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Integrative Genomic and Expression Analysis of Zinc Transporter (ZIP) Genes in Sunflower (Helianthus annuus L.) under Drought Stress

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Abstract

Among micronutrients, zinc serves predominantly as a cofactor of many enzymes involved in important biochemical reactions. Vigorous sunflower growth in response to zinc application points towards the existence of an efficient zinc transportation system in this crop. ZIP protein is responsible for the availability of zinc and iron in all plant cells. The absence of comprehensive ZIP gene characterization in sunflower was the driving force for conducting this research work. The objective of the current study was to explore and characterise all ZIP genes across the sunflower genome. A total of 19 ZIP genes were identified and designated as HaZIPs in ascending order. All 19 HaZIP proteins were predicted to exist in the plasma membrane. The HaZIPs family was clustered into 3 groups based on phylogenetic assessment. Not much diversity in structural features of genes belonging to the same group was observed; the genes belonging to different groups exhibited variations in motif configuration. The genes were unevenly mapped on 9 chromosomes, with the maximum genes (7) found on chromosome 15. Two paralog pairs showed segmental duplication, while tandem duplication was witnessed in 4 paralog pairs. Sunflower exhibited no phylogenetic association with other crops, except Arabidopsis thaliana, where a single ortholog was witnessed. Significant increase in expression of HaZIP-1, HaZIP-3, HaZIP-5 and HaZIP-19 was recorded upon exposure of sunflower to drought stress, compared to control, for all 1h, 3h, 6h and 9h. Overall, maximum expression of all 4 genes was witnessed after 3h treatment, while minimum expression was recorded after 9h exposure to drought stress. The cis-acting ABRE could be involved in higher expression of HaZIP genes under drought stress.

Keywords: Sunflower, ZIPs, Zinc transporters, genome-wide, drought

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Introduction

Zinc is one of the most important micronutrients that serves as an integral component of more than 300 enzymes in plants (Castillo-González et al., 2018). Many of these enzymes catalyse essential metabolic processes. Zinc acts as a cofactor for carbonic anhydrase, which is primarily involved in the conversion of CO2 and water into carbonic acid and thus ensuring the continuity of oxygen and CO2 cycles in the atmosphere (Sofo et al., 2018). Similarly, Zinc plays an important role in the synthesis of growth-promoting auxin and chlorophyll, which traps the solar energy and hence initiates the process of photosynthesis (Gupta et al., 2016). Zinc contributes to the stability of the cell membrane to avoid any injury during drought and thermal stress (Zhou et al., 2019). Inadequate Zinc is associated with low pollen viability, resulting in poor fertilisation and seed production. Stunted growth, chlorosis and delayed flowering are the other symptoms that frequently appear during zinc deficiency (Li et al., 2013**).** Thus, zinc is essential for optimal plant growth, productivity and combating different stresses**.** Zinc availability in soil, competition with other nutrients and plant-mycorrhizal association are some of the factors which influence **Z**inc uptake from the soil (Moreno-Lora and Delgado, 2020). Zinc availability depends on soil pH and the presence of low molecular weight chelating substances in the soil. Plant roots take up Zn mostly in divalent form (Zn2+) by increasing its availability via the efflux of H+ ions and organic acids to the soil. Higher concentration of the H+ ions makes the soil acidic and hence enhances Zinc availability. Similarly, low molecular weight organic acids make stable complexes with zinc, facilitating its uptake by the root cells (Zhang et al., 2012). Elevated concentration of phosphorus and iron compete with zinc for the same transportation pathways and may decline its uptake. Zinc ions are transported across the plasma membrane of root cells via specific transporter proteins (Rudani et al., 2018). ZIP proteins are a family of membrane proteins responsible for the uptake of zinc and iron (Ullah et al., 2023). These proteins, located in epidermal root cells, help in the transportation of zinc from the rhizosphere into the cytoplasm of root cells (Marschner, 2012). As an optimum zinc concentration is required for normal physiological functions of the plant, expression of ZIP proteins is tightly regulated, ensuring their adequate availability in different tissues. ZIP1 and ZIP3 have been reported to be closely associated with the maintenance of zinc homeostasis. Zinc availability and transportation are affected under various abiotic stresses, including water scarcity, salinity and high temperature. ZIP4 is upregulated at the onset of zinc deficiency, thus enhancing zinc uptake from the rhizosphere (Verma et al., 2021). ZIP1 and ZIP3 play a role in loading xylem with zinc and routing it from roots to shoots, while ZIP7 facilitates intracellular zinc transportation (Ullah et al., 2023).

Sunflower (Helianthus annuus L.), for several reasons including source of edible oil, vitamin E and biofuel production, is gaining attention (Adeleke and Babalola, 2020; Barontini et al., 2015). Globally, sunflowers rank fourth, while it occupies first position among oilseed crops grown in Pakistan (Rodriguez et al., 2002; Ahmad et al., 2011). Sunflower responds more efficiently to zinc application in the form of enhancing plant height, leaves and dry matter production (Torun, 2013). Such ameliorations in crop parameters not only highlight the metabolic role of this

micronutrient but also help in formulating a strong hypothesis for the existence of the ZIP gene family in sunflower. ZIP has been reported as one of the major families responsible for zinc transportation, and no attempt has previously been made for its exploration in sunflowers. The present study was carried out to uncover all ZIP genes across the genome of sunflowers.

Material and Methods

Identification of ZIP genes across the sunflower's genome

ZIP sequence of Arabidopsis thaliana (NP_001318977.1) was isolated from NCBI (https://www.ncbi.nlm.nih.gov/) and inserted in Pfam finder (http://pfam.sanger.ac.uk, Punta et al., 2012) for detecting the ZIP domain. AtZIP domain was blasted against the sunflower genome using phytozome v.13 (https://phytozome-next.jgi.doe.gov., Goodstein et al., 2012) for extracting HaZIP transcripts.

Physical characterisation and predicting the location of HaZIPs proteins

Molecular weight, protein length and CDS of HaZIPs were taken from phytozome. Similarly, other features of HaZIP proteins, including PI, GRAVY, instability and aliphatic indices, were obtained from Expasy Protparam (https://web.expasy.org/protparam, Gasteiger et al., 2003). HaZIP protein sequences were inserted in CELLO Life (http://cello.life.nctu.edu.tw/) for exploring their sub-cellular location.

Conserved domain and interspecific phylogeny of ZIP Proteins

Two files, including rename and hitdata files, mandatory for finding conserved domains, were obtained from TBtool.v1.09854 (Chen et al., 2018) and conserved domain database (Marchler-Bauer et al., 2015), respectively. The files were subjected to TBtool.v1.09854 for generating domain architecture (Chen et al., 2018).

The phylogenetic relationship of ZIP proteins across host plants, model plants, and 5 other plants, including Oryza sativa, Zea mays, Setaria italica, Hordeum vulgare, and Triticum aestivum, was explored by inserting their sequences into MEGA7.0.26. HvZIP sequences were inserted in MEGA 7 (Kumar et al., 2016) for exploring phylogeny between H.vulgare and Arabidopsis thaliana based on maximum likelihood (ML, 1,000 bootstrap replicates). Tajiima Relative rate test with P value (0.05) used as threshold value. A p-value less than 0.05 showed no differences in the sequences, and a P-value greater than 0.05 exhibited differences in the sequences. Two ZIPs from the same plant associated with the same clade of the phylogenetic tree were termed as paralogs, while those belonging to different plants were named as orthologs.

Mapping and duplication of HaZIP genes

The information pertaining to chromosome number, position and length of HaZIP genes was extracted from phytozome (Goodstein et al., 2012) and inserted in the PhenoGram Plot for mapping HaZIP on the chromosomes (http://visualization.ritchielab.psu.edu/phenogram s/plot,Wolfe et al., 2013). Homology between any 2 HaZIP genes was detected using SIAS (http://imed.med.ucm.es › Tools › sias). Two genes having more than 50% homology and covering more than 90% protein length were termed duplicated genes. (Wang et al., 2017). The duplicated HaZIPs with physical distance of less than and more than 50 kb were designated as tandemly duplicated and segmentally duplicated genes, respectively (Cannon et al., 2004).

Intraspecific phylogeny, conserved motif and structure of HaZIP genes

HaZIP sequences were inserted in MEGA 7.0 (Kumar et al., 2016) for exploring phylogeny. Conserved motifs were visualised in HaZIP proteins using Multiple Em for Motif Elicitation (http://memesuite.org, Bailey et al., 2006). A maximum of ten motifs per HaZIP sequence were annotated using Pfam (http://pfam.sanger.ac.uk, Punta et al., 2012). CDS and genomic sequences of HaZIP genes were extracted from Pytozome and subjected to Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/) for visualising the gene structure.

Promoter Region Analysis

A sequence of 1500bp upstream to the translation initiation site (ATG) was taken from Phytozome. PlantCARE was used for exploring cis-regulatory elements in HaZIP genes. (Lescot et al. 2002).

Transcriptomic analysis of HaZIP genes

Transcriptomic data of sunflower against drought stress were extracted from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/). The data was filtered across TBtool for detecting transcripts linked with the ZIP family.

Growing sunflower, drought treatments and qRT-PCR

Sunflowers were dipped in tap water for 20 seconds and then treated with calcium hypochlorite (10%) for 12 min. Then seeds were grown on a basic salt medium for 3 days. The medium was changed to Hoagland solution (20%) and further grown in a chamber for a week in a growth room with 14/10 hours photoperiod, 200 ± 25 µmol $m^{-2} s^{-1}$ light intensity, 22/17°C (day/night) and 58% relative humidity. Some seedlings were treated with PEG-induced drought stress, while the other lying in Hoagland solution (20%) were labelled as control. Sample collection was carried out after 1 h, 3 h, 6h and 9 h of drought exposure to drought stress.

Plant samples were subjected to RNeasy plant mini kit (Qiagen, code# 74904) for RNA extraction. First-strand cDNA was synthesised from total RNA (1 µg) using Super Script Transcriptase III Kit and oligo (dT) primers. A reaction mixture, consisting of SYBR Green (5µl), cDNA (2µl), 0.3 µl each of forward and reverse primers and water (2.4µl), was subjected to qRT-PCR with 42 cycles for amplification (Roche, Basel, Switzerland). The reaction was adjusted as denaturation (98 °C for 10 sec), annealing (55 °C for 30 sec) and extension (72 for 2 min). The Ct values were incorporated in the Livak formula ($2^{-\triangle\triangle CT}$) for the determination of relative gene expression after normalisation with actin as an internal control. The primers used for transcriptomic analysis are given in Table S2.

Statistical analysis

A two-factorial CRD design with 3 replications was used to get valid estimates of treatment effect, having minimum experimental error. Statistical software was used for the analysis of data. ANOVA or F test was used for determining the level of treatment effect, while LSD test was applied to compare means after ANOVA.

Results

Detection and Physico-chemical characterization of HaZIP in sunflower

Nineteen ZIP genes across the genome of sunflower were detected and named as HaZIP1- HaZIP19. HaZIP18 was recorded as the largest gene with 1491 bp CDS, 497 amino acids protein length (PL) and 53.61 kDa protein molecular weight (PMW), while HaZIP11was found as the smallest one with only 849 CDS, 283 PL and 30.74 kDa PMW, respectively (Table 1). The cell

membrane was predicted as the residence for all 19 HaZIP proteins. Four HaZIPs proteins, including HaZIP6, HaZIP7, HaZIP10 and HaZIP18, were declared unstable for showing an instability index greater than 40. HaZIP17 exhibited a maximum aliphatic index (120.84). Isoelectric points, GRAVY and transmembrane of HaZIP were found in the range of 5.35- 8.91, 0.269-0.746 and 8-9, respectively (Table S2).

Conserved domain and interspecific phylogeny of ZIP Proteins

Conserved ZIP domains were detected in all HaZIP proteins (Figure 1). Sixty-five ZIP proteins across different species, including Helianthus annuus, Arabidopsis thaliana, Oryza sativa, Zea mays, Setaria italica, Hordeum vulgare and Triticum aestivum, were clustered into 5 groups. Maximum ZIP proteins (18) were found in Group I, while minimum ZIP proteins (8) were explored in Group II (Figure.1). Likewise, 10 paralogous pairs (maximum) were observed in H. vulgare, H. annuus and A. thaliana. Maximum orthologs (5) were observed in Z. mays and S. itlica. Only 1 ortholog of sunflower was recorded with A.thaliana (Figure 2)

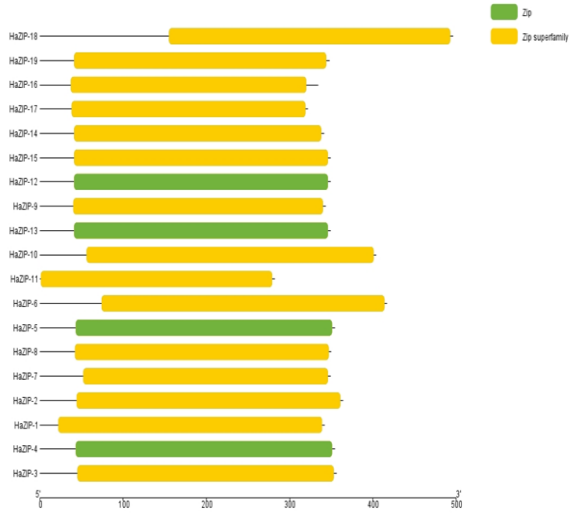

Figure 1: Detection of ZIP domains in HaZIP Proteins

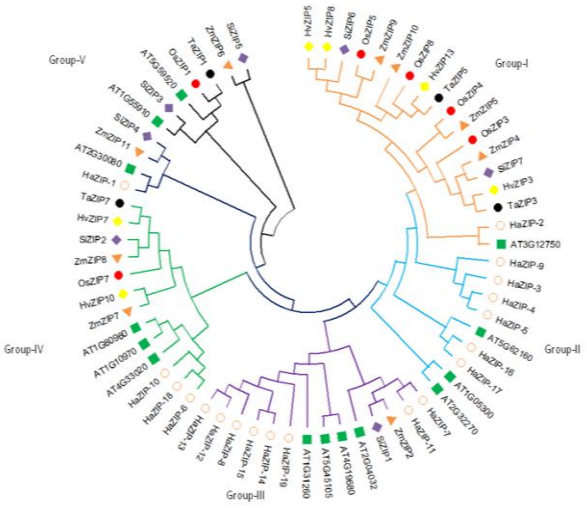

Figure 2. Phylogenetic analysis of the ZIP family protein from sunflower (HaZIP)

Chromosomal mapping and duplication of HaZIP genes

Nineteen HaZIP genes were unequally distributed on 10 chromosomes, while no gene was mapped on any of the 7 chromosomes, including 2, 7, 9, 10, 11, 12 and 14. Seven HaZIP genes (maximum) were placed on chromosome number 15, while only 1 HaZIP (minimum) was found on each of chromosomes 1, 3, 6 and 13. Segmental duplication was witnessed in 2 paralog pairs of HaZIPs, while the rest of the 4 exhibited tandem duplications (Figure 3). The Ka/Ks ratio was less than 1 for four paralogs, so they were under purifying selection (Table 1).

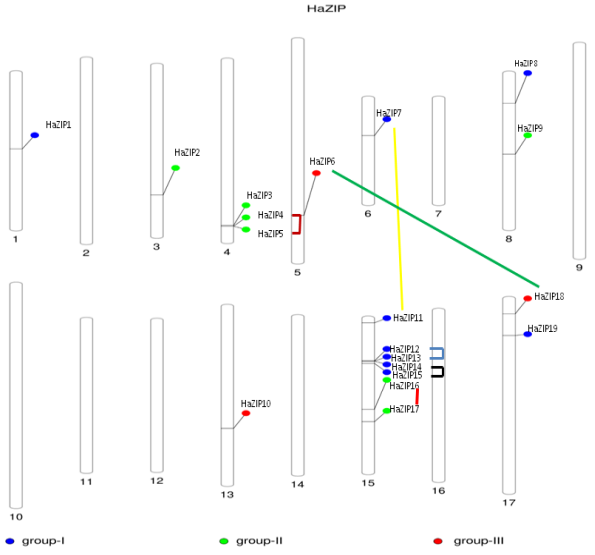

Fig. 3: Mapping of ZIP genes on chromosomes and its duplications pattern across the Sunflower genome.

Segmentally and tandemly duplicated genes have been linked with lines and boxes, respectively.

Groups	Paralogous pair	Ka	Ks	Ka_Ks	Duplications
I	HaZIP-12–HaZIP-13	0	0	NaN	tandem
	HaZIP-14–HaZIP-15	0.011610638	0.181576717	0.063943431	tandem
	HaZIP-7–HaZIP-11	0.023754955	0.194271264	0.122277245	segmental
II	HaZIP-16–HaZIP-17	0.067579982	0.19916481	0.339316883	tandem
	HaZIP-4–HaZIP-5	0	0.027891 66	0	tandem
III	HaZIP-6–HaZIP-18	0.215179247	1.145169918	0.187901589	segmental

Table 1. Duplicated HaZIP genes and their pattern in Helianthus annuus

Intraspecific phylogeny, motif configuration and structural analysis of HaZIP genes

Nineteen HaZIPs proteins were clustered into group-I, group-II and group-III with 8, 7 and 4 members, respectively (Figure 4A). Maximum exons (4) were found in HaZIP14, followed by HaZIP4 and HaZIP5 with 2 exons each, while 3 exons were recorded in the rest of the 16 HaZIP genes (Figure 4C). As introns are the intervening sequences between two exons, the number of introns is marked as 1 less than the number of exons in each HaZIP (Figure 4C). Group-II was explored as the largest one, with respect to the number of motifs per HaZIP, ranging from 7-10, followed by group-III (6-10) and group-I (5-9), respectively (Figure 4B). The number of amino acids per motif varied, with the overall number ranging from 15 to 50 amino acids. Maximum amino acids (50) were witnessed in motifs 1, 2, 3 and 4, while only 15 amino acids were observed in motifs 8 and 10 (minimum). All motifs showed association with zinc transportation (Table 2). ZIP domain sequence was detected in all HaZIPs.

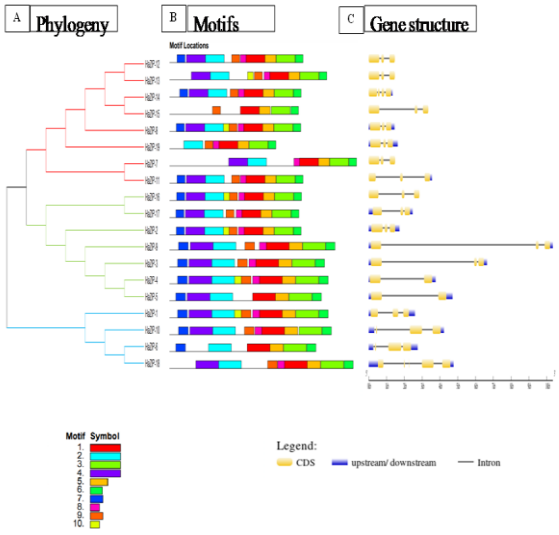

Fig 4. Phylogenetic, motif and gene structure analysis of the ZIP family in sunflower.

1. Phylogenetic tree of all genes in the ZIP family of sunflower. The genes are highlighted in different colours: Group I in red, Group II in olive and Group III in blue
2. Motifs are shown in colored boxes in front of the respective HaZIP proteins.
3. Yellow boxes show exons, black lines represent introns, while the untranslated region (UTR, down/upstream) is highlighted with blue boxes.

Motif #	Sequence	Width	Discription
1	VLELGIVVHSVVIGLSLGASNDPCTIKPLVAALCFHQMFEGMGLGGCILQ	50	ZIP Zinc transporter
2	FMHVLPDAFDMLTSPCLPDNPWSKFPFTGFIAMLAAIFTLMVDSMATSYY	50	ZIP Zinc transporter
3	KTYKENSPTALIVEGVLNASSAGJLIYMALVDLLAADFMGPKLQGSIKLQ	50	ZIP Zinc transporter
4	KJIAIAAILIAGIIGVCJPLIGRSIPALSPDRSLFVIVKAFAAGVILATG	50	ZIP Zinc transporter
5	KJIAIAAILIAGIIGVCJPLIGRSIPALSPDRSLFVIVKAFAAGVILATG	29	ZIP Zinc transporter
6	KSYVALLLGAGGMSLLAKWA	20	ZIP Zinc transporter
7	CTKALAZCEDETNNPCNNKSK	21	ZIP Zinc transporter
8	GAIGQQLLRYRVVAQ	15	ZIP Zinc transporter
9	TRDQEMAVASGGAMHFHGHAH	21	ZIP Zinc transporter
10	TSKNNAIAAEGGEVV	15	ZIP Zinc transporter

Analysis of cis-acting regulatory elements

From PlantCARE, a total of 1545 cis-acting regulatory elements were explored in the 1500bp upstream region of 19 HaZIP genes. Cis regulatory elements were dominated by promoter responsive elements (1126), followed by Light responsive elements (212) (Figure 5A), hormone responsive elements (110) (Figure 5B), environmental stress (49) (Figure 5C), developmental (42) (Figure 5D), site binding (4) and other elements (2).

The putative cis-acting elements associated with upregulation of ZIP genes include zinc deficiency-related elements (ZDRE) (GTCGAC), ABA-responsive elements (ABRE) (ACGTG), dehydration-responsive elements (DRE/CRT) (G/ACCGCC), and low-temperature-responsive element (LTRE) (CCGAC) motifs. PlantCARE result revealed the presence of ABRE elements in 13 HaZIPs, while LTRE elements were detected in 2 genes. Results obtained from the PLACE database exhibited the occurrence of at least 1 of the 4 mentioned cis-elements in each HaZIP gene.

Annexure (A)

A) Light-responsive elements, (B) Hormonal-responsive elements, (C) Environmental stress-responsive elements, and (D) Developmental-responsive elements

Expression profiling of selected HaZIP genes under drought stress

Significant increase in expression of HaZIP-1 and HaZIP-3 genes was recorded upon treating sunflower with drought stress for all 1h, 3h, 6h and 9h, compared to the control. LSD results revealed no significant difference between 1h and 3h treatment, but after 6h and 9h treatment significant decline was witnessed compared to initial 1h and 3h treatments (Fig 6, A & B). Sunflower treated with drought stress for different duration including 1h, 3h, 6h and 9h exhibited significant increase in expression of HaZIP-5 compared to control plants. LSD results showed no significant difference among 1h, 3h and 6h drought stress treatment; however, a significant decrement in expression was observed after 9h of drought stress (Fig 6, C). Similarly, significant elevation in expression in HaZIP-19 was observed

under all treatments of drought stress as compared to control plants (Fig 6, D).

Annexure (B)

Annexure (C)

Discussion

Efficient response of sunflower to zinc application in terms of enhanced grain, fodder and protein production has led to the assumption of sunflower as a potential repository for ZIP genes. Nineteen ZIP genes explored across the genome of sunflower were found in line with those detected in common bean (Astudillo et al., 2013). However, this number is more compared to foxtail millet, barely (Alagarasan et al. 2017) and less than wheat (Evens et al., 2017). Mapping of 19 HaZIPs over 9 chromosomes was found in agreement with those reported in Malus domestica. The detection of a wider range of theoretical PI value (5.35-8.91) for HaZIPs, strengthened earlier reports in cowpea (Ullah et al, 2023) and maize (Mondal et al., 2014).

The broader range of molecular weight associated with HaZIP proteins (30.7-53.6 kDa) was found in line with Ullah et al (2023). Localisation of all HaZIP proteins in the cell membrane strengthened its role as transporting channels across the plasma membrane and was in agreement with reports of Pedas et al (2008) and Lee et al. (2010) in rice and trifoliate orange, respectively.

Intraspecific diversity observed in the phylogeny of HaZIP proteins suggested the possible role of chromosomal aberration associated with HaZIPs, and is in agreement with earlier reports of Ma et al. (2019) and Fu et al (2017). Model plant (Arabidopsis thaliana) exhibited some similarity with the host plant by sharing a single artholog, while rests of the characterised plants were marked as out-groups due to the existence of arthologs in sunflowers. Among the out groups, strong phylogenetic association was witnessed in Z.mays and S.itlica by sharing one third of the total artholgs detected in the present genome-wide study. Detection of many paralog in sunflower (6) advocated the possible role of gene duplication events in the extension of the ZIP family in sunflower (Liu et al., 2018; Timko et al., 2008).

The cis-elements are important molecular switches involved in the transcriptional regulation of genes during gene expression and may be induced through ABA-dependent and ABA-independent signal transduction pathways (Yamaguchi, Shinozaki, and Shinozaki, 2005).

The higher number of ABRE, as detected in the current study, could be involved in the elevated expression of HaZIP genes under drought stress. Water deficit triggers phosphorylation of transcription factors (ABF), which bind to the CIS-acting ABREs, resulting in higher expression of HaZIP genes. The enhanced expression of HaZIP facilitates zinc uptake, leading to overall improvement in cell membrane stability, hormonal biosynthesis, photosynthetic and ROS scavenging processes during drought. The presence of at least 1 of the 4 putative cis-elements (ZDRE, ABRE, DRE and LTRE), as explored in the PLACE database analysis, strengthened the earlier report of Mondal et al (2014) in ZmZIP genes. Zinc Deficiency Response Element (ZDRE) detected in 3 HaZIPs (HaZIP6, HaZIP16 and HaZIP17) has been reported previously in Arabidopsis thaliana (Assunção et al., 2010).

Statements & Declarations

Data availability

The data is available in the respective web links and accession numbers

Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

ZS and AU designed the research. SZ and AU extracted the data under the supervision of ZS. ZK, IS, AM and KT helped in manuscript writing and figure setting. All the authors approved the submission of the manuscript.

Ethical standards:

This article does not contain any studies with human participants or animals performed by any of the authors

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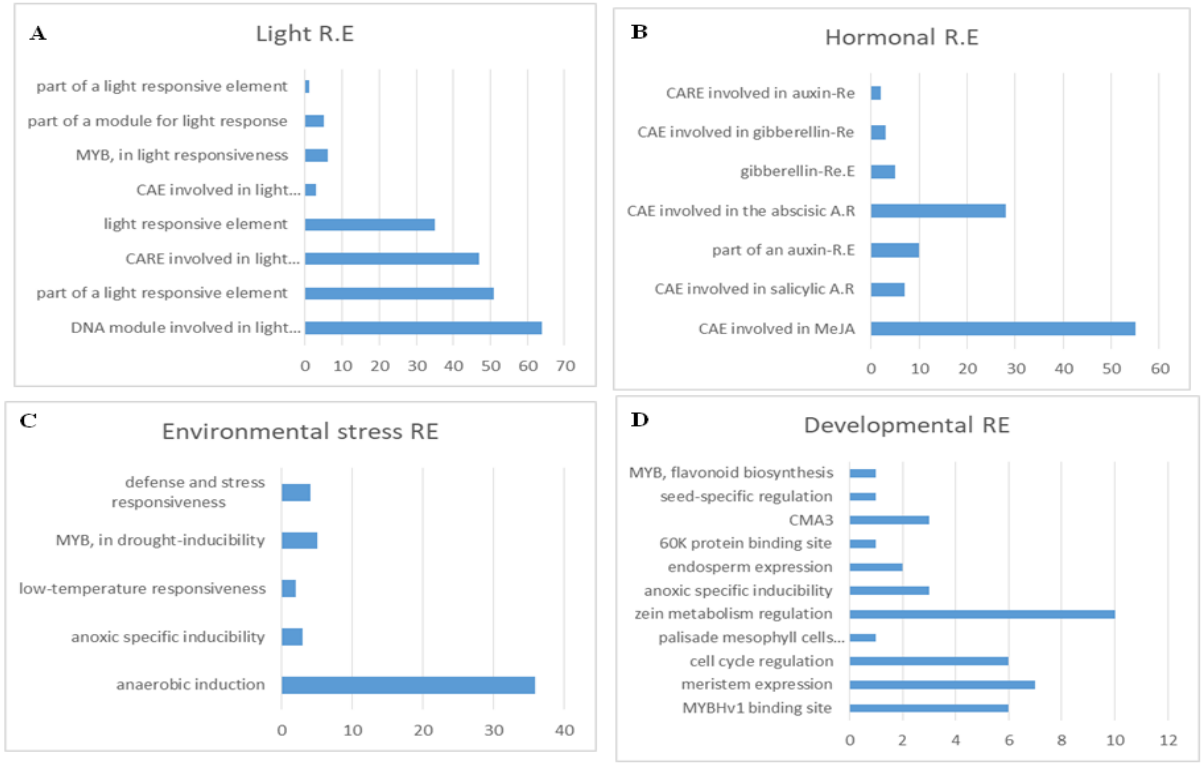
Table S2: Physicochemical properties of HaZIP protein family

Groups	Transcript ID	Gene Name	Chromosome number	CDS (bp)	Protein length (A.A)	Protein Molecular Weight (kDa)	pI	GRAVY	Instability index	Aliphatic index
I	HanXRQChr15g0476541	HaZIP-12	15	1050	350	37.05187	8.72	0.633	33.24	109.37
	HanXRQChr15g0476581	HaZIP-13	15	1050	350	37.05187	8.72	0.633	33.24	109.37
	HanXRQChr15g0476591	HaZIP-14	15	1026	342	36.19304	8.91	0.645	36.14	111.61
	HanXRQChr15g0477221	HaZIP-15	15	1050	350	37.10102	8.91	0.598	37.4	109.37
	HanXRQChr08g0216701	HaZIP-8	8	1053	351	37.33443	8.86	0.671	35.93	110.71
	HanXRQChr17g0545111	HaZIP-19	17	1047	349	37.18619	8.73	0.567	35.64	110.14
	HanXRQChr06g0176091	HaZIP-7	6	1050	350	37.77965	8.66	0.484	41.86	108.11
	HanXRQChr15g0465031	HaZIP-11	15	849	283	30.74832	8.58	0.457	39.93	101.99
II	HanXRQChr15g0487451	HaZIP-16	15	1005	335	36.31988	6.11	0.587	25.91	114.16
	HanXRQChr15g0489781	HaZIP-17	15	1491	323	34.609	6.33	0.746	24.99	120.84
	HanXRQChr03g0080281	HaZIP-2	3	1095	365	39.3068	6.4	0.464	34.29	109.92
	HanXRQChr08g0226331	HaZIP-9	8	1032	344	36.1406	6.54	0.671	33.84	117.46
	HanXRQChr04g0122791	HaZIP-3	4	1071	357	38.14778	6.22	0.486	27.76	112.36
	HanXRQChr04g0123051	HaZIP-4	4	1065	355	37.90142	6.30	0.490	29.42	111.07
	HanXRQChr04g0123071	HaZIP-5	4	1065	355	37.90142	6.30	0.490	29.42	111.07
III	HanXRQChr01g0011661	HaZIP-1	1	1029	343	36.95839	5.75	0.513	33	107.25
	HanXRQChr13g0410531	HaZIP-10	13	1215	405	43.75466	5.35	0.393	45.96	104.31
	HanXRQChr05g0152071	HaZIP-6	5	1254	418	44.61935	6.30	0.277	40.22	100.62
	HanXRQChr17g0538351	HaZIP-18	17	1491	497	53.61902	6.14	0.269	52.27	95.56

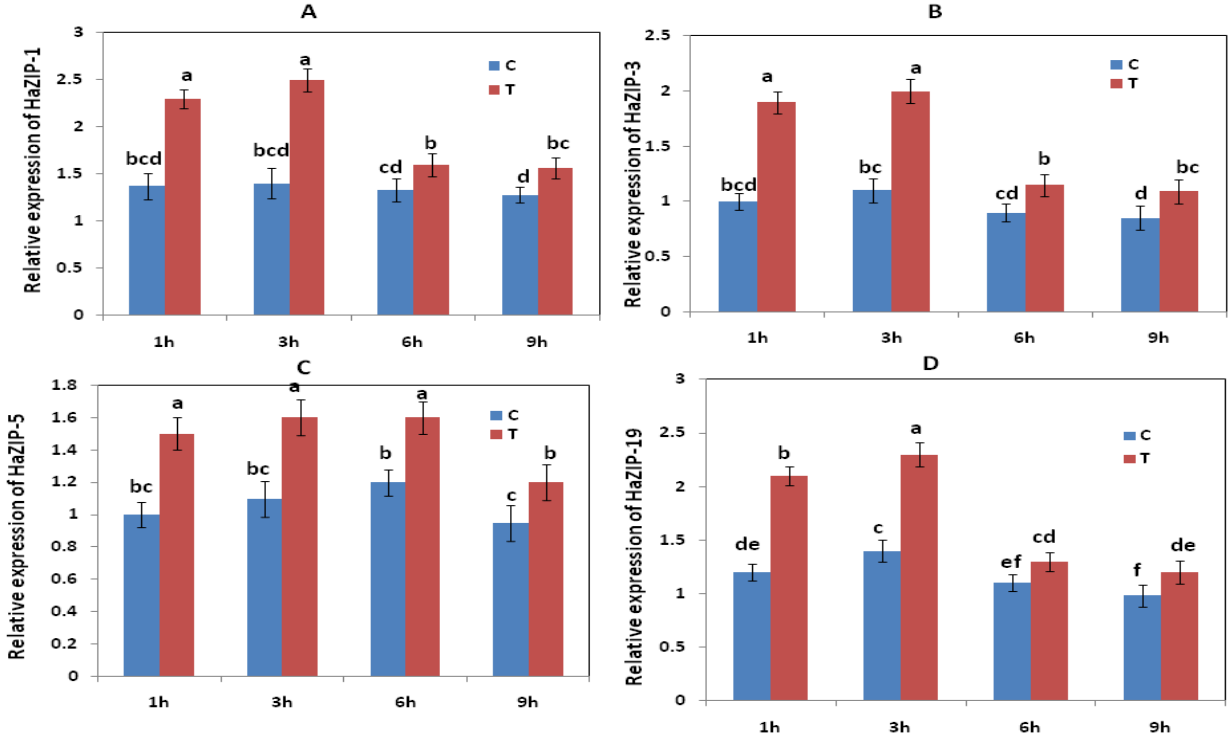
Annexure (A)

Figure 5: Cis-elements in genes belong to the ZIP family of sunflowers



Annexure (B)

Figure 6. Expression profiling of HaZIP genes after drought treatment for (A) one hour, (B) three hours, (C) six hours, and (D) nine hours



Annexure (C)

Table S1: List of the primers used for functional validation (qPCR) of selected HaZIP genes.

No.	Sequence ID	FP sequence	RP sequence	FP length	RP sequence	FP Tm	RP Tm	FP GC %	RP GC %	Product size
1	HanXRQChr01g001166_HaZIP-1	AAGACGGTGGTGGAGATAGAGA	ACGGCTTTATCGTACACTGGTT	22	22	60.025	60.029	50	45.455	184
2	HanXRQChr04g012279_HaZIP-3	TACATGTCCACTGGAAAGGAGC	ATCTTCCTTATGGGCAAAGGCA	22	22	60.027	60.024	50	45.455	176
3	HanXRQChr04g012307_HaZIP-5	TACATGTCCACTGGAAAGGAGC	TACTTGCAGCCATCCCTTGAAT	22	22	60.027	60.025	50	45.455	141
4	HanXRQChr05g015207_HaZIP-6	GGGTTGGAGGACAACCCTATCA	CCTCATGCTTGATGATTCCATA	22	22	60.023	60.025	50	45.455	184
	Primers for qRT-PCR									
	Genes	**Primers**	**Sequences (5' -> 3')**							
	HaZIP-1	Forward primer	AAGACGGTGGTGGAGATAGAGA							
		Reverse primer	ACGGCTTTATCGTACACTGGTT							
	HaZIP-3	Forward primer	TACATGTCCACTGGAAAGGAGC							
		Reverse primer	ATCTTCCTTATGGGCAAAGGCA							
	HaZIP-5	Forward primer	TACATGTCCACTGGAAAGGAGC							
		Reverse primer	ACTTGCAGCCATCCCTTGAAT							
	HaZIP-6	Forward primer	GGGTTGGAGGACAACCCTAT							
		Reverse primer	CCTCATGCTTGATGATTCCA							
	actin	Forward primer	GACTCTGGTCATGGTGTCAGC							
		Reverse primer	GGCTGGAAGAGGACCTCAGG							