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Genome-wide analysis of bHLH and bZIP Transcription factors and their temporal expression under abiotic stress conditions in Groundnut (*Arachis hypogaea* L.)

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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). Transcription factors can control the expression of many target genes through specific binding to the cis-acting elements in the promoters of the target genes. The basic leucine zipper (bZIP) and basic helix-loop-helix (bHLH) represents one of the largest as well as most diverse transcription factor (TFs) families. They are known to play role in both stress as well as in various plant developmental processes. In this study, a comprehensive phylogeny, chromosomal location, conserved motif identification and expression profiles under high temperature and drought stress. of bZIP and bhLH TF gene family was carried in groundnut. A total of 151 bZIP and 39 bHLH transcription factors have been identified from groundnut. Expression analysis during high temperature and heavy metal stress conditions. Gene expression studies revealed differential expressions of bZIP and bhLH TFs suggesting the possible role in various stress mitigation and can serve as a candidate genes for improving abiotic stress tolerance and can be helpful in enhancing the crop productivity under stress conditions.

Key words: Groundnut, bZIP, bHLH, Abiotic stress

Plants are frequently being exposed to abiotic stresses such as drought, high salinity, high osmolarity, nutrient deficiency etc. These environmental factors negatively affect the plants leading to reduced growth and yield. Plants have evolved several defence mechanisms start from the alteration of gene expression and cellular metabolism to changes in plant growth, development, and crop yield (Akula Ramakrishna *et al.*, 2011). Following exposure to abiotic stress specific ion channels and kinase cascades are activated, reactive oxygen species (ROS), phytohormones like abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) accumulate, and a reprogramming of the genetic machinery results in adequate defense reactions and an increase in plant tolerance in order to minimize the biological damage caused by the stress (Ines Ben Rejeb *et al.*, 2014). Under stress conditions, plants synthesize ABA in various organs and initiate defense mechanisms, such as the regulation of stomatal aperture and expression of defense related genes conferring resistance to environmental stresses. Expression of functional proteins is largely controlled by specific transcription factors (TFs). Recent studies demonstrated that DREB1/ CBF, DREB2, AREB/ABF, and NAC have important roles in response to abiotic stresses in rice (Kazuo Nakashima *et al.*, 2009). TFs like MYB, AP2/ERF, NAC, bZIP, bHLH and WRKY families act as the early responders to environmental signals and trigger the expression of stress-induced genes that are important for plants to be tolerant to abiotic stress.

Groundnut is one of the important legume crops of tropical and semiarid tropical countries (annual production of ~ 46 million tons) where it provides a major source of edible oil and protein. Groundnut kernels contain 47-53% oil and 25-36% protein. The genus Arachis belongs to family Fabaceae, sub family Papilionaceae, Tribe Aeschynomeneae, Subtribe Stylosanthinae. The genus Arachis has more than 70 wild species, of which only Arachis hypogaea L is domesticated and commonly cultivated. The *Arachis* genus is composed mostly of diploid species ($2n = 2x = 20$). *A. hypogaea* is an allotetraploid (AABB-type genome; $2n = 4x = 40$), derived from a hybridization event between two diploid species and polyploidization. Chromosomes are of mostly similar size and divided into A and B sub-genomes. Cytogenetic, phylogeographic and molecular evidence indicate *A. duranensis* and *A. ipaensis* as the donors of the A and B sub-genomes, respectively. In plant genomes approximately 7% of the coding sequences are assigned to transcription factors (TFs) (Soren Lindemose *et al.*, 2013), and many of these are immediate-early abiotic stress-responsive genes (Kilian *et al.*, 2012). A TF can control the expression of many target genes through specific binding to the *cis*-acting elements in the promoters of the target genes.

The basic leucine zipper (bZIP) transcription factor family is one of the largest and most conserved families, named according to the conserved bZIP domain that is composed of 60-80 amino acids and contains two functional regions: a basic region and a leucine zipper. The basic region is conserved and responsible for nuclear localization and DNA binding. The leucine zipper motif that consists of several repeats of leucine or other hydrophobic amino acids is involved in recognition and dimerization of bZIPs (Wei Hu *et al.*, 2016). Recent studies show that bZIP TFs play crucial roles in various aspects of biological processes, including organ differentiation, embryogenesis, seed maturation, flower and vascular development. Increasing evidences have also indicated that bZIP TFs take part in the regulation of plants' response to biotic and abiotic stress. The basic helix–loop–helix (bHLH) proteins are a large superfamily of eukaryotic transcription factors, and play a central role in a wide range of metabolic, physiological, and developmental processes (Sonnenfeld *et al.*, 2005). Their bHLH domain contains approximately 60 amino acids, including a basic region and a HLH region (Murre *et al.*, 1989). The basic region, which consists of approximately 17 amino acids and is located at the N-terminus of the domain, is a DNA-binding region that allows HLH proteins to bind to a consensus hexanucleotide E-box (CANNTG) (Mark Eben Massari *et al.*, 2000). The HLH region is composed of two amphipathic helices consisting of hydrophobic residues linked by a divergent (both in length and primary sequence) loop, and functions as a dimerization domain

(Ferré D'Amaré *et al.*, 1994). The HLH domain promotes protein–protein interactions and allows for the formation of homodimeric or heterodimeric complexes. Several previous studies showed that bHLH plays an important role in protecting plants from abiotic stresses. A novel bHLH transcription factor, PebHLH35, enhanced the drought tolerance of Populus euphratica (Dong *et al.*, 2014). BrabHLH from Chinese cabbage participated in cold stress (Song *et al.*, 2014), and the grapevine bHLH transcription factor confers tolerance to cold stress in Arabidopsis (Xu *et al.*, 2014). Thus, bHLH TFs play an important role in various abiotic stresses. Agricultural production and quality are adversely affected by various abiotic stresses world-wide and this will be exacerbated by the deterioration of global climate. To feed a growing world population, it is very urgent to breed stress-tolerant crops with higher yields and improved qualities against multiple environmental stresses. Our study provides detailed characterization of bZIP and bHLH TFs which can be used as candidate genes to develop stress tolerant varieties in groundnut.

MATERIALS AND METHODS

***Plant materials and* stress treatment**

Seeds of groundnut (ICGV1119) 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, heavy metal stress was imposed hydroponically for 3 days with 300µM $CdCl_2$ 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 tissues were harvested immediately and stored at -80 °C for further analysis.

Identification, characterization and sub-cellular localization of bHLH and bZIP proteins

The bHLH and bZIP domain containing protein sequences of groundnut 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 bHLH and bZIP domain downloaded from the Peanut 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 bHLH and bZIP 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, WOLF PSORT (http://www.genscript.com/psort/wolf_psort.html) tool was used to predict the subcellular localizations.

Multiple Sequence Alignment and Phylogenetic Analysis

Amino acid sequences of bHLH and bZIP TFs belonging to groundnut were imported to BioEdit v7.2.5 (Hall 1999) and multiple sequence alignment was performed with bHLH and bZIP protein sequences using ClustalW with default parameters. The bHLH and bZIP sequences were imported into MEGA v6.06 (Tamura *et al.*, 2013) to construct a phylogenetic tree.

Genome wide distribution, Gene structure and Conserved Motif analysis

The chromosomal location of *bHLH* and bZIP 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 Centre 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 *bHLH* and bZIP these 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) was utilized for analysis of the conserved motifs.

Total RNA isolation and cDNA Synthesis and PCR amplification of *bHLH* and *bZIP* genes

Total RNA was isolated from control and stress treated shoot tissues using Trizol reagent and 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 for *AdbHLH48, AibHLH22, AdbZIP12*

and *AibZIP15* are listed in Table 1. cDNA concentration was checked using Nanodrop 2000 (Thermo Scientific). PCR reactions were setup using Taq DNA Polymerase. Each PCR reaction included 2 µl cDNA (1µg), 1 unit Taq DNA Polymerase, 10mM dNTPs, 2.5 µl Taq Assay Buffer (10X), 0.5 µl gene specific forward primer (10 µM), 0.5 µl reverse primer (10 µM), and made upto 25 µl with sterile water. The reactions conditions were 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 54 °C for 45s and 72 °C for 30s; 72 °C for 2 min.

Expression analysis of *bHLH* and bZIP genes

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 for AdbHLH48, AibHLH22, AdbZIP12 and AibZIP15 were designed using Primer3 software (Table 1). qRT- PCR reactions were performed using SYBR Green PCR Master mix (Takara) on CFX96 Real Time PCR (Biorad). Each PCR reaction (10 µl) included 2 µl cDNA (100ng), 5µ1 1x SYBR Green Master mix, 0.5 µl gene specific forward primer (10 µM), 0.5 µl reverse primer (10 µM), and 2 µl sterile water. The *bHLH* and bZIP 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 10 s, 54 °C for 45s and 72 °C for 30s. All reactions were run with three technical and the data was analyzed using $2^{-\Delta\Delta CT}$ method.

RESULTS AND DISCUSSION

Identification of bHLH, Protein features, multiple sequence alignment and Phylogenetic analysis

To identify all the bHLH transcription factors, we retrieved all the predicted bHLH genes from Plant TFDB and Peanut Base (http://peanutbase.org/). The keyword, HMM profile and BLAST search predicted that the groundnut genome encodes about 151 bHLH proteins. A total of 151 bHLH genes were identified from both *A. duranensis* and *A.ipaënsis*. They were named as *AdbHLH1* to *AdbHLH79*, and *AibHLH1* to *AibHLH72* respectively. Basic information like molecular weight and pI of AdbHLH are depicted in Table 2. The average polypeptide length was 351.21 residues with the length ranging from 181aa (AdbHLH 77) to 665 aa (AdbHLH 67). The pI values range from 4.69 to 9.76. The sub-cellular localization results revealed that majority of the proteins were localized to nucleus and 2/76 were predicted to be localized in chloroplast and 1 in cytoplasm. Basic information like molecular weight and pI of AibHLH are depicted in Table 3. The average polypeptide length was 362.90 residues with the length ranging from 168aa (AibHLH 70) to 663 aa (AibHLH 18). The pI values range from 4.61 to 9.77. The sub- cellular localization results revealed that majority of the proteins were localized to nucleus and 1/76 were predicted to be localized in chloroplast. The multiple alignment of *AdbHLH and AibHLH, proteins* indicated that they share a highly conserved 7-9 domains consisting of N-terminal DNA binding domain and a variable C-terminal transcriptional regulation domain (Fig 1 and 2).

To examine the structure and phylogenetic relationships of groundnut bHLH TFs identified in our study, a combined phylogenetic tree was constructed with the aligned bHLH domains from groundnut. The relationship among the 79 AdbHLH and 72 AibHLH TFs was investigated through constructing phylogenetic trees using Neighbour Joining method and the tree topology revealed several pairs of bHLH proteins with a high degree of homology in the terminal nodes of each subfamily Fig 3 and 4. Examination of the phylogenetic tree emphasis that the groundnut AdbHLH TFs can be classified into seven major groups: Group 1 (17) Group 2 (15), Group 3 (11), Group 4 (7), Group 5 (11), Group 6 (13), Group7 (5). AibHLH can be classified into nine major groups: Group 1(17) , Group 2 (11), Group 3 (18), Group 4(4), Group 5(3),Group 6(7), Group7(3), Group8(1), Group 9(8).

Identification of bZIP, Protein features, Multiple sequence alignment and Phylogenetic analysis

To identify all the bZIP transcription factors, we retrieved all the predicted bZIP genes from Plant TFDB and Peanut Base (http://peanutbase.org/). The keyword, HMM profile and BLAST search predicted that the groundnut genome encodes about 39 bZIP proteins. A total of 39 bZIP genes were identified from both *A. Duranensis* and *A.ipaënsis*. They were named as *AdbZIP*1 to *AdbZIP*18 and *AibZIP*1 to *AibZIP*21

respectively. Basic information like molecular weight and pI of AdbZIP are depicted in Table 4. The average polypeptide length was 293.4 residues with the length ranging from 146aa (AdbZIP 15) to 495 aa (AdbZIP 1). The pI values range from 5.03 to 9.9. The sub- cellular localization results revealed that majority of the proteins were localized to nucleus and 1/76 were predicted to be localized in endoplasmic reticulum. Basic information like molecular weight and pI of AibHLH are depicted in Table 5. The average polypeptide length was 296.4 residues with the length ranging from 145aa (AibZIP 18) to 800aa (AibZIP 8). The pI values range from 4.86 to 9.36. The sub- cellular localization results revealed that majority of the proteins were localized to nucleus and 2/76 were predicted to be localized in endoplasmic reticulum. The multiple alignment of *AdbZIP* and *AibZIP* indicated that they share 5 to 6 highly conserved domains consisting of N-terminal DNA binding domain and a variable C-terminal transcriptional regulation domain (Fig 5 and 6).

To examine the structure and phylogenetic relationships of groundnut bZIP TFs identified in our study, a combined phylogenetic tree was constructed with the aligned bZIP domains from groundnut. The relationship among the 18AdbZIP and 21AibZIP TFs was investigated through constructing phylogenetic trees using Neighbour Joining method and the tree topology revealed several pairs of bZIP proteins with a high degree of homology in the terminal nodes of each subfamily Fig 7 and 8. Examination of the phylogenetic tree emphasis that the groundnut AdbZIP is classified into 8 groups: Group1 (4), Group2 (1), Group3 (3), Group4 (2), Group5 (1), Group6 (1), Group7 (1), Group8 (5). AibZIP TFs are classified into 8 groups: Group1 (4), Group2 (2), Group3 (1), Group4 (4), Group5 (2), Group6 (1), Group7 (1), Group8 (6).

Chromosomal distribution and gene structure of bHLH and bZIP members

The genome of groundnut comprises of 20 chromosomes (10 from *duranensis* and 10 from *ipaensis*) varying in their length in which shortest being chromosome 8 and longest is the chromosome 3 in *A. duranensis while in A.ipaënsis*, shortest being chromosome 4 and longest being chromosome 9. *In silico* mapping of *bHLH* and *bZIP* indicated an uneven distribution of the genes on all the chromosomes. (Fig 9, 10, 11 and 12). The exact position (in bp) of each *bHLH* and *bZIP genes* on groundnut chromosomes is given in Table 2, 3 and 4. The gene structures were investigated through genomic annotation to determine the structural diversity. All *bHLH* and *bZIP* genes harbored at least two exons except few being the shortest not having intron. In addition, a separate phylogenetic tree was generated from the complete protein sequences of all the *bHLH* and *bZIP* genes (Fig 13, 14, 15 and 16).

Identification of conserved motifs

The MEME (Multiple Expectation Maximization for Motif Elicitation) server was used for exploring motif distribution in 79 AdbHLH, 72 AibHLH, 18 AdbZIP, and 21 AibZIP (Fig 17, 18 and 19). Five different conserved motifs were identified, of which most of them had at least three highly conserved motifs. The motif sequence logos are depicted in the Table 6, 7, 8 and 9. Some of these motifs have been characterized in animals regarding specificity in the DNA-binding sequence recognition and dimerization activities responsible for the activation or repression of target genes or for binding to small molecules. Multiple sequence alignment and identification of conserved motifs using MEME tool indicates that most of the bHLH and bZIP proteins possessed 5 to 6 sub-domains in the N termini that conferred the DNA-binding activities. The motif composition of these TF 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.

PCR amplification of bHLH and bZIP genes

Total RNA was isolated from stress treated tissues (Fig 20 and 21). The PCR reaction mixtures were run on 1.5% Agarose gel prepared using 1X TAE buffer along with 100 bp ladder. A single band of *AibZIP15* (704bp) and *AdbZIP12* (709bp) was observed on gel. There was no PCR product for *AdbHLH48* and *AibHLH22* under high temperature stress (Fig 22b). Under heavy metal stress, *AdbHLH48* (450bp) and *AibZIP15* (709bp) were amplified and single band was observed on gel (Fig 22a). The band pattern is comparatively similar to the

results obtained by quantitative PCR analysis.

Expression profiles bHLH genes during high temperature and high metal stress

The plant specific *bHLH TFs* play important role 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 signalling transduction pathways (Rosa M. Pérez-Clemente *et al.*, 2013). bHLH proteins are plant-specific TFs that have been shown to function in abiotic stress responses (Marie Pireyre *et al.*, 2015). To investigate the responses of *bHLH* genes to high metal and high temperature stress, we analysed the expression profiles of one *bHLH* gene from each genome and expressed the results as fold changes with respect to the control. During heavy metal stress, bHLH belonging to Group 5 and Group 1 such as *AdbHLH48 and AibHLH22* genes were down regulated with folds 4.49 and 1.56 respectively (Fig 23). During high temperature, bHLH genes were up-regulated and AdbHLH48 and AibHLH22 were found to be induced by 16.9 and 0.93 folds respectively (Fig 24). During heavy metal stress, bZIP genes belonging to Group 4 and Group 1 such as *AdbZIP12* and *AibZIP15* showed up regulation by 9.25 and 10.01 folds respectively. Out of 2 bZIP genes *AdbZIP12* was down regulated by 12.9 fold and *AibZIP15* was up-regulated by 10.68 fold (Fig 24). *AibZIP* showed increased expression under both drought and high temperature stress.

Table 1. List of primers for RT-qPCR

Gene Name	Forward Primer	Reverse Primer	Length (bp)	Tm (°C)
AdbHLH48	ACGGATCCTGACCTGTTCCAACTGCTTG	TACTCGAGTCTATGAGCTCCGGGATGAG	28	63.0
AibHLH22	ACGGATCCTCTCTTGCAGAGGGGAAAGA	TACTCGAGCAAGTCTTGGGTTCACAGCA	28	63.0
AdbZIP12	ACGGATCCACCACCAGCAAATGTTCTCC	TACTCGAGAGGCGCAAGAATTAGGAACA	28	63.0
AibZIP15	ACGGATCCGGCGTCTTCAAGTGGAACAT	TACTCGAGAACTGGCTCCATGAATGACC	28	63.0

Table 2. AdbHLH genes identified in Peanut, Chromosomal location, protein features and its localization prediction.

Protein	Chromosome Number	Chromosomal Location (bp)		Deduced Polypeptide			Subcellular Localization
		Start	End	Length	pI	MW	
AdbHLH1	A01	11180803	11184851	450	5.9	56349.39	nucleus
AdbHLH2	A01	23818826	23821600	387	7.18	42810.87	Nucleus
AdbHLH3	A01	24107575	24110292	268	7.03	29696.69	Nucleus
AdbHLH4	A01	33747530	33755680	297	4.91	33725.91	Nucleus
AdbHLH5	A01	76355573	76358681	255	8.73	27535.72	Nucleus
AdbHLH6	A01	92963020	92966444	405	5.7	43265.18	Nucleus
AdbHLH7	A02	4990418	4992478	447	6.04	50189.76	Nucleus
AdbHLH8	A02	11490442	11491724	516	5.45	55840.51	Nucleus
AdbHLH9	A02	65874875	65881124	338	6.42	37356.38	Nucleus
AdbHLH10	A02	65875202	65880387	345	7.7	37894.06	Nucleus
AdbHLH11	A02	66678616	66681794	407	9.24	44298.24	Nucleus
AdbHLH12	A02	89000583	89007871	694	5.1	77631.15	Nucleus
AdbHLH13	A03	3170735	3173105	465	6.38	51017.44	Nucleus
AdbHLH14	A03	4396954	4399636	349	6.1	38798.89	Nucleus
AdbHLH15	A03	12136351	12138613	216	6.84	24041.54	Nucleus
AdbHLH16	A03	107170665	107172739	406	6.43	44670.22	Nucleus
AdbHLH17	A03	117229922	117230891	338	7.14	38190.66	Nucleus
AdbHLH18	A03	120673179	120676797	258	7	29055.83	Nucleus
AdbHLH19	A03	123213398	123215577	337	4.69	37951.67	Nucleus
AdbHLH20	A03	131184949	131187253	471	5.52	52619.98	Nucleus
AdbHLH21	A04	29987499	29997525	247	5.34	28285.34	Nucleus
AdbHLH22	A05	757006	758358	349	5.16	39414.5	Nucleus
AdbHLH23	A05	4471852	4474149	334	6.9	36880.17	Nucleus
AdbHLH24	A05	86030089	86031635	220	9.53	24900.43	Nucleus
AdbHLH25	A05	104078105	104082033	367	6.07	40422.45	Nucleus

AdbHLH26	A05	105232729	105237613	539	5.16	58266.45	Nucleus
AdbHLH27	A06	11815778	11820126	357	5.69	39118.08	Nucleus
AdbHLH28	A06	109557765	109560470	419	5.65	45509.36	Nucleus
AdbHLH29	A06	110137735	110139757	277	5.85	31321.61	Nucleus
AdbHLH30	A07	9293133	9294428	256	8.76	28456.33	Nucleus
AdbHLH31	A07	55234108	55235523	417	6.44	52504.98	Nucleus
AdbHLH32	A07	57713413	57715466	397	7.08	43397.54	Nucleus
AdbHLH33	A07	68460163	68461453	321	4.83	36494.42	Nucleus
AdbHLH34	A07	70853348	70871615	350	4.88	39768.86	Nucleus
AdbHLH35	A07	75244942	75246494	313	9.18	34370.98	Nucleus
AdbHLH36	A08	15304566	15307528	343	4.86	39261.21	Nucleus
AdbHLH37	A08	17382662	17385316	393	5.85	42849.76	Nucleus
AdbHLH38	A08	23771934	23774264	328	5.57	37212.97	Nucleus
AdbHLH39	A08	24276717	24279598	401	5.68	44863.38	Nucleus
AdbHLH40	A08	29959576	29960976	236	6.01	26574.31	Nucleus
AdbHLH41	A08	43112521	43115242	400	8.88	44306.71	Nucleus
AdbHLH42	A09	1141726	1145405	357	6.77	40041.13	Nucleus
AdbHLH43	A09	4405214	4406928	366	5.31	41366.62	Nucleus
AdbHLH44	A09	9616546	9619558	580	6.45	63460.81	Nucleus
AdbHLH45	A09	116722167	116723907	291	7.76	31338.87	Chloroplast
AdbHLH46	A10	4762764	4765178	368	7.66	41676.67	Nucleus
AdbHLH47	A10	22120099	22122002	236	9.28	26260.28	Nucleus
AdbHLH48	A10	104379283	104380857	238	7.86	26566	Nucleus
AdbHLH49	A01	90749358	90752534	256	4.87	29223.97	Nucleus
AdbHLH50	A03	19592294	19594435	221	6.67	25350.15	Nucleus
AdbHLH51	A03	38984268	38987093	528	9.04	59651.37	Nucleus
AdbHLH52	A03	121854601	121856521	327	6.41	36595.78	Nucleus
AdbHLH53	A04	20876962	20880191	487	5.13	54204.89	Nucleus
AdbHLH54	A05	1099767	1101296	336	6.28	37941.85	Nucleus
AdbHLH55	A05	5650995	5652478	335	4.96	37474.88	Nucleus
AdbHLH56	A05	5676022	5677733	322	6.57	36193.44	Nucleus
AdbHLH57	A06	1704105	1705517	307	5.86	35063.41	Nucleus
AdbHLH58	A06	4585188	4586562	278	5.36	31533.59	Nucleus
AdbHLH59	A07	63884568	63887247	332	6.06	36849.39	Nucleus
AdbHLH60	A07	70852467	70853604	193	9.76	21638.45	Nucleus
AdbHLH61	A08	13571762	13573816	325	7.22	35758.02	Nucleus
AdbHLH62	A08	31926538	31927699	228	7.07	26186.85	Nucleus
AdbHLH63	A09	36737760	36739645	303	9.3	34193.65	Nucleus
AdbHLH64	A10	57409973	57414243	311	6.38	35382.97	Nucleus
AdbHLH65	A02	4340844	4342187	473	5.51	53353.28	Nucleus
AdbHLH66	A03	6936674	6945870	279	7.7	31084.6	Nucleus
AdbHLH67	A06	2305528	2307525	665	6.22	72541.1	Nucleus
AdbHLH68	A07	66084895	66087281	262	8.84	28851.52	Nucleus
AdbHLH69	A08	32827123	32829163	272	8.61	30093.18	Nucleus
AdbHLH70	A09	96870471	96872697	223	6.33	25042.36	Nucleus
AdbHLH71	A09	112385877	112387278	311	5.3	34671.55	Nucleus
AdbHLH72	A09	112389260	112390463	302	5.2	33731.5	Nucleus
AdbHLH73	A09	120376856	120378791	361	6.31	40610.33	Nucleus
AdbHLH74	A03	19352315	19353746	208	5.69	23649.96	Cytoplasm
AdbHLH75	A05	105520640	105522451	262	6.01	29705.07	Nucleus
AdbHLH76	A09	110546064	110547184	275	6.99	31371.18	Nucleus
AdbHLH77	A10	2767037	2768803	181	9.26	20830.97	Nucleus
AdbHLH78	A04	62752020	62756877	662	5.54	74386.17	chloroplast
AdbHLH79	A06	14352907	14359399	572	8.25	64663.36	Nucleus

Table 3: AibHLH genes identified in Peanut, Chromosomal location, protein features and its localization prediction.

Protein	Chromosome Number	Chromosomal Location		Deduced Polypeptide			Subcellular Localization
		Start	End	Length	pI	MW	
AibHLH1	B01	636283	640342	520	5.9	56387.44	nucleus
AibHLH2	B01	30217938	30223799	354	4.75	39553.40	nucleus
AibHLH3	B01	107608827	107611906	255	8.73	27506.73	nucleus
AibHLH4	B01	136640565	136641988	255	4.87	29061.79	nucleus
AibHLH5	B02	77755308	77758153	413	9.27	44795.72	nucleus

AibHLH6	B02	94625362	94627028	189	6.5	21513.15	nucleus
AibHLH7	B03	108120840	108123374	271	8.77	29901.1	nucleus
AibHLH8	B03	121291052	121294003	258	7.04	28944.73	nucleus
AibHLH9	B03	123867236	123869445	347	4.7	39115.85	nucleus
AibHLH10	B03	132161677	132163387	508	5.45	56536.24	nucleus
AibHLH11	B04	28150372	28160597	219	6.86	25266.9	nucleus
AibHLH12	B05	746211	747857	350	5.06	39482.53	nucleus
AibHLH13	B05	4501469	4503100	304	7.2	33763.84	nucleus
AibHLH14	B05	98520553	98525231	536	5.15	57911.12	nucleus
AibHLH15	B05	109099855	109104474	367	6.07	40381.4	nucleus
AibHLH16	B06	3996822	4000058	209	9.69	23867	nucleus
AibHLH17	B06	4110261	4113865	362	5.85	39529.32	nucleus
AibHLH18	B06	18357835	18358780	663	6.22	72369.88	nucleus
AibHLH19	B06	134206069	134209265	420	5.65	45537.41	nucleus
AibHLH20	B07	9285262	9287119	256	8.6	28370.24	nucleus
AibHLH21	B07	33652692	33654351	357	4.71	40780.16	nucleus
AibHLH22	B07	62509004	62511083	395	7.07	43147.23	nucleus
AibHLH23	B07	123477966	123480961	311	4.88	35544.09	nucleus
AibHLH24	B07	125315732	125318601	380	5.74	41449.09	nucleus
AibHLH25	B08	990433	991857	405	6.35	52694.21	nucleus
AibHLH26	B08	1799227	1801832	330	5.44	37429.29	nucleus
AibHLH27	B08	2084005	2086453	402	5.68	44927.41	nucleus
AibHLH28	B08	7512500	7513857	180	8.8	20429.6	nucleus
AibHLH29	B08	89807832	89809286	329	4.91	37237.77	nucleus
AibHLH30	B08	128695301	128698133	369	8.19	41646.29	nucleus
AibHLH31	B09	276697	278046	344	4.61	39789.54	nucleus
AibHLH32	B09	1342273	1346258	242	9.2	27208.04	nucleus
AibHLH33	B09	139937689	139941958	327	8.82	34926.71	nucleus
AibHLH34	B09	6839044	6841764	354	6.97	39996.66	nucleus
AibHLH35	B10	131077378	131078628	238	8.46	26579.05	nucleus
AibHLH36	B01	636822	639231	520	5.9	56387.44	nucleus
AibHLH37	B01	773401	775837	451	5.37	51213.51	nucleus
AibHLH38	B01	29853579	29855816	365	7.18	40438.28	nucleus
AibHLH39	B01	134857606	134860895	407	5.7	43503.43	nucleus
AibHLH40	B01	137028861	137032298	416	9.75	46883.57	chloroplast
AibHLH41	B02	6253314	6255779	446	6.13	50118.64	nucleus
AibHLH42	B02	102553396	102557165	661	4.89	73703.12	nucleus
AibHLH43	B02	105759050	105761496	338	6.81	38166.63	nucleus
AibHLH44	B03	5875560	5878134	463	6.38	50834.15	nucleus
AibHLH45	B03	10090832	10092450	279	7.7	30985.46	nucleus
AibHLH46	B03	14817318	14819570	217	5.99	24186.71	nucleus
AibHLH47	B03	41339015	41343427	539	8.88	60474.21	nucleus
AibHLH48	B03	122440911	122442823	327	6.03	36538.68	nucleus
AibHLH49	B04	20530498	20533734	487	5.13	54204.89	nucleus
AibHLH50	B05	1081535	1082506	342	6.24	38412.28	nucleus
AibHLH51	B05	5834411	5835652	335	5	37457.8	nucleus
AibHLH52	B06	13767672	13769322	289	5.2	32933.15	nucleus
AibHLH53	B06	20280531	20282138	530	6.11	58644.05	nucleus
AibHLH54	B06	134873226	134875351	362	4.9	41028.16	nucleus
AibHLH55	B07	38152793	38153907	266	8.24	29235.88	nucleus
AibHLH56	B07	42703139	42704374	332	6.06	36819.32	nucleus
AibHLH57	B07	121797374	121799212	310	8.77	34239.27	nucleus
AibHLH58	B07	125783661	125785901	272	4.79	30831.18	nucleus
AibHLH59	B09	269215	270201	192	9.77	21471.28	nucleus
AibHLH60	B09	44277247	44279136	303	9.15	34152.55	nucleus
AibHLH61	B09	131488064	131489865	369	6.16	41501.24	nucleus
AibHLH62	B09	146220285	146221793	272	6.53	31041.7	nucleus
AibHLH63	B10	72699993	72703002	305	6.56	34721.31	nucleus
AibHLH64	B02	4649893	4651764	656	6.38	72947.22	nucleus
AibHLH65	B02	14875849	14877865	516	5.45	55794.42	nucleus
AibHLH66	B04	76925964	76930950	662	5.59	74255	nucleus
AibHLH67	B06	2329181	2336236	569	7.11	64242.7	nucleus
AibHLH68	B08	7512297	7514586	180	8.8	20429.6	nucleus

AibHLH69	B09	118317111	118319428	220	6.4	24837.11	nucleus
AibHLH70	B03	21876207	21877320	168	8.31	19027.77	nucleus
AibHLH71	B05	145697971	145699460	264	5.75	29877.09	nucleus
AibHLH72	B03	125089589	125093842	480	5.93	54008.05	nucleus

Figure 1. Multiple alignment of 79 AdbHLH TFs of groundnut

Figure 1. Continued

Figure 1. Continued

Figure 2. Multiple alignment of 72 AibHLH TFs of groundnut

Figure 2. Continued

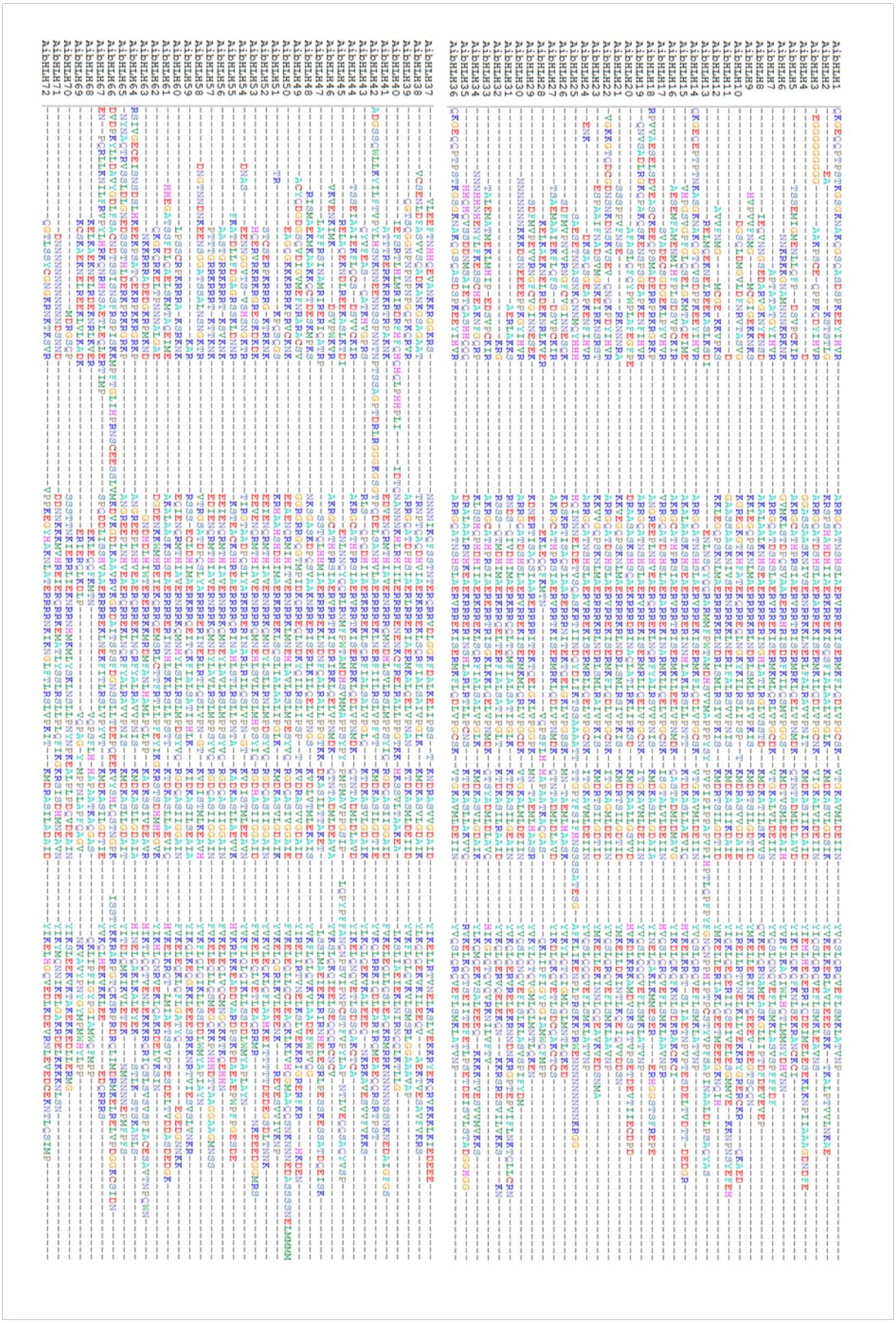


Figure 2. Continued

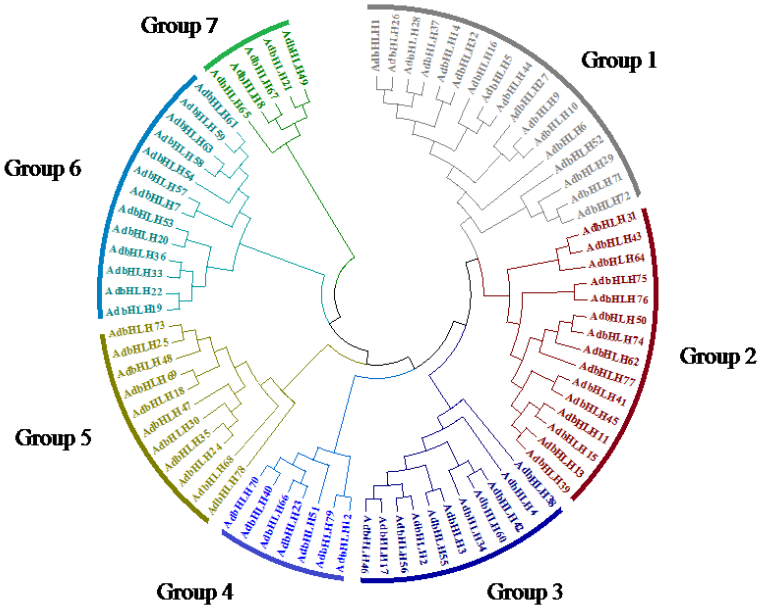


Figure 3. Phylogenetic relationship and gene structure of the bHLH genes. Phylogenetic tree was constructed with MEGA 6.0 on a multiple alignment of 79 amino acid sequences of bHLH genes from *Arachis duranensis* Exon/ intron structure of bHLH genes are represented by boxes and black lines, respectively.

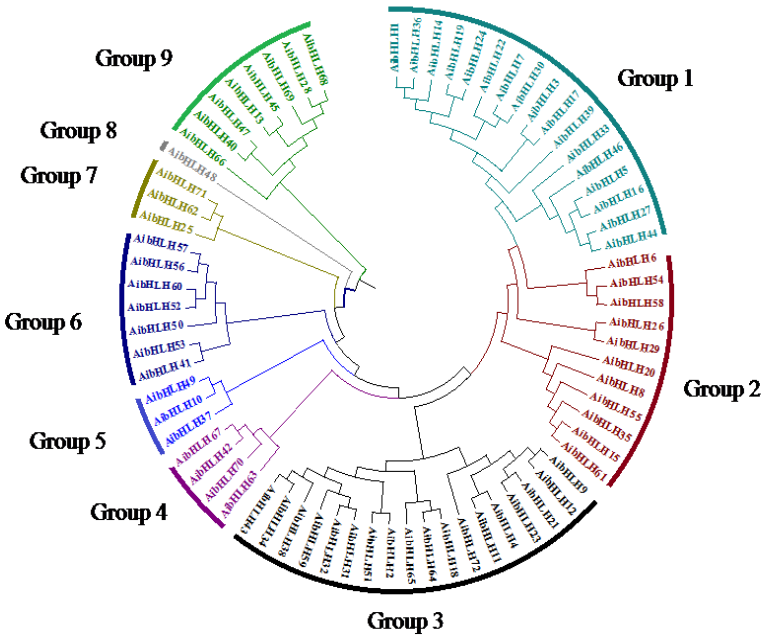


Figure 4. Phylogenetic relationship and gene structure of the bHLH genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 72 amino acid sequences of bHLH genes from *Arachis ipaënsis.*

Table 4. AdbZIP and AibZIP proteins identified in Peanut, Chromosomal location, protein features and its localization prediction.

Protein	Chromosome Number	Chromosomal Location (bp)		Deduced Polypeptide			Subcellular Localization
		Start	End	Length (aa)	pI	MW	
AdbZIP1	A06	67093845	67094858	495	7.07	54713.19	Nucleus
AdbZIP2	A07	72015371	72018639	322	5.94	35914.02	Nucleus
AdbZIP3	A10	95293270	95294273	316	5.03	33962.22	Nucleus
AdbZIP4	A10	104633025	104633360	212	5.7	24810.41	Nucleus
AdbZIP5	A01	104038428	104038856	217	8.48	24519.65	Nucleus
AdbZIP6	A02	45495034	45497618	443	6.06	48807.58	Nucleus
AdbZIP7	A10	104137408	104138555	800	5.84	86930.94	E.R
AdbZIP8	A03	21151650	21152954	304	5.65	33778.3551	Nucleus
AdbZIP9	A08	1090955	1091461	168	7.1	19345.84	Nucleus
AdbZIP10	A08	32533691	32536893	332	5.3	37390.56	Nucleus
AdbZIP11	A05	4161574	4162581	224	9.9	24845.75	Nucleus
AdbZIP12	A06	104086507	104089154	373	5.18	40440.98	Nucleus
AdbZIP13	A07	23354880	23357810	225	9.13	25954.56	Nucleus
AdbZIP14	A03	4,793,469	4,793,906	163	6.12	18187.23	Nucleus
AdbZIP15	A06	7,343,727	7,344,167	146	5.78	16384.81	Nucleus
AdbZIP16	A03	134,132,232	134,132,612	160	6.14	17947.95	Nucleus
AdbZIP17	A05	4,161,574	.4,162,581	224	9.9	24845.75	Nucleus
AdbZIP18	A06	16,421,562	16,422,038	158	8.91	18493.87	Nucleus
AibZIP1	B03	135171129	135171510	155	6.22	17528.52	Nucleus
AibZIP2	B04	130023980	130024291	224	4.98	26262.69	Nucleus
AibZIP3	B05	101761857	101762086	234	4.86	26920.53	Nucleus
AibZIP4	B05	1567450	1572508	388	5.72	41074.03	Nucleus
AibZIP5	B07	106034150	106034580	164	7.1	18889.34	Nucleus
AibZIP6	B08	25788300	25790824	307	7.21	34005.71	Nucleus
AibZIP7	B10	119022617	119023360	327	5.11	35139.44	Nucleus
AibZIP8	B10	130798698	130800592	800	5.89	86979.07	E .R
AibZIP9	B10	131256375	131256710	216	6.04	25239.89	Nucleus
AibZIP10	B03	8405762	8407205	271	6.21	29420.98	Nucleus
AibZIP11	B10	130798698	130799838	800	5.89	86979.07	E .R
AibZIP12	B01	704446	705030	194	5.85	22774.32	Nucleus
AibZIP13	B01	26679598	26679918	183	9.36	21497.39	Nucleus
AibZIP14	B02	54142154	54144616	296	5.72	33288.03	Nucleus
AibZIP15	B03	7502032	7502526	164	6.12	18274.30	Nucleus
AibZIP16	B03	23484533	23487428	344	5.66	39240.76	Nucleus
AibZIP17	B08	11006331	11009532	331	5.38	37397.60	Nucleus
AibZIP18	B09	21764782	21765285	145	5.61	16555.98	Nucleus
AibZIP19	B06	128409215	128411175	372	5.18	40414.98	Nucleus
AibZIP20	B06	:9,088,845	9,089,285	146	5.78	16384.81	Nucleus
AibZIP21	B07	:106,033,813	106,034,307	164	7.10	18889.34	Nucleus

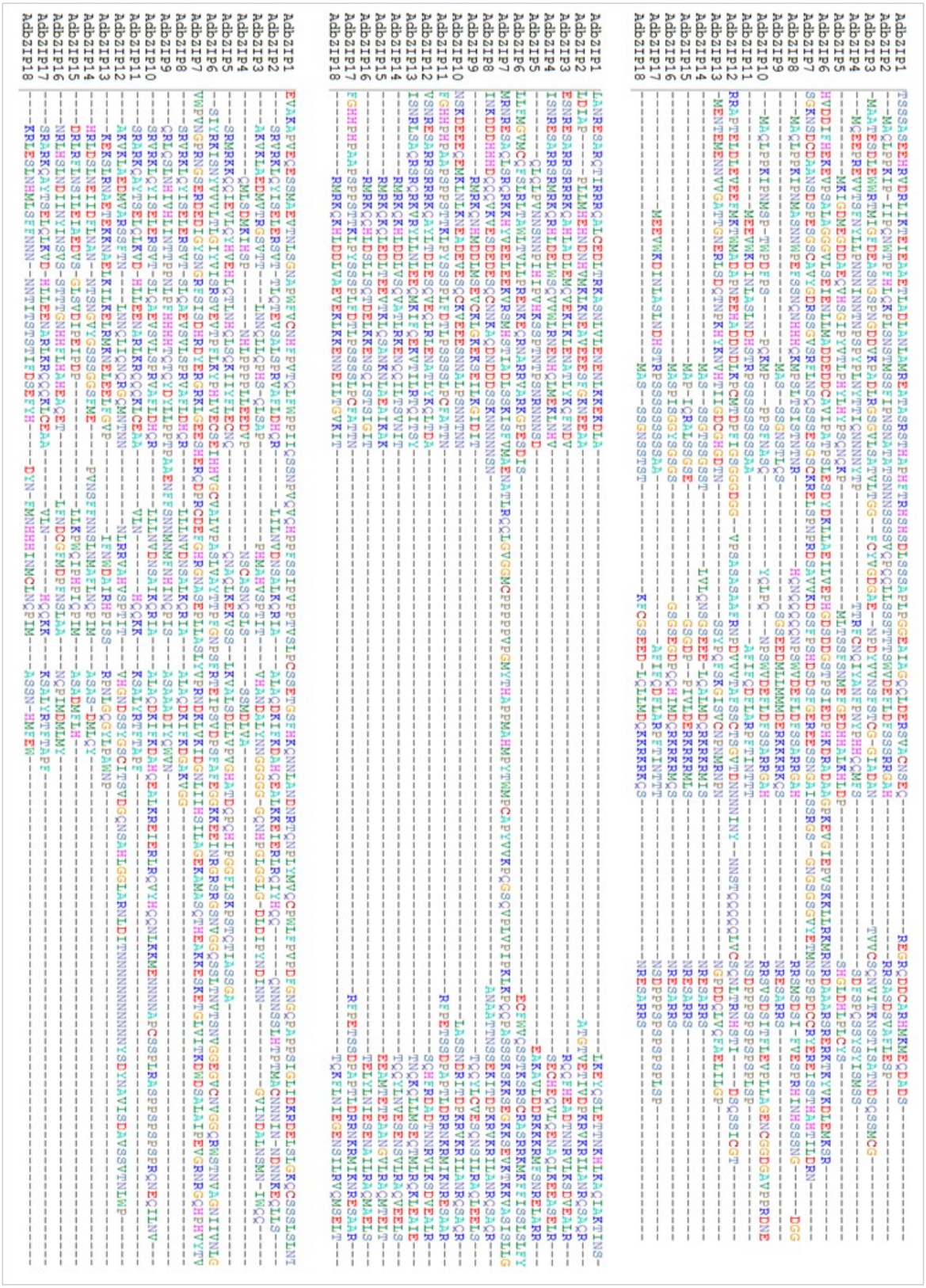


Figure 5. Multiple alignment of 18 AdbZIP TFs of groundnut

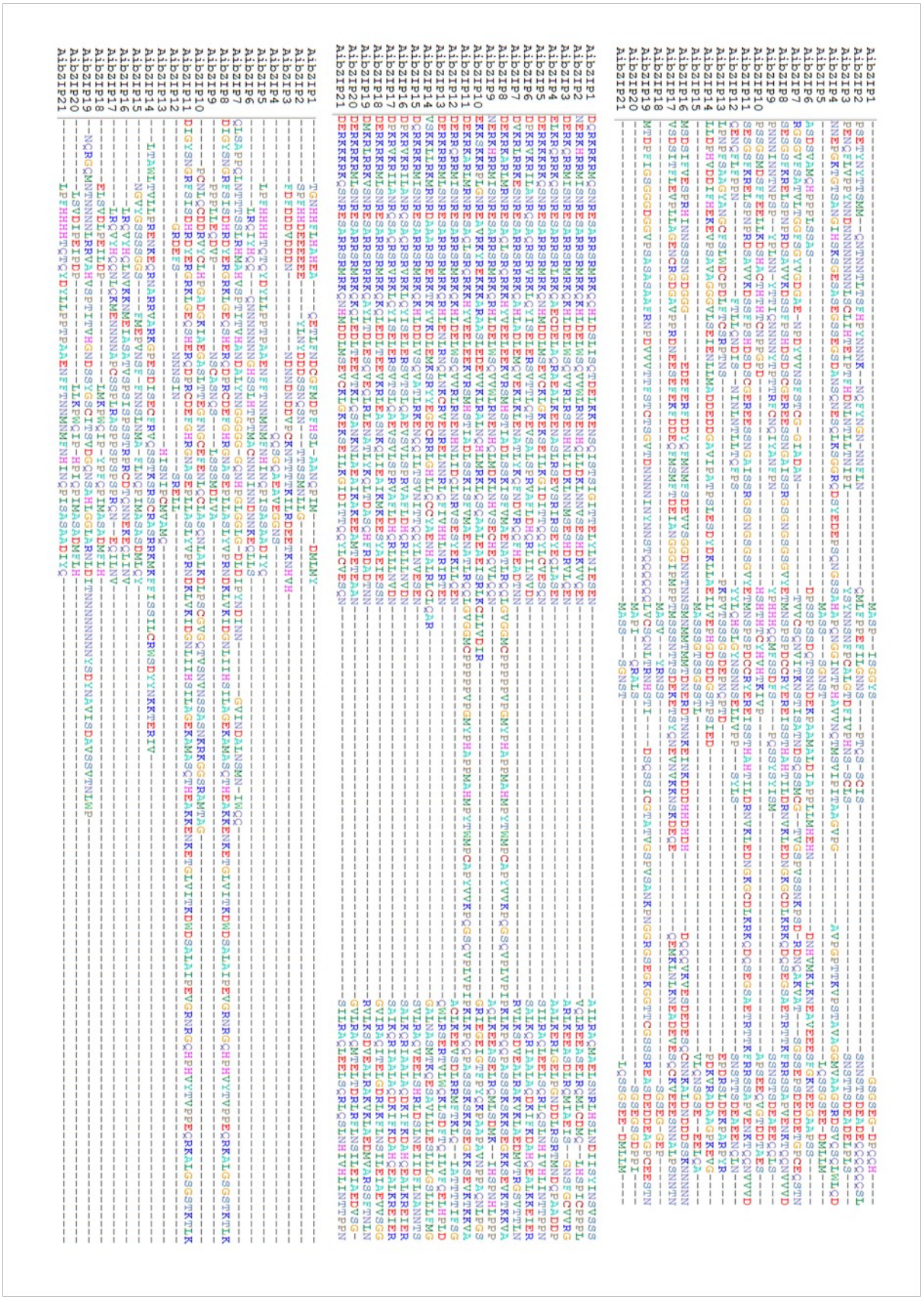


Figure 6. Multiple alignment of 21 AibZIP TFs of groundnut

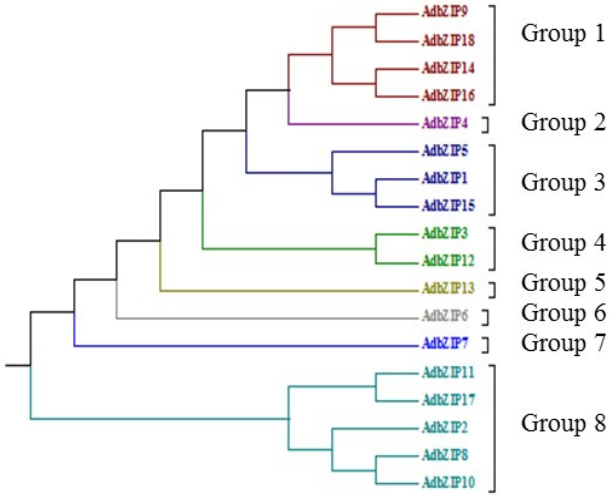


Figure 7. Phylogenetic relationship and gene structure of the bZIP genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 18 amino acid sequences of bZIP genes from *Arachis duranensis* Exon/intron structure of bZIP genes are represented by boxes and black lines, respectively.

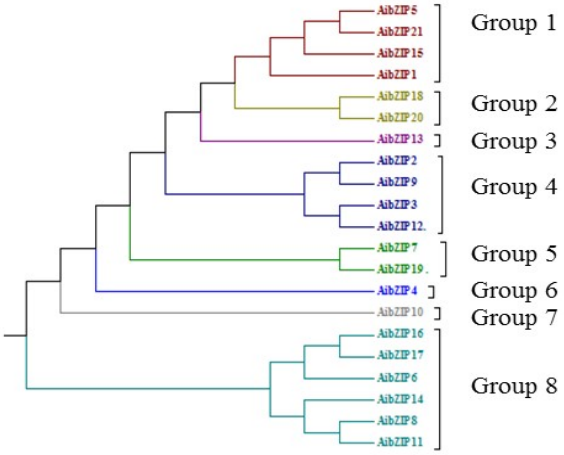


Figure 8. Phylogenetic relationship and gene structure of the bZIP genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 21 amino acid sequences of bZIP genes from *Arachis ipaënsis*

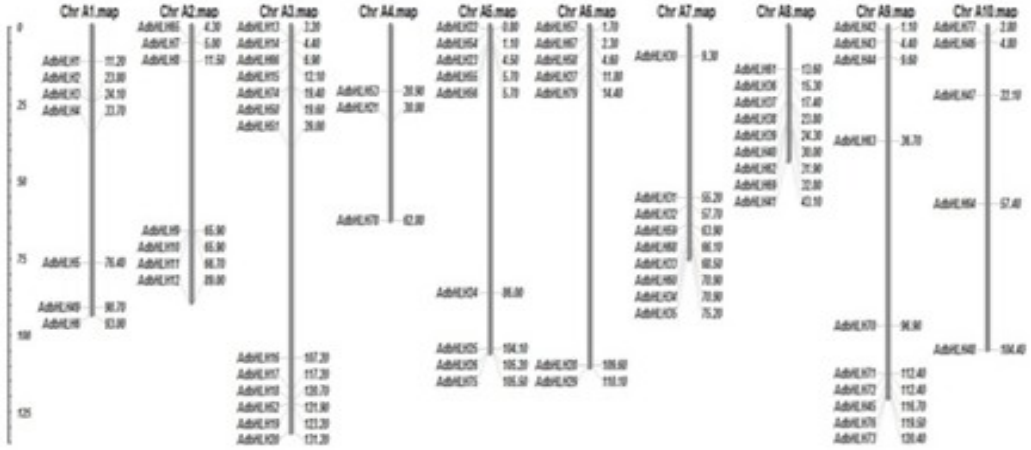


Figure 9. Distribution of 79 bHLH genes from *Arachis duranensis* on peanut chromosomes and physical location of each bHLH gene on the ten chromosomes from each species (positions in cM).

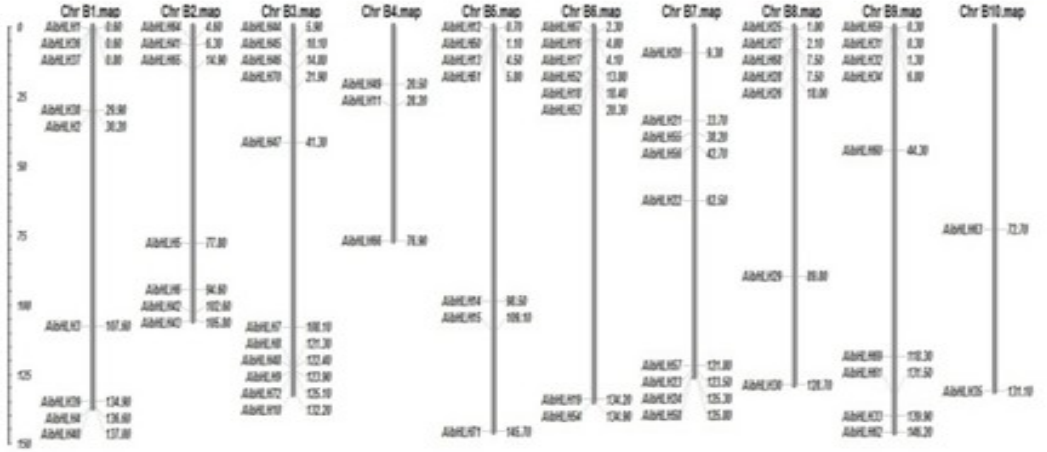


Figure 10. Distribution of 72 bHLH genes from *Arachis ipaensis* on Peanut chromosomes and physical location of each bHLH gene on the ten chromosomes from each species (positions in cM).

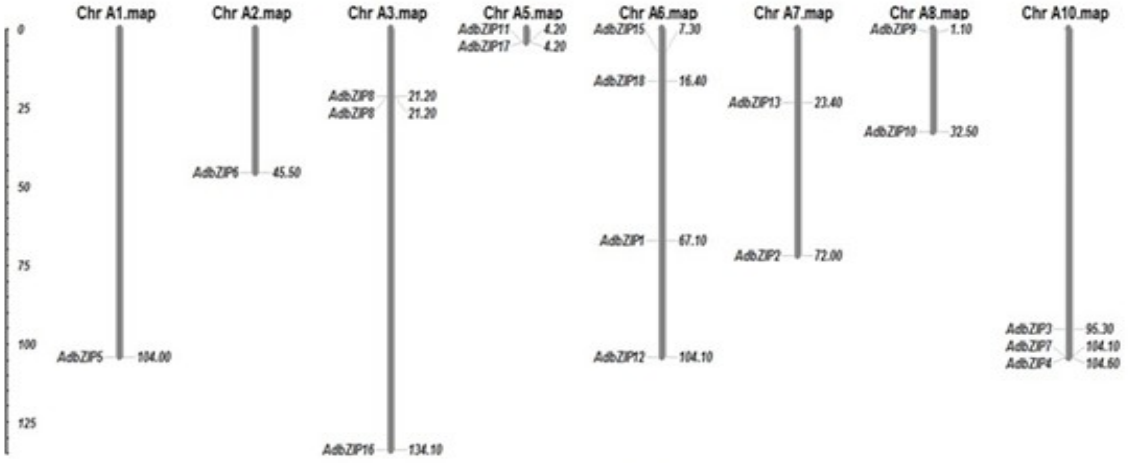


Figure 11. Distribution of 18 bZIP genes from *Arachis duranensis* on Peanut chromosomes and physical location of each bZIP gene on the ten chromosomes from each species (positions in cM).

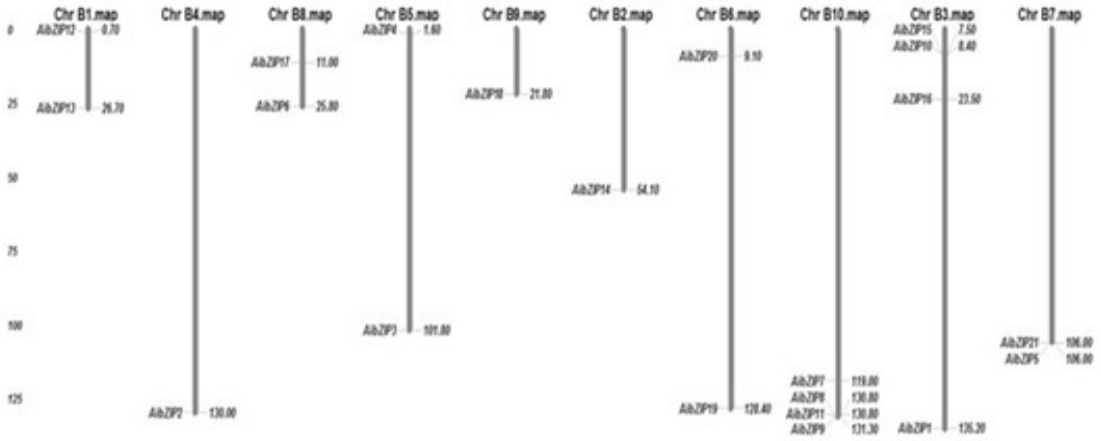


Figure 12. Distribution of 21 bZIP genes from *Arachis ipaensis* on Peanut chromosomes and physical location of each bZIP gene on the ten chromosomes from each species (positions in cM).

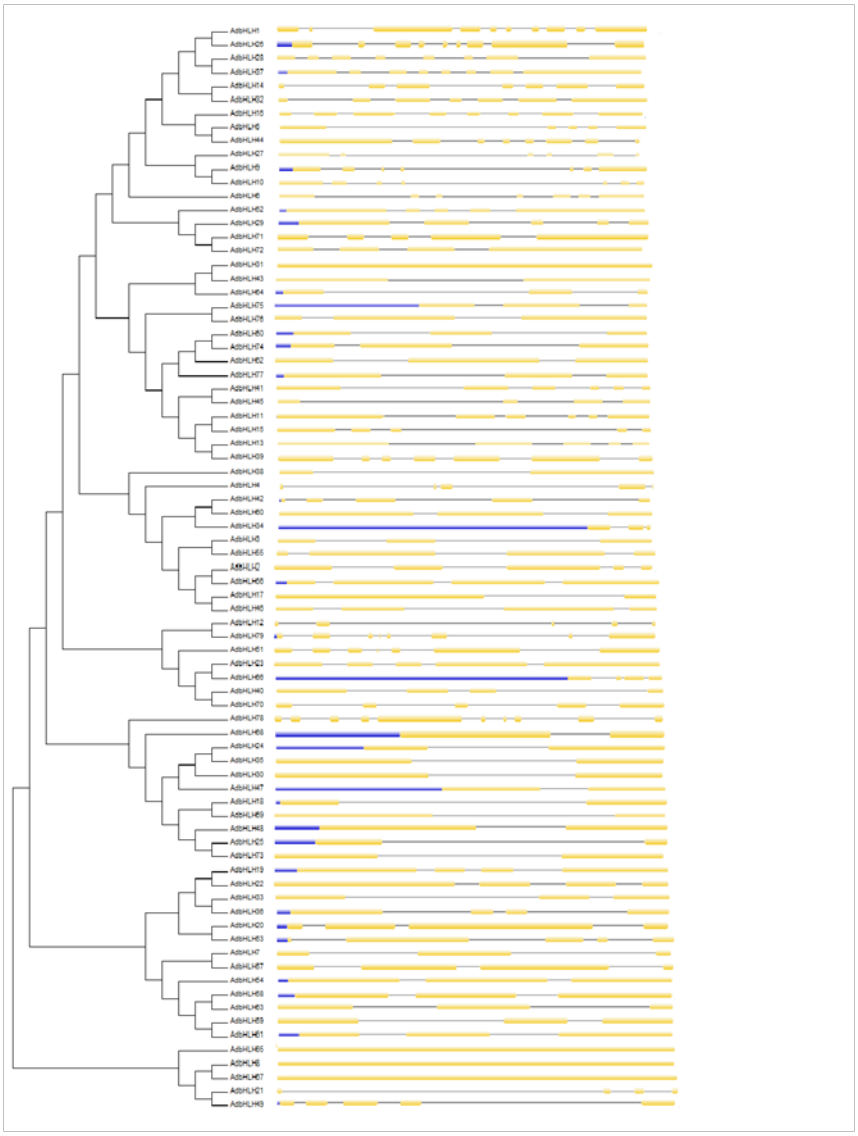


Figure 13. Phylogenetic relationship and gene structure of the bHLH genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 79 amino acid sequences of bHLH genes from *Arachis duranensis*. Exon/ intron structure of bHLH genes are represented by boxes and black lines, respectively.

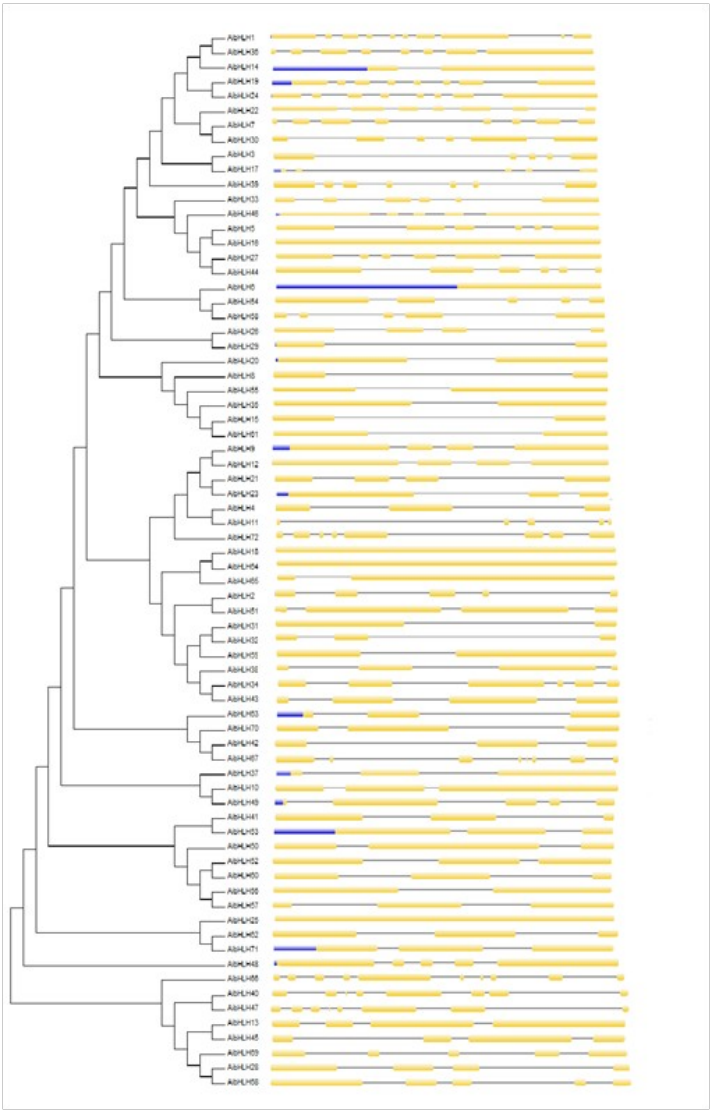


Figure 14. Phylogenetic relationship and gene structure of the bHLH genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 72 amino acid sequences of bHLH genes from *Arachis ipaënsis* Exon/intron structure of bHLH genes are represented by boxes and black lines, respectively

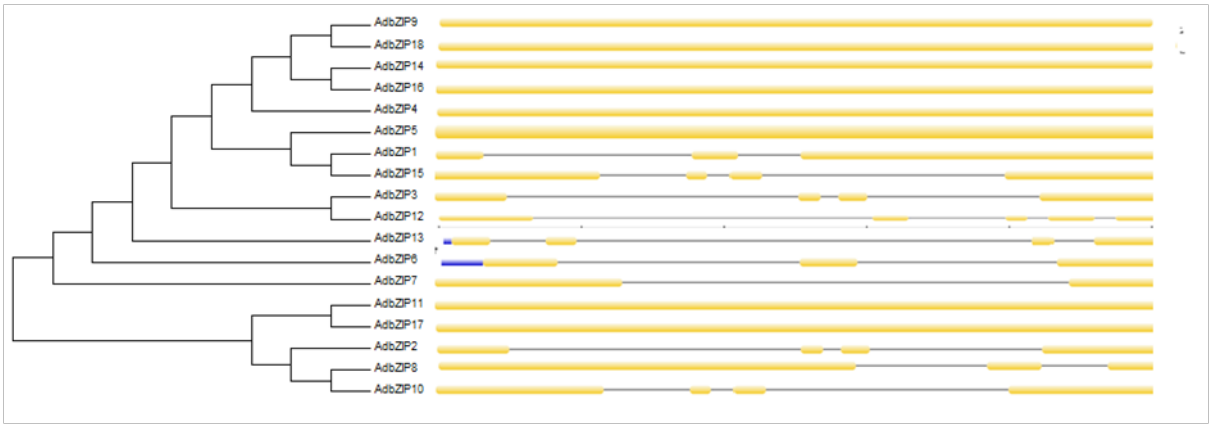


Figure 15. Phylogenetic relationship and gene structure of the bZIP genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 18 amino acid sequences of bZIP genes from *Arachis duranensis* Exon/intron structure of bZIP genes are represented by boxes and black lines, respectively.

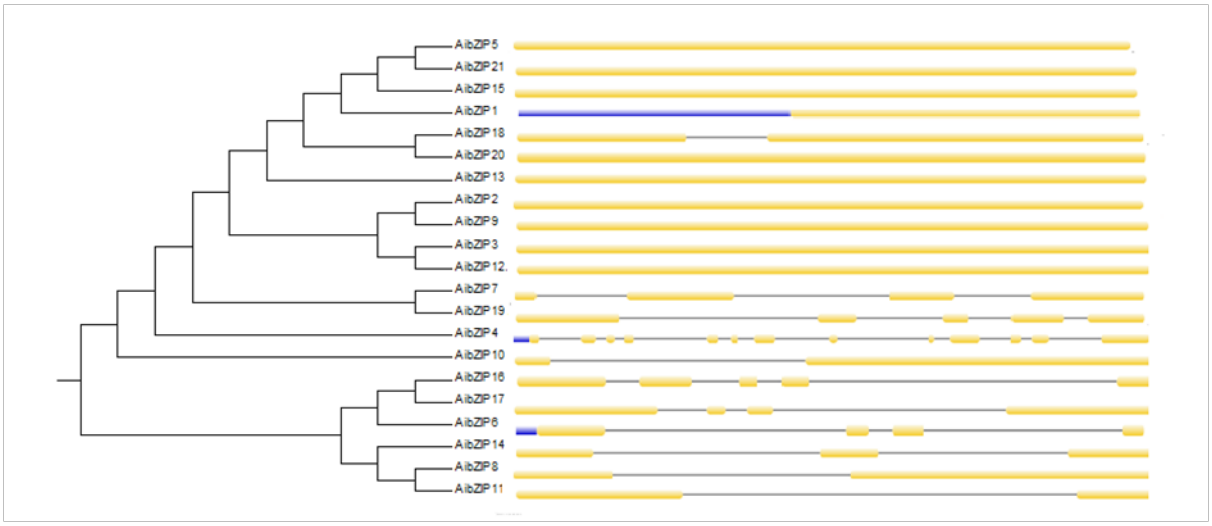


Figure 16. Phylogenetic relationship and gene structure of the bZIP genes. Phylogenetic tree was constructed with MEGA6.0 on a multiple alignment of 21 amino acid sequences of bZIP genes from *Arachis ipaënsis* Exon/intron structure of bZIP genes are represented by boxes and black lines, respectively

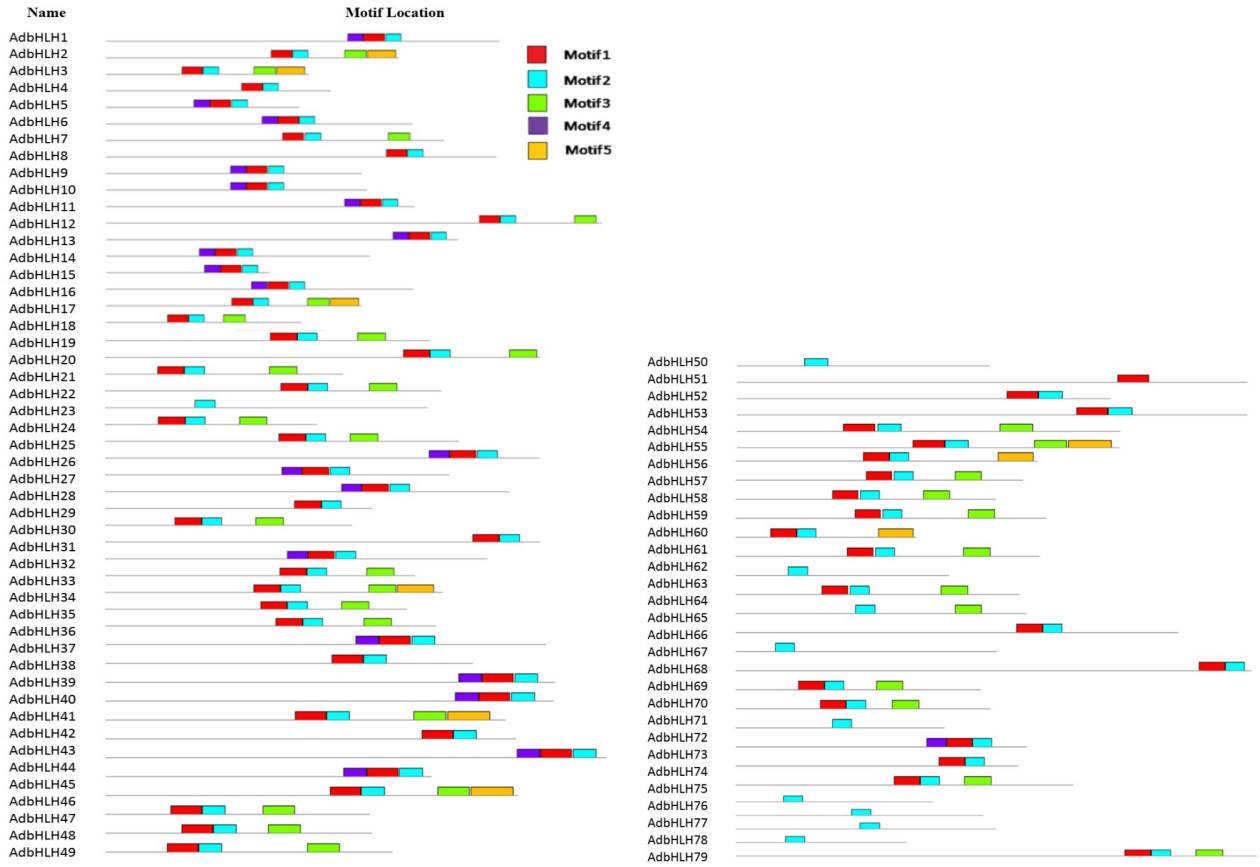


Figure 17. Schematic representation of conserved motifs in the AdbHLH proteins predicted by MEME. Each motif is represented by a number in the colored box. The black lines represent non-conserved sequences.

Figure 18. Schematic representation of conserved motifs AibHLH proteins predicted by MEME. Each motif is represented by a number in the colored box. The black lines represent non-conserved sequences.

Figure 19. Schematic representation of conserved motifs in the AdbZIP and AibZIP proteins predicted by MEME. Each motif is represented by a number in the colored box. The black lines represent non-conserved sequences.

Table 6: Conserved motif logos identified in AdbHLH proteins using MEME tool

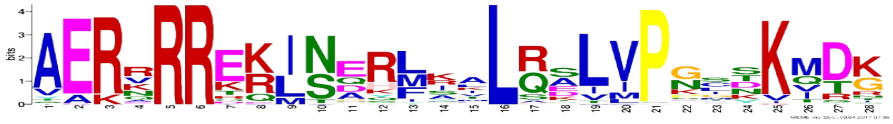
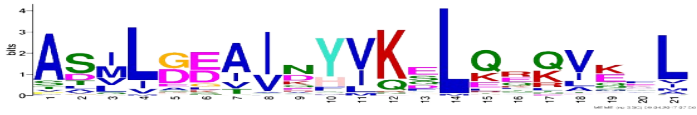
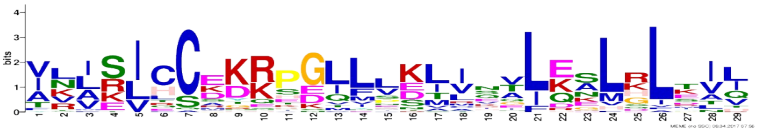
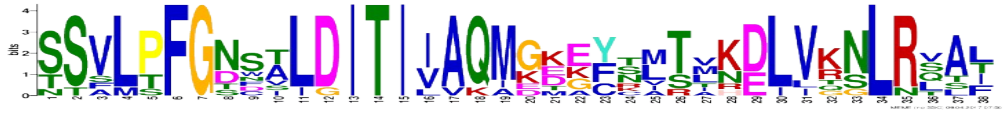
Sl.No	Motif Logo
1	
2	
3	
4	
5	

Table 7: Conserved motif logos identified in AibHLH proteins using MEME tool

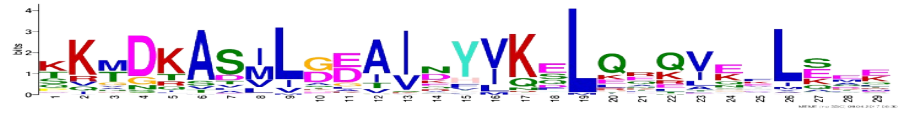
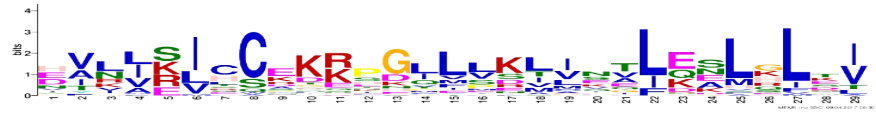
Sl.No	Motif logo
1	
2	
3	
4	
5	

Table 8: Conserved motif logos identified in AdbZIP proteins using MEME tool

Name	Motif Logo
1	
2	
3	
4	
5	

Table 9: Conserved motif logos identified in AibZIP proteins using MEME tool

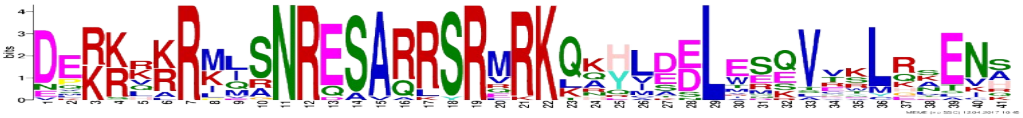
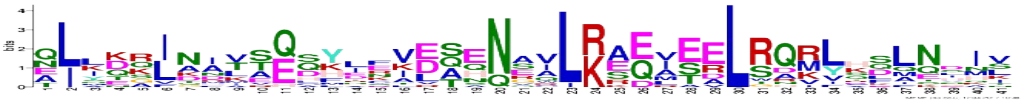
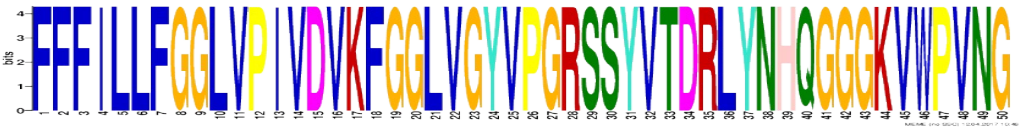
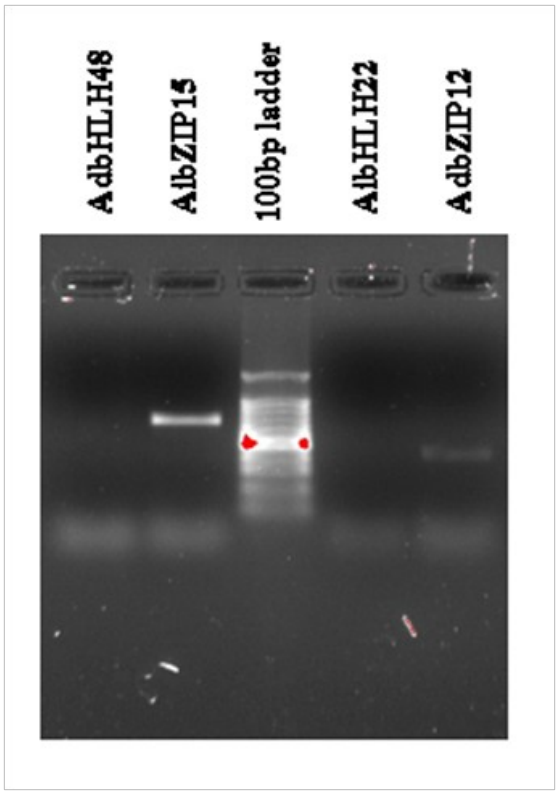
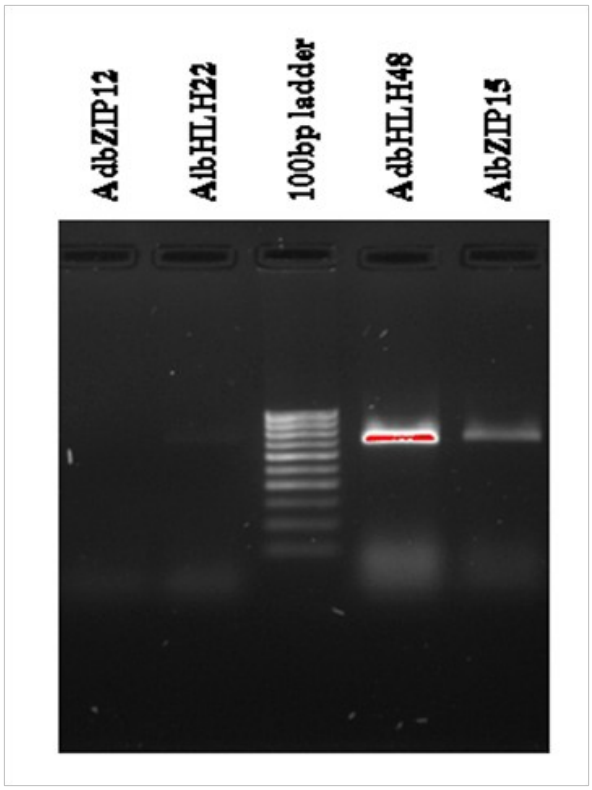
Name	Motif Logo
1	
2	
3	
4	
5	

Figure 20: Control and heavy metal treated seedlings

Figure 21: Control and high temperature stress treated seedlings



A



B

Figure 22: A) Agarose gel electrophoresis of PCR amplified products under high temperature stress. B) Agarose gel electrophoresis of PCR amplified products under heavy metal stress

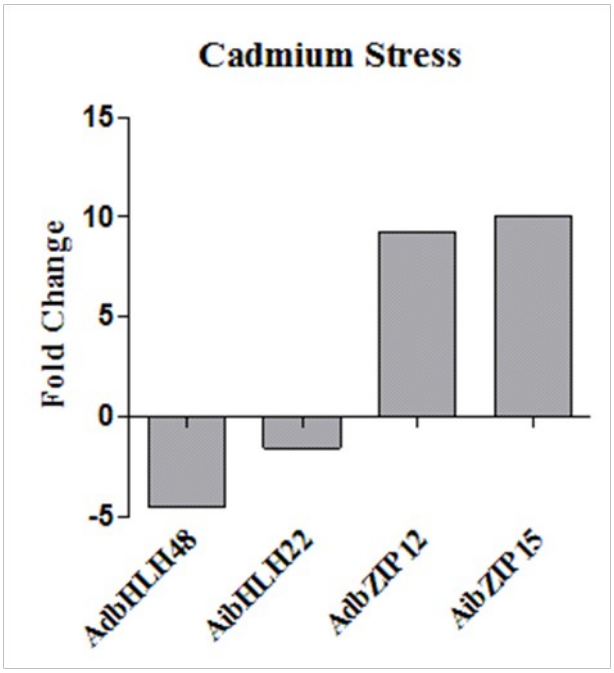


Figure 23: Expression profile of *AdbHLH* and *AibHLH* genes obtained by RT-qPCR of Cadmium chloride treated shoot samples.

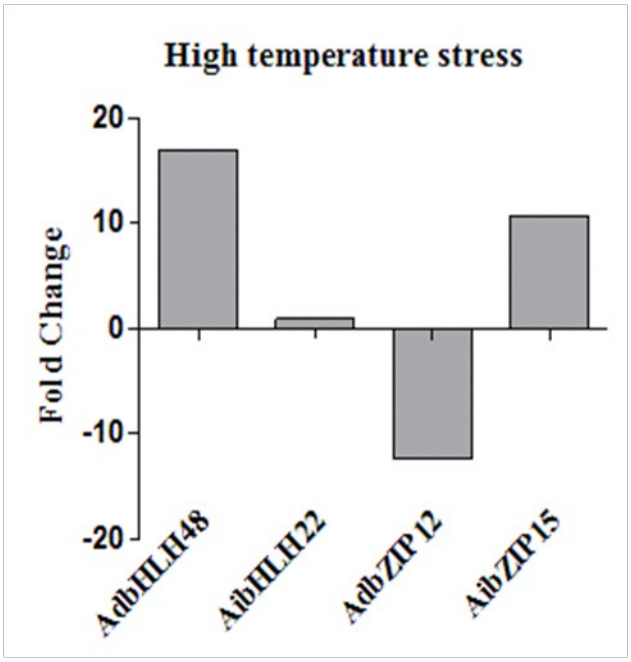


Figure 24: Expression profile of *AdbZIP* and *AibZIP* genes obtained by RT-qPCR of high temperature treated shoot samples.

CONCLUSION

Plants growing in their natural habitats are often challenged simultaneously by multiple stress factors, both abiotic and biotic. Several families of plant TFs play significant roles in translating abiotic stress signals into changes in gene expression. So far, research into TFs that regulate abiotic stress responses has mainly focused on single TFs and their isolated function. The present study comprising genome- wide analysis of bHLH and bZIP TFs, detailed protein features, motif composition, multiple sequence alignment, phylogenetic analysis, gene structure, chromosomal location and expression analysis under high temperature and heavy metal stress provides valuable data for further functional analysis to develop multi stress tolerant varieties in groundnut.

CONFLICTS OF INTEREST

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

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