

Original Research Article**Characterization and expression analysis of WRKY transcription factors in Groundnut (*Arachis hypogaea* L.,)****Abstract**

Groundnut (*Arachis hypogaea* L.), is an important subsistence oil yielding crop of the semi-arid tropics and often exposed to several environmental cues (high temperature, drought & heavy metal). The WRKY transcription factor (TF) is one of the master regulator, and play vital role in stress responses. However, far less information is available on functional characterization and tolerance mechanism of stress responsive WRKY genes in Groundnut till date. In this study, a comprehensive phylogenetic, protein features, gene structure and motif analysis of WRKY TF gene family was carried out. In addition, we conducted expression profiling of 10 WRKY genes under high temperature, drought and heavy metal (CdCl_2) in various tissues. Majority of the AhWRKYs were clustered and share close relationship with *Arabidopsis* and *Glycine max*. RT-qPCR analysis of AhWRKY genes revealed that differential expression either in their transcript abundance or in their expression patterns in response to at least one abiotic stress. Of the 10 WRKY genes, AhWRKY41 level was found to be maximum in all the stress conditions. On other hand, AhWRKY20 and AhWRKY22 levels were decreased. The obtained data demonstrate that AhWRKY41 may act as a positive regulator in drought/ high temperature/heavy metal and would exhibit stress tolerance mechanism by activation of stress-associated gene expression.

Key words: WRKY, abiotic stress, RT- qPCR, Groundnut

1. Introduction

Groundnut (*Arachis hypogaea* L.) is an oil yielding crop cultivated worldwide and one of the major grain legumes in tropical and subtropical regions, often exposed to maximum

temperature of >40 °C for a short periods during growing season and low or erratic rainfall results in reducing its yield by 40%. However, its productivity is majorly affected by high temperature, drought, and heavy metal, associated with increased population, inherit food demand and global climate change. WRKY superfamily of transcription factors (TFs) are composed of several proteins involved in transcriptional regulation of developmental processes and stress responses. The WRKY TFs characterized by unique WRKYGQK motif followed by a zinc-finger-like motif C₂H₂ or C₂HC and evolutionarily highly conserved [1]. A single WRKY transcription factor might mediate transcriptional reprogramming associated with several signaling pathways. However, recent advances revealed the enormous significance in eliciting responses induced by abiotic stress conditions. For instance, over-expression of *OsWRKY45* and *11* in *Arabidopsis* results in enhanced tolerance to salt and drought [2, 3]. Niu and Wang et al [4, 5] identified 53 TaWRKYs through wheat ESTs and demonstrated, over-expression of TaWRKY2, 10 and 19 exhibiting increased stress tolerance. Recent studies demonstrated WRKY proteins are also involved in regulating developmental processes via auxins, cytokinins, and steroids in the downstream of hormone signaling in the antagonistic functions of salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) [6]. In *Arabidopsis* expression of *TaWRKY79* produced longer primary roots than the wild type in the presence of either NaCl or ABA. *OsWRKY31* (Rice) and *AtWRKY70* (*Arabidopsis*) were reported to be involved in regulation of disease resistance, root growth, auxin and immune responses, senescence, defense signaling pathways respectively, suggesting their involvement in synchronization of multiple biological processes [7, 8]. GmWRKY21 over-expressing transgenic *Arabidopsis* were more tolerant to cold, and induced GmWRKY54 displayed more salt and drought than those of wild type,

whereas over-expression of GmWRKY13 results in increased sensitivity to salt and mannitol stress [9].

More recently, over-expression of GsWRKY20, a member of WRKY subgroup III, in alfalfa enhanced to both drought and salt tolerance of the transgenic plants [1]. In *Populus simonii*, 20 WRKY genes showed differential response to various biotic and abiotic stress conditions suggesting they could play vital role in imparting stress tolerance [10]. Extensive research on WRKY TFs has been carried out in the recent past in several crop plants such as rice [11], cucumber [12] maize [13], tomato [14] etc., due to their important role in various biological, physiological and molecular processes. However, expression and characterization analysis of essential members of these TFs under different abiotic stress in Groundnut is yet to be investigated. In the present study, we have analyzed 10 WRKY encoding transcripts for gene structure, evolutionary relationship, conserved motifs and expression analysis under high temperature, drought and heavy metal stress in Groundnut. For the first time we demonstrated the transcriptional activation of AhWRKY41 that would confer drought/ high temperature/ heavy metal tolerance by activation of cellular antioxidant systems or stress associated genes in Groundnut.

2. Material and Methods

2.1. Identification, characterization and sub- cellular localization of WRKY genes

We retrieved WRKY genes from Plant Transcription Factor Database which shared homology with stress responsive WRKY genes from Arabidopsis and Glycine max. The length, molecular weight and pI of each deduced polypeptide were calculated using the ExpasyProtParam tool (<http://web.expasy.org/protparam/>). The putative WRKY homologs were

determined by BLASTP (<https://www.arabidopsis.org/Blast/index.jsp>). Further, CELLO (<http://cello.life.nctu.edu.tw/>) and WOLF PSORT (http://www.genscript.com/psort/wolf_psort.html) programs were used to predict the sub-cellular localizations. Multiple sequence alignment of candidate domains from Peanut, *Arabidopsis* and *Glycine max* was performed using BioEdit. To study the homology among the 10 WRKY genes, phylogenetic tree was constructed using Neighbour joining method.

2.2. Conserved motifs and Gene structure analysis

The MEME Suite tool v4.9.1 (<http://meme.nbcr.net/meme>) [15] was used for analysis of the conserved motifs of AhWRKY protein sequences. Gene Structure Display Server from Center for Bioinformatics, Peking University, was used to display the intron exon junctions (<http://gsds.cbi.pku.edu.cn/index.php>). The genomic and mRNA sequences of WRKY genes were downloaded and used as query for generating its gene structure. The number of introns and exons were estimated based on this alignment and confirmed by the coordinates given in the sequences.

2.3. Plant material and abiotic stress treatments

Seeds of Peanut (ICGV1999) were surface-sterilized and grown under controlled conditions at 28 °C day/25 °C night with a 12-h light/12-h dark photo period. After 10 days of germination, seedlings (Shoot, Leaf, Cotyledon, Stem and Root) were exposed to high-temperature [42 °C for 2h (induction) followed by 48 °C for 6h]. Drought stress was stimulated by withholding water for 5 days and for heavy metal stress, seedlings were hydroponically exposed to 300µM CdCl₂ for 72h. After the stress treatment, control and stress exposed tissues were harvested immediately and stored at -80 °C for further analysis.

2.4. Gene Expression Analysis by qPCR and Pearson correlation

Total RNA was isolated from control and stress treated tissues (Shoot, leaf, cotyledon, stem and root) using TRIzol (Invitrogen) according to the manufacturer's instructions and then treated with RNAase-free DNAase I (Promega). All RNA samples were quantified by Nanodrop 2000 (Thermo Scientific). cDNA was synthesized by reverse transcription with 500ng of total RNA using PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. Gene specific primers were designed using Primer3 software (Table 1). qRT- PCR reactions were performed using SYBR Green PCR Master mix (Takara) on Lightcycler96 Real time PCR (Roche). Each PCR reaction (20 µl) included 2 µl cDNA, 1x SYBR Green Master mix, 0.5 µl sequence specific forward primer (10 µM), 0.5 µl reverse primer (10 µM), and 7 µl sterile water. Actin was used as a reference for quantitative the expression of *AhWRKY* genes. The reactions conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30s and 72 °C for 15s. Three biological replicates were used. The $\Delta\Delta^{Ct}$ method was used for quantification. To analyse the qPCR results of stress inducible *AhWRKY* genes for statistical significance, Pearson correlation coefficient was used to calculate R and p values at the 0.05 level of significance. STRING 10 (<http://string.embl.de/>), computational tool was used to predict the protein- protein interaction of the stress inducible *AhWRKY* proteins in Arabidopsis with the default parameters.

Table 1: Primers used for Real time PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Length (bp)	Tm (°C)
Actin	TTGGAATGGGTCAGAAAGGATGC	AGTGGTGCCTCAGTAAGAAGC	196	57.13
AhWRKY41	CCTCTGAGGGAGGACAAATCA	AAGGCCACTCTCAAAAGCTCA	108	60.19
AhWRKY20	AATGCTGGTTGCCCTGTTAG	CCATCCCAAGATCAAGGCTA	205	60.13
AhWRKY4	GGTGGAGAAATCCGATGAGAA	ATCAGCCAAATCCTCTCTCCCT	147	60
AhWRKY40	TGGCTGAAATGCTTTCTGTG	TCTCGGAGTTCCCATTTGTTT	180	60
AhWRKY12	GCTACAGCCACAGTCACAGC	TCGGATCCCATGCTTCTAACC	106	60
AhWRKY15	TGCAGCAGTGTAAGAGGGTG	CAGCTGCAGTGAGAGAGTGG	115	59.91
AhWRKY69	GAAGCAAGTTGAACGAAGCC	GAGGAGGAAGAGGAGGAGGA	114	59.83
AhWRKY3	AGGGTTGAGTCCTTCTGGGT	GGAGCTGTCGGAGCTGTTAC	177	60
AhWRKY2	GCTAGAGCAGGGTTCAATGC	GGGTGGTAGGACTGAGACCA	124	59.9
AhWRKY44	ATCAGCCATGAAAGATTCCGG	GCGGATGTTAAAGCCTTCTG	140	60

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3. Results

3.1. Characterization of *AhWRKY* genes and Sub- cellular Localization

We obtained 10 *AhWRKY* genes and their corresponding protein sequences from Plant Transcription Factor database. Basic information like molecular weight and pI are depicted in Table 2. The average polypeptide length was 340.8 residues with the length ranging from 199 aa (AhWRKY44) to 568 aa (AhWRKY3). The pI values range from 5.2559 to 10.2566. The metal chelation zinc finger motif pattern of the three groups were C-X₄₋₅-C-X₂₂₋₂₃HxH (C2H2) (I), C-X₅CX₂₂₋₂₃-HxH (C2H2) (II) and C-X₇-CX₂₃-HXC (C2HC) (III). The sub-cellular localization of 10 *AhWRKY* proteins were analyzed using WOLF PSORT (http://www.genscript.com/psort/wolf_psort.html) and CELLO (<http://cello.life.nctu.edu.tw/>) [16] programs. The results showed 9/10 *AhWRKY* proteins were localized to nucleus and 3/10 was predicted to be localized in cytoplasm and chloroplast.

3.2. Multiple sequence alignment and phylogenetic analysis

To analyze the features of *AhWRKY* domain in Groundnut, we performed multiple sequence alignment of the conserved domains derived from *Arabidopsis* and *Glycine max*. The alignment reveals, all the 10 *AhWRKY* proteins share the conserved domain (WRKYGQK) and zinc finger motifs (Figure. 1). To examine their evolutionary relationship we constructed phylogenetic tree and showed well- organized classification of 10 *AhWRKY*s falls into group I (AhWRKY20, AhWRKY3 and AhWRKY2), group IIa (AhWRKY40), group IIc (AhWRKY12), group IId (AhWRKY15), group IIe (AhWRKY22, AhWRKY44 and AhWRKY69) and group III (AhWRKY41) (Figure. 2).

Protein	TF ID	GmWRKY homolog	Deduced polypeptide			Subcellular localization		Group	Domain
			L (aa)	pI	MW (kDa)	PSORT	CELLO		
AhWRKY41	Ahy002014	41	218	9.7727	25.211	Nuclear	Nuclear	GIII	C-X ₇ -CX ₂₃ -HXC
AhWRKY20	Ahy003521	20	358	7.9025	39.382	Nuclear	Nuclear	GI	C-X ₄₋₅ -C-X ₂₂₋₂₃ HxH
AhWRKY22	Ahy008917	22	370	5.2559	40.371	Nuclear	Nuclear	GIle	C-X5CX22-23-HxH
AhWRKY40	Ahy011981	40	320	8.2094	35.704	Nuclear	Nuclear	GIla	C-X ₅ CX ₂₂₋₂₃ -HxH
AhWRKY12	Ahy014145	12	218	8.0863	24.62	Cytoplasm	Nuclear	GIlc	C-X ₅ CX ₂₂₋₂₃ -HxH
AhWRKY15	Ahy014372	15	363	10.2566	39.276	Nuclear	Nuclear	GIId	C-X ₅ CX ₂₂₋₂₃ -HxH
AhWRKY69	Ahy014637	69	235	5.3279	24.885	Nuclear, cytoplasm	Nuclear	GIle	C-X ₅ CX ₂₂₋₂₃ -HxH
AhWRKY3	Ahy017324	3	568	7.9178	61.9	Nuclear	Nuclear	GI	C-X ₄₋₅ -C-X ₂₂₋₂₃ HxH
AhWRKY2	Ahy018168	2	559	5.175	60.538	Nuclear	Nuclear	GI	C-X ₄₋₅ -C-X ₂₂₋₂₃ HxH
AhWRKY44	Ahy020733	44	199	8.57	22.147	Nuclear, choloroplast	Nuclear	GIle	C-X ₅ CX ₂₂₋₂₃ -HxH

133 **Table 2: Features of 10 WRKY Proteins in Peanut**

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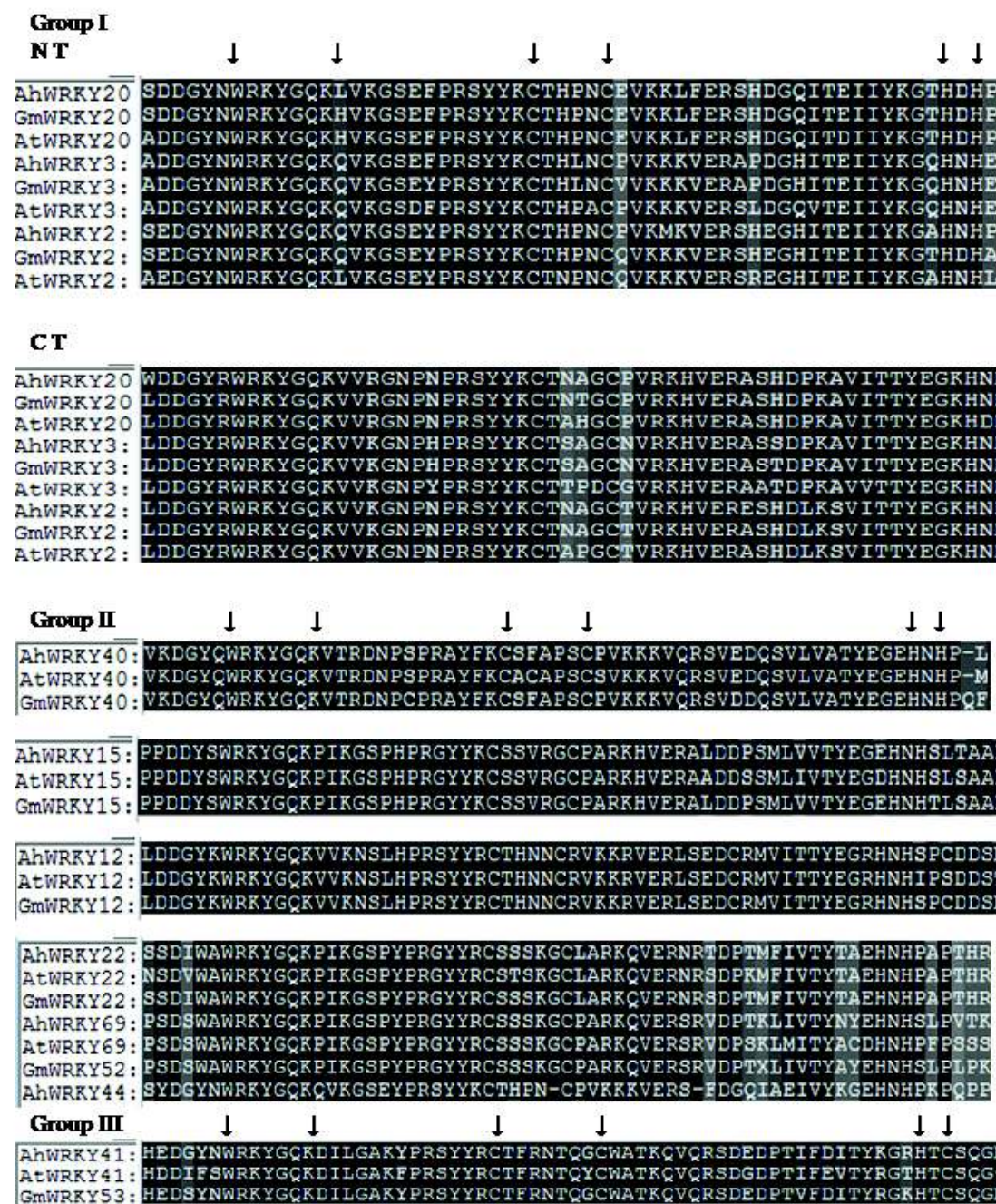


Figure. 1: Multiple sequence alignment of conserved WRKY domains from Peanut, Arabidopsis and Glycine max. NT and CT represent N termini and C termini of Group I WRKY domains respectively. The highly conserved WRKYGQK domain is indicated between the arrows. Cystein (C) and Histidine (H) are represented by arrows which indicate the zinc finger motifs.

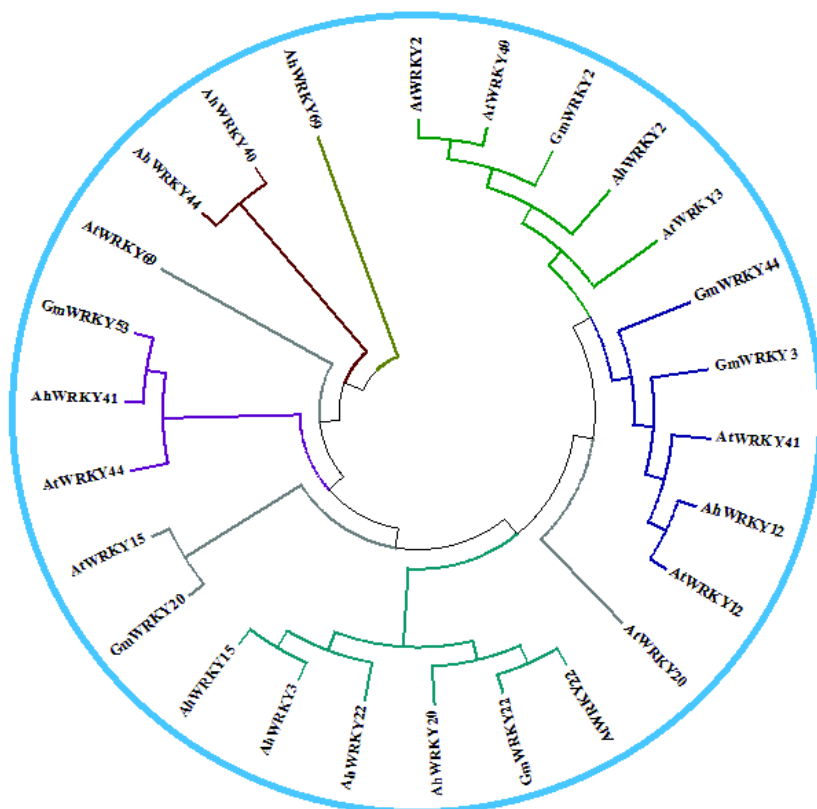


Figure. 2: Unrooted Phylogenetic tree of Peanut, *Arabidopsis* and *Glycine max* WRKY sequences. The tree was constructed by the MEGA v6.0 program with the Neighbor-Joining algorithm. The evolutionary relations were calculated using the p-distance method.

3.3. Analysis of motif composition and Gene structure

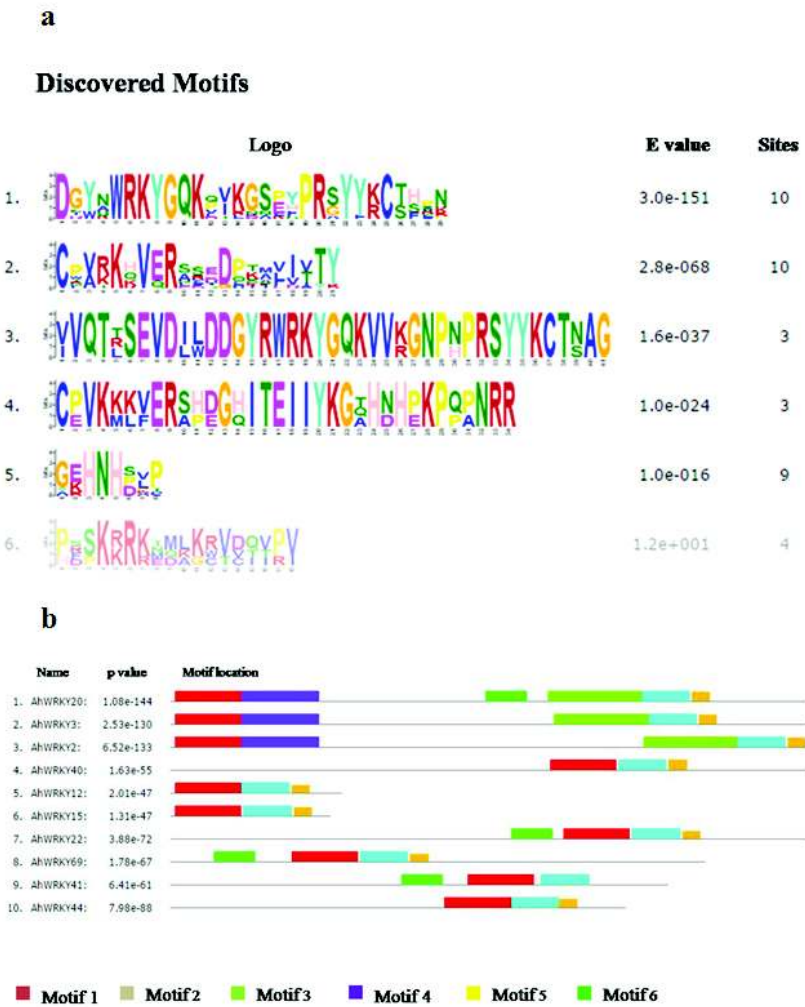
The WRKY share significant sequence conservation within domain regions and to investigate the homologous sequence and frequency of the most prevalent amino acids, sequence logos were produced using the amino acid sequences of AhWRKY proteins in MEME Suite tool and six motifs were defined (Figure. 3a & b). Group I consist of AhWRKY20 shares all the six conserved motifs while AhWRKY3 and AhWRKY2 share five motifs. Group II members (AhWRKY40, 12, 15, 22, 69, 44) shares motifs 1, 2 and 5. Group III member AhWRKY41 shares motifs 1, 2 and 6. The gene structure analysis revealed that the WRKY genes harbored at least two exons with varying length. In addition, a separate phylogenetic tree was generated from

the complete protein sequences of all the WRKY genes. The most closely related members in the same subfamilies shared similar exon/ intron structures in terms of intron number and exon length (Figure. 4).

3.4. Expression patterns of *AhWRKY* genes under different abiotic stress treatments

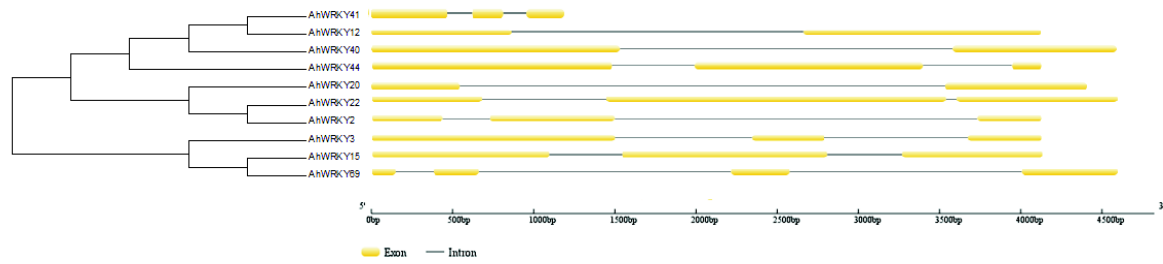
In order to characterize the relative expression of WRKY genes under different abiotic stress, we conducted qPCR. Under high temperature stress, *AhWRKY2*, *40*, *41*, *69* and *44* were found to be up-regulated by 2.9, 1.3, 4.7, 5.2 and 1.02 folds respectively. On the other hand, *AhWRKY20*, *22*, *12*, *15* and *3* were down regulated by 0.26, 0.36, 0.91, 0.73 and 0.59 fold respectively (Figure. 5). During drought, *AhWRKY41* and *2* showed 2.4 and 2 fold up regulation while, *AhWRKY40* shows significant down regulation by 0.48 fold (Figure. 5). Under heavy metal stress, most of the genes were down regulated while *AhWRKY3* is significantly up regulated by 6.85 fold. To summarize the overall expression patterns, *AhWRKY41* showed induced expression in all three stress conditions (Figure. 5). *AhWRKY2* and *69* showed enhanced expression under high temperature and drought while *AhWRKY15* shows up-regulation during drought and heavy metal stress. *AhWRKY20* gene was repressed in all the three stress conditions. The other genes show differential expression patterns under different abiotic stress which may indicate the involvement of these genes in complex stress regulatory mechanisms. The tissue specific expression (leaf, cotyledon, stem and root) data of 10 *AhWRKY* genes under abiotic stress were depicted in the Figure. 5. Analysis of Pearson correlation coefficient of qPCR results with R value of 0.997 and p-value of 0.00001, indicating that the results are significant at <0.05. The prediction of protein- protein interaction between stress inducible *AhWRKY* orthologs in *Arabidopsis* was studied by STRING 10 program. The interaction network shows that out of 10 *AhWRKY* stress inducible proteins, *AhWRKY40*, *AhWRKY15*, *AhWRKY22* and *AhWRKY44*

179 shows their association with WRKY18, GUN5, WRKY33, STZ, RHL41, DIC2, CZF1 and
180 CML38 (Figure. 6).



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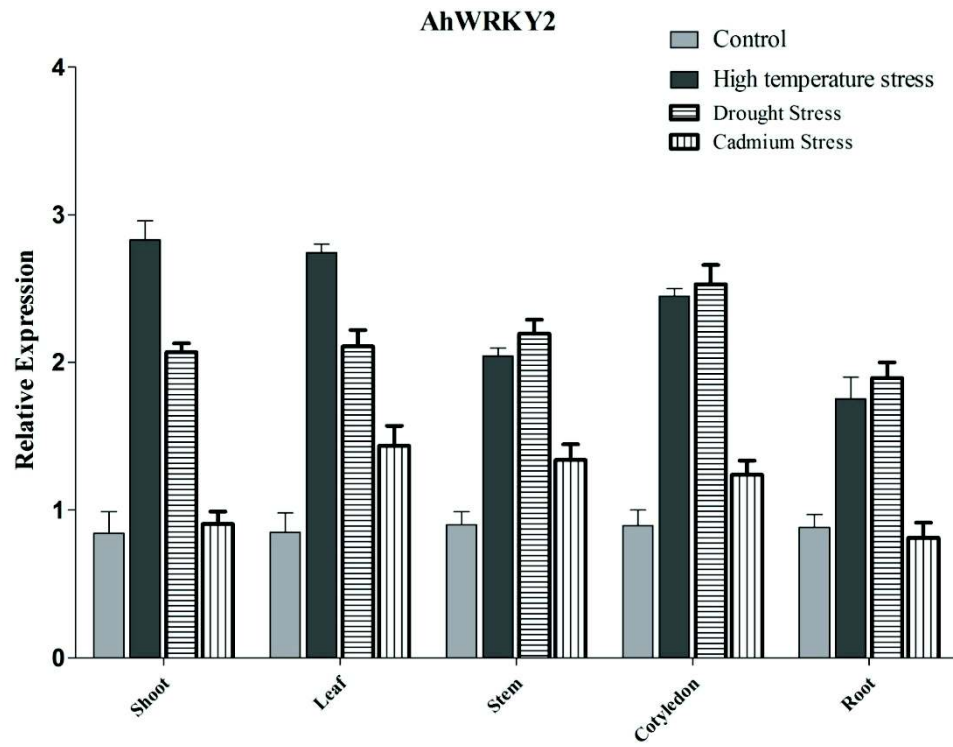
182 **Figure. 3: a)** The domain prediction of ten *A. hypogaea* WRKY protein sequences was
183 performed using MEME software, which generated a letter logo to represent the WRKY domain,
184 zinc finger and other motifs. The height of the letters in the y-axis represents the degree of
185 conservation and relative frequency of each amino acid at that position. **b)**The distribution of
186 conserved motifs among the putative AhWRKY proteins is shown and different motifs are
187 represented by different color blocks as indicated at the bottom of the figure.
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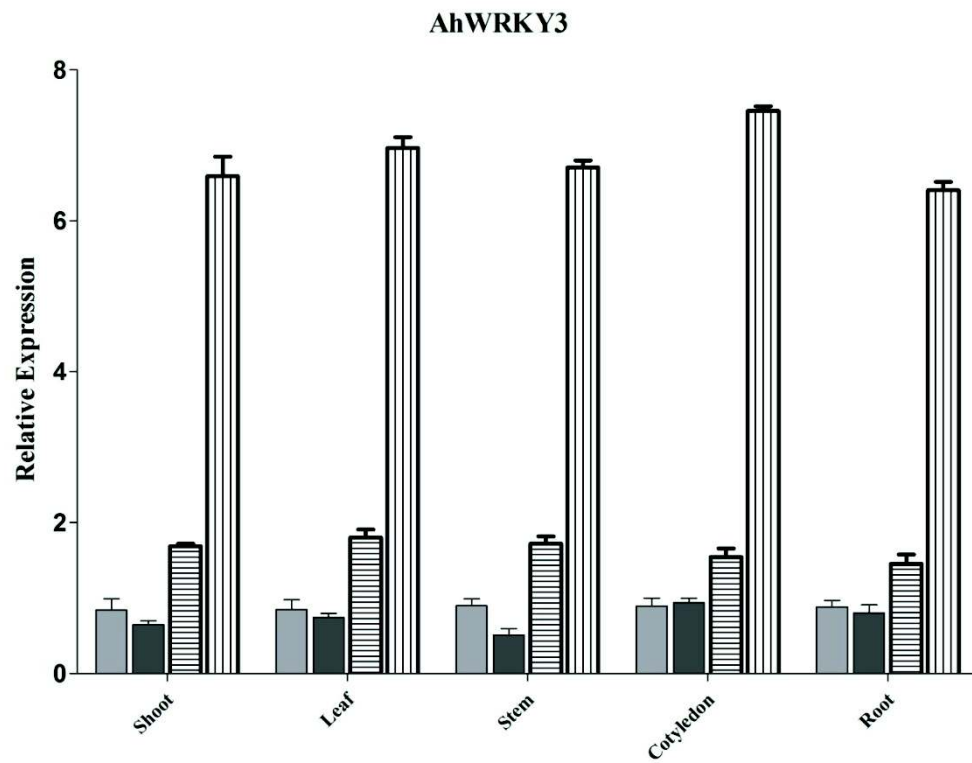
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190 **Figure. 4:** Phylogenetic relationship and gene structure of the *WRKY* genes. Yellow box indicate
 191 exons and black line indicates introns.

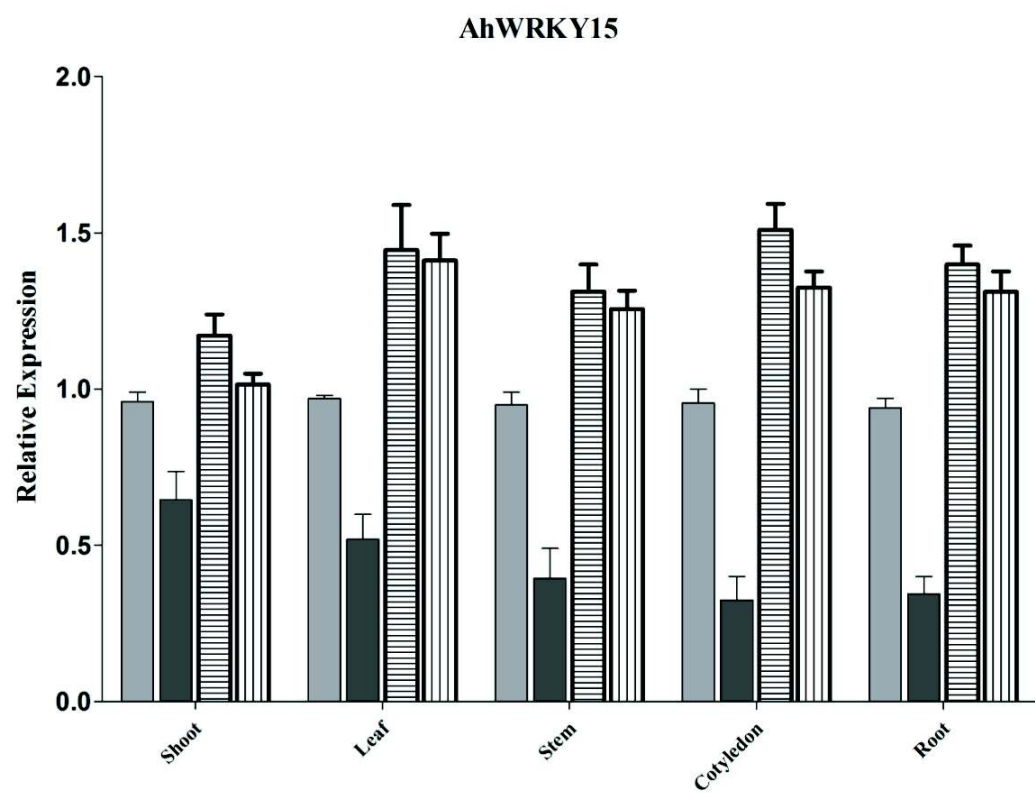
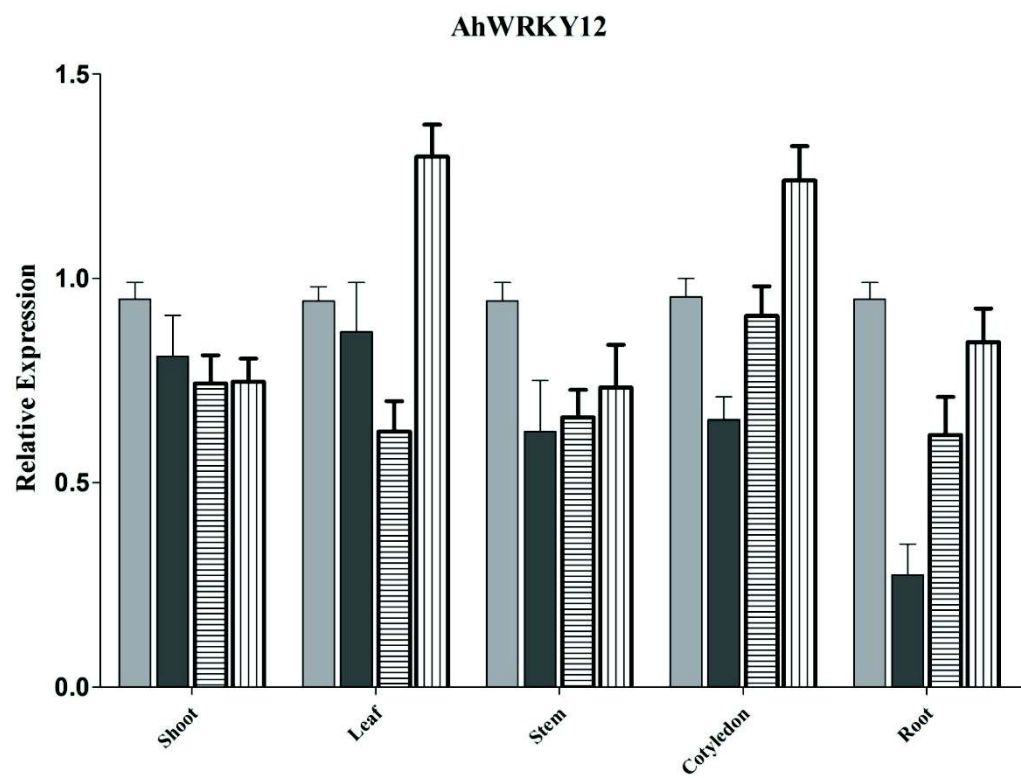
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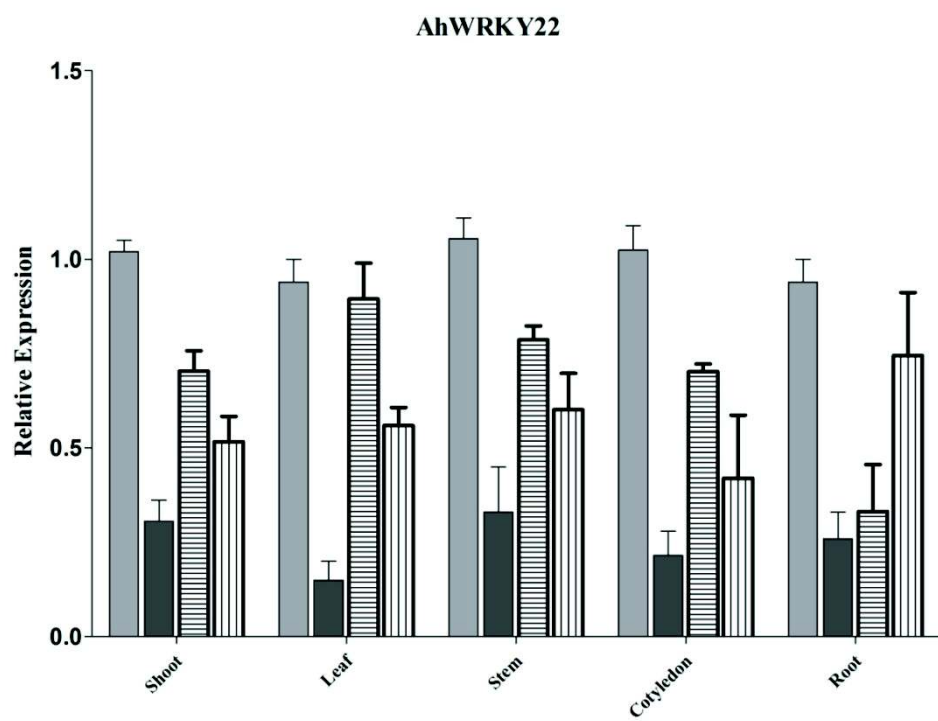
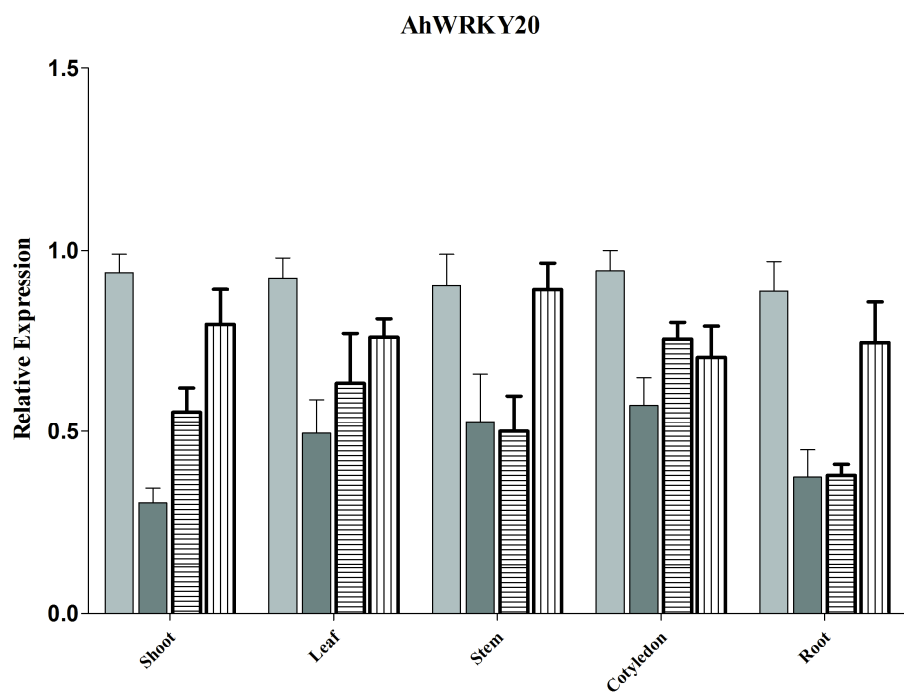


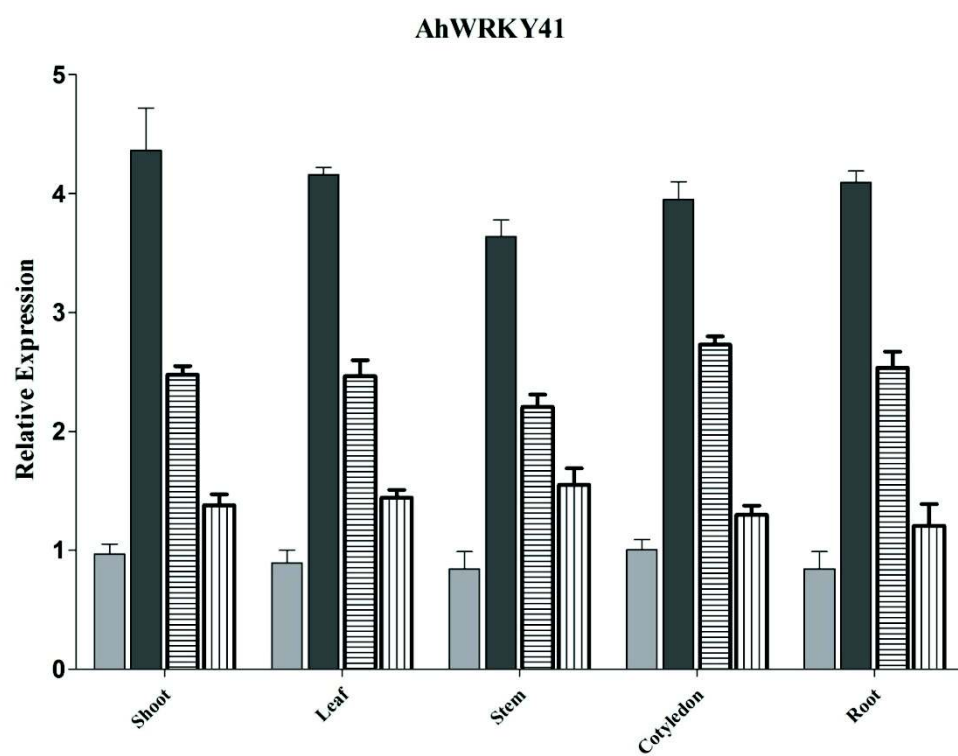
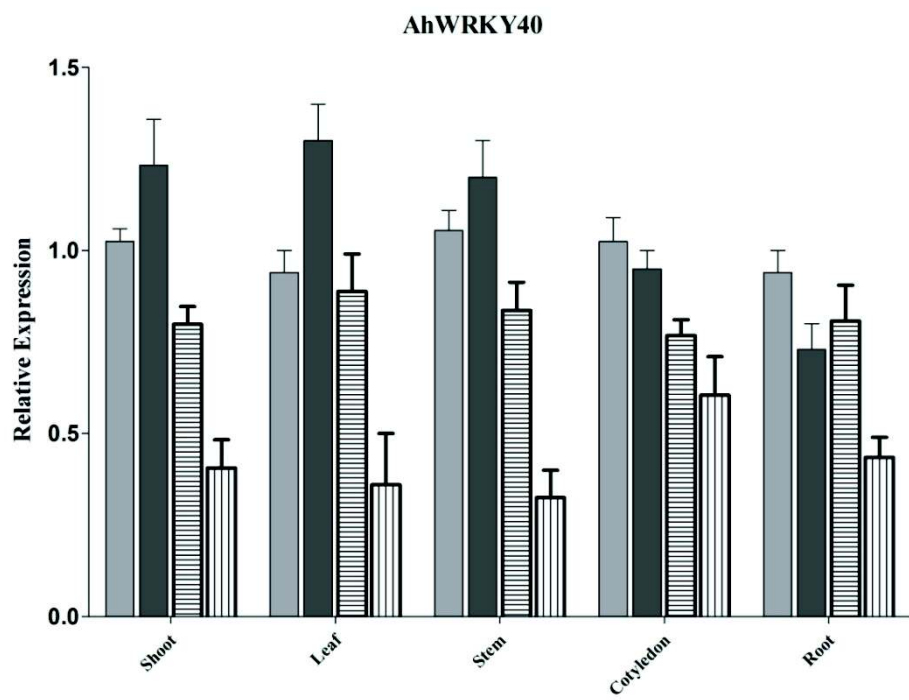
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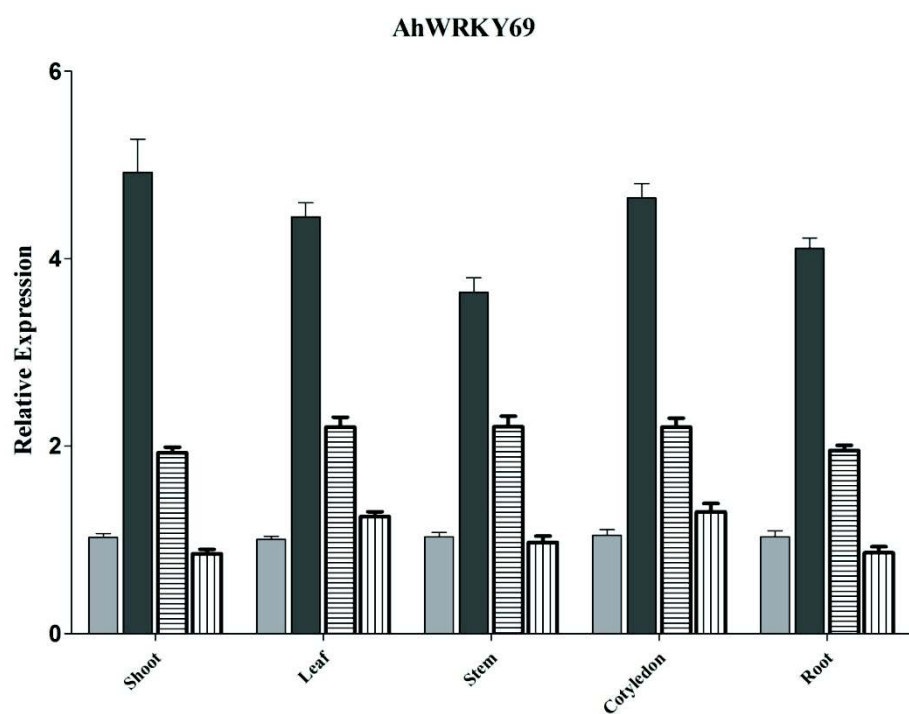
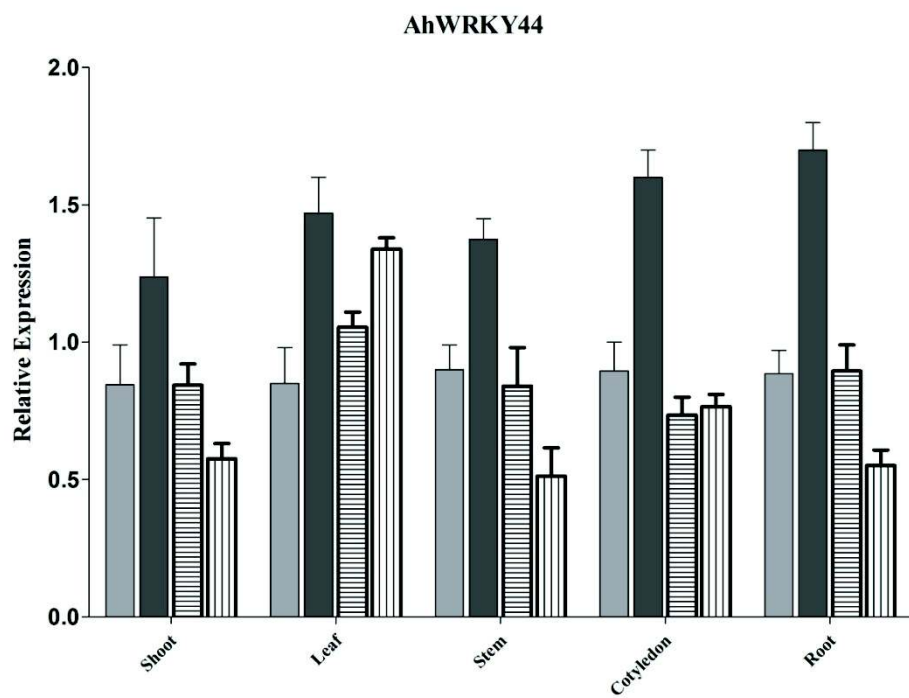


Figure 5: Expression pattern of 10 *AhWRKY* genes under high temperature, drought and heavy metal stress in different tissues.

224 Functional characterization and deciphering the stress tolerance mechanism by WRKY was
225 elucidated only in model crops. In groundnut, no reports on expression and functional
226 characterization of stress associated WRKYs are found till date. Multiple sequence alignment of
227 the Peanut WRKY domain shows homology with *Arabidopsis* and *Glycine max*. Hence, we
228 predict that the functional annotations of AhWRKYs may be fairly similar to AtWRKYs and
229 GmWRKYs. Phylogenetic analysis reveals that 10 WRKYs were classified into three groups. Of
230 the three, the Group II were found to be abundant, which includes *AhWRKY22*, *AhWRKY40*,
231 *AhWRKY12*, *AhWRKY15*, *AhWRKY69* and *AhWRKY44*, were further assembled into four sub-
232 groups (IIa, IIc, IId and IIe). The WRKY protein belonging to Group I identified in every
233 ancestral organism has two WRKY domains and represents the ancestral form and evolved early
234 [13]. In addition, motifs analysis using MEME tool confirms the presence of WRKY domain and
235 zinc finger motifs. Previously, it was reported that, among the two domains only the C- terminal
236 domain belonging to Group I has sequence specific DNA binding activity with W- box while N-
237 terminal domain showed weaker binding activity [26]. However, the N-terminal WRKY domain
238 might alternatively provide an interface for protein- protein interactions that coincide with the
239 function of zinc- finger like domains [13]. It was assumed that, due to the variability in the N-
240 terminal domain during evolution, it may be evolved into another pattern to accomplish other
241 regulatory functions or deleted from the sequence. As WRKY genes are themselves
242 transcriptionally regulated, understanding the regulatory processes governed by these genes is a
243 challenge. However, their distinct expression pattern in various tissues under specific biological
244 condition might unfold the regulatory functions of these transcription factors. Several studies
245 have described the essential roles of WRKY TFs in the regulation of gene expression [27- 30]. In
246 various plants such as *Arabidopsis*, rice, *Glycine max*, wheat, cotton, maize and *Populus*, a

number of WRKY genes are characterized that function as key regulators in signaling pathways for resistance to abiotic stress [31- 35, 13, 10]. In present study, we imposed three abiotic stress treatments such as high temperature, drought and heavy metal on 10 day old seedlings and studied expression analysis of *AhWRKY* genes. The results indicated that most of the *AhWRKY* genes were induced in various tissues. Compared to the drought and heavy metal stress, there are few reports on response of WRKY genes to high temperature stress. *AhWRKY41* is induced in all the three stresses in various tissues including leaf, cotyledon, stem and root indicating that it may be involved in multi abiotic stress response. Zhong et al. [36] reported the regulation of ABI3 expression and seed dormancy by WRKY41 and ABA in Arabidopsis. Luo et al. [37] demonstrated the tolerance of *GsWRKY20* over-expression lines to drought which exhibited decreased water loss rate and stomatal density in Arabidopsis and also showed the transgenic lines mediated ABA signaling by selectively promoting the expression of negative regulators and repressing the positive regulators. Our study emphasize, *AhWRKY20* shares homology with *GmWRKY20* is repressed in all three abiotic stress conditions and *AhWRKY40* is repressed in drought and heavy metal stress suggests these genes might play an important role in drought stress response and ABA signaling. Xiang et al. [38] analysed the physiological function of the *Arabidopsis* WRKY22 during dark-induced senescence which was suppressed by light and promoted by darkness, thus evidences the participation of AtWRKY22 in the dark-induced senescence. Our results showed that *AhWRKY22* repressed in all three stress conditions which gives a clue that it could be involved in senescence. Further, *AhWRKY2* was induced in response to high temperature and drought stress and evidences shows its homology in Arabidopsis, WRKY2 transcription factor mediates seed germination and post germination developmental arrest by ABA [39]. Lai et al. [40] demonstrated the positive role of WRKY3 and WRKY4 in

270 plant resistance to necrotrophic pathogens. Interestingly, *AhWRKY3* is highly induced under
 271 cadmium and drought stress which gives evidence for its role in stress responses in Peanut.
 272 WRKYs TFs were implicated integrating ROS homeostasis regulation and abiotic stress
 273 resistance in many crops. A recent study explored APTEALA 2 is regulated by different
 274 members of WRKY and NAC family, in addition, the link between the ROS-response ZAT12
 275 zinc finger protein and iron regulation in cells [41, 42]. The protein- protein interaction network
 276 identified WRKY18, GUN5, WRKY33, STZ, RHL41, DIC2, CZF1 and CML38 which show
 277 association with *AhWRKY* proteins. WRKY18 and WRKY33 interacting with elicitor-
 278 responsive *cis*-acting element, positively modulates defense- related gene expression and disease
 279 resistance [43, 44]. GUN5 (GENOMES UNCOUPLED 5), a multifunctional protein was
 280 involved in chlorophyll synthesis, plastid-to-nucleus retrograde signalling and ABA perception
 281 [45]. STZ (salt tolerance zinc finger), was found to repress the stress responsive genes DREB1A
 282 and LTI78 and could be involved in jasmonate (JA) early signaling response. Similarly, RHL41
 283 is involved in light acclimation, cold and oxidative stress responses [46], while DIC2 could be
 284 involved in protecting plant cells against oxidative stress. CZF1 (zinc finger CCCH domain-
 285 containing protein) is involved in salt stress response.

286 **5. Conclusion**

287 Various abiotic stress such as high temperature, drought and heavy metal adversely affect
 288 plant growth and development. WRKY Transcription factors are known to regulate plant stress
 289 responses by altering the expression of stress responsive genes. In this study we have
 290 characterized the expression of 10 WRKY transcription factors from Groundnut. The findings
 291 demonstrate that *AhWRKY41* would be a positive regulator involved multi abiotic stress
 292 tolerance. The tissue specific expression analysis of WRKY TFs under different abiotic stress

and their *insilico* characterization may help in complete understanding of regulatory role of these factors.

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