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Unraveling *NAC* family transcription factors and their expression analysis under high temperature and drought stress in Peanut

Suchithra B.[1], Shafia Hoor F.[1]. Puspha T. C.[2] and Nagesh Babu R.[1]*

[1] *Department of Chemistry & Biochemistry, Maharani Cluster University, Bengaluru - 560001, Karnataka, India*

[2] *Department of Zoology, Maharani Cluster University, Bengaluru - 560001, Karnataka, India*

*E-Mail: ***nageshbabur@gmail.com***

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Transcription factors play pivotal roles in the conversion of stress signal perception to stress-responsive gene expression. NAC (NAM, ATAF1/2 and CUC2) domain proteins are plant-specific transcriptional factors known to play diverse roles in various plant developmental processes and received considerable attention as regulators in stress signaling. Considering the relatively large number of NAC transcription factors from different plants and their diverse roles under complex environmental stimuli remains a challenge. In this study, phylogenies, genome localizations, gene structure and expression profiles of NAC genes under high temperature and drought treatments, with a focus on Peanut (*Arachis hypogaea*) genotypes (*A. duranensis & A. ipaensis*) was performed. Thirty eight NAC genes from each genotype were detected, including eight membrane-bound members which includes AdNAC26, AdNAC36, AiNAC16, AiNAC17, AiNAC37, AdNAC14, AiNAC12 & AiNAC29. Majority of the identified NAC proteins had atleast four NAC domain containing conserved motifs and were found to be localized to nucleus. *AdNAC21* and *AiNAC3* were found to be positive regulators in drought and high temperature responses. Our results provides foundation for selection of promising stress- responsive NAC candidates for detailed plant functional analysis, leading to development of transgenic Peanut varieties with improved productivity under drought and high temperature.

Abbreviations: TFs- Transcription factors; TMHs- Trans-membrane helices; NTL- NTM like; NTM-NAC with Trans-membrane Motifs

Key words: NAC transcription factor, phylogenetic analysis, qRT-PCR, Peanut

Abiotic stresses such as high temperature and drought greatly limit the growth and crop productivity worldwide. Several NAC (NAM, ATAF1/2, and CUC2) proteins have been documented as important regulators in stress responses. The NAC TFs function as important components in complex signaling progresses during plant stress responses. Until recently, the possible involvement of TF NAC proteins in abiotic stress responses was deduced indirectly from transcription profiling; recent functional analyses, however, have provided some direct evidence. The tight regulation and fine-tuning of NAC genes during plant stress responses contribute to the establishment of complex signaling webs, and the important roles of NAC genes in stress responses make them potential candidates for imparting stress tolerance. Plant-specific NAM, ATAF1/2, and CUC2 (NAC) proteins constitute one of the largest transcription factor (TF) families and are characterized by a well-conserved N terminal NAC domain (Olsen *et al.*, 2005, Puranik *et al.*, 2012). The NAC domain, which comprises nearly 160 amino acid residues, can be divided into five sub domains (A to E) based on its motif distribution (Aida *et al.*, 1997, Ooka et al, 2003). The highly conserved sub-domains C and D may be responsible for binding to DNA, sub domain A may be involved in homo- and hetero- dimerization, and the divergent sub-domains B and E may be implicated in the functional diversity of NAC proteins (Ooka *et al.*, 2003; Jensen *et al.*, 2010; Chen *et al.*, 2011). Genes encoding NAC proteins were regulated (i) transcriptionally by upstream TFs such as ABREs (ABA-responsive elements) and DREs (Dehydration-responsive elements), (ii) post transcriptionally by micro-RNAs or alternative splicing, and (iii) post-translationally by ubiquitinization, dimerization, phosphorylation or proteolysis (Puranik *et al.*, 2012). As an additional feature, some NAC proteins comprise a helical transmembrane motif for anchoring to plasma membrane or endoplasmic reticulum NAC proteins have been implicated in a wide range of plant developmental processes, including lateral root formation (He *et al.*, 2005), shoot branching (Mao *et al.*, 2007), flowering (Sablowski, Meyerowitz, 1998) and leaf senescence (Guo, Gan, 2006). In particular, numerous NAC domain proteins have also been implicated in various defense responses such as drought (Jeong *et al.*, 2010), salinity (Zheng *et al.*, 2009), cold (Aslam *et al.*, 2012), fungal and bacterial pathogens (Wang *et al.*, 2009). Extensive investigation aided by the availability of several complete plant genomic sequences has identified more than 100 NAC genes in Arabidopsis, rice, soybean, foxtail millet, Chinese cabbage, 74 in grape (*Vitis vinifera*) and 88 in pigeonpea (Wu *et al.*, 2015) etc. Abiotic stress triggers a wide range of plant responses, from the alteration of gene expression and cellular metabolism to changes in plant growth, development, and crop yield. Thus, understanding the complex mechanism of drought and high temperature tolerance is important for agriculture production (Nuruzzaman *et al.*, 2013). Although the genes encoding the transcription factors just accounts for a little portion in the whole genome, transcription factors are important in the regulated networks (Hobert, 2008).

Peanut (*Arachis hypogaea L*.) is an oilseed crop cultivated worldwide and one of the major grain legumes in tropical and subtropical regions. However, its productivity is strongly affected by drought one of the most serious constraints to crop production and, associated with the predicted consequences of global climate change, increases the need for drought-adapted varieties. To date, 80 species in genus *Arachis* were identified and have been classified into nine taxonomic sections (Bertiol *et al.*, 2011). Wild species are diploid, but cultivated peanut is allotetraploid (AABB). The wild ancestral species of cultivated peanut are generally considered to be *duranensis* and *ipaënsis*, which contributed the A and B sub-genomes, based on morphology, cytology, fertility of the interspecific hybrid and molecular studies (Kochert *et al.*, 1996; Ramos *et al.*, 2006). NAC genes in plants have been investigated in great detail for their role in various stress and developmental aspects; thus, they could be important candidates for the improvement programs. Detailed analysis of NAC domain genes using the assembled Peanut genome including prediction of gene functions, evolutionary significance and investigation of their expression profiles is necessary to understand function

of NAC gene family. Our results provide a comprehensive genome-wide knowledge of NAC proteins in peanut and a preliminary knowledge of specific NAC proteins potentially involved in drought and high temperature response in peanut. Through these analyses, we have increased knowledge concerning the evolution and function of Peanut NAC genes.

MATERIALS AND METHODS

Plant materials and treatment

Seeds of Peanut (Luhua14) 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, drought was imposed by withholding water for 5 days and for high temperature stress seedlings were exposed to high-temperature [42 °C for 2h (induction) followed by 48 °C for 6h]. After the stress treatment, control and stress exposed shoot were harvested immediately and stored at -80 °C for further analysis.

Identification, characterization and subcellular localization of NAC genes

The NAC domain containing protein sequences of Peanut were retrieved from the Plant Transcription Factor Database ver. 2.0. and *Arachis* genome (Peanut Base) for the hidden Markov model (HMM) profile of the NAC domain downloaded from the Pfam database using HAMMER (ver. 3.0). All redundant sequences were removed and the collected data were further curated by examining the presence of the conserved NAC domain with the help of Pfam (http://pfam.sanger.ac.uk/), SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) web server. The length, molecular weight and pI of each deduced polypeptide were calculated using ExpasyProtParam tool (http://web.expasy.org/protparam/). Further, CELLO (http://cello.life.nctu.edu.tw/) and WOLF PSORT (http://www.genscript.com/psort/wolf_psort.html) tools were used to predict the subcellular localizations. Amino acid sequences of NAC TFs belonging to Peanut were imported to BioEdit v7.2.5 (Hall, 1999) and multiple sequence alignment was performed with NAC protein sequences from Arabidopsis and Glycine max using ClustalW with default parameters. The NAC sequences along with Arabidopsis sequences were imported into MEGA v6.06 (Tamura *et al.*, 2013) to construct a phylogenetic tree by Neighbor-Joining method and the bootstrap test was performed with 1000 iterations. Finally, TMHMM Server ver. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the membrane bound NAC members.

Genome wide distribution pattern, Gene structure and identification of conserved motifs

The chromosomal location of *AdNAC* and *AiNAC* genes were obtained from peanut base website (http://peanutbase.org/) and the map was generated using MapInspect (http://mapinspect.software.informer.com/). 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 these NACs were downloaded and used as query for generating its gene structure. A number of introns and exons were estimated based on this alignment and confirmed by the coordinates given in the sequences. The MEME Suite tool v4.9.1 (http://meme.nbcr.net/meme) (Bailey *et al.*, 2009) was utilized for analysis of the conserved motifs. STRING 10 (http://string.embl.de/), computational tool was used to predict the functional protein association network gene ontology annotation (GO) of the NAC proteins in Arabidopsis with the default parameters. These interactions were derived from genomic context, high-throughput experiments and co-expressions studies.

Expression analysis of NAC genes

Total RNA was isolated from control and stress tissue 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 (Additional Resource 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 µlcDNA, 1x SYBR Green Master mix, 0.5 µl sequence-specific forward primer (10 µM), 0.5 µl universal reverse primer (10 µM), and 7 µl sterile water. The NAC expression was normalized against actin as reference gene. 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. All reactions were run with three technical and two biological replicates and the data was analyzed using $2^{-\Delta\Delta CT}$ method.

RESULTS

Protein features, Multiple sequence alignment and Phylogenetic analysis

To identify all the NAC transcription factors, we retrieved all the predicted NAC genes from Plant TFDB and Peanut Base (http://peanutbase.org/). Basic information like molecular weight and pI are depicted in Additional Resource 2. The average polypeptide length was 347.1 residues with the length ranging from 158 aa (AdNAC17) to 698 aa (AiNAC37). The pI values range from 4.43 to 9.46. The subcellular localization results revealed that majority of the proteins were localized to nucleus and 9/76 were predicted to be localized in cytoplasm. The multiple alignment of AdNACs, AiNACs and NACs from Arabidopsis and Glycine max indicated that all of the Peanut NACs shared a highly conserved N-terminal DNA binding NAC domain, which consists of five consensus sub-domains (A-E), and a variable C-terminal transcriptional regulation domain. Additionally, a conserved bipartite nuclear localization signal was also found in the D subdomain of the majority of Peanut NACs, suggesting that these NACs may be localized to the nucleus (Additional Resource 3) (Greve *et al.*, 2003). To examine the structure and phylogenetic relationships of Peanut NAC TFs identified in our study, a combined phylogenetic tree was constructed with the aligned NAC domains from Peanut, Arabidopsis and soybean (Fig. 1). Examination of the phylogenetic tree analysis emphasis that the Peanut NAC TFs can be classified into nine major groups: Group I (14) Group II (8), Group III (17), Group IV (5), Group V (14), Group VI (4), Group VII (2), Group VIII (4) and Group IX (8). Phylognetic trees constructed from the AdNACs, AiNACs and AdNAC-AiNACs together are depicted in the Additional Resource 4A & Fig. 2 respectively.

Identification, chromosomal distribution and gene structure of NAC members

The keyword, HMM profile and BLAST search predicted that the Peanut genome encodes about 38 NAC proteins. A total of 38 NAC genes were identified from both *A. duranensis* and *A. ipaënsis*. They were named as *AdNAC1* to *AdNAC38*, and *AiNAC1* to *AiNAC38*, respectively. The genome of Peanut comprises of 20 chromosomes (10 from *duranensis* and 10 from *ipensis*) varying in their length, shortest being chromosome 8 (48.94 Mb) and longest is the chromosome 3 (133.14 Mb) in *A. duranensis while in A. ipaënsis*, shortest being chromosome 2 with 108.64 Mb and longest being chromosome 5 with 149.44 Mb in size (Fig. 3). *In silico* mapping of NACs indicated an uneven distribution of the genes on all the chromosomes. The exact position (in bp) of each *AdNACs* and *AiNACs* on Peanut chromosomes is given in Additional Resource 2. The gene structures were investigated through genomic annotation to determine the structural diversity. All NAC genes harbored at least two exons except *AiNAC16* being the shortest not having intron. In addition, a separate phylogenetic tree was generated from the complete protein sequences of all the NAC genes (Fig. 2).

Identification of conserved motifs and Gene annotation

The MEME (Multiple Expectation Maximization for Motif Elicitation) server was used for exploring motif distribution in 38 AdNAC and 38 AiNAC proteins. Nine different conserved motifs were identified, of which most of them had at least four NAC domain-encoding motifs, and 54 shared a highly conserved typical NAC domain containing five consensus sub domains (motifs 2, 4, 1, 5 and 3) in the same order (Fig. 4). The motif sequence logos are depicted in the Additional Resource 4C. Prediction of functional protein association network of NAC proteins in Arabidopsis using STRING program revealed the interaction of NAC083 (AdNAC15) with VND1, VND7, NAC1, NAC41, ANAC026 and NAC007; XND1 (AdNAC5) with NAC073 and NAC010; NAC090 (AiNAC14) with NAC044 and NAC036; BTF3 (AiNAC2)

with NACA2 and AT3G12390 (Additional Resource 4D). Gene ontology (GO) annotation of NAC proteins showed the involvement of these proteins in different biological processes, cellular and molecular functions (Additional Resource 4E). The membrane transcription factors proteins are stored in their dormant forms in association with the intracellular membranes. When plants are exposed to abrupt environmental changes, they are released from the membranes through proteolytic cleavage events and enter the nucleus, where they regulate expression of genes involved in perception of stress signals, stress signaling (Kim *et al.*, 2010). Among 76 NACs (38 AdNACs& 38 AiNACs), 08 members (AdNAC14, 26, 36, AiNAC16, 17, 29 and 37) were identified as membrane-associated NTLs using the TMHMM v2.0 (Table 1), of which 5 (AdNAC26, AdNAC36, AiNAC16, AINAC17 and AiNAC37) and 3 (AdNAC14, AiNAC12 and AiNAC29) members contain one and two TMHs, respectively. The phylogenetic tree constructed with membrane associated NTLs identified from Peanut, *Arabidopsis* and Rice indicated that the Peanut NTLs were scattered into different groups (Additional Resource 4B).

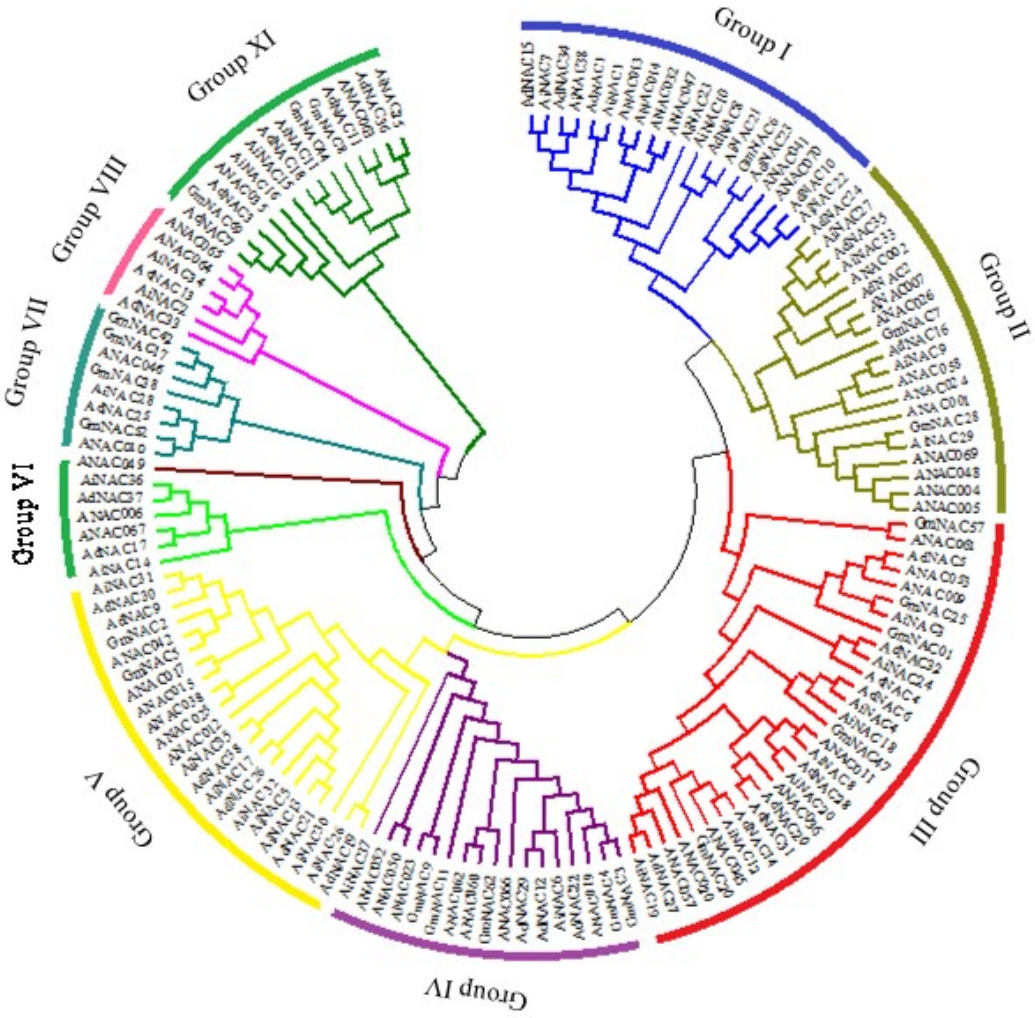

Figure 1: Unrooted phylogenetic tree constructed using the neighbor-joining (NJ) method, and the bootstrap test was carried out with 1,000 iterations representing the relationships between the Peanut, *Arabidopsis* and *Glycine max* NAC domain proteins.

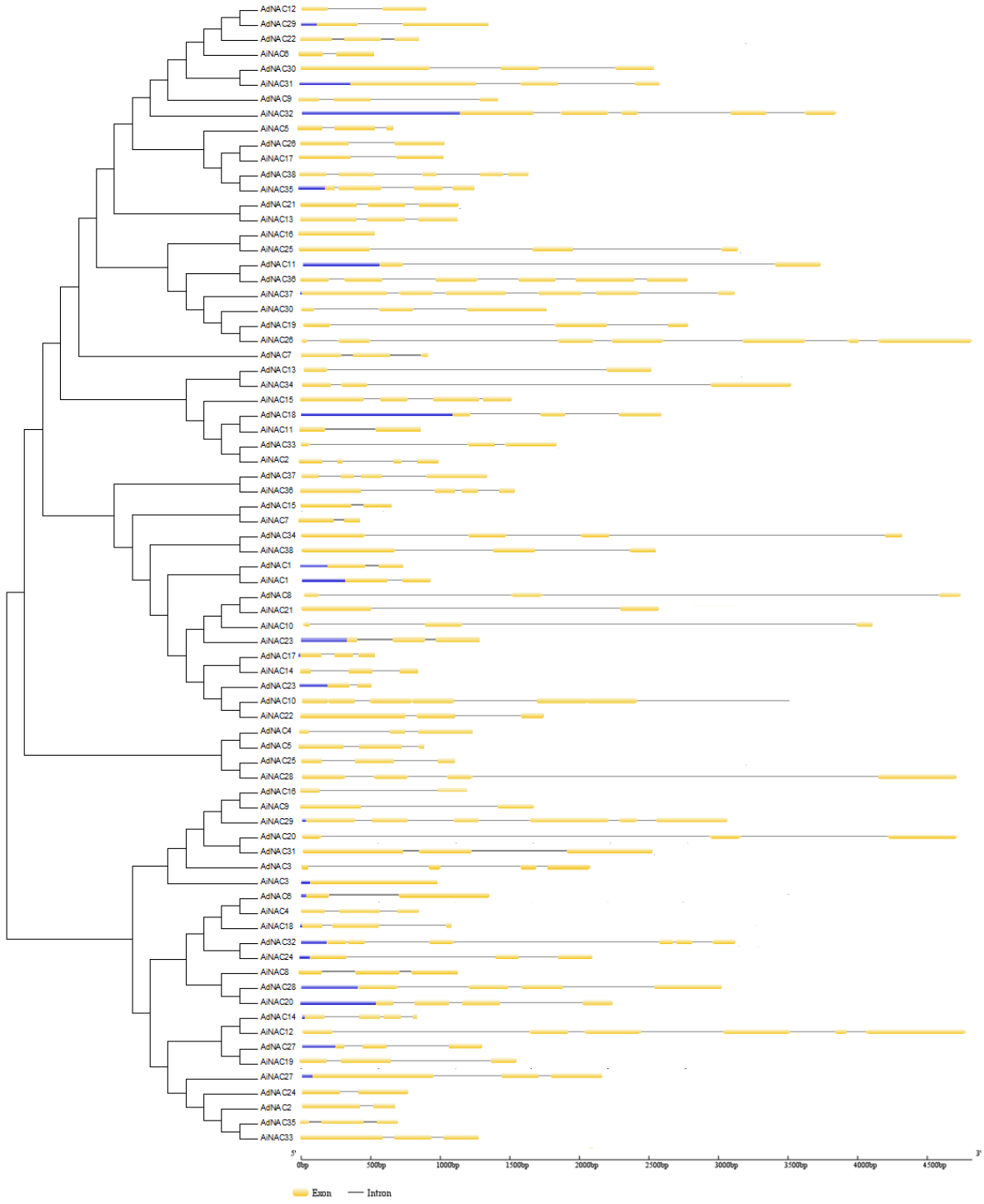

Figure 2. Phylogenetic relationship and gene structure of the NAC genes. Phylogenetic tree was constructed with MEGA6.0 using the neighbor-joining (NJ) method with 1,000 bootstrap replicatesbased on a multiple alignment of 76 amino acid sequences of NAC genes from Arachis duranensis & Arachis ipaënsis. Exon/ intron structure of NAC genes are represented by boxes and black lines, respectively.

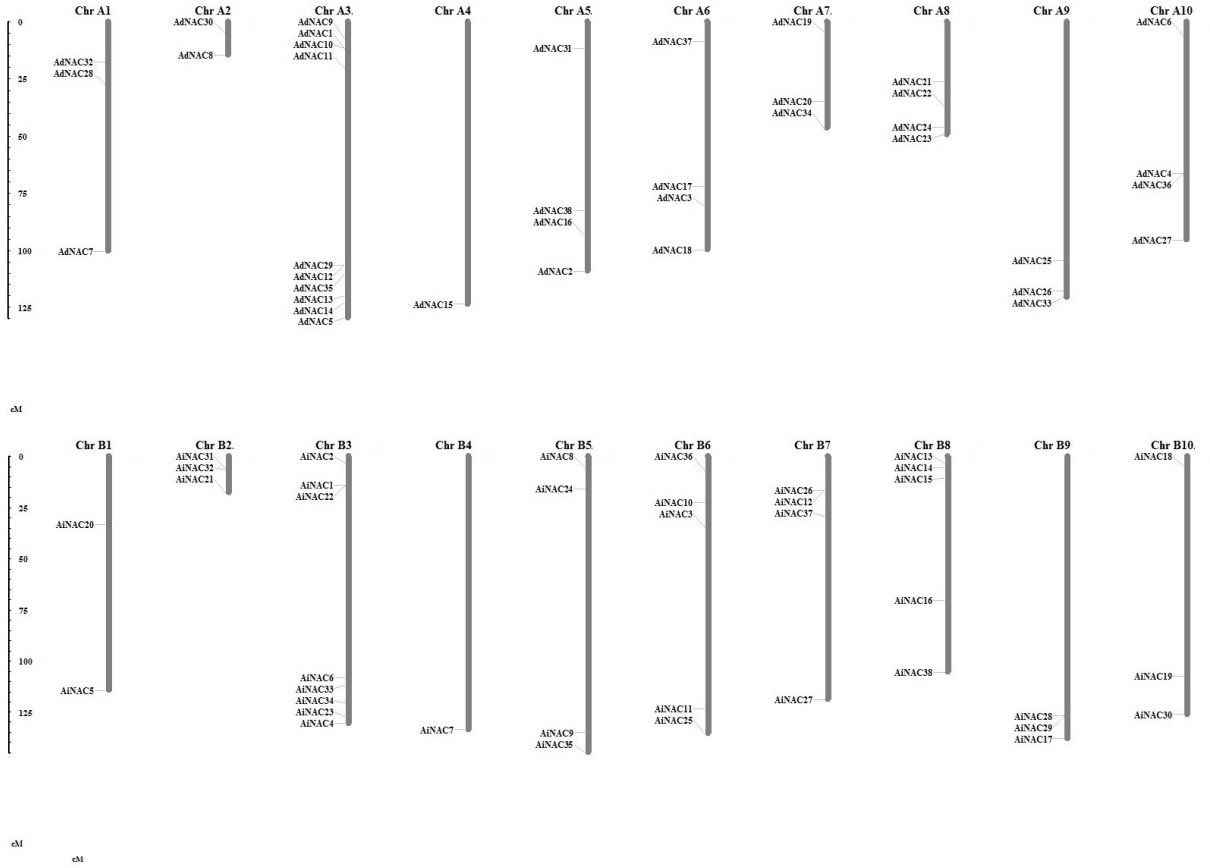

Figure 3: Distribution of 76 NAC genes on Peanut chromosomes and physical locations of each NAC gene on the ten chromosomes from each species (positions in cM).

Table 1: Putative membrane- bound Peanut NTLs

Gene Name	Membrane bound member	Length (aa)	Transmembrane sequences	Expected number of AAs in TMHs	Expected number, first 60 AAs
AdNAC14	AdNTL1, AdNTL2	633	525…..544 612…..631	40.88286	0
AdNAC26	AdNTL3	551	523…..545	22.13992	0.00091
AdNAC36	AdNTL4	592	569…..591	22.56383	0.01596
AiNAC12	AiNTL5, AiNTL6	678	582…..604 656…..675	41.84687	0
AiNAC16	AiNTL7	216	5…..27	20.81172	20.80519
AiNAC17	AiNTL8	559	531…..553	22.14053	0.00084
AiNAC29	AiNTL9, AiNTL10	583	531…..553 558…..580	40.01905	0
AiNAC37	AiNTL11	698	669…..691	22.56604	0.01787

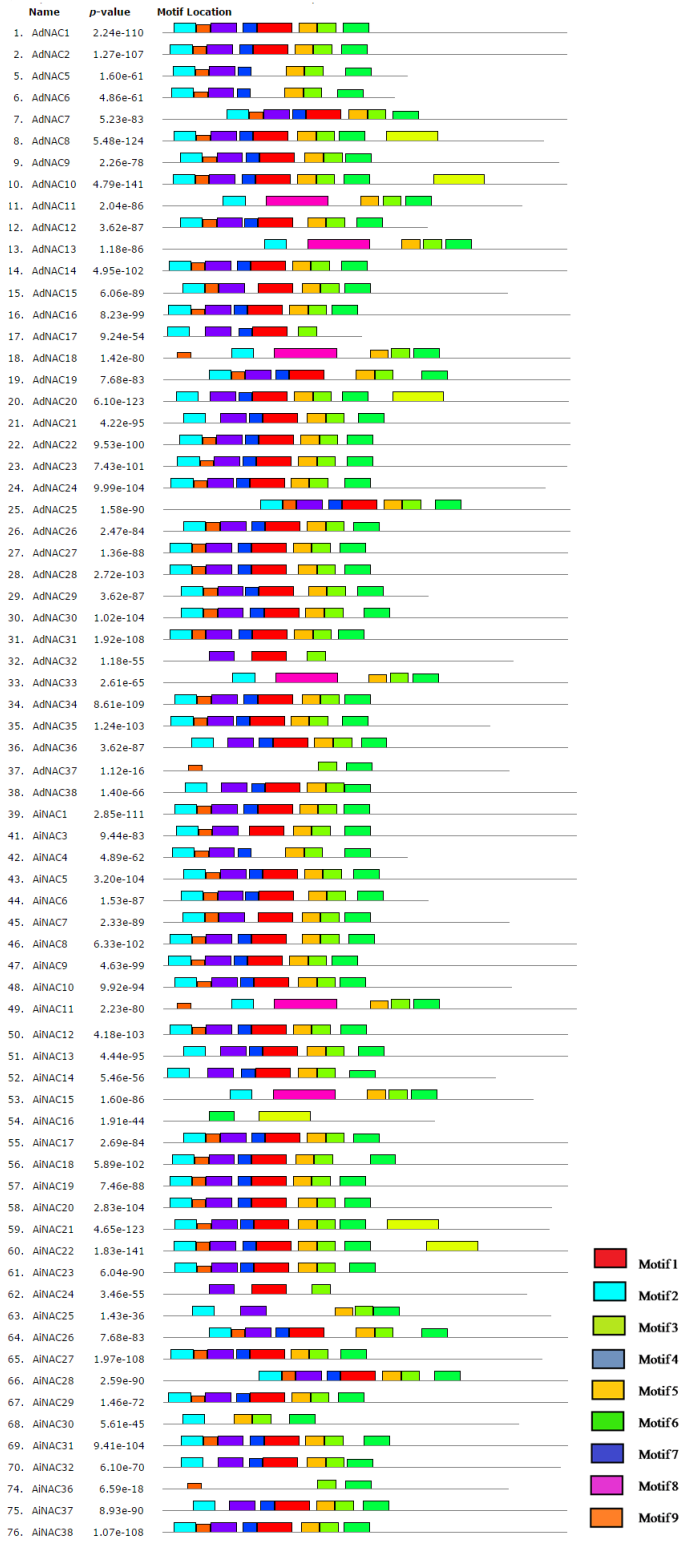

Figure 4: Schematic representation of conserved motifs in the AdNAC and AiNAC proteins predicted by MEME. Each motif is represented by a number in the colored box. The black lines represent non-conserved sequences.

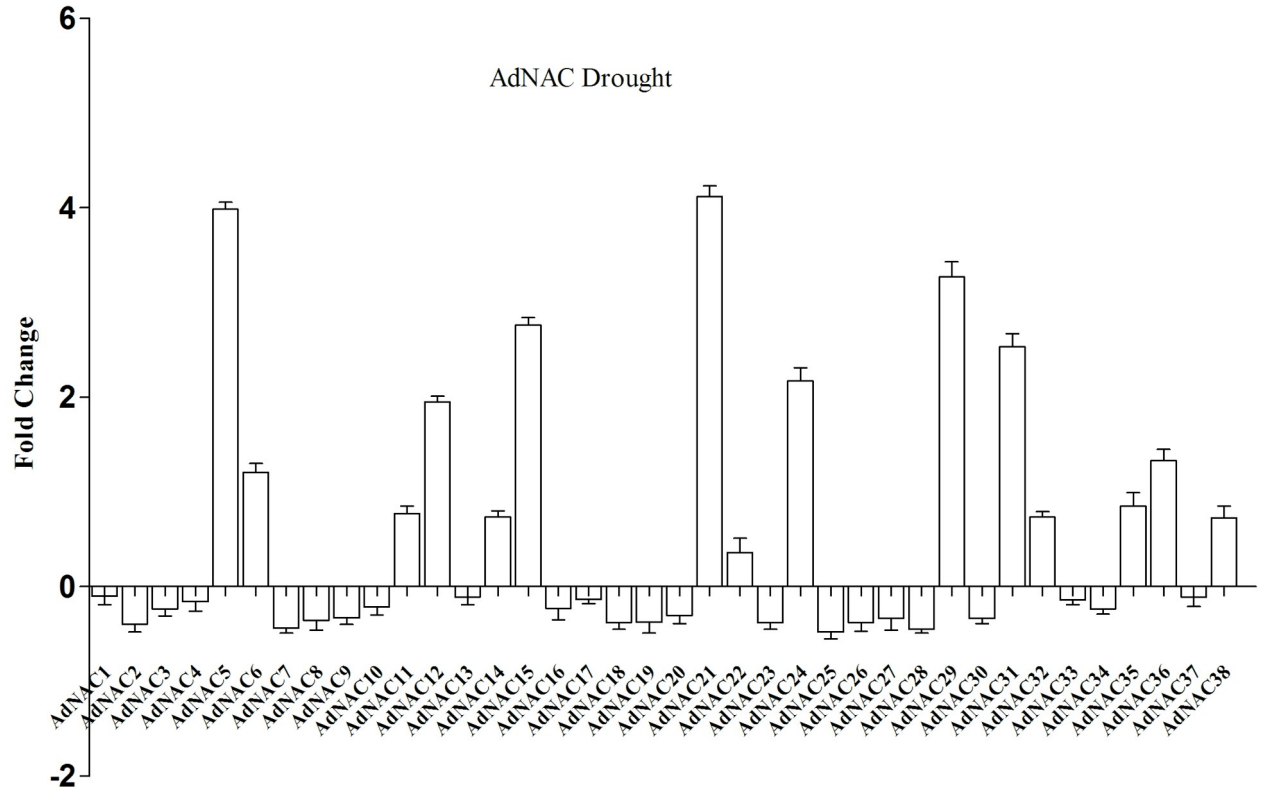

Figure 5: Expression profile of *AdNAC* genes obtained by RT-qPCR of treated (drought) and well watered (WW) control shoot samples

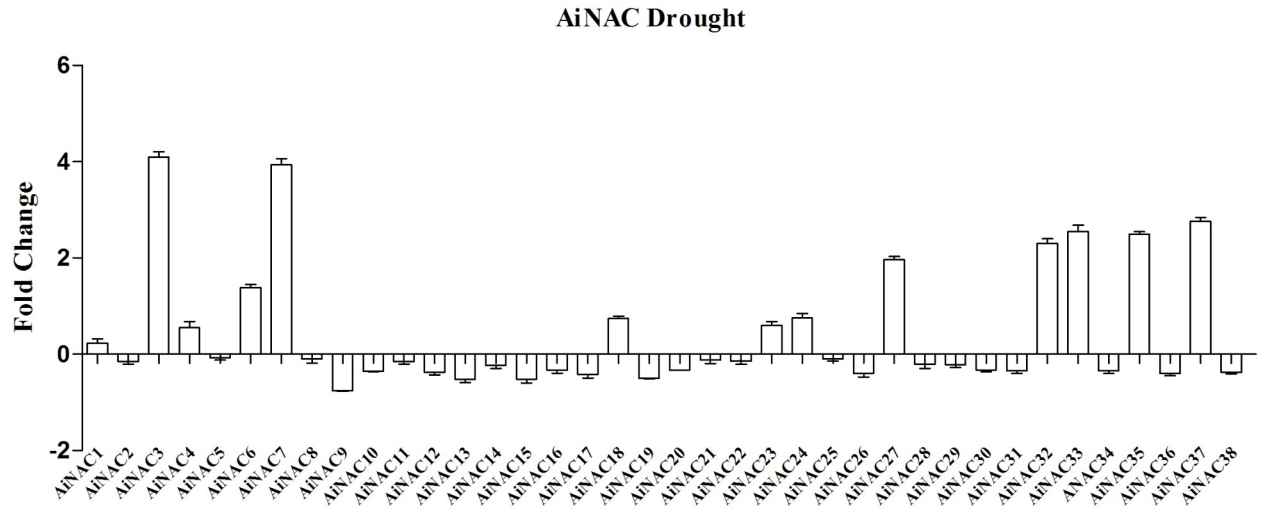

Figure 6: Expression profile of *AiNAC* genes obtained by RT-qPCR of treated (drought) and well watered (WW) control shoot samples

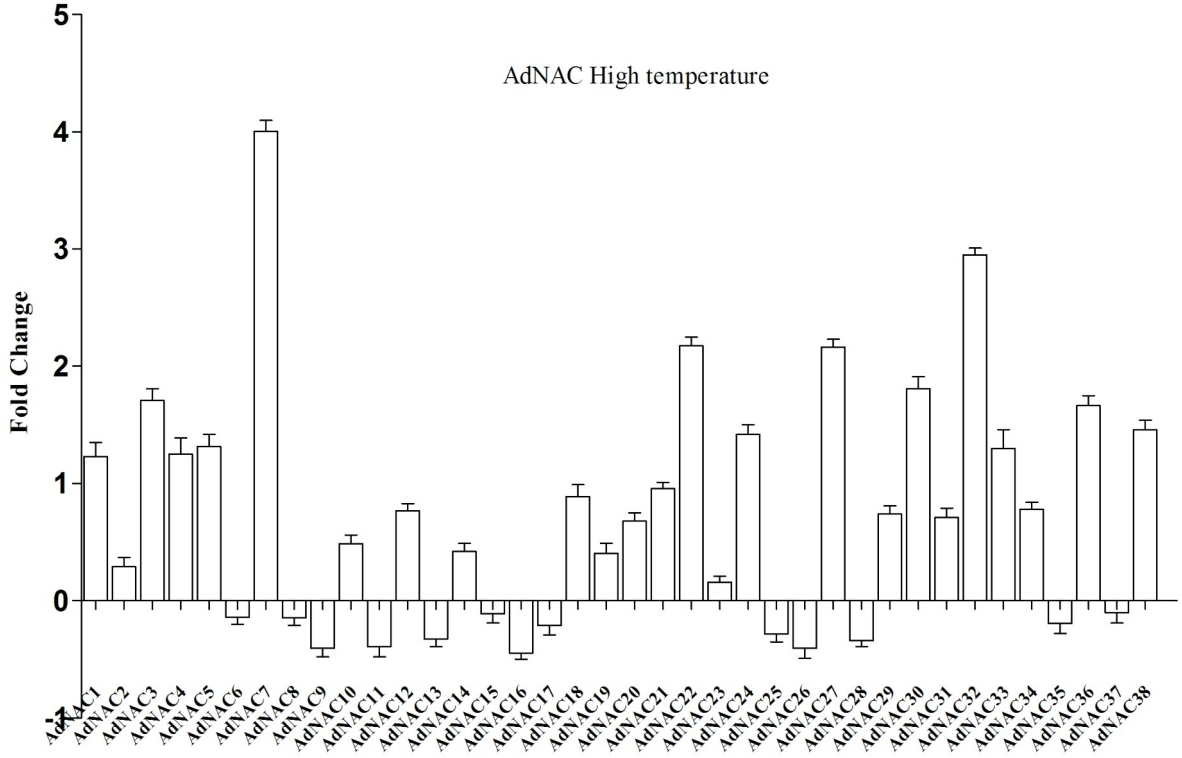

Figure 7: Expression profile of *AdNAC* genes obtained by RT-qPCR of treated (high temperature) and well watered (WW) control shoot samples

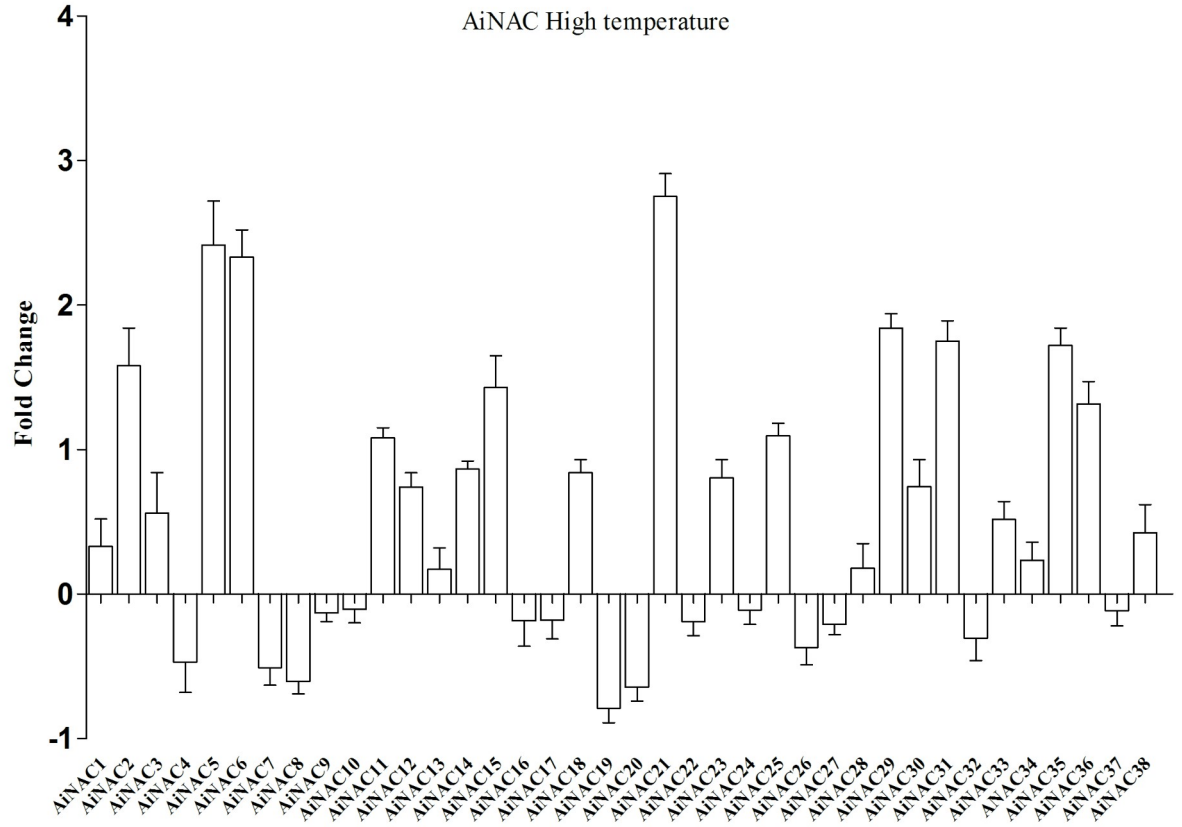

Figure 8: Expression profile of *AiNAC* genes obtained by RT-qPCR of treated (high temperature) and well watered (WW) control shoot samples

Expression profiles of AdNAC and AiNAC genes during high temperature and drought stress

NAC proteins are plant-specific TFs that have been shown to function in abiotic stress responses (Nakashima *et al.*, 2014; Puranik *et al.*, 2012). To investigate the responses of NAC genes to drought stress, we investigated the expression profiles of NAC genes in seedlings and expressed the results as fold changes with respect to the controls. During drought stress NACs belonging to Group V NACs such as *AdNAC32, AdNAC36, AdNAC38, AiNAC24, AiNAC32, AiNAC35* and *AiNAC37* were found to be up regulated by 0.68, 1.45, 0.85, 0.75, 2.4, 2.49 and 2.76 folds respectively. ANAC069 belonging to the same group encodes a membrane-bound NAC protein that integrates auxin and salt signals to regulate seed germination in Arabidopsis. In Group VI, *AdNAC5, AdNAC6, AdNAC12, AdNAC15, AdNAC21, AdNAC22, AdNAC24, AdNAC29, AdNAC35* were induced by 4.06, 1.3, 2.01, 2.84, 4.01, 0.51, 2.03, 2.43 and 0.71 folds respectively (Fig. 5 & 6). During high temperature, 48 genes were up-regulated and among them *AdNAC7, 22, 32, AiNAC5, 6, 21* were found to be induced by 4.1, 2.1, 3.01, 2.11, 2.52, 2.59 folds respectively. The other up-regulated genes showed fold change between 1 and 3. All the other genes were down regulated with the fold changes ranging between 0.5 to 1 (Fig7 & 8). *AdNAC21* and *AiNAC3* showed increased expression under both drought and high temperature stress.

DISCUSSION

The plant-specific NAC transcription factors (TFs) play important roles in regulation of diverse biological processes, including development, growth, cell division and responses to environmental stimuli. To cope with these stresses, plants have evolved a range of physiological and biochemical responses and a complex of signaling transduction pathways (You *et al.*, 2015). *AdNAC* and *AiNAC* genes were mapped to the Peanut genome according to their position information from Peanut Base. Among all, chromosome 3 contains the highest number of NACs (26%), while only one gene was located on chromosome 4 (0.026%). There was no positive correlation between the chromosome length and the number of *NAC* genes. The ends of chromosome exhibited stronger synteny than the central regions of chromosomes (Nagy *et al.*, 2012). It is well known that gene structural diversity is a possible mechanism for the evolution of multi-gene families. Investigation of gene structures reveals that the most closely related members in the same subfamilies shared similar exon/ intron structures in terms of intron number and exon length suggesting, these members may be evolved early and represent the ancestral form. All well-known NAC domain proteins bind specifically to the CATGTG motif of the promoter region (Tran *et al.*, 2004) or act as a functional motif or activation domain (Oh *et al.*, 2005). The relationship among the 76 Peanut NAC genes was investigated through constructing phylogenetic trees using Neighbour Joining method and the tree topology revealed several pairs of NAC proteins with a high degree of homology in the terminal nodes of each subfamily. According to Fig. 1, 76 NAC genes formed 9 clades and they were designated groups I to IX, the largest group was Group III which has 17 members. Several putative trans-membrane helices have been identified in other plant species such as Chickpea (Ha *et al.*, 2014), Glycine max (Le *et al.*, 2011), Arabidopsis (Kim *et al.*, 2010), Rice (Kim *et al.*, 2007), Maize (Shiriga *et al.*, 2014), Potato (Singh *et al.*, 2013), Foxtail millet (Puranik *et al.*, 2013), Chinese cabbage (Liu *et al.*, 2014) and Tomato (Kou *et al.*, 2014). Out of 11 putative GmNTLs of soybean and 08 CaNTLs of Chickpea, 2 and 4 members possess two TMHs respectively whereas all the NTLs predicted in other plant species possess only one TMH, suggesting that the existence of doubled TMHs might be specific to leguminous plants. A phylogenetic tree constructed from the NTLs from Peanut, Arabidopsis (NTLs/ANACs) and rice (OsNTLs/ONACs) indicated that the Peanut NTLs were scattered into 4 major groups (Additional Resource 4B). The membrane bound TF can immediately regulate the downstream genes upon stress perception and activation. In Arabidopsis a membrane bound NAC protein, NTL6, has been to shown to get activated upon cold stress as the membrane fluidity changes and induces the expression of pathogenesis related proteins. In addition, the plant hormone ABA also activates the NTL6 (Seo, Park, 2010), thus indicating the involvement

in biotic and abiotic stress responses. Membrane bound NAC proteins have been implicated as major players in biotic and abiotic stress response affects major physiological processes like flowering (Kim *et al.*, 2007), seed germination (Kim *et al.*, 2008), leaf senescence (Lee *et al.*, 2012) and also cell division (Kim *et al.*, 2006). Considering the varied functions of membrane bound NAC genes in crops, the identification of four membrane bound NAC genes would be useful in understanding their specific function in Peanut. Gene ontology annotation reveals that, a majority of these proteins were predicted to be involved in response to stress as well as cellular, metabolic and biosynthetic processes. The molecular functions of these proteins corresponded to transcription regulator activity. Further, cellular component analysis revealed the localization of these gene products in nucleus. Multiple sequence alignment and identification of conserved motifs using MEME tool indicates that most of the NAC proteins possessed A to E subdomains in the N termini that conferred the DNA-binding activities. The motif composition of these NAC sequences may provide clues for further functional analysis of these TFs. However, the biological significance of most of the putative motifs remains to be elucidated.

Several reports demonstrated that NAC genes were involved in regulating plant development at different growth stages causing us to further associate the biological functions of NAC genes (Wang *et al.*, 2013). In Arabidopsis, ANAC002/ ATAF1 was induced by long-term treatment with ABA and/ or during age-dependent senescence. ANAC019, ANAC055, and ANAC072 in Arabidopsis showed up-regulation at transcription levels after drought, high salinity and abscisic acid (ABA) treatments, and those over-expression results in increased tolerance to drought (Tran *et al.*, 2004). In Glycine max, GmNAC2, GmNAC3 and GmNAC4 were strongly induced by osmotic stress. GmNAC3 and GmNAC4 were also induced by ABA, JA and salinity but differed in their response to cold (Guilherme *et al.*, 2009). In addition, GmNAC2-overexpressing tobacco lines were developed and found to be hypersensitive to drought, high salinity, and cold stress indicating GmNAC2 functions as a negative regulator during abiotic stress, and participates in ROS signaling pathways through modulation of the expression of genes related to ROS-scavenging (Jin *et al.*, 2013). *TaNAC2L* over-expression activated the expression of heat-related genes in the transgenic Arabidopsis, suggesting that TaNAC2L may improve heat tolerance by regulating the expression of stress-responsive genes (Guo *et al.*, 2015). In Rice, *SNAC3* was ubiquitously expressed and its transcript level was induced by drought, high temperature, salinity stress, and abscisic acid (ABA) treatment. Over-expression of *SNAC3* in rice resulted in enhanced tolerance to high temperature, drought, and oxidative stress caused by methyl viologen (MV), whereas suppression of *SNAC3* by RNAi resulted in increased sensitivity to these stresses (Fang *et al.*, 2015). *AdNAC21* and *AiNAC3* were found to be up-regulated under both drought and high temperature stress, suggesting these genes may be positive regulators of abiotic stress responses in Peanut. Overall, qRT-PCR analysis demonstrated that all the genes displayed variations in their expression behavior in response to drought and high temperature stress, suggesting these genes may be involved in regulation of stress responsive genes and further characterization will help in understanding their role in imparting abiotic stress tolerance in Peanut.

CONCLUSIONS

The NAC TFs has been proposed as important arbitrators of various plant processes and have been subjected to intensive characterization, especially in well-known model plants. Our study to identify and characterize NAC TFs in Peanut genome using genome-wide survey, expression analysis coupled with molecular tools provides foundation of our understanding of their regulatory roles. The comprehensive genome-wide analysis led to identification of 76 NAC TF genes. A uniform nomenclature was provided to the identified genes and proteins, followed by their comparative phylogenetic analysis with *Arabidopsis* and *Glycine max* NAC TFs. Functional conservation within a sub-family and serves as an initial platform in facilitating a better understanding of the structure-function relationship between individual members. The variability in gene expression patterns

implies that NACs may regulate a complex web of pathways to perform different physiological functions for acclimatizing towards multiple challenges. This understanding will prove useful in improving drought and high temperature tolerance since these differentially expressed genes are probably involved in abiotic tolerance in Peanut. Thus, the analysis provides preliminary indications of putative function of several Peanut NAC genes, which would help in channelizing directional efforts for their functional characterization.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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Additional Resource 1: List of primers for qRT-PCR

Sl No	Gene Name	Forward (5'- 3')	Reverse (5'- 3')	Product Size
1	AdNAC1	ATGGCCTCAAATCAAACTGG	TCCTTGCATGAAGGGAAGTT	149
2	AdNAC2	ACTTCGGCTGCTACTCCTCA	ATCTGGTTGTTGGTGCATGA	150
3	AdNAC3	CCCAACATCCGACACATACA	CGTCATCTTGGGCAACACTA	152
4	AdNAC4	AGGAGCACAGGCTGAAGAAG	GATGGCACCAACAATGTCAC	151
5	AdNAC5	ATAGCAGCAGCTGGTTTGGT	ACATCAGGGTGGCAAGGTAG	151
6	AdNAC6	CTGGGTTTTGCTTCTCTCCA	CCACTTGAAAACGCCTTACC	151
7	AdNAC7	GAGTGACGACGTCGGGTTAT	GGCAAACGGTACTCGTTCAT	160
8	AdNAC8	CTGGGTTTCGGTTCTATCCA	GCATTGAGCTTAGCCACCTC	154
9	AdNAC9	TCCAGGATTCAGGTTTCACC	TCCCACTTTAGCCAATCCTG	154
10	AdNAC10	CTCTTATACACCCGCGCTTC	TTCACATGATCCAACCTCCA	145
11	AdNAC11	CGGATTGGCTAGGTTTACCA	CCCATCTTCACCTTCAATGG	149
12	AdNAC12	GCTATTGGAAGGCAACAGGA	TTCATGCAAGACCCAGTGAG	149
13	AdNAC13	AAAGGTTCACACCGACCAAG	GGTCTTCTCGGGCTTCTTCT	151
14	AdNAC14	GTTTCAAGCCAAAGCAGACC	CCTGTAAACCGGTGGTGTCT	145
15	AdNAC15	CGATCCATGGGATTTACCAG	AGTTGCGATGTGTTTGTCCA	148
16	AdNAC16	AAGTCTGAGCCTTGGGACCT	GTCTTTGCCTGTGGTCTTCC	147
17	AdNAC17	ACCGGTCGCAAAACTAAATG	CGTCGATCAAATGCTCTGAA	152
18	AdNAC18	AACCCCAGGCTACAATTTCC	AGCCATTGCAGAGAGGAAGA	150
19	AdNAC19	CCACCCAACAGATGAAGAGC	ATTCCTTCTCCCCCATTGAT	152
20	AdNAC20	CACGGGCTCTTCATCTCTTC	ATAGGGCTTGTTTGGGTGTG	144
21	AdNAC21	TCCACCAGGTTTCAGATTCC	AAGCCTTCTTTGGCAAATCC	149
22	AdNAC22	ACGACCGAAGGAAGGAAAGT	ACAGAACCCAATCGTCAAGC	157
23	AdNAC23	AGGTCTGAGCCTTGGGATTT	TGTCTTTTCCAGTGGCCTTC	148
24	AdNAC24	GAGGCTTGATGATTGGGTGT	TTGGTCTCATGCTCATGCTC	153
25	AdNAC25	TCTACGCATTGAAAGCAACG	CTCTCCCTTTTGCAGACAGC	150
26	AdNAC26	ATTCTCGTACCCCGAGGATT	ACCGAAACCCCCAAAAATAG	149
27	AdNAC27	TTGGGATGATGATGATGACG	CCCGACTCACATGAAACAGA	153
28	AdNAC28	ATCACTGCCACCTGGATTTC	TTCCCAAGGATCAAACTTGC	139
29	AdNAC29	GCTATTGGAAGGCAACAGGA	TTCATGCAAGACCCAGTGAG	149
30	AdNAC30	CGTCAGCGTCAATAAATCCA	CAATGGCTCAGAGGATGACA	152
31	AdNAC31	AATCATCGTGTGTCCCACCT	GATCCCAGGGTTCAATACGA	147
32	AdNAC32	CCATGCAGCGAAATTACTGA	GGGCCACTGAAGATTGTTGT	148
33	AdNAC33	TCCTCAGGGTCCTCAGACAG	TCCCACCATTTTTGTTCTCC	150
34	AdNAC34	CCACCACTTGCAACATCATC	ATGGCATTCATGGAAGTGTG	150
35	AdNAC35	ACGTGGGAGAGTAGGGAGGT	GTCCTGATGCCAAGGAGAGA	147
36	AdNAC36	AGGTTTCCGATTTCGACCTT	ACCGACAAATCTGGCAAGTC	144
37	AdNAC37	AGGCGAATGTTGTTGGAAAG	GGCCTTTTCCACCTCTTCTT	145
38	AdNAC38	AAGGGACAGCAAACAGGAGA	AAGCATATGGCAGGAAGGTG	150
39	AiNAC1	ATGGCCTCAAATCAAACTGG	TCCTTGCATGAAGGGAAGTT	149
40	AiNAC2	GCTAAGCGGTGTGAGTAGGG	CTGTGGTGGTTGTCTTGTGG	147
41	AiNAC3	TGGAGTGAAGAAAGCCCTTG	GCATAGCACCCACTCATCAA	151
42	AiNAC4	ATAGCAGCAGCTGGTTTCGT	ACATCAGGGTGGCAAGGTAG	148
43	AiNAC5	TCCATCCATAACCTCCCAAA	AAGGGAGGTAGACCGGAAGA	152
44	AiNAC6	GCTATTGGAAGGCAACAGGA	TTCATGCAAGACCCAGTGAG	149
45	AiNAC7	GCATCAGAAGAGGCCTTCAC	GATTCGGTGGTGATTCCACT	149
46	AiNAC8	TTGGAAAGCCACTGGAAAAG	ACTCGTGCATAACCCAATCC	149
47	AiNAC9	CCACCCAACAGATGAAGAGC	CATTCCTTCTCACCCATGCT	150
48	AiNAC10	GAACAGAGCGACGACAAGTG	TGATCCAGCCAGTTTTGATG	149
49	AiNAC11	AACCCCAGGCTACAATTTCC	AGCCATTGCAGAGAGGAAGA	150
50	AiNAC12	TAGGTTCACCATGCCTCCAT	GCAACCGAAAGAATTTCCTG	150
51	AiNAC13	TCCACCAGGTTTCAGATTCC	AAGCCTTCTTTGGCAAATCC	149
52	AiNAC14	ACCGGTCGCAAAACTAAATG	CGTCGATCAAATGCTCTGAA	152
53	AiNAC15	CTTGCCAGGAGTAAGCAAGG	ACTGGTCTGGTCTTGCCTGT	153
54	AiNAC16	GCTGCGTACAAACAGAGCAA	CTCAGTCTTACGGCCTTTGG	145
55	AiNAC17	GTGGGCTGATGATGAATGTG	GGGCAAGCTCATTATCCAAA	149
56	AiNAC18	AGCATCATCACCCTCAGCTT	GCTCCCATGGATCGAACTTA	153
57	AiNAC19	CTCTTTCCTTCCCTGGGTTC	AAGTCCAGTGGCGAAAGAAA	148
58	AiNAC20	ATCACTGCCACCTGGATTTC	TTCCCAAGGATCAAACTTGC	139
59	AiNAC21	CTTCTTCAGCTTCCGTGACC	ACACCAAAGTCTTCCGCATC	149

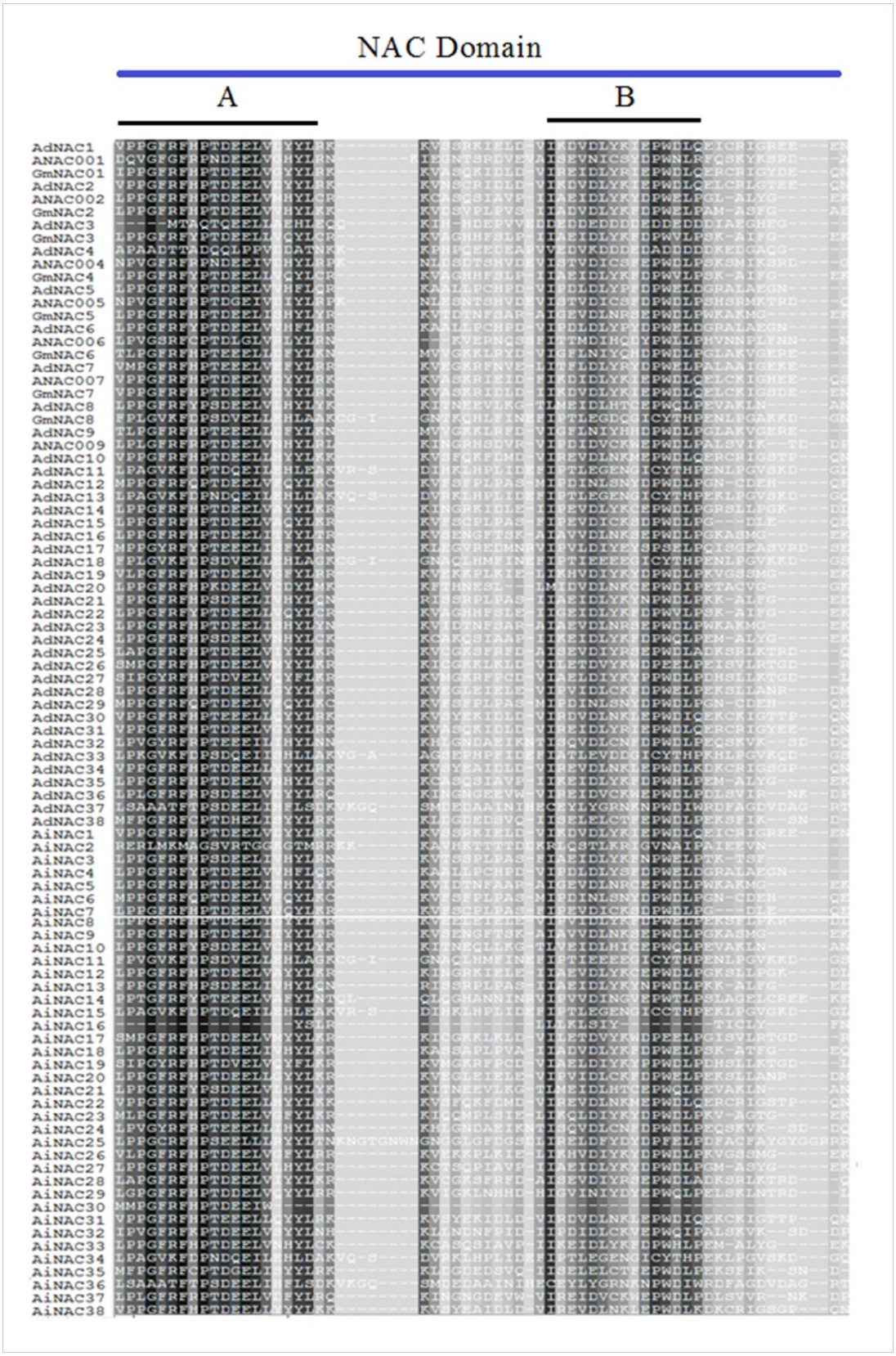
60	AiNAC22	TTCCAATGATCCACAAGCAA	GGCCGTTGAGTTGTTGAGTT	148
61	AiNAC23	GCTTCTGATTCATGGGCAAT	GGGTGTTGGTGTTGTTGATG	152
62	AiNAC24	TGAACCATGCAGCGAAATTA	AGGCCACTGAAGATCGTTGT	152
63	AiNAC25	TTAACGGTGTTGGGGAGAAC	CCGGCAAAGAACATAAGAGG	152
64	AiNAC26	TAGGTTCACCATGCCTCCAT	GCAACCGAAAGAATTTCCTG	150
65	AiNAC27	GTCAACGAGATGGAACAGCA	AGCGACTACCCAACATCCAC	151
66	AiNAC28	TCTACGCATTGAAAGCAACG	CTCTCCCTTTTGCAGACAGC	150
67	AiNAC29	AAGCAAATTCTAGCCGTGGA	TGCAGAATGAAGCTGGAGTG	149
68	AiNAC30	GCTTCTGATTCATGGGCAAT	GGGTGTTGGTGTTGTTGATG	152
69	AiNAC31	TCCCAAGCCTAGAGAGTCCA	CACATGTGAATGACCCTTCG	147
70	AiNAC32	GCCGTGTTCCTAATGGTGTC	CACAGGCTTGTGCATCAGTT	150
71	AiNAC33	ACGTGGGAGAGTAGGGAGGT	GTGATGAACGTGTCCTGGTG	158
72	AiNAC34	AAAGGTTCACACCGACCAAG	GGTCTTCTCGGGCTTCTTCT	151
73	AiNAC35	CTTGGGATTTGCCTGAAAAA	TCTGTCGCTCATTTCCTGTG	147
74	AiNAC36	ACCAAAAAGCACCATTCCAC	TGCCACTCTTGTCAAAGACG	151
75	AiNAC37	GAGAATACGGTTGGCACGAT	ACTCTCACCAGCAGCAAGGT	148
76	AiNAC38	GCCTCCTCTTTGCTTGTTTG	TTGTCCACTACCTGCCATCA	151

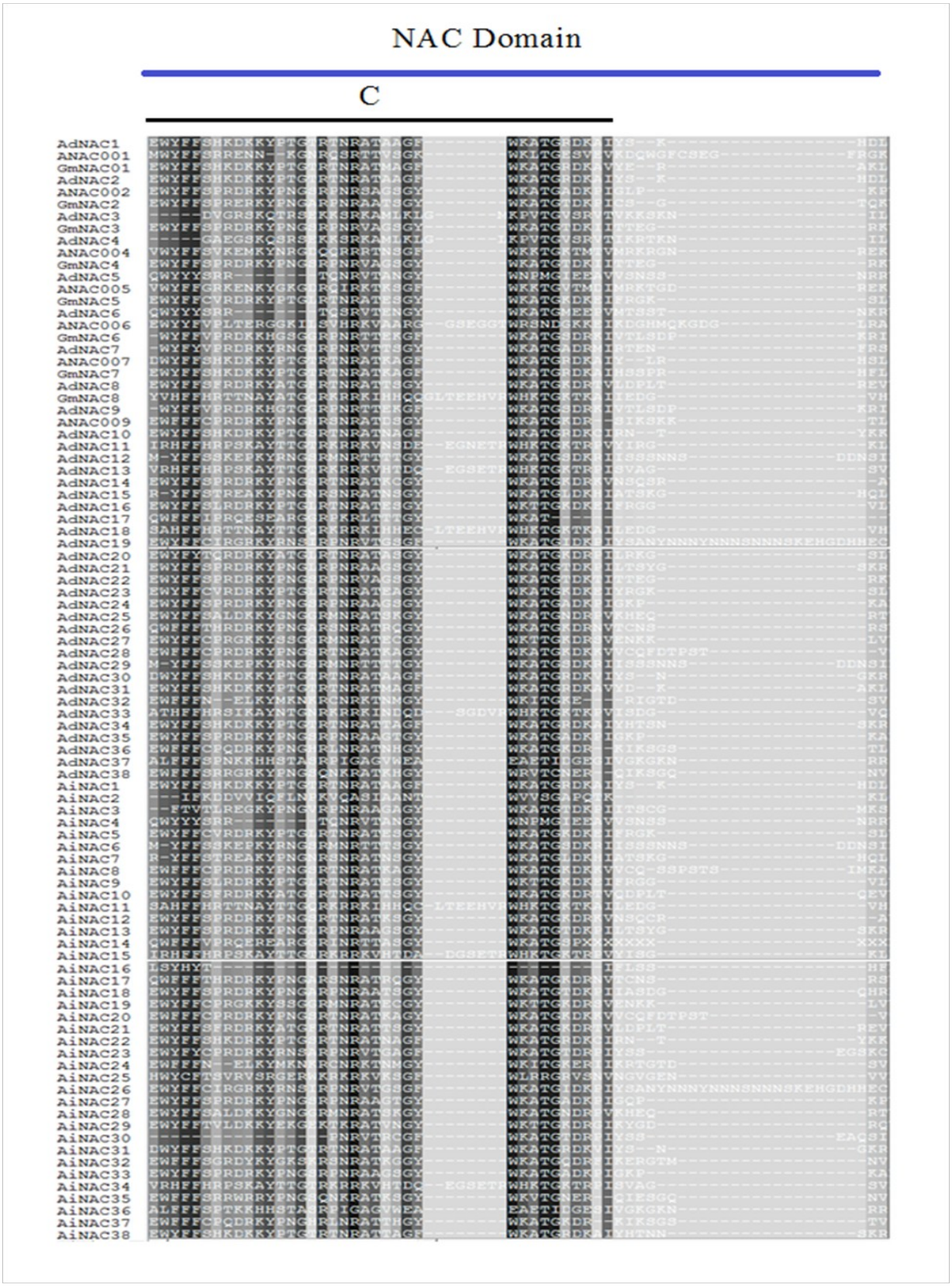
Additional Resource 2: NAC genes identified in Peanut, Chromosomal location, protein features and its localization prediction

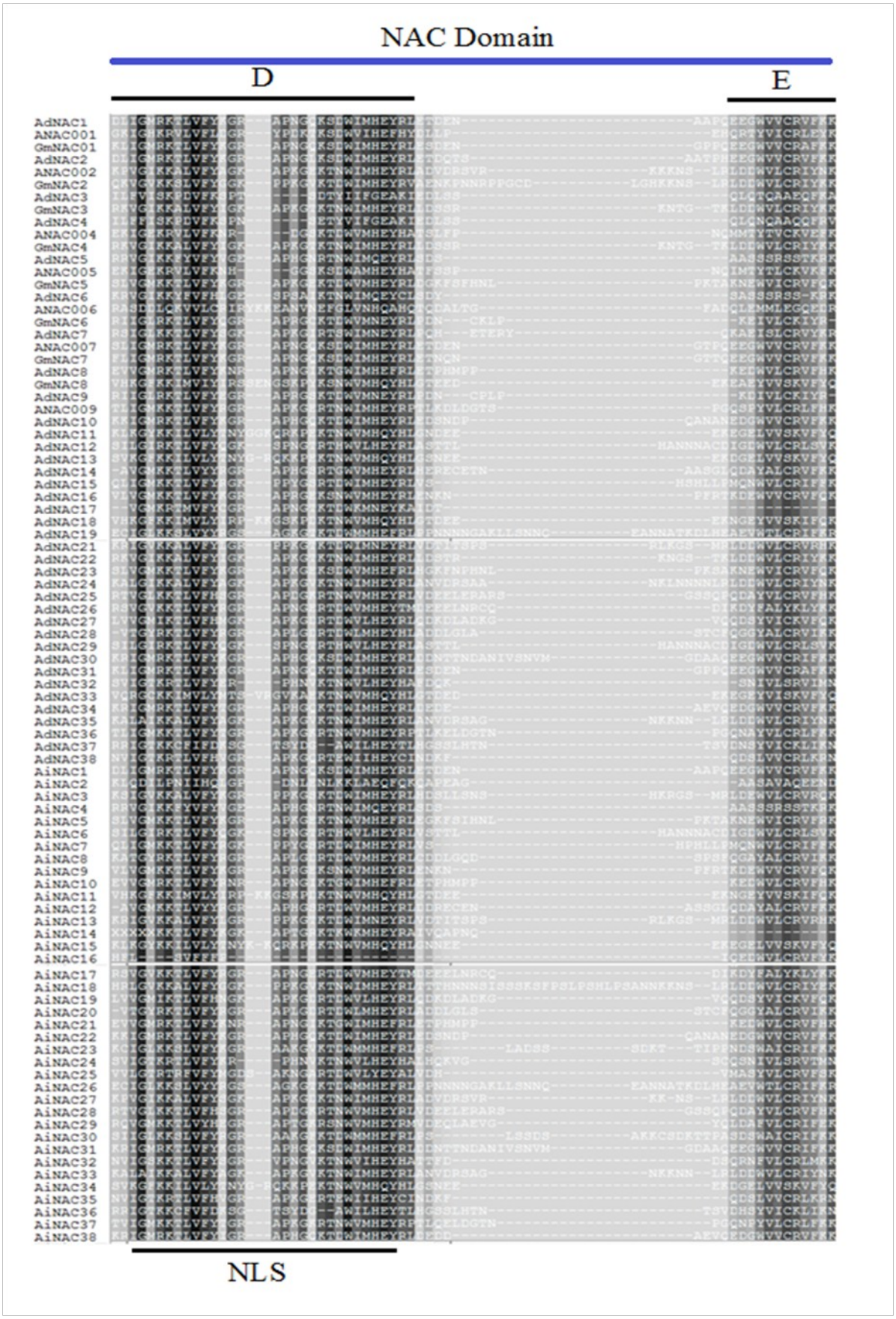
Protein	Chromosome no	Chromosomal location		Deduced polypeptide			Subcellular localization	
		Start	End	L (aa)	pI	MW (kDa)	PSORT	CELLO
AdNAC1	A3	11454354	11455035	345	0.83	39826.91	Nucleus	Nucleus
AdNAC2	A5	108981314	108981928	341	6.3	39322.81	Nucleus	Nucleus
AdNAC3	A6	80396908	80399152	200	4.43	21895.11	Nucleus	Nucleus
AdNAC4	A10	66153332	66154679	220	4.47	23646.1	Nucleus	Nucleus
AdNAC5	A3	129693299	129694115	195	4.77	22268.5	Nucleus	Nucleus
AdNAC6	A10	5998028	5999322	185	4.83	21399.83	Nucleus	Nucleus
AdNAC7	A1	100229992	100230922	396	6.86	45253.17	Nucleus	Nucleus
AdNAC8	A2	14425971	14431568	303	6.57	39680.56	Nucleus	Nucleus
AdNAC9	A3	7358062	7359492	315	6.62	36289.21	Cytoplasm	Nucleus
AdNAC10	A3	11724750	11725893	384	7.33	43616.33	Nucleus	Nucleus
AdNAC11	A3	20188288	20192205	286	8.19	32587.37	Nucleus	Nucleus
AdNAC12	A3	106298658	106299356	211	9.45	23638.54	Nucleus	Nucleus
AdNAC13	A3	119828375	119830946	333	8.67	37720.54	Cytoplasm	Nucleus
AdNAC14	A5	5429578	5430695	633	6.34	71663.95	Nucleus	Nucleus
AdNAC15	A4	123543593	123544127	274	9.46	31056.99	Nucleus	Nucleus
AdNAC16	A5	93368779	93370121	362	7.21	41028.72	Nucleus	Nucleus
AdNAC17	A6	71790277	71790797	158	7.82	18406.9	Nucleus	Nucleus
AdNAC18	A6	99555217	99557900	398	4.84	44766.62	Nucleus	Nucleus
AdNAC19	A7	4217527	4220338	367	6.19	42710.4	Nucleus	Nucleus
AdNAC20	A7	34924711	34930461	322	7.57	36404.96	Nucleus	Nucleus
AdNAC21	A8	26150292	26151482	360	5.95	41040.72	Nucleus	Nucleus
AdNAC22	A8	36880883	36881761	349	8.2	39130.85	Nucleus	Nucleus
AdNAC23	A8	46083457	46084425	321	8.99	36330.98	Nucleus	Nucleus
AdNAC24	A8	49202615	49203371	304	6.42	35117.57	Nucleus	Nucleus
AdNAC25	A9	104514590	104515729	513	6.85	56824.02	Nucleus	Nucleus
AdNAC26	A9	118019296	118020327	551	4.58	62431.12	Nucleus	Nucleus
AdNAC27	A10	95255804	95258481	367	4.75	40434.33	Cytoplasm	Nucleus
AdNAC28	A1	27306712	27309985	331	4.82	37221.31	Nucleus	Nucleus
AdNAC29	A3	106298424	106299692	211	9.45	23638.54	Nucleus	Nucleus
AdNAC30	A2	4630146	4632702	463	6.05	51131.07	Nucleus	Nucleus
AdNAC31	A5	11520982	11522773	370	5.72	42053.82	Nucleus	Nucleus
AdNAC32	A1	17654261	17657374	278	4.95	32114.67	Nucleus	Nucleus
AdNAC33	A9	120443393	120445227	417	4.99	46396.41	Nucleus	Nucleus
AdNAC34	A7	46474184	46478634	350	6.93	40443.03	Nucleus	Nucleus
AdNAC35	A3	110320453	110321103	260	7.71	29831.7	Nucleus	Nucleus
AdNAC36	A10	66508937	66511725	592	5.52	66957.8	Nucleus	Nucleus
AdNAC37	A6	8759634	8760991	275	6.6	30814.72	Cytoplasm	Nucleus
AdNAC38	A5	82433773	82435384	363	9.35	40163.1	Nucleus	Nucleus

AiNAC1	B3	14030606	14031364	345	5.82	39895.92	Nucleus	Nucleus
AiNAC2	B3	3397200	3398172	159	7.93	17385.82	Nucleus	Nucleus
AiNAC3	B6	34263503	34264948	339	6.33	38553.64	Nucleus	Nucleus
AiNAC4	B3	130519432	130520236	194	4.7	22170.39	Nucleus	Nucleus
AiNAC5	B1	114413189	114413851	330	8.16	37207.1	Nucleus	Nucleus
AiNAC6	B3	107857973	107858464	211	9.45	23678.6	Nucleus	Nucleus
AiNAC7	B4	133562785	133563191	275	9.21	31227.2	Nucleus	Nucleus
AiNAC8	B5	5593767	5594848	352	5.26	39942.63	Nucleus	Nucleus
AiNAC9	B5	134972984	134974696	363	7.21	41082.72	Nucleus	Nucleus
AiNAC10	B6	22593847	22597864	277	6.7	31980.62	Nucleus	Nucleus
AiNAC11	B6	123070548	123071415	398	4.87	44763.58	Nucleus	Nucleus
AiNAC12	B7	16494646	16499500	678	5.51	77254.89	Nucleus	Nucleus
AiNAC13	B8	3509212	3510399	360	5.95	41047.75	Nucleus	Nucleus
AiNAC14	B8	5732628	5733422	264	7.82	28212.34	Nucleus	Nucleus
AiNAC15	B8	10780723	10782775	294	8.82	33464.63	Nucleus	Nucleus
AiNAC16	B8	70326651	70327366	216	5.61	24401.27	Cytoplasm	Nucleus
AiNAC17	B9	138001768	138002822	559	4.57	63262.12	Cytoplasm	Nucleus
AiNAC18	B10	4890841	4891946	345	6.51	39364.7	Nucleus	Nucleus
AiNAC19	B10	107295672	107296861	367	4.72	40432.26	Cytoplasm	Nucleus
AiNAC20	B1	33384497	33387453	309	5.37	34992.16	Nucleus	Nucleus
AiNAC21	B2	17820383	17822891	307	6.59	34673.92	Nucleus	Nucleus
AiNAC22	B3	14391486	14393315	375	7.28	42546.28	Nucleus	Nucleus
AiNAC23	B3	127136415	127138105	405	6.86	45210.02	Nucleus	Nucleus
AiNAC24	B5	15820770	15822869	289	5.65	33148.19	Nucleus	Nucleus
AiNAC25	B6	135601579	135604741	308	5.61	34840.84	Nucleus	Nucleus
AiNAC26	B7	16494344	16499499	367	6.19	42710.4	Nucleus	Nucleus
AiNAC27	B7	118782790	118785019	301	6.54	33737.99	Nucleus	Nucleus
AiNAC28	B9	127070525	127076074	512	6.85	56758.9	Nucleus	Nucleus
AiNAC29	B9	127100455	127103707	583	4.67	65014.53	Cytoplasm	Nucleus
AiNAC30	B10	126350924	126352571	283	5.87	32172.67	Cytoplasm	Nucleus
AiNAC31	B2	5953378	5956019	577	8.62	64412.11	Nucleus	Nucleus
AiNAC32	B2	6650206	6654171	316	5.08	36332.42	Nucleus	Nucleus
AiNAC33	B3	111878870	111880200	260	7.09	29859.71	Nucleus	Nucleus
AiNAC34	B3	120758782	120762349	330	8.47	37327.96	Nucleus	Nucleus
AiNAC35	B5	144854743	144856564	306	5.48	34445.9	Nucleus	Nucleus
AiNAC36	B6	7136978	7138554	275	6.73	30990.83	Nucleus	Cytoplasm
AiNAC37	B7	29146175	29149324	698	4.74	77755.42	Nucleus	Nucleus
AiNAC38	B8	105359002	105361311	350	6.73	40504.11	Nucleus	Nucleus

Additional Resource 3: Multiple alignment of 76 NACs of Peanut, Arabidopsis and Glycine max. Conserved NAC domain and subdomains (A-E) are indicated by thick blue line and black thin black lines, respectively, above the sequences. The putative nuclear localization signal (NLS) is shown by a black line below the sequence.

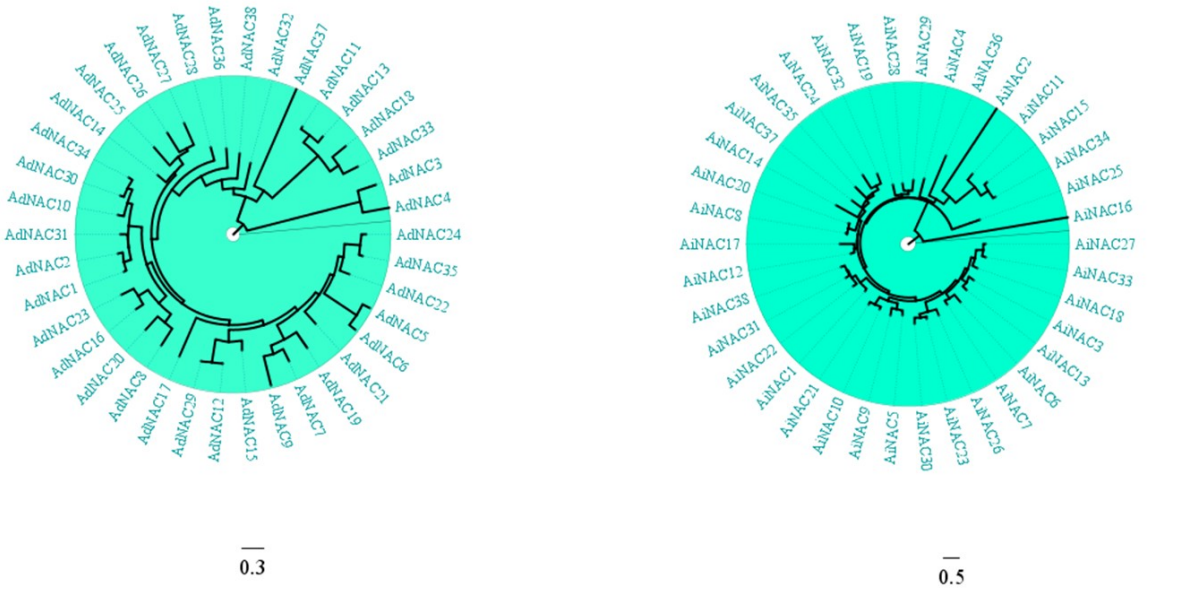



NAC Domain
C

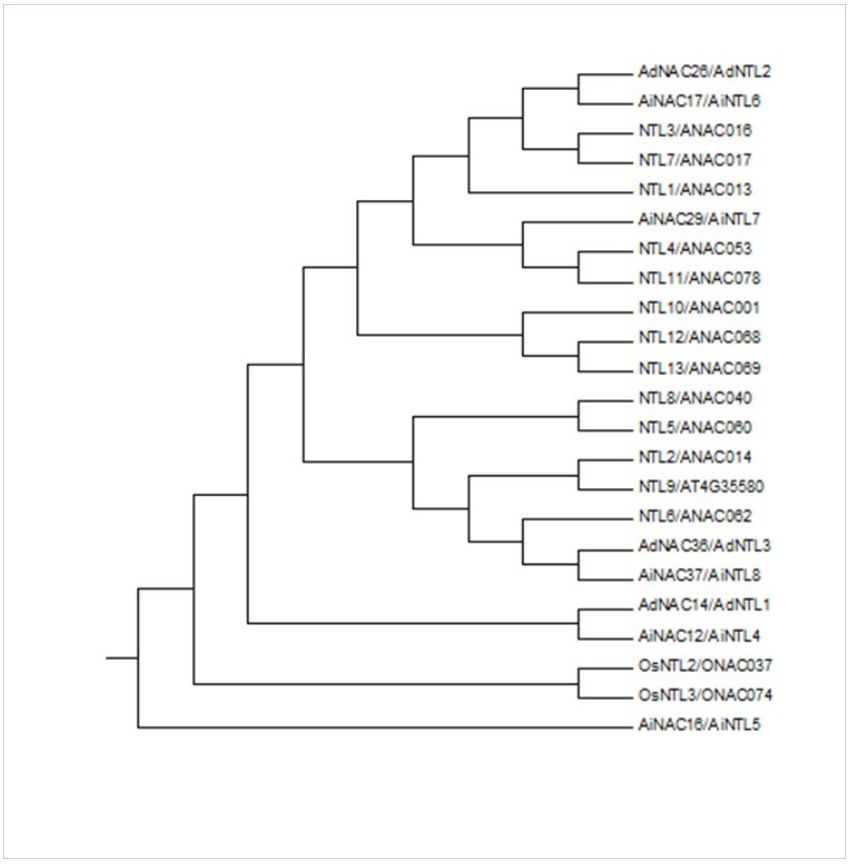

NAC Domain
D
E
NLS

Additional Resource 4

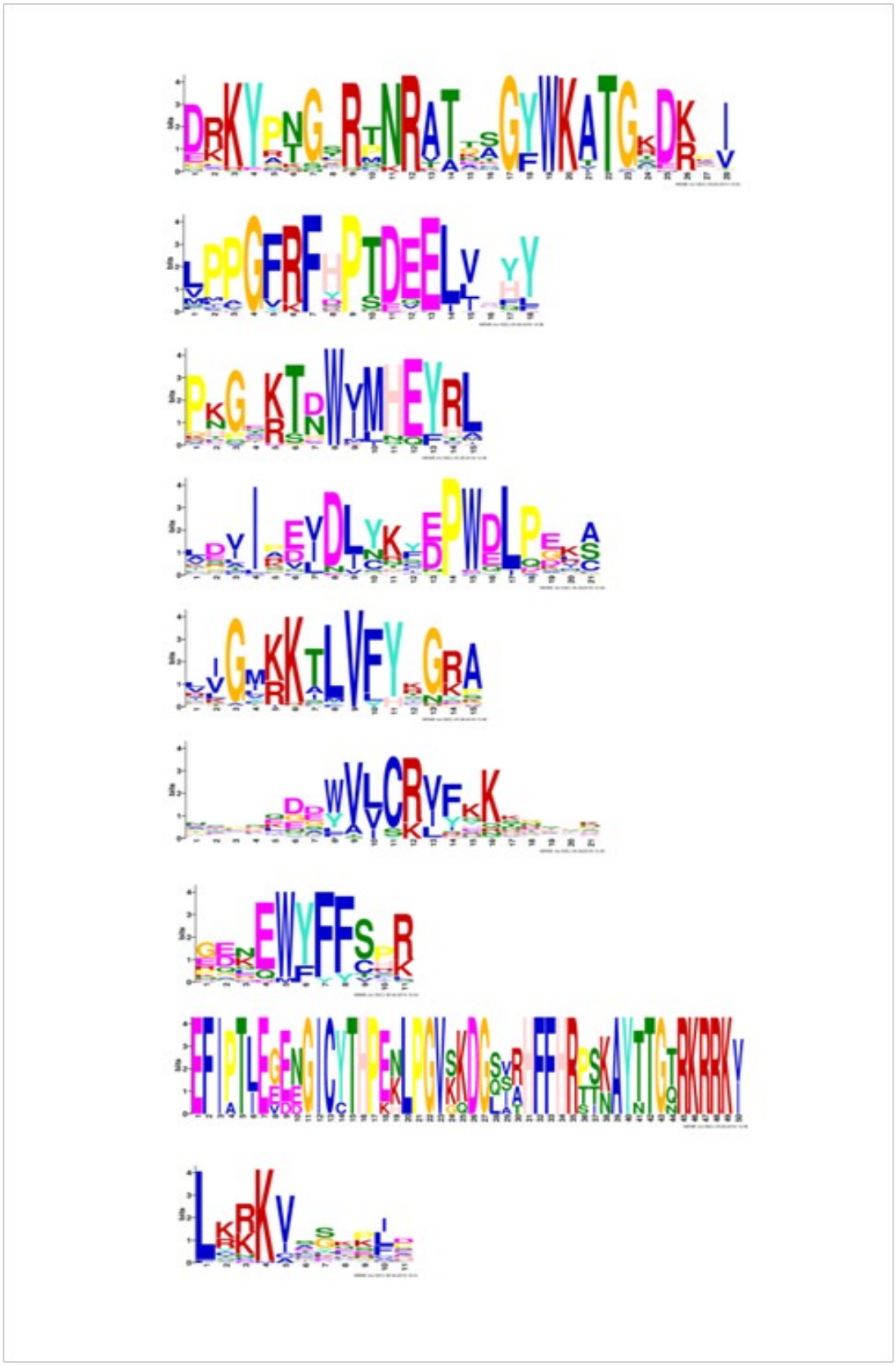
A. Unrooted phylogenetic tree constructed using the neighbor-joining (NJ) method, and the bootstrap test was carried out with 1,000 iterations representing the relationships between AdNACs and AiNACs



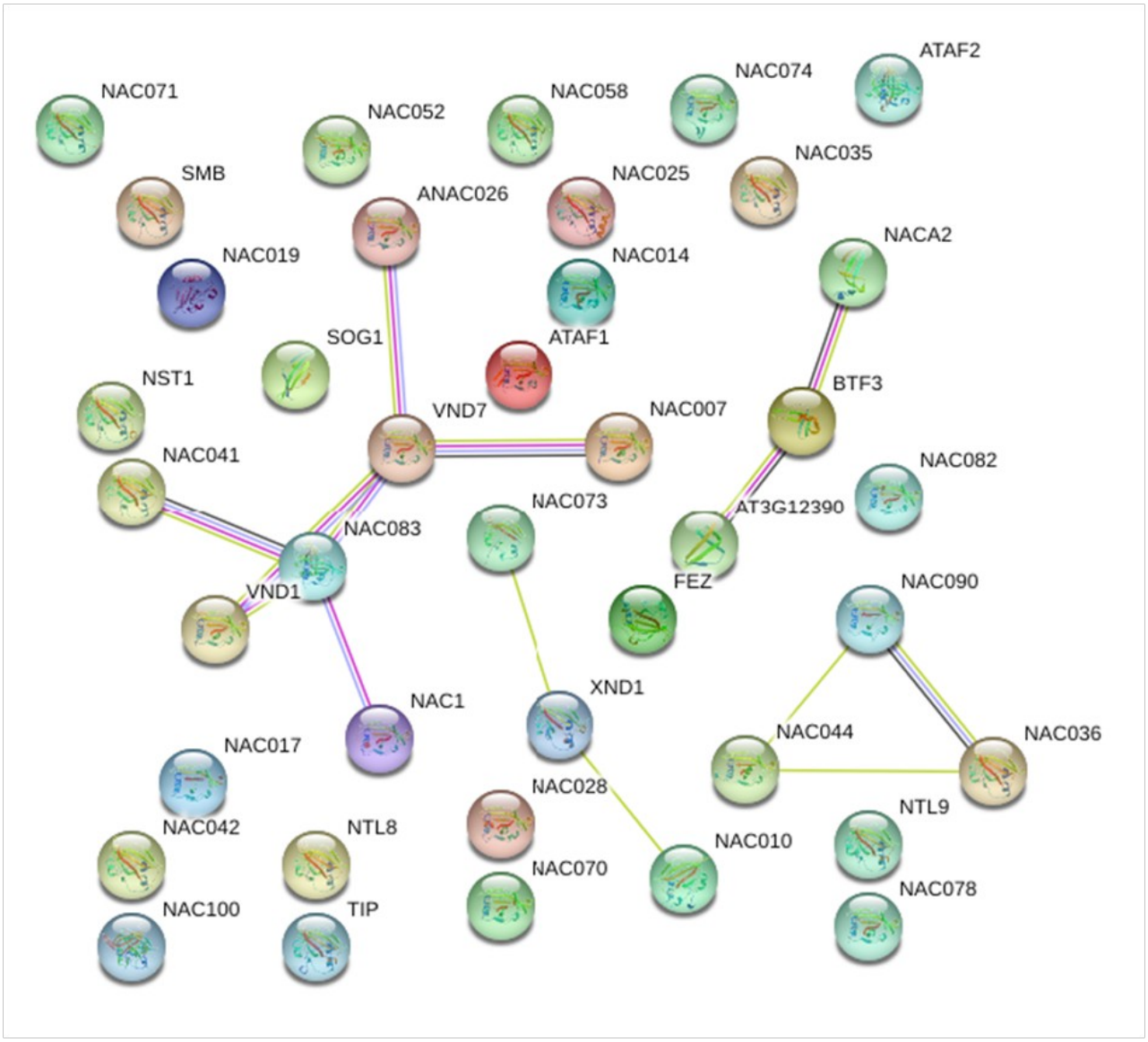
B. Phylogenetic tree of membrane-bound NACs from Peanut (AdNTLs & AiNTLs/ AdNACs & AiNACs), Arabidopsis (NTLs/ANACs) and rice (OsNTLs/ONACs). The unrooted phylogenetic tree was constructed using the full protein sequences.



C. Conserved motif logos identified using MEME tool



D. Functional protein association network of Peanut NAC proteins in Arabidopsis



E. Gene Ontology annotation of NAC proteins