY 18, 40, 60. It acts as negative regulator of ABA signalling during germination and post germination process (Shang Y., 2010) [24]. These WRKY 18,40,60 binds to W-box region of ABI 4 and 5 gene and inhibits its expression, however exact molecular mechanism is not clear (Liu *et al*., 2012) [17]. Hence in our study, expression of PM_WRKY 3 may be due to reduction of ABA signalling to favour embryogenesis and germination.

Gene expression pattern of PM_WRKY 17 primer showed similarity with WRKY transcription factor in our result and showed amplification in all the samples. Cycling threshold

values for respective gene was 25.73 which indicated higher level of gene expression. qRT-PCR analysis revealed similar expression pattern in both the genotype where leaf tissue showed reduction while root tissue showed increase in gene expression fold change when compared with seven day to fourteen day stage. Amongst all the samples P-7-4 14 day root showed higher level of fold change (1.67) while P-7-4 fourteen day leaf had lower fold change (0.54). As no detailed information is available for current transcription factors, further characterization is necessary which could help to enhance our understanding regarding the role of given transcription factor during different vegetative stage.







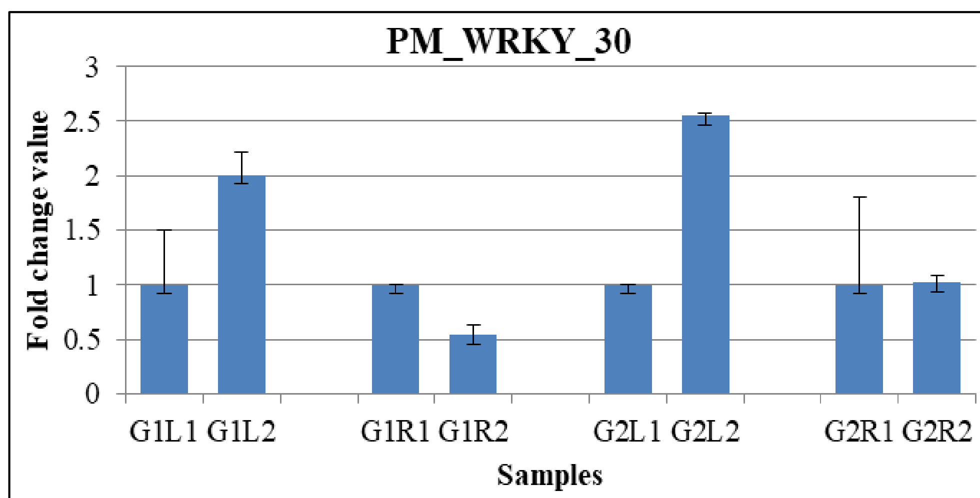


Fig. 1: qRT-PCR analysis of different WRKY TFs of leaf and root tissues of two pearl millet genotypes with reference to endogenous gene TIP-41. Here the bar represents value of standard deviation

BLAST result of primer PM_WRKY 28 showed similarity to protein wrky1-like isoform x2 and amplification analysis of respective transcription factor indicated highest fold changes in 7042 S seven day root tissue while P-7-4 seven day leaf showed lowest level of fold change value using 7042 S seven day leaf as a calibrator. Fourteen day stage showed slight increase in fold change value as compare to respective seven day stage except 7042 S root tissue. WRKY1 is a member of the WRKY transcription factors in plants involved in disease resistance, abiotic stress and senescence as well as in some developmental processes. WRKY1 is also involved in the salicylic acid signaling pathway. The crystal structure of the WRKY1 C-terminal domain revealed a zinc-binding site and identified the DNA-binding residues of WRKY1. It is expressed at 2-10 leaves stage, flowering, mature plant embryo, petal differentiation and expansion stage, plant embryo bilateral stage, globular as well as leaf senescence stages.

Gene expression pattern of PM_WRKY 30 has similarity to WRKY DNA-binding domain superfamily protein. Obtained result indicated 7042 S 14 day root showed highest level of fold change while P-7-4 fourteen day leaf had lowest fold change value. WRKY DNA-binding domain also known as W-box is found exclusively in all plant species and not present in non-plant species (Schluttenhofer *et al.,* 2015) [23]. Generally transcription factors have one or two WRKY protein domains with DNA binding activity containing highly conserved core WRKYGQK motif and zinc finger region (Eulgem *et al*., 2000) [7]. WRKY transcription factor interaction with DNA binding domain and activates respective genes which mostly are responsible for plant development and defence (Johnson *et al*., 2002, Luo *et al*., 2005) [13, 18]. For specific activity of respective transcription factor further characterization of respective transcription actor is required in our current study.

Conclusion

Overall result revealed expression of some of the WRKY transcription factors during important vegetative stages in pearl millet leaves and roots tissues in current study. As nearly equal amplification values were obtained during qRT-PCR, greater fold change difference was not observed. The result obtained indicated that some WRKY transcription factors are involved in seed growth and development of pearl millet and hence there is a definite role of transcription factors for vegetative development of pearl millet. The current study may help to identify importance of WRKY transcription factor during early stages of development in Pearl millet.

Conflict of Interest

The authors declare no conflict of interest

Acknowledgement

We thank Centre of Excellence in Biotechnolgy, Aanad Agricultural University for their support in research work.

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