Journal of Stress Physiology & Biochemistry, Vol. 21, No. 1, 2025, pp. 206-221 ISSN 1997-0838

ORIGINAL ARTICLE

OPEN ACCESS

Deciphering ROS and ABA mediated WRKY transcription factors under abiotic stress conditions in Groundnut

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Received September 11, 2024

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 ($CdCl_2$). 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 N-terminal of *AhWRKY41* showed transcriptional activation in yeast cells. Higher levels of proline content and activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), but reduced malondialdehyde (MDA), and H_2O_2 levels were observed in all the stress conditions. 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 by ABA mediated cellular antioxidant systems.

Key words: WRKY, Abiotic stress, RT- qPCR. Transactivation, ROS

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 C_2H_2 or C_2HC and evolutionarily highly conserved (Tang *et al.*, 2014). A single WRKY transcription factor might mediate transcriptional reprogramming associated with several signalling 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 (Qiu, Yu. 2009, Wu *et al.*, 2009). Niu with co-authors and Wang with co-authors (Niu *et al.* 2012, Wang *et al.*, 2013) 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) (Bakshi and Oelmüller. 2014). 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 (Zhang *et al.*, 2008, Ulker *et al.*, 2007). 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 (Zhou *et al.*, 2008). 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 (Tang *et al.*, 2014). 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 (Zhao *et al.*, 2010). Extensive research on WRKY TFs has been carried out in the recent past in several crop plants such as rice (Ross *et al.*, 2007), cucumber (Ling *et al.* 2011) maize (Wei *et al.*, 2012), tomato (Huang *et al.* 2012) 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.

MATERIALS AND METHODS

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.

Conserved motifs and Gene structure analysis

The MEME Suite tool v4.9.1 (http://meme.nbcr.net/meme) (Bailey *et al.*, 2009) 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.

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 $CdCl_2$ for 72h. After the stress treatment, control and stress exposed tissues were harvested immediately and stored at -80 °C for further analysis.

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 (Supplementary file 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.

Transcription Activation of *AhWRKY41*

For the transactivation assay, complete sequence of *AhWRKY41-N* open reading frame (ORF) sequence were generated by PCR with primers (Supplementary file 1). The PCR products was fused with yeast GAL4 DNA binding domain in frame with the in the vector pGBTK7 (Clontech) between the NcoI and BamHI sites and the recombinant plasmid was transformed into yeast (AH109), harboring the HIS3 and LacZ reporter genes. Further, yeast strain was plated on SD/Trp- medium and cultured at 30°C. HIS3 activity was assessed for viability test on a histidine-lacking medium with 10 mM 3-AT (3-amino- 1,2,-triazole, Sigma, USA). LacZ activity was tested by β-galactosidase according to the manufacturer's instructions (Clontech).

Electrolyte leakage, MDA, Proline and Antioxidant enzyme activity assays

Electrolyte leakage was determined using relative conductivity as previously described (Cao *et al.*, 2007). Lipid peroxidation was estimated as the MDA content (Cui, Wang, 2006), and the free proline content (Shan *et al.*, 2007). The antioxidant enzyme activities of SOD, POD, CAT in the shoots were estimated as described (Qiu *et al.*, 2011). Each assay was replicated at least three times per sample.

RESULTS

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 Supplementary file 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 from5.2559 to 10.2566. The metal chelation zinc finger motif pattern of the three groups were C-X_{4-5}-C-X_{22-23}HxH (C2H2) (I), C-X_5CX_{22-23}-HxH (C2H2) (II) and C-X_7-CX_{23}-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/) (Yu *et al.*, 2006) programs. The results showed 9/10 AhWRKY proteins were localized to nucleus and 3/10 was predicted to be localized in cytoplasm and chloroplast.

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 AhWRKYs 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).

Analysis of motif composition, Gene structure and Phylogenetic tree

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 AhWRKY20shares 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).

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. 5a). During drought, *AhWRKY41* and *2* showed 2.4 and 2 fold up regulation while, *AhWRKY40* shows significant down regulation by 0.48 fold (Figure. 5b).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. 5c). *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 Supplementary file 3a. Analysis of Pearson correlation co- efficient 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 shows their association with WRKY18, GUN5, WRKY33, STZ, RHL41, DIC2, CZF1 and CML38 (Supplementary file 3b).

Transcription Activation of *AhWRKY41*and antioxidant enzymes

To verify AhWRKY41 transcriptional activation of full length AhWRKY41 ORF, Yeast strain AH109 was transformed with fusion plasmids pGBKT7-AhWRKY41-N, and pGBKT7 as a control. As shown in Figure. 6, the yeast cells transformed with pGBKT7- AhWRKY41- N grew well in His^- medium. Meanwhile, yeast cells transformed with other plasmids could only survive on SD/-Trp medium. The result of *LacZ* staining showed that the yeast cells transformed with pGBKT7-AhWRKY41-N turned blue in the presence of X-gal. These results indicated that the N-terminal region of AhWRKY41 has transcription activation activity. More than 2 fold expression of Ah*WRKY41* suggests abiotic stress tolerance in Groundnut and exhibited a higher levels proline and superoxide dismutase (SOD) content, as well as higher activities of catalase (CAT) and peroxidase (POD), but less ion leakage (IL), lower contents of malondialdehyde (MDA) and H_2O_2.

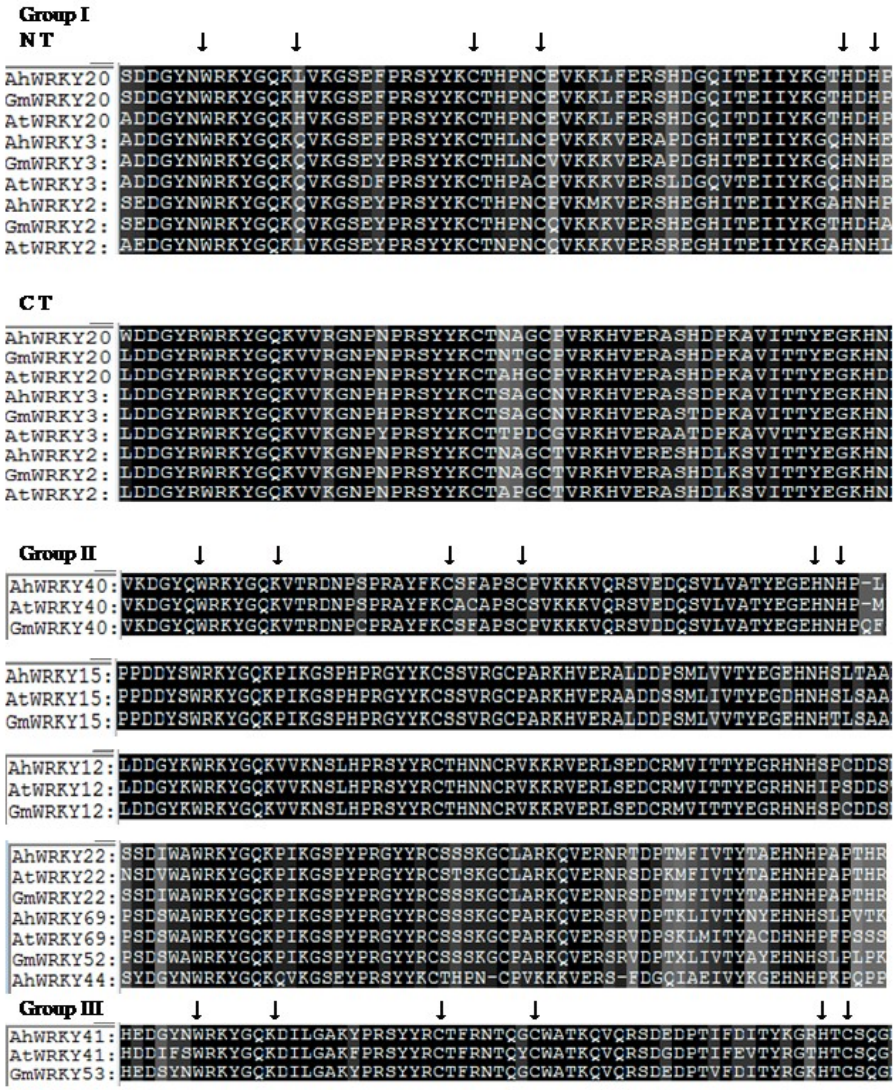

Figure 1: Multiple sequence alignment of conserved WRKY domains from Peanut, *Arabidopsis* and *Glycine max*. NT and CT represent N termini and C termini of Group I WRKY domains respectively. The highly conserved WRKYGQK domain is indicated between the arrows. Cystein (C) and Histidine (H) are represented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

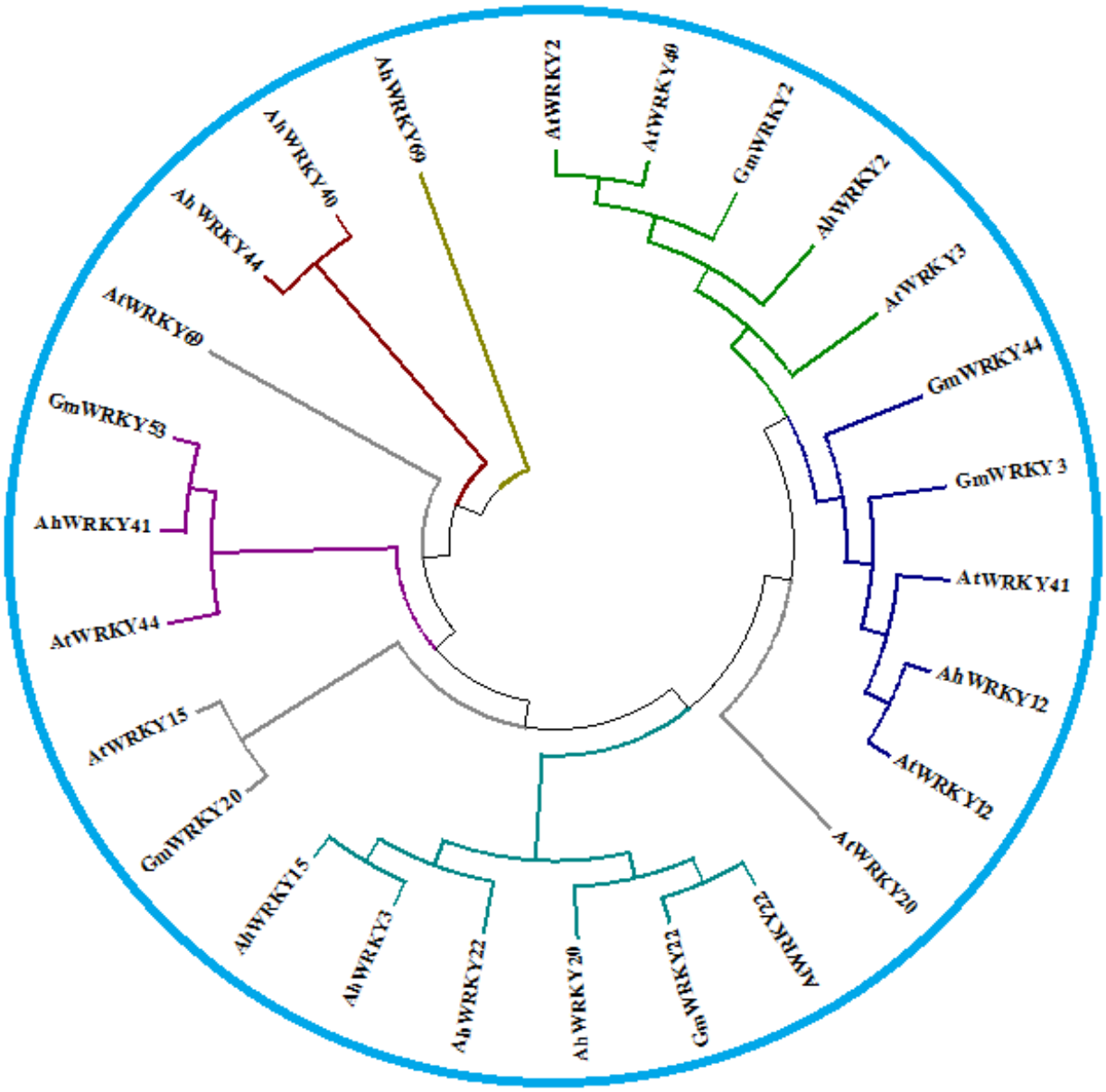

Figure 2. Un-rooted Phylogenetic tree of Peanut, *Arabidopsis* and *Glycine max* WRKY sequences. The tree was constructed by the MEGA v6.0 program with the Neighbor-Joining algorithm. The evolutionary relations were calculated using the p-distance method.

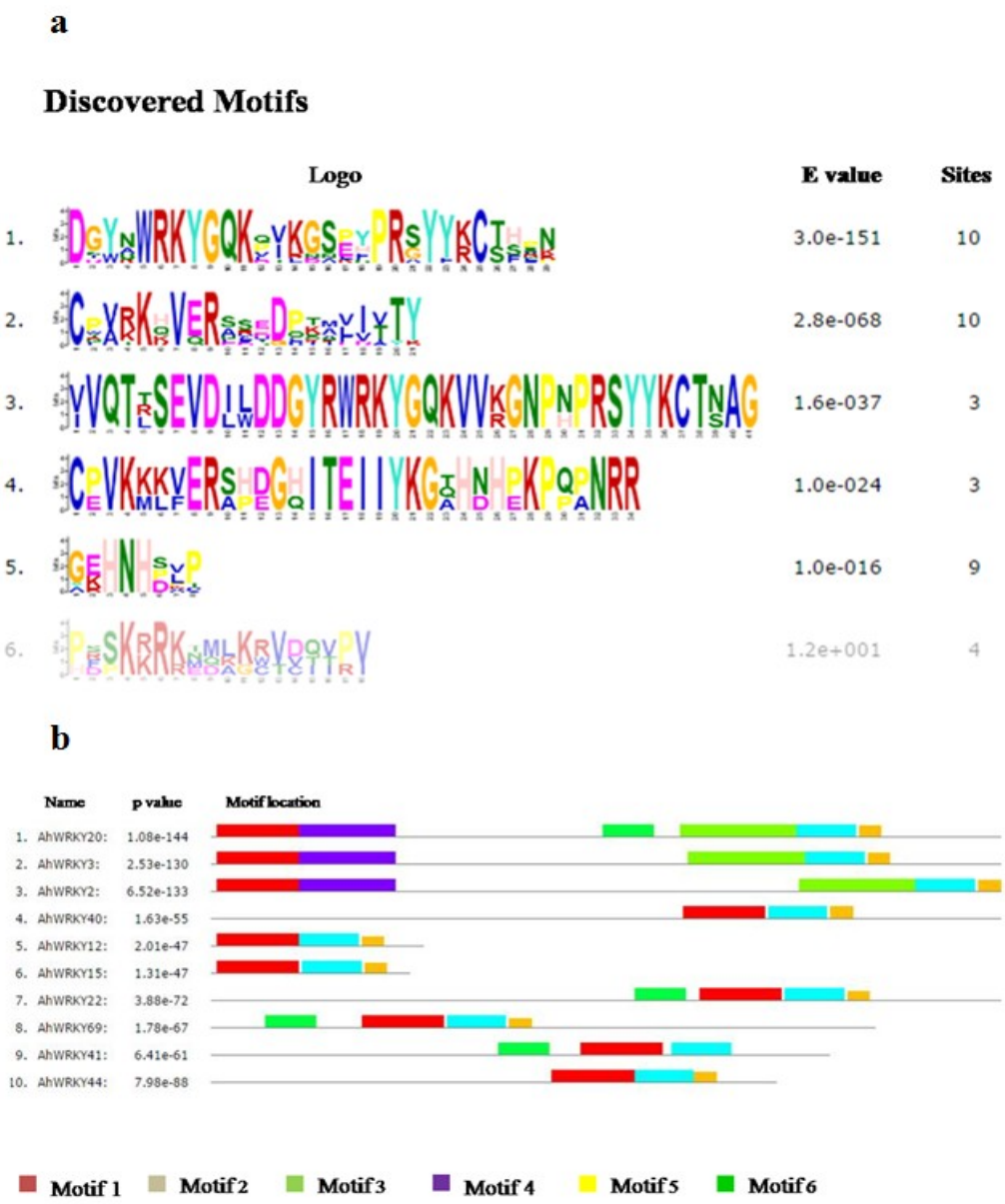

Figure 3: a) The domain prediction of ten *A. hypogaea* WRKY protein sequences was performed using MEME software, which generated a letter logo to represent the WRKY domain, zinc finger and other motifs. The height of the letters in the y-axis represents the degree of conservation and relative frequency of each amino acid at that position. b) The distribution of conserved motifs among the putative AhWRKY proteins is shown and different motifs are represented by different color blocks as indicated at the bottom of the figure.

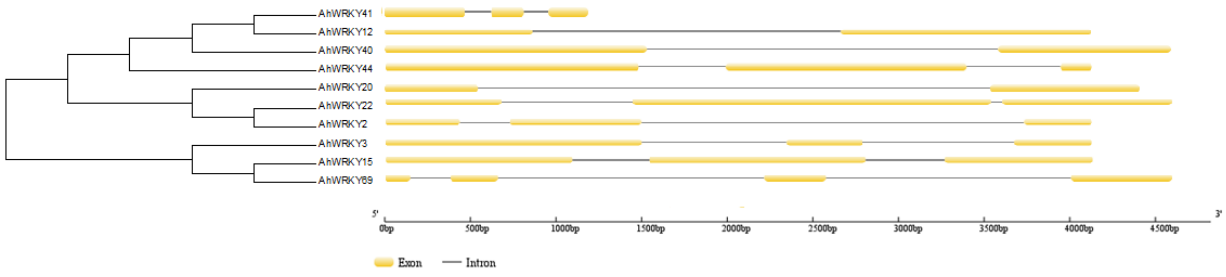

Figure 4: Phylogenetic relationship and gene structure of the WRKY genes. Yellow box indicate exons and black line indicates introns.

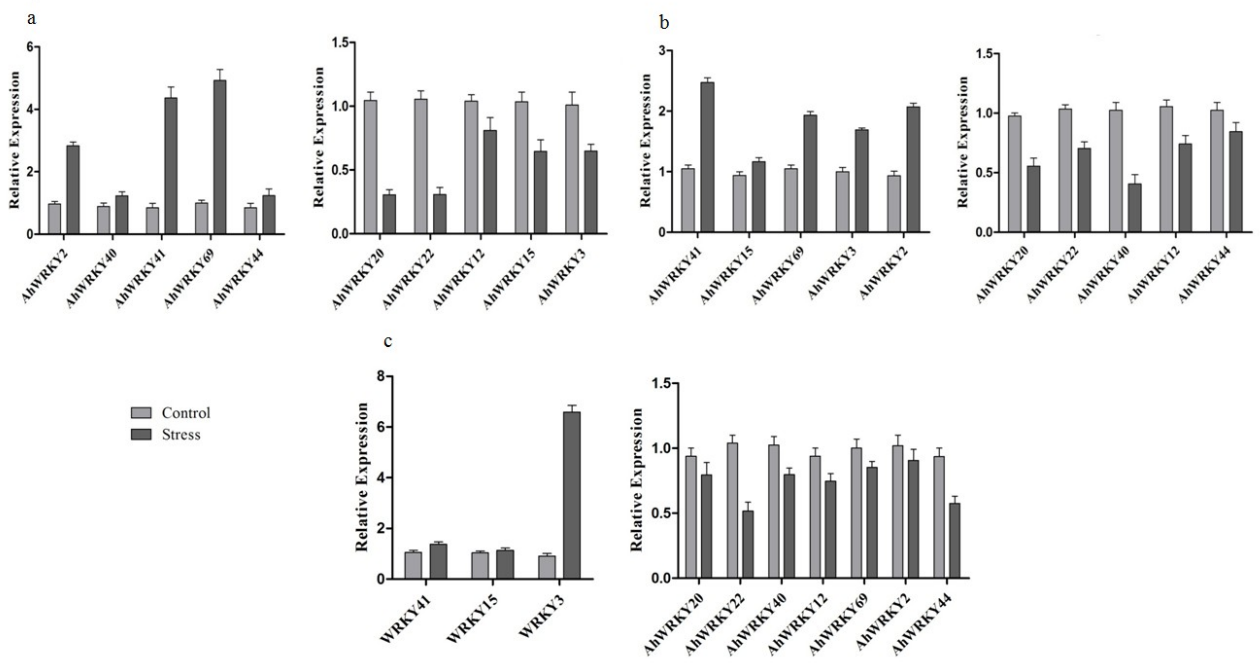

Figure 5: Expression pattern of 10 AhWRKY transcription factors. a) High temperature b) Drought c) Heavy metal

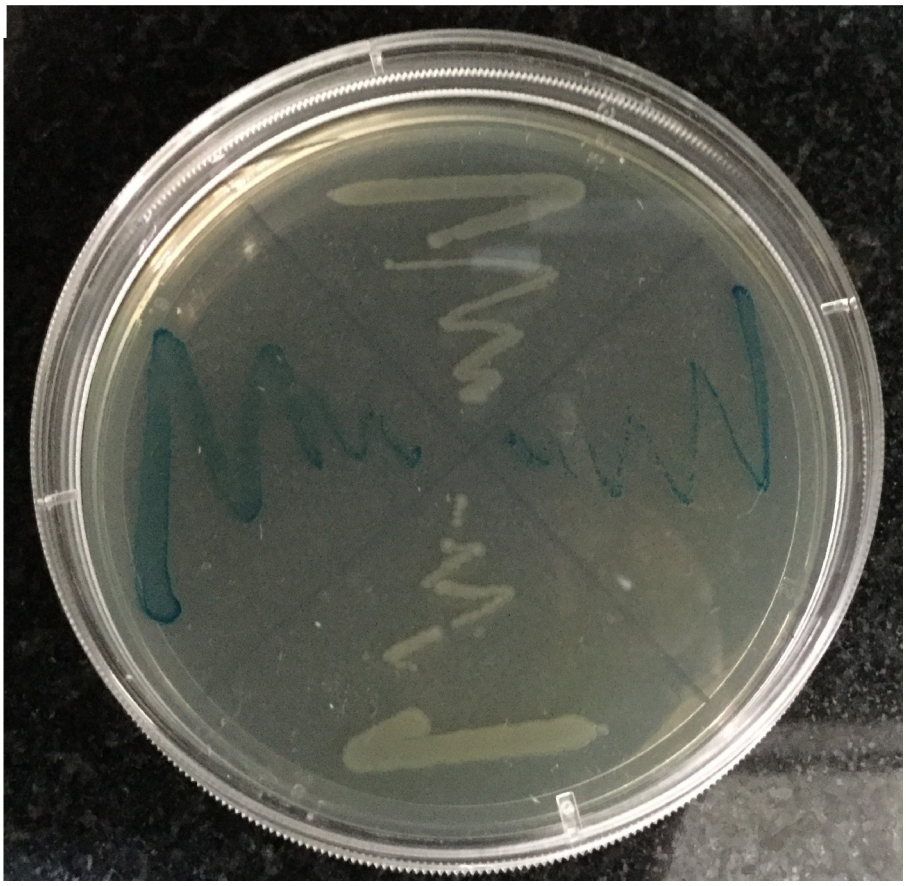

Figure 6: Transactivation assay of AhWRKY41 in yeast. The plasmids contains the fusion genes and the control plasmid (pGBTK7) (White) were expressed in yeast strain AH109. The transformants (Blue) were streaked on plates containing SD/Trp- and X- gal.

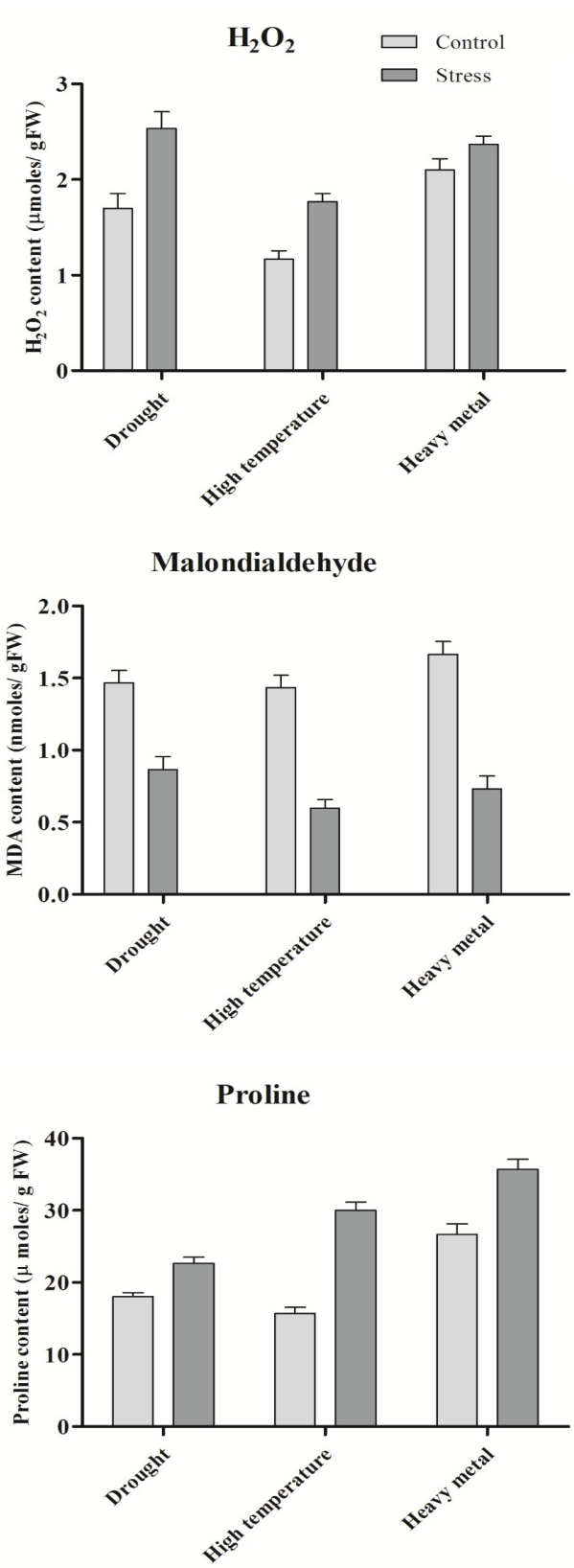

Figure 7: Changes in a) H_2O_2 (µmoles/ g FW) b) MDA (nmoles/ g FW) and c) Proline content (µmoles/ gFW). Values are mean of three replicates ±SE.

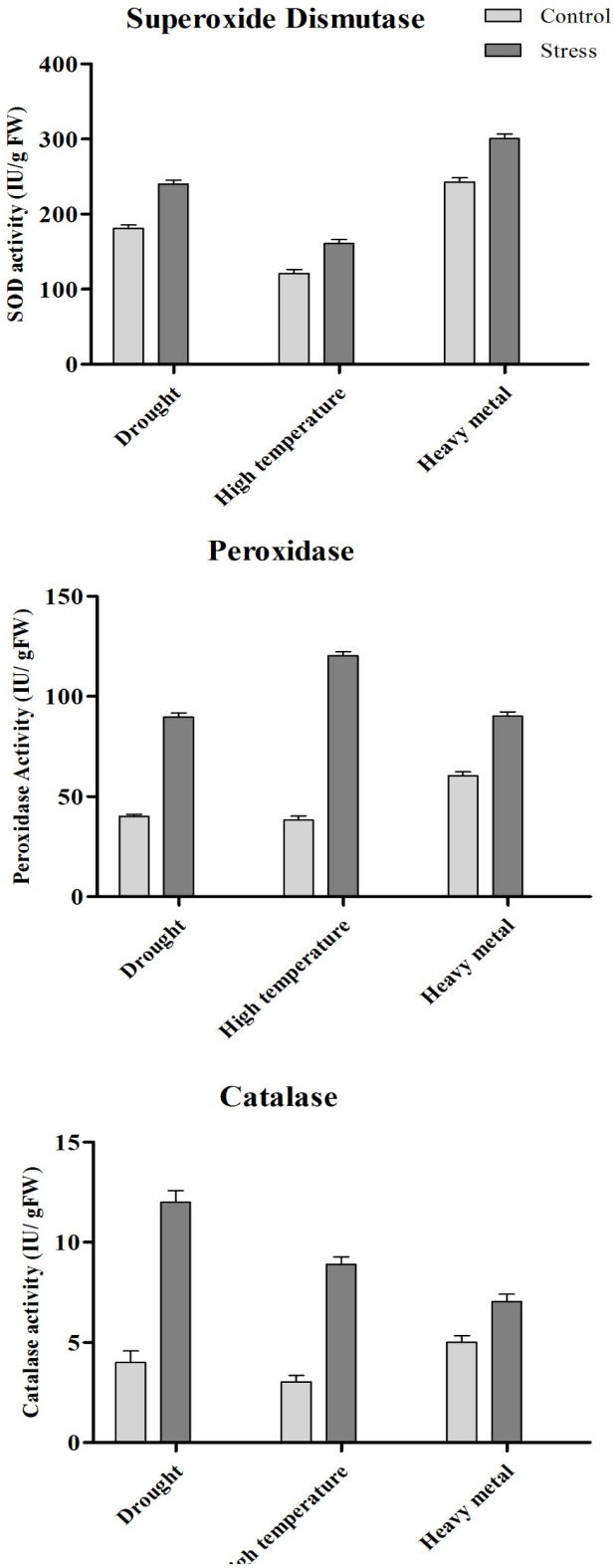

Figure 8: Changes in activities of a) SOD (IU/ g FW) b) POD (IU/ gFW) and c) CAT (IU/ gFW). Values are mean of three replicates ±SE.

DISCUSSION

Plants have developed a great ability to reprogram their transcriptome in a highly dynamic and temporal approach through an integrated network of transcription factors to adapt to the changing environmental stress

conditions. Among these, WRKY proteins are important members of transcription factors involved in regulation of plant stress responses (Pandey, Somssich, 2009). Although it has been well documented that WRKY TFs were connected with various plant defense mechanisms under abiotic stress conditions. Meanwhile, non-*WRKY* genes that enhance plant drought and salt stress by either efficient ROS elimination through the activation of the cellular antioxidant systems or the activation of stress-associated genes have been extensively reported in *Arabidopsis* (Moon *et al.* 2003, Luo *et al.* 2009), *Oryza sativa* (Ning *et al.*, 2010, Kumar *et al.* 2012), *Poncirus trifoliate* (Huang *et al.*, 2010, Huang *et al.*, 2011), and wheat (Hu *et al.*, 2012, Hu *et al.*, 2013). Functional characterization and deciphering the stress tolerance mechanism by WRKY was elucidated only in model crops. In groundnut, no reports on expression and functional characterization of stress associated WRKYs is found till date. Multiple sequence alignment of the Peanut WRKY domain shows homology with *Arabidopsis* and *Glycine max*. Hence, we predict that the functional annotations of AhWRKYs may be fairly similar to AtWRKYs and GmWRKYs. Phylogenetic analysis reveals that 10 WRKYs were classified into three groups. Of the three, the Group II were found to be abundant, which includes *AhWRKY22, AhWRKY40, AhWRKY12, AhWRKY15, AhWRKY69 and AhWRKY44*, were further assembled into four sub-groups (IIa, IIc, IId and IIe). The WRKY protein belonging to Group I identified in every ancestral organism has two WRKY domains and represents the ancestral form and evolved early (Wei *et al.*, 2012). In addition, motifs analysis using MEME tool confirms the presence of WRKY domain and zinc finger motifs. Previously, it was reported that, among the two domains only the C- terminal domain belonging to Group I has sequence specific DNA binding activity with W- box while N-terminal domain showed weaker binding activity (Llorca *et al.*, 2014). However, the N-terminal WRKY domain might alternatively provide an interface for protein- protein interactions that coincide with the function of zinc- finger like domains (Wei *et al.*, 2012). It was assumed that, due to the variability in the N- terminal domain during evolution, it may be evolved into another pattern to accomplish other regulatory functions or deleted from the sequence. As WRKY genes are themselves transcriptionally regulated, understanding the regulatory processes governed by these genes is a challenge. However, their distinct expression pattern in various tissues under specific biological condition might unfold the regulatory functions of these transcription factors. Several studies have described the essential roles of WRKY TFs in the regulation of gene expression (Raineri *et al.*, 2015). In 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 (Dong *et al.*, 2003, Wei *et al.*, 2012, Zhao *et al.*, 2015). 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. Ding *et al.* (2014) reported the regulation of ABI3 expression and seed dormancy by WRKY41 and ABA in Arabidopsis. Luo *et al.* (2013) 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. Zhou *et al.* (2011) 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 (Jiang and Yu, 2009). Lai *et al.* (2008) demonstrated the positive role of WRKY3 and WRKY4 in plant resistance to necrotrophic pathogens. Interestingly, *AhWRKY3* is highly induced under cadmium and drought stress which gives evidence for its role in stress responses in Peanut. WRKYs TFs were implicated integrating ROS homeostasis regulation and abiotic stress resistance in many crops. A recent study explored APTEALA 2 is regulated by different members of WRKY and NAC family, in addition, the link between the ROS-response ZAT12 zinc finger protein and iron regulation in cells (Wang *et al.* 2013, Wang *et al.* 2016). Le *et al.* (2016) reported that ZAT12 interacts with and suppresses the function of a central regulator of iron deficiency responses, the basic helix-loop-helix transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR. The iron uptake is suppressed and risk of hydroxyl radical formation is prevented by up- regulation of ZAT12 in response to ROS accumulation (Dietz *et al.* 2016). Similarly W-box promoter of RBOH, and WRKY is phosphorylated by MAPK, linking MAPK phosphorylation events in response to pathogen recognition with accumulation in tobacco. Recently ROS-response regulatory proteins were explored by underlying the APETALA2/ethylene response transcription factor redox responsive transcription factor 1 regulated WRKYs (Matsuo *et al.* 2015). GhWRKY involved in stress response by regulating ABA signalling and cellular levels of ROS. Sun *et al.*, (Sun *et al.*, 2015) isolated WRKY gene BdWRKY 36 from B. distachyon and found its functions as a positive regulator of drought by controlling ROS homeostasis and regulating stress related genes. The protein- protein interaction network identified WRKY18, GUN5, WRKY33, STZ, RHL41, DIC2, CZF1 and CML38 which show association with AhWRKY proteins. WRKY18 and WRKY33 interacting with elicitor-responsive *cis*-acting element, positively modulates defense-related gene expression and disease resistance (Chen *et al.* 2010, Birkenbihl *et al.* 2012). GUN5 (GENOMES UNCOUPLED 5), a multifunctional protein was involved in chlorophyll synthesis, plastid-to-nucleus retrograde signalling and ABA perception (Mochizuki *et al.*, 2001). STZ (salt tolerance zinc finger), was found to repress the stress responsive genes DREB1A and LTI78 and could be involved in jasmonate (JA) early signalling response. Similarly, RHL41 is involved in light acclimation, cold and oxidative stress responses (Iida et ak., 2000), while DIC2 could be involved in protecting plant cells against oxidative stress. CZF1 (zinc finger CCCH domain-containing protein) is involved in salt stress response.

CONCLUSION

AhWRKY41, a group of III WRKY family member was annotated from Groundnut for the first time, which was significantly up- regulated by abiotic stress conditions and exhibits transcriptional activation in yeast cells. Further, accumulation of proline and H_2O_2, decreased MDA and improved ROS system emphasise AhWRKY41 may serve as a positive regulator and synergistic regulation by ROS homeostasis through direct or indirect activation of the cellular antioxidant systems or activation of stress-associated gene expression.

CONFLICTS OF INTEREST

The author declare that he has no potential conflicts of interest.

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Supplementary file 1: Primers used for Real time PCR and amplification of AhWRKY41- N

Supplementary Table 1: Primers used for Real time PCR

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Amplicon Length (bp)	Tm (°C)
Actin	TTGGAATGGGTCAGAAGGATGC	AGTGGTGCCTCAGTAAGAAGC	196	57.13
AhWRKY41	CCTCTGAGGGAGGACAATCA	AAGGCCACTCTCAAAGCTCA	108	60.19
AhWRKY20	AATGCTGGTTGCCCTGTTAG	CCATCCCAAGATCAAGGCTA	205	60.13
AhWRKY4	GGTGGAGAATCCGATGAGAA	ATCAGCCAAATCCTCTCCCT	147	60
AhWRKY40	TGGCTGAAATGCTTTCTGTG	TCTCGGAGTTCCCATTGTTC	180	60
AhWRKY12	GCTACAGCCACAGTCACAGC	TCGGATCCATGCTTCTAACC	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	ATCAGCCATGAAAGATTCGG	GCGGATGTTAAAGCCTTCTG	140	60
AhWRKY41- N	TA**CCATGG**CAAGCAACAGCAACAGCAAC	AC**GGATCC** TTTGTACTTGCTTCGTGGCC	301	59.06

Supplementary file 2: Features of 10 WRKY Proteins in Peanut

Supplementary Table 2: Features of 10 WRKY Proteins in Peanut

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	GIIe	C-X5CX22−23-HxH
AhWRKY40	Ahy011981	40	320	8.2094	35.704	Nuclear	Nuclear	GIIa	C-X_5CX_{22-23}-HxH
AhWRKY12	Ahy014145	12	218	8.0863	24.62	Cytoplasm	Nuclear	GIIc	C-X_5CX_{22-23}-HxH
AhWRKY15	Ahy014372	15	363	10.2566	39.276	Nuclear	Nuclear	GIId	C-X_5CX_{22-23}-HxH
AhWRKY69	Ahy014637	69	235	5.3279	24.885	Nuclear, cytoplasm	Nuclear	GIIe	C-X_5CX_{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	GIIe	C-X_5CX_{22-23}-HxH

Supplementary file 3: Expression pattern of 10 *AhWRKY* genes under abiotic stress in different tissues.