



OPEN Genome-wide identification of the *TIFY* gene family in *Helianthus annuus* and expression analysis in response to drought and salt stresses

Kangping Zhang^{1,3}, Yaxi Zhou^{1,3}, Yuanyuan Jiang¹, Yaqi Zhou², Jinhui Song¹, Jingjing Zhang¹, Jianzhong Guo¹, Lin Li¹ & Xianping Zhang^{1✉}

Given the increasing recognition of frequent drought problems associated with global warming, sunflower (*Helianthus annuus* L.) has been widely studied as a model plant tolerant to drought and salt stresses. However, there is a lack of information on the systematic identification of the sunflower *HaTIFY* gene family. In the present study, 21 *HaTIFY* genes in sunflower were identified and the members of *HaTIFY* family were divided into four subfamilies, i.e., *TIFY*, *JAZ*, *ZML* and *PPD*. Gene duplication is a major driver for the expansion of the gene family. Here, three segmental and two tandem duplicated gene pairs were identified via duplication and synteny analysis. Furthermore, five paralogous *TIFY* gene pairs might have undergone purifying selective pressure during evolution based on Ka/Ks ratio. *HaJAZ2/4/5/9/12* from *JAZ V* subfamily were highly expressed in the majority of tissues. In the analysis of promoter elements of *HaTIFYs*, more than half of 21 *HaTIFY* genes contained the drought induction elements. Notably, *HaPPD1* and *HaPPD4* were significantly upregulated at the early stages of both drought and salt treatments, highlighting their potential roles in enhancing sunflower resistance to abiotic stresses. In conclusion, the *HaTIFY* gene family plays a crucial role in the positive regulation of sunflower's response to abiotic stresses, offering key candidate genes for enhancing resistance in sunflower breeding programs.

Keywords *TIFY* genes, Bioinformatics, *Helianthus annuus* L., Abiotic stress, Expression profiles

Sunflower (*Helianthus annuus* L.) is the fifth-largest commercial oilseed crop in the world after peanut, soybean, sesame, and rapeseed, and is widely cultured more than 22.9 million hectares on a global scale¹. In the context of global warming, more frequent and intense drought and high salinity events in more geographic areas are an alarming issue, resulting in enormous losses in seed yield and oil production². It has been reported that sunflower is extremely vulnerable to drought and salt stress caused by early spring drought, especially in the young growth stage¹. Many reports have shown that sunflowers exposed to abiotic stress have evolved complex signal perception and transduction systems, which is related to the large-scale reprogramming of downstream regulated and co-regulated gene expression by transcription factors (TFs) in response to stress^{3–5}. Therefore, the identification of key resistance genes in the sunflower genome and clarifying the potential TFs regulatory mechanisms by bioinformatic and transcriptomic approaches are crucial for enriching resistance gene resources in sunflower to cope with unpredictable and extreme environments.

The plant-specific *TIFY* proteins were reported to regulate plant development including a highly conserved *TIFY* domain with the characteristic alpha-alpha-beta fold^{6,7}. The *TIFY* domain contains 36 amino acids with a conserved motif TIF[F/Y]XG, which is originally known as *ZIM* (Zinc-finger protein expressed in Inflorescence Meristem). The *ZIM* domain was initially discovered in the *ZIM* proteins in *Arabidopsis thaliana*, and then, to characterize the most conserved amino acid motifs of this family members, Vanholme et al.⁸ suggested it should be annotated as *TIFY*. *TIFY* domain has shuffled or formed new subfamilies by merging with other domains in evolutionary processes⁷. The *TIFY* family is typically divided into four clades, i.e., *TIFY*, *ZIM*-like

¹Department of Horticulture and Landscape Architecture, Taiyuan University, Taiyuan 030032, Shanxi, China.

²Fenyang College of Shanxi Medical University, Fenyang 032299, Shanxi, China. ³Kangping Zhang and Yaxi Zhou contributed equally to this work. ✉email: 18584564192@163.com

(ZML), JAZ (JASMONATE ZIM) and PEAPOD (PPD)⁸. The members of TIFY subfamily are characterized by one TIFY domain, whereas the other subfamilies possess multiple domains. For instance, the JAZ subfamily proteins contain highly conserved TIFY and Jas (CCT_2) domains⁹. Previous reports showed that Jas motif is specific of the JAZ subfamily, serving as a negative feedback regulator in the jasmonic acid (JA) signaling pathway via mediating hormone-dependent interactions between JAZ proteins and F-box protein COI1, thereby contributing greatly to environmental adaptation^{6,9,10}. In addition to TIFY domain, the ZML subfamily members are characterized by CCT domain and C2C2-GATA zinc-finger domain¹⁰, whereas the PPD subfamily proteins consist of characteristic N-terminal PPD domain and partially Jas domain defecting the conserved Proline-Tyrosine (PY) residue at the C-terminus^{11,12}.

To date, *TIFY* genes have been identified across the multiple varieties, including 26 in *Zea mays*¹², 18 in *Fagopyrum tataricum*¹³, 16 in *Dendrobium huoshanense*¹⁴, and 77 in *Brassica napus* L.¹⁵ and other plants. Indeed, the multiple regulatory effects of the *TIFY* gene family on plant growth, development¹⁶ and abiotic resistance^{17,18} indicate that this gene family contains a wealth of valuable gene resources. Previous studies across various species suggested that TIFY members have also been showed to engage in increasing plant tolerance to abiotic stresses such as drought, high salinity, or alkali stress^{15,19}. For example, some researchers have highlighted the significant positive role of *ClJAZ1/7* genes in watermelon under drought and salinity conditions²⁰. In rice, overexpression of *OsJAZ8* has been showed to improve drought resistance²¹. Additionally, the overexpression of *FtJAZ10/12* enhanced the heat stress tolerance in *Fagopyrum tataricum*¹³. According to a recent survey, *EjJAZ2/4/9* responded to salt stress, which might enhance salinity tolerance through the JA pathway in *Eriobotrya japonica*¹⁸. Moreover, except for the JAZ subfamily proteins, it has been reported that *DhTIFY1*, *PPD1*, and *ZLM1* had prompt and strong responses in *Dendrobium huoshanense*¹⁴. Previous studies reported that the expression of sunflower *HaJAZ* gene tended to downregulate under drought and salt stress patterns⁵. However, extensive studies of other *HaTIFY* family members and their function involved in drought and salt tolerance are still limited. It is, therefore, hypothesized that the *TIFY* gene family members in sunflower are involved in regulating the responses to drought and salt stresses.

In the present study, we identified the *TIFY* gene family from the sunflower genome and conducted an extensive analysis of the gene structure and conserved motifs, physicochemical properties, conserved domain, promoter *cis*-elements to shed new light on *HaTIFY* genes evolutionary relationships and potential functions. Subsequently, the dynamic expression levels of key *HaTIFY* genes involved in drought and salt stress response were analyzed by RNA-seq and qRT-PCR validation. This study provides a basic foundation for further exploration of the functions of *HaTIFY* genes in resistance to abiotic stresses and may provide insights into the regulatory mechanisms of sunflower resistance.

Results

Identification and characterization of TIFY genes in *Helianthus annuus*

A total of 21 *HaTIFY* genes were identified by screening the sunflower protein database. The *HaTIFY* proteins can be further categorized into four subfamilies (namely JAZ, PPD, ZML, and TIFY) according to the motifs and domains composition. *HaTIFY1* and *HaTIFY2* with one TIFY domain belonged to the TIFY subfamily, and the remaining 19 TIFY proteins possessed TIFY and Jas domains. *HaJAZ1*–*HaJAZ12* were grouped as members of the JAZ subfamily; whereas the four proteins without the conserved PY amino acids in the Jas motif were named *HaPPD1*–*HaPPD4*. Three protein sequences contained GATA zinc-finger and CCT domains, which is characteristic of ZML subfamily (designated as *HaZML1* to *HaZML3*).

The essential characteristics of the sunflower *HaTIFY* proteins were subsequently presented in Table 1. Our analysis revealed that the length of CDS exhibited variation, with values spanning from 366 (*HaPPD3*) to 2254 (*HaZML1*). Correspondingly, the largest protein (*HaZML1*) contained 738 amino acids, whereas *HaPPD3* was the smallest with 119 amino acids. The molecular weight ranged from 12.95 kDa (*HaPPD3*) to 81.37 kDa (*HaZML1*) in these proteins. The fat index of the *HaTIFYs* were 60.11 to 82.50. Moreover, the average instability index of the *HaTIFY* proteins was 54.54, indicating that these proteins may be relatively unstable. The values of theoretical pI in 14 out of 21 proteins were more than 7, while the other seven proteins were acidic (pI < 7). All proteins with negative GRVAY values displayed hydrophilic properties because of negative scores. Subcellular localization prediction revealed that nine TIFY proteins localized in the nucleus and chloroplasts, respectively, two in the peroxisomal, and one in the cytoplasm. This result indicated that *HaTIFY* genes mainly function in the nucleus and chloroplasts, and they may be involved in multiple regulatory pathways. In addition, irregular curling was the main secondary structure mode, the order of proportion was irregular curling > α -helix > extended chain > β -folding. The results of tertiary structures predictions of *HaTIFY* proteins indicated that the tertiary structures of three pairs of proteins (e.g., *HaJAZ3/11*, *HaPPD1/4* and *HaJAZ7/8*) were highly similar (Fig. S1).

Phylogenetic analyses and classification of *HaTIFY* gene family

Based on the full length amino acid sequences of 59 TIFY proteins among three species (21 TIFYs in *Helianthus annuus*, 18 in *Arabidopsis thaliana* and 20 in *Oryza sativa*), a phylogenetic tree was constructed using the NJ method (Fig. 1). The result showed that the 59 TIFYs across these species were classified into four subfamilies: JAZ, ZML, TIFY, and PPD. For each group, the number of TIFY proteins varied. Specifically, the JAZ subfamily was the largest group with 37 differential members. This result indicated that the extensive evolution history may be prone to appear in JAZ subfamily to adapt multiple stressors. The JAZ subfamily could be further clustered into five subgroups (JAZ I–JAZ V), in which JAZ I, JAZ II, and JAZ V were universally present in three species. Notably, the JAZ IV subgroup only contained seven *OsJAZ* proteins, while other species did not include any JAZ proteins in this subgroup. In addition, PPD members only included dicotyledonous plants, indicating that the PPD subgroup might be occurred after differentiation in monocotyledonous plants. The difference in

Gene ID	Gene Name	Fat index	CDS length (bp)	Protein length (aa)	Molecular weight (kDa)	pI	Instability index	Alpha helix (%)	Extended strand (%)	Beta turn (%)	Random coil (%)	Subcellular localization	GRAVY
Ha2_00027795	HaTIFY1	60.11	1083	354	36.99	9.00	52.24	9.32	7.34	1.69	81.64	Peroxisomal	-0.523
Ha6_00048490	HaTIFY2	82.50	382	124	13.30	4.96	46.62	9.68	16.94	3.23	70.16	Peroxisomal	-0.001
Ha1_00043659	HaIAZ1	63.21	1059	346	38.27	8.83	60.15	19.94	4.05	2.31	73.70	Nucleus	-0.870
Ha4_00023692	HaIAZ2	76.63	609	199	22.27	8.90	38.26	19.10	6.53	5.03	69.35	Chloroplast	-0.690
Ha4_00025152	HaIAZ3	75.48	382	124	14.16	9.27	76.72	20.97	7.26	3.23	68.55	Chloroplast	-0.738
Ha4_00026095	HaIAZ4	62.22	568	185	20.65	9.16	47.80	20.54	9.19	3.78	66.49	Nucleus	-0.732
Ha6_00048868	HaIAZ5	66.67	571	186	20.50	9.37	69.07	21.51	8.06	3.23	67.20	Nucleus	-0.634
Ha8_00040962	HaIAZ6	73.72	455	148	16.81	9.92	65.75	29.05	6.76	3.38	60.80	Chloroplast	-0.627
Ha10_00001139	HaIAZ7	79.08	665	217	23.77	6.76	44.19	18.43	4.61	2.76	74.19	Nucleus	-0.505
Ha10_00003867	HaIAZ8	79.08	665	217	23.77	6.76	44.19	18.43	4.61	2.76	74.19	Nucleus	-0.505
Ha11_00031214	HaIAZ9	73.57	610	199	22.17	8.63	35.77	20.60	6.53	3.52	69.35	Chloroplast	-0.469
Ha13_00015061	HaIAZ10	71.30	705	230	26.19	7.26	33.36	18.26	8.26	2.61	70.87	Chloroplast	-0.687
Ha15_00038833	HaIAZ11	71.50	385	125	14.19	9.69	84.15	24.00	10.40	3.20	62.40	Nucleus	-0.854
Ha15_00040674	HaIAZ12	75.29	632	206	22.21	9.05	60.45	17.96	7.77	3.40	70.87	Chloroplast	-0.558
Ha10_00003584	HaPPD1	66.61	732	239	25.88	6.21	58.21	10.04	5.86	1.67	82.43	Chloroplast	-0.344
Ha12_00032229	HaPPD2	71.79	836	273	28.60	8.38	43.29	11.36	6.96	2.56	79.12	Nucleus	-0.197
Ha12_00033789	HaPPD3	61.60	366	119	12.95	9.98	72.53	18.49	6.72	2.52	72.27	Chloroplast	-0.621
Ha14_00017726	HaPPD4	66.61	732	239	25.88	6.21	58.21	10.04	5.86	1.67	82.43	Chloroplast	-0.344
Ha15_00040692	HaZML1	72.87	2254	738	81.37	9.29	39.70	13.14	13.55	3.79	69.51	Plasma	-0.390
Ha16_00020739	HaZML2	74.97	1019	334	36.80	6.52	59.53	14.37	4.49	0.60	80.54	Nucleus	-0.486
Ha16_00020745	HaZML3	64.89	949	311	33.24	5.18	55.21	9.00	5.47	1.61	83.92	Nucleus	-0.626

Table 1 . Characteristics of identified 21 HaTIFY genes in sunflower.

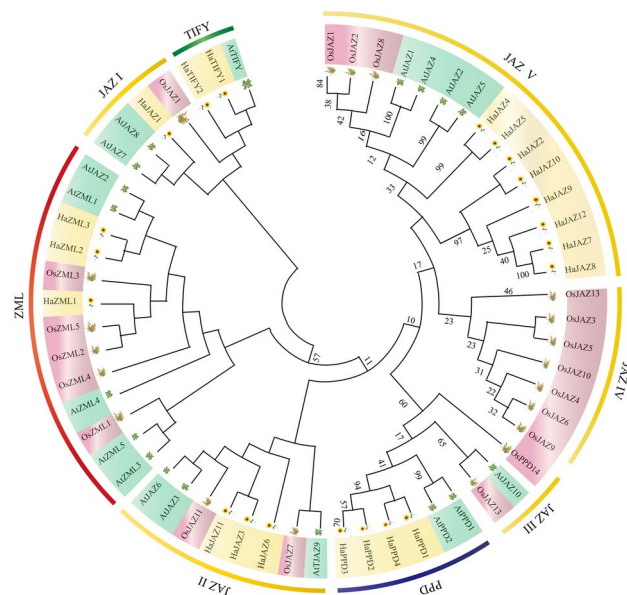


Fig. 1. Phylogenetic analysis of TIFY proteins from *Helianthus annuus* (Ha), *Arabidopsis thaliana* (At), *Oryza sativa* (Os). Neighbor-Joining phylogeny of 59 TIFY proteins of the three species, was determined by MEGA 7.0 program with 1000 bootstrap replicates.

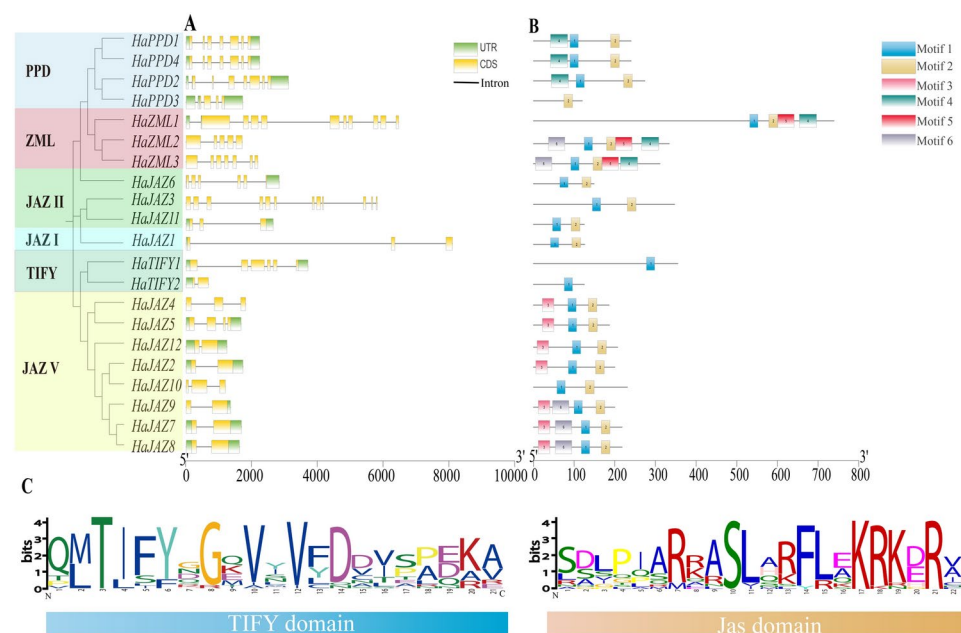


Fig. 2. Phylogenetic relationships, gene structure and motifs of HaTIFYs in *Helianthus annuus*. **(A)** The exon-intron structure of *HaTIFYs*. **(B)** Distribution of the conserved motifs in *HaTIFY* proteins. The scale bar indicates 2000 aa. **(C)** The sequences of TIFY and Jas domains.

the number of clades members between sunflower and rice was probably that they undergone expansion or contraction since the differentiation from monocots and eudicots.

Gene structure and conserved motif analysis of *HaTIFY* gene family

The exon-intron structure could also be used to provide additional evidence for understanding *HaTIFY* genes structure evolution to further support phylogenetic grouping. The results revealed that the loss or gain phenomenon of introns or exons was identified in several branches of *HaTIFY* genes (Fig. 2A). Specifically, *HaPPD3* consisted of 4 exons, while others in PPD subfamily contained 7 exons, indicating that exons may be lost during evolution. In addition, *HaJAZ2/7/8/9/12* were composed of the same number of exons with two, and

HaPPD1/2/4 with 7, and the exon length of these genes displayed almost the same. These *TIFY* genes might be the product of repeated events. All *HaTIFY* genes possessed intron. The number of introns belonging to the PPD subfamily members was more than that of the other subfamilies; whereas the members of JAZ V subfamily (only 1–3 introns) were least. Our analysis revealed that there is a strong correlation between the phylogeny of *TIFY* gene family members and the exon–intron structure, and the genes of the same subfamily usually have similar gene structures.

The online website MEME (<http://meme-suite.org/>) was used to annotate the conserved domain in *HaTIFY*s. A total of 6 motifs (named motif 1 to motif 6) were identified from the conserved regions in 21 *HaTIFY*s (Fig. 2B). Although the number of amino acids of *TIFY* proteins varied, the clustered *HaTIFY* proteins contained the same number of amino acids and similar conserved domain distribution, such as *HaPPD1/4* and *HaZML2/3*. It was observed that each *HaTIFY* member contained 1–5 motifs. It is worth mentioning that the members of ZML group generally contained most motifs, while members of *TIFY* clade only contained one motifs. However, some of which were unique to specific subgroups, such as motif 3 limited to JAZ V, motif 5 distributed only in ZML subfamily; motif 4 was showed in PPD and ZML subgroups. This finding conforms to phylogeny analysis, and they explain together that the members of clustered at the one branch generally possessed a similar conserved sequence motifs. Furthermore, *TIFY* and Jas domains were performed by drawing their sequence logos. As presented in Fig. 2C, *TIFY* domain had a conserved TIF[F/Y]XG motif and amino acid sites Val-10 and Val-12. Jas domain was highly conserved, particularly in location sites 7, 10, 11, 14, 17, 18, 19, and 21.

Analysis of cis-acting element in *HaTIFY* gene family

The *cis*-elements in the promoter region of *TIFY* family members were shown in Fig. 3, based on the PlantCARE database. The phytohormone elements were found widely in each *HaTIFY* gene promoter (Fig. 3A). *HaPPD4* had up to 15 phytohormone responsive elements; *HaPPD2* and *HaJAZ11* had up to 11 and 10 elements, respectively. Further, the relatively abundant *cis*-elements involved hormone signaling pathways were ABRE and TCA-motif, TGACG-motif. ABRE was present in almost all *HaTIFY*s promoters (Fig. 3B, C). Another important finding was that more than two-thirds of *HaTIFY* genes contained TGACG-motif element, which is known to modulate responses to various necrotizing pathogens. This finding underscores the potential involvement of these genes in the JA signaling pathway. The most numerous and widely distributed in stress-responsive elements was ARE, followed by MBS. Interestingly, *HaJAZ10*, *HaPPD4* and *HaPPD1* had more drought-induced elements than others, which infers that they may also have the same function in response to drought stress. Conversely, a relatively small number of genes were found to contain TC-rich repeats *cis*-elements.

Chromosome localization and duplication analysis

The detailed chromosome localization of 21 *HaTIFY* genes linked to tandem replications events were plotted in Fig. S2. They were mapped irregularly across 12 chromosomes. Among them, Chr4, Chr10, and Chr15 contained the largest number of *HaTIFY* genes (3 each), followed by Chr6, Chr12, and Chr16 with two *HaTIFY*s, respectively. Chr1, Chr2, Chr8, Chr11, Chr13, and Chr14 had the least gene distribution, with only one gene each. It is interesting to note that more than half of *HaTIFY* genes were close to chromosome terminal. Furthermore, the duplication events of the sunflower *HaTIFY* genes showed that three segmental (*HaJAZ2/4*, *HaJAZ3/11*, and

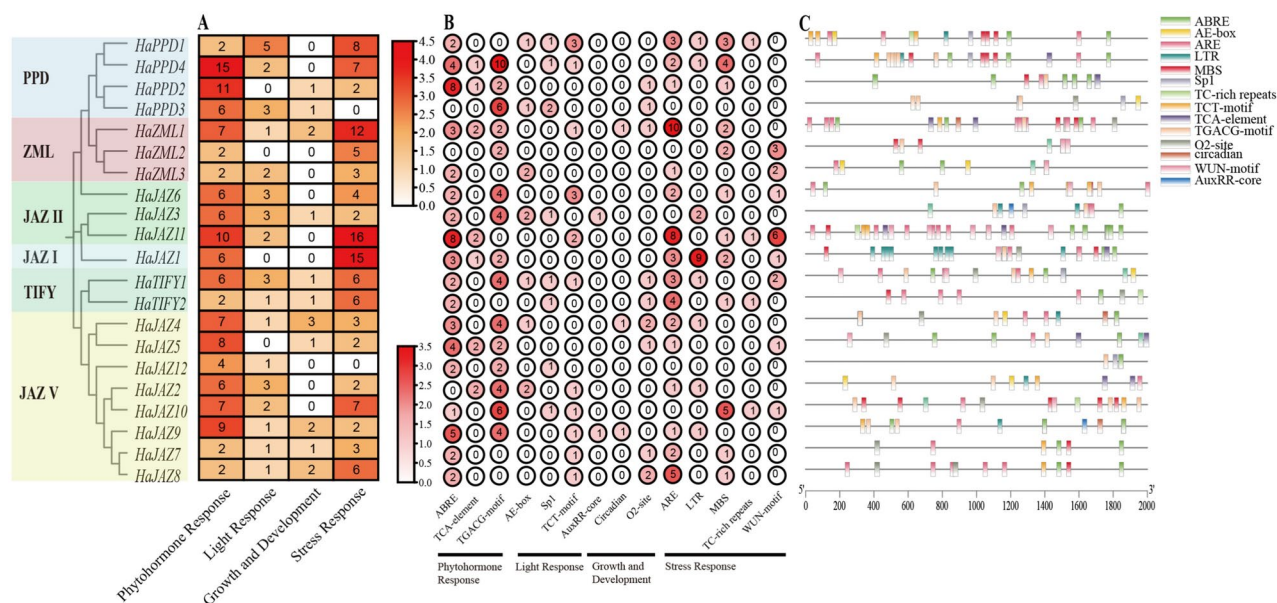


Fig. 3. Prediction of *cis*-acting elements in *HaTIFY*s promoters. (A) Each *HaTIFY* contains the number of *cis*-acting elements detected which were divided four types. (B) The number of different elements of each *HaTIFY* gene in four types of elements. (C) Visualization of four types of elements in *HaTIFY*s promoters by TBtools, including position, kind and quantity of elements.

HaPPD3/4) and two tandem duplicated gene pairs (*HaJAZ12/HaZML1* and *HaZML2/3*) were identified (Fig. S3). This finding had illustrated the importance of both whole-genome duplication and segmental duplication events in the evolution and expansion of the *HaTIFY* genes in sunflower. It is worth noting that as a pair of segmental duplicated genes, *HaJAZ3/11* exhibited a highly comparable tertiary structure (Fig. S1).

Due to the chromosomal localization lacking of the nature of *TIFY* genes origin, the gene duplication analysis between sunflower and two model plants (*Arabidopsis thaliana* and *Oryza sativa*) were constructed (Fig. S4). Notably, sunflower and *Arabidopsis* have three pairs of homologous genes, while there are two homologous gene pairs between sunflower and rice. This is consistent with the fact that both belong to dicotyledonous plants. *HaJAZ2* exhibited collinear relationships in both monocotyledons and dicotyledons, indicating that this gene might play crucial roles in evolution. In addition, the selection pressure of homologous *TIFY* genes was assessed using the ratio of Ka/Ks. The Ka/Ks ratios of the five paralogous pairs were less than 1 (*HaJAZ2/AT1G72450.1*, *HaJAZ8/AT1G72450.1*, *HaPPD2/AT1G70700.3*, *HaJAZ2/Os03t0180800-01*, and *HaJAZ4/Os09t0439200-01*) (Table S1). The analysis demonstrated that *HaTIFY* genes were primarily constrained by purifying selective pressure during their evolutionary development.

Protein–protein interaction (PPI) network

In the study of the interactions between *HaTIFY* proteins and their potential partners, we constructed protein–protein interaction network based on STRING database (Fig. 4). A total of 17 *HaTIFY* proteins were presented in the PPI network, including all proteins of the JAZ V subgroup. However, there were only one or two members in other subgroups. Notably, the interaction between all *HaJAZ* proteins and COI1 could have occurred as a result of JAZ proteins as a transcriptional repressor, by inhibiting downstream gene expression in the JA pathway. Further, the JAZ V subfamily also displayed multiple interactions with MYC2 to inhibit the JA signaling pathway, likely due to the members of JAZ group with the C-terminal jasmonic acid (JA)-related (Jas, CCT_2) domain. Regarding the results, the members of PPD and JAZ V subfamily had more interaction partners, which may play a remarkable role in coordinating hormone signals. The above results reveal the complex network of protein level interactions, and predict their potential functions.

Microarray analysis of expression profiles

Based on previous RNA-seq data, the expression patterns of sunflower *TIFY* gene family in ten main tissues have varying profiles of expression. As showed in Fig. 5, members of the *HaTIFY* family into four clusters (I to IV) by cluster analysis, according to the varied expression levels. Compared with other groups, *HaPPD2* and *HaJAZ4/5/12* belonged to the group I showed strong expression in numerous organs. More than one-third of the *HaTIFY* genes were highly expressed in the majority of tissues, with *HaJAZ* genes accounting for 75% of them. For example, the expression levels of *HaJAZ2/4/5/9/12*, members of the JAZ V subfamily, were particularly prominent, in bract, corolla, ligule, ovary, stamen, stem, and style. These results may reveal the important roles of these genes in flower development of sunflower. In contrast, the expression levels of some *HaJAZ* genes (such as *HaJAZ6/10/11*) were low or not expressed.

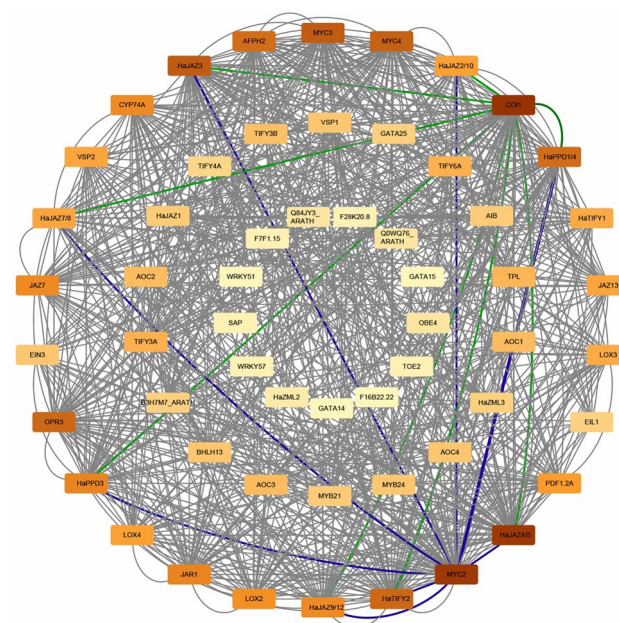


Fig. 4. Interaction network of *TIFY* proteins in sunflower. The green lines represent the interaction between COI1 and *HaTIFY* proteins; and the blue lines represent the interaction between MYC2 and *HaTIFY* proteins. The network was constructed using the STRING tool and Cytoscape, and confidence score is 0.4.

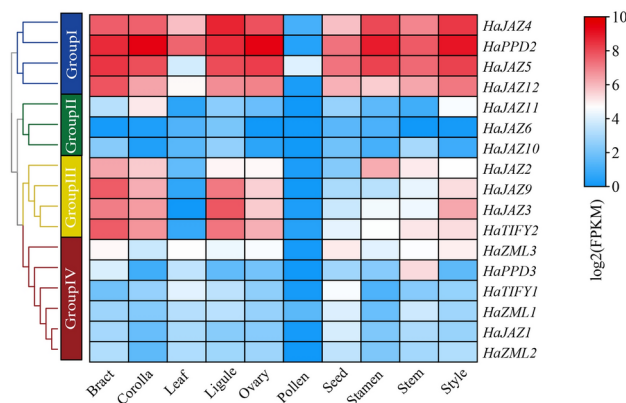


Fig. 5. Expression profiles of *HaTIFY* genes in different tissues and organs. The normalized $\log_2(\text{FPKM})$ was used to create a heatmap. The indicator ranges in the upper right corner represent the range of the values after normalization. Detailed information is showed in Table S2.

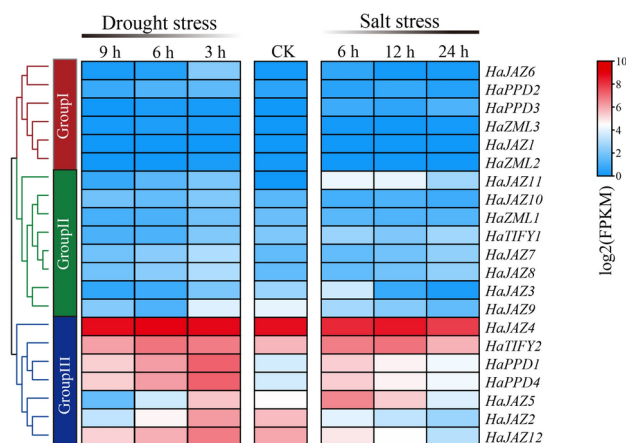


Fig. 6. Expression patterns of *HaTIFY* genes following 200 mM NaCl and 15% PEG6000 treatments at different times. The normalized $\log_2(\text{FPKM})$ was used to create a heatmap. The indicator ranges in the upper right corner represent the range of the values after normalization. Detailed information is showed in Table S3.

Expression patterns of *TIFY* genes under abiotic stresses

The transcriptome data was employed to further verify the expression of *HaTIFY* genes under drought (15% PEG6000) and salt (200 mM NaCl) treatments by extracted the total RNAs from sunflower leaves (Fig. 6). Among them, *HaJAZ2/4/5/12*, *HaPPD1/4*, and *HaTIFY2* were highly expressed in the control and different stress environments. Moreover, *HaJAZ5* and *HaPPD1/4* showed a similar up–down–down pattern under both drought and salt stresses, respectively. On the contrary, the expression values of *HaJAZ6/10/11*, *HaPPD2/3*, and *HaZML1/2/3* were close to or equal to 0. As expected, the homologous genes (such as *HaZML2/3* or *HaPPD1/4*) shared similar expression patterns under the same stress. In general, most of *HaTIFY* genes with a tendency to express in response to stimuli were from the JAZ V subfamily, which may reveal the potential roles of the JAZ V subfamily in dealing with drought or salt stress.

Based on transcriptome data, six *HaTIFY* genes (*HaJAZ2/5/12*, *HaPPD1/4* and *HaTIFY2*) were selected to further validate by qRT-PCR analysis. Overall, the expression levels of the same gene in different stress treatments and stages were found to be highly consistent with transcriptome data (Fig. 7). There were statistically significant upward or downward trends of their expression levels at the initial stage of stresses ($P < 0.05$). For example, *HaJAZ2/5*, *HaPPD1/4*, and *HaTIFY2* were significantly induced by drought at 3 h post treatments. Additionally, the expression levels of *HaJAZ5*, *HaPPD1/4*, and *HaTIFY2* were shown to be significantly up-regulated after 6 h of salt condition compared to the CK group, and then gradually decreased with the increased duration of salt stress.

The expression levels of *HaJAZ2* and *HaJAZ12* experienced a massive decline during the whole process of salt condition, but the opposite trends appeared in drought condition. Differing from the above two genes, a similar expression pattern with increasing at first and then decreasing was observed the expression levels of other four genes (*HaJAZ5*, *HaPPD1/4* and *HaTIFY2*) at different periods following the two types of stress. Interestingly, the extent of *HaPPD1* and *HaPPD4* upregulation is higher compared to other genes, indicating

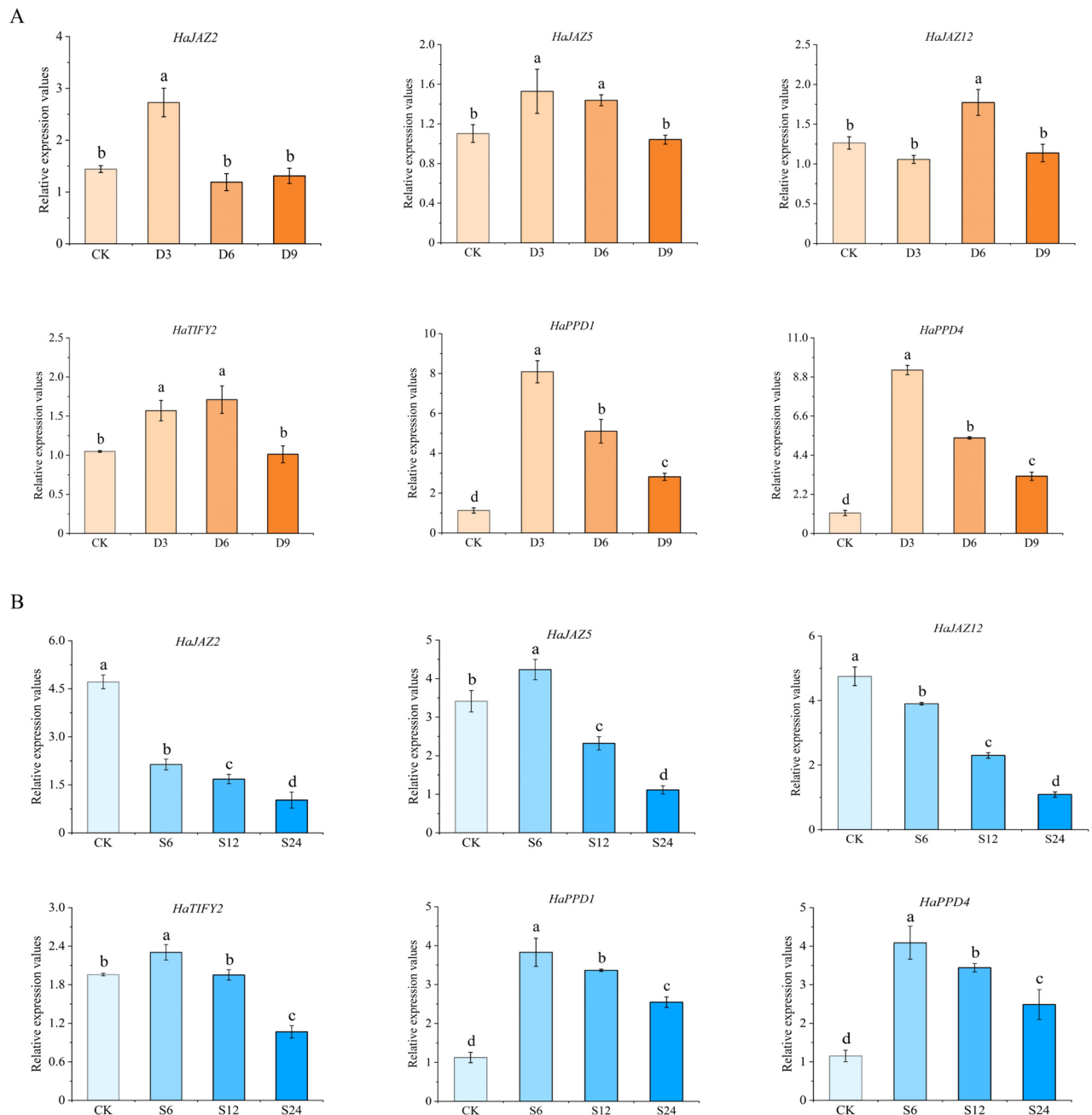


Fig. 7. qRT-PCR analysis of differences in *HaTIFYs* expression levels under drought (A) and salt (B) stresses. (A) CK, D3, D6 and D9 represent the expression levels of *HaTIFYs* at 0, 3, 6 and 9 h under drought stress condition in X-axis; (B) CK, S6, S12 and S24 represent in 0, 6, 12 and 24 h exposed to salt treatment. Statistically significant differences were analyzed by Duncan's multiple-range test. Different lowercase letters above the bars indicate a significant difference between these treatments ($p < 0.05$).

that they have potentially function roles in drought and high salinity tolerance. For instance, the peak expression levels of *HaPPD1* and *HaPPD4* were significantly upregulated under drought and salt treatments compared to CK. This phenomenon may be related to the presence of numerous drought induction elements in the promoter of *HaPPD1* and *HaPPD4* genes. In summary, these results provide useful clues for specific mechanism that *HaTIFY* genes involved in the response to several abiotic stresses tolerance, which is an intriguing issue to deserve further study.

Discussion

Numerous studies have documented that *TIFY* genes encode plant-specific transcription factors, which not only participate in the regulation of plant growth and development^{9,22}, but also play an important role in abiotic/biotic stress responses^{23–28}. With an increasing number of genome sequences being released, *TIFY* gene family

has been widely identified in oilseed crops, including *Brassica napus*¹⁵, *Zea mays*¹², *Juglans regia*²⁸ and *Glycine soja*¹⁷. Sunflower has been well known for its outstanding resistance to environmental stresses², while the functional characterizations of *TIFY* genes in sunflower have not been reported yet. Accordingly, we conducted an identification of the *TIFY* gene family in the sunflower genome and investigated their structure and expression levels with a focus on identifying sensitive members responsive to abiotic stresses.

In the present study, 21 *HaTIFY* genes unevenly distributed on 12 chromosomes were identified, and *HaTIFY* proteins were divided into four subfamilies, namely JAZ, PPD, ZML, and *TIFY* (Fig. 1), which were consistent with the classification methods used in *Juglans regia*²⁸, *Fagopyrum tataricum*¹³, and *Solanum lycopersicum*¹². In agreement with our findings, other published results showed that there is no PPD subfamily in monocotyledonous plants²⁹. It might be linked with the fact that neofunctionalization or loss of function driven by differences in selective constraints between monocots and dicots²⁹. Previous study has provided evidence that the PPD subfamily was formed from the merging of *TIFY* domain and PPD domain before vascular plant formation³⁰. It was worth noting that *HaTIFYs* within the same subfamily typically had similar conserved motif and exon–intron structure, as shown by gene structure and phylogenetic analysis (Fig. 1 and Fig. 2). Similar observations were reported in *Arabidopsis* and other plants, that a strong correlation existed between phylogenetics and exon–intron structure^{23,31}.

Furthermore, the genes from the same subfamily have similar gene structures based on the information contained in expression pattern and phylogenetic analysis (Fig. 1 and Fig. 7). For example, the genes located in the closest phylogenetic branches, such as *HaPPD1/4* and *HaJAZ2/12* with similar tendency under drought and salt stresses, respectively. Moreover, *HaJAZ11* and *HaJAZ6* were clustered, and these two genes both tended to be not or less expressed illustrates, presumably due to pseudogenization⁵. Among groups JAZ I–JAZ V, JAZ V subfamily had the maximum *HaJAZs* members, 8 *HaJAZs* were clustered, possibly leading to species-specific adaptation. It has been reported that *OsJAZ8* was overexpressed in leaves of seedlings of rice to eventually enhanced salt stress tolerance²¹. Meanwhile, *AtJAZ1* can directly interact with the negative regulator of ABA signaling to regulate the response to salt and drought treatments³². Considering this aspect, *OsJAZ8*, *AtJAZ1* and 8 *HaJAZ* proteins from one clade might share a similar biological function. As expected, the majority of JAZ V subfamily members (i.e., *HaJAZ2/4/5/9/12*) were highly expressed in floral tissues, including bract, corolla, ligule, ovary and style (Fig. 5), which indicates that their functions in flower development of sunflower are indispensable³³. Furthermore, our findings showed that the expression levels of *HaJAZ2/4/5/12* tended to be highly were observed under drought and salt stresses. This result suggests that the genes in JAZ V subfamily might play a more important role than other subfamilies in responding to abiotic stresses.

Tandem and segmental duplications are widely recognized as key mechanisms in plant evolution, gene family expansion, and functional differentiation, particularly in response to abiotic stress³⁴. In our study, three pairs of segmental duplication genes from the JAZ subfamily (*HaJAZ2/4*, *HaJAZ3/11*, and *HaPPD3/4*), as well as two tandem replication events (*HaJAZ12/ZML1* and *HaZML2/3*) (Fig. S3), revealing that these duplication events might contribute to the evolution and expansion of the *HaTIFY* gene family³¹. Researchers have showed that the functions and expression patterns of segmentally duplicated genes were similar³⁵. This viewpoint only partially supports by our observation that the expression level of *HaJAZ2* and *HaJAZ4*, which underwent segmental duplications, showed similar up-and-down expression patterns during drought stress (Fig. 6). This result suggested that *HaJAZ2* and *HaJAZ4* might have similar biological functions. However, the opposite trend was observed in kiwifruit²⁹ and sunflower⁵ that repeated the expression patterns of segmentally duplicated gene pairs were not completely consistent, resulting in neofunctionalization to regulating abiotic tolerance. Our result showed that *HaJAZ* genes were conserved in sequence but varied in expression among duplicated genes. Specifically, *HaJAZ11* tended to be not expressed in most of tissues, while *HaJAZ3* tended to be divergently expressed among tissues or stress treatments (Fig. 5 and Fig. 6). Meanwhile, despite the fact that *HaPPD3/4* was duplicated, the expression patterns of them varied in response to drought and salt stresses. Accordingly, we imply that duplicated *HaTIFY* genes may undergo neofunctionalization to breakdown functional redundancy in the adaptation to abiotic stresses. Furthermore, the inter-species collinearity analysis showed that there were more pairs of homologous genes in sunflowers and *Arabidopsis*, compared those existed between sunflowers and rice (Fig. S4). It is possible due to the difference between monocotyledonous and dicotyledonous plants³³. The evolutionary selection pressure analysis also showed that five duplicated paralogous gene pairs were strongly constrained by purification selection as *Ka/Ks* ratio was less than 1 (Table S1), which might have resulted in preserved functionality or pseudogenization^{12,33}.

It is important to mention that MYC2 as the direct target of JA receptor *COI1* genes, interacts with the C-terminal domain of JAZ protein and then induce a set of JA responsive genes after degradation of JAZ protein³⁶. Increasing evidence supports the argument that JAZ proteins through bind to bHLH TFs repressed the expression of the early JA-responsive genes^{36,37}. Our results obtained from the PPI analysis align with above findings, in which the multiple proteins in JAZ V subfamily interacting with *COI1* and MYC2 may play an important role in the JA pathway (Fig. 4). Furthermore, with the increase in time of salinity stress, the expression level of *HaJAZ2/12* continuous significant declined (Fig. 7), which can be clearly demonstrated the fact that the JAZ gene as a “repressor” response to environmental stresses in sunflower⁵. Subsequently, the expression patterns of six differentially expressed genes in the continuous drought or salt environment were quantified by qRT-PCR (Fig. 7). Noteworthy, *HaJAZ5*, *HaPPD1/4* and *HaTIFY2* exhibited the similar expression patterns that initially increased and then declined, whether exposed to drought or salt treatment. Interestingly, *HaPPD1* and *HaPPD4* were significantly upregulated at the early stages of both drought and salt treatments, highlighting their potential role in enhancing sunflower resistance to abiotic stresses.

In summary, 21 *HaTIFY* genes were identified in sunflower and *HaTIFY* proteins were divided into four subfamilies, namely JAZ, PPD, ZML, and *TIFY*. The proteins from the same subfamily were similar in conserved motifs and domains. Segmental and tandem duplication events contributed to the expansion of *HaTIFY* gene

family. Phylogenetic analysis and the synteny comparisons between sunflower and other plant species (*Arabidopsis thaliana* and *Oryza sativa*) uncovered the vital evolution information of *TIFY* gene family. Furthermore, more than half of 21 *HaTIFY* genes contained the drought induction elements. Subsequently, RNA-seq and qRT-PCR analysis showed that most members of PPD and JAZ V groups had relatively high expression levels. *HaPPD1* and *HaPPD4* tend to be sensitive to drought and salt stresses. In conclusion, the *HaTIFY* gene family plays a crucial role in the positive regulation of sunflower's response to abiotic stresses, offering key candidate genes for enhancing resistance in sunflower breeding programs.

Materials and methods

Identification and analysis of *HaTIFY* gene family

To identify the members of *TIFY* gene family in sunflower genome, the whole-genome, protein sequence, and annotation files of sunflower genome were downloaded from the Sunflower Genome Sequence database (<http://www.sunflowergenome.org>). The Hidden Markov Model (HMM) profile of the conserved functional domain of TIFY (PF06200) was obtained from the Pfam Database v34.0 (<http://pfam.xfam.org/>). Firstly, the HMM profile was used to screen the sunflower proteomes to obtain the all potential TIFY genes in sunflower using the *hmmsearch* software in the HMMER package v3.0²⁹. After removing redundant and incomplete sequences ($E\text{-value} < 1e^{-5}$), the conserved domain architectures of the acquired sequences were further validated using the online SMART tool (<http://smart.embl-heidelberg.de/>). The amino acid sequences containing the conservative TIFY domain were identified as the candidate TIFY members, while proteins without the typical functional domain of TIFY were manually excluded³¹.

Subsequently, the properties of the TIFY proteins, including theoretical isoelectric point (pI), molecular weight (Mw), and grand average of hydropathy (GRAVY), were analyzed using the ExPASy server (<https://web.expasy.org/protparam/>). The predictive analysis of the subcellular localization of sunflower TIFY protein was predicted by the ProtComp v9.0 tool (<http://www.softberry.com/>)¹⁵. The secondary structures and tertiary structures were performed via SOPMA web-server (<https://npsa.lyon.inserm.fr/>) and SWISS-MODEL server (<https://swissmodel.expasy.org/>), respectively. Next, the STRING web-server (<https://cn.string-db.org/>) and Cytoscape_v3.10.1 were used for protein–protein interaction network analysis.

Phylogenetic analysis, motif recognition, and promoter cis-elements

We compared 59 TIFY proteins including 18 protein sequences from *Arabidopsis thaliana*, 20 protein sequences from *Oryza sativa* and 21 protein sequences from *Helianthus annuus*. A rootless phylogenetic tree was constructed using MEGA 7.0 software with the neighbor-joining (NJ) method and setting bootstrap analysis with 1000 bootstrap replicates³⁸. TIFY protein information in *Arabidopsis thaliana* (TAIR, <https://www.arabidopsis.org/>) and *Oryza sativa* (TIGR, <http://rice.plantbiology.msu.edu/>) was available at a publicly-accessible website, respectively⁴.

To further understand the structural diversity of *TIFY* genes in sunflower, the Gene Structure Display Server 2.0 (GSDS 2.0) (<http://gsds.cbi.pku.edu.cn/>) was used to determine their intron–exon organizations. The conserved motifs of full-length TIFY proteins were identified by MEME Suite v5.4.1 (<https://meme-suite.org/meme/>), using the default parameters and set the conserved motif number to 6. The promoter sequences of *HaTIFY* genes were obtained in Phytozome v13 database (<https://phytozome-next.jgi.doe.gov/pz/portal.html>). Further, 2000 bp upstream of CDS was extracted each *HaTIFY* promoter. The *cis*-acting regulatory elements of the plant promoter sequence were predicted through PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and visualized via TBtools²⁶.

Chromosomal distribution, gene duplication, and synteny analysis

The chromosomal location and potential tandem duplications of the *HaTIFY* genes were analyzed using TBtools³⁹. The entire sunflower genome sequence was retrieved from the Genome Database for Sunflower and all the predicted *TIFY* genes were mapped onto the corresponding chromosomes to visualize their distribution patterns. Genes were considered to be duplicated when there was more than 85% identity at their nucleotide sequences, which were manually marked on the chromosomal location¹². To analyze the duplication events of the *TIFY* genes, we used MCScanX in TBtools. The selection pressure of the duplicated pairs and dividing of homologous *TIFY* genes was estimated based on the synonymous (Ks) and non-synonymous (Ka) rate among the gene pairs using the KaKs_Calculator 2.0 software⁴⁰.

Expression profiles of *HaTIFY* genes in sunflower tissues and organs

The transcriptome data of sunflower *TIFY* genes in different tissues and organs were obtained from the Sunflower Genome Database (<https://www.sunflowergenome.org/>). The expression data of 10 tissues and organs were selected, including bract, corolla, leaf, ligule, ovary, pollen, seed, stamen, stem, and style. All the relative expression levels of *HaTIFY* genes were normalized as FPKM values (Fragments Per Kilobase of transcript per Million mapped reads). Finally, the heatmaps of the expression patterns of *HaTIFY*s were created by TBtools based on the normalized $\log_2(\text{FPKM})$ ⁴⁰.

Plant materials, stress treatments and RNA-seq

The sunflower material used in this study was local cultivated variety (NWS-567) in Taiyuan, Shanxi Province, China. This material was identified in 2018 by provincial agricultural regulatory department in accordance with relevant institutional guidelines and regulations. The plants were grown in the mixed substrate of fertile soil and vermiculite (1:1 v/v) in plastic pots (170 × 220 mm) with in a 16 h light/8 h dark greenhouse located in Taiyuan University (Taiyuan, Shaanxi province, China) under a condition of 28 °C and the relative humidity of 65%. Four-leaf-old plantlets were treated with 200 mM NaCl (sampled at 0, 6, 12 and 24 h) and 15% polyethylene

glycol (PEG) 6000 (collected at 0, 3, 6 and 9 h). Three biological replicates were used for each sample. Afterwards, the sunflower leaves samples at different durations of stimuli were quick-frozen in liquid nitrogen and stored at -80°C for RNA extraction and RNA-seq.

Total RNA was extracted using OmniPlant RNA Kit (DNase I) (CW2598S, Kangwei Century Biotechnology Co., Ltd, Beijing, China). Sequencing was performed at the illumina Novseq Xplus (Illumina Biotech Co, Ltd., San Diego, CA, USA). Trim the original paired end readings and quality control were conducted using fastp v0.23.4 software. Then, the clean reads were localized to reference genome with orientation mode using HISAT2 v2.2.1 software⁵. The mapping results were subjected to String Tie to assemble transcripts in each sample²⁵. The heatmap was generated using the log2 (FPKM) through TBtools.

Quantitative real-time PCR (qRT-PCR) analysis

Select genes with high expression levels and significant changes for qRT-PCR analysis. First-strand cDNA synthesis was performed using the PrimeScript[™] RT Reagent Kit with gDNA Eraser (Wuhan Servicebio Technology Co, Ltd.). The primer sequences listed in Table S4. qRT-PCR was conducted using Universal Blue SYBR Green qPCR Master Mix, and the parameters were as follows: pre-denaturation at 95°C for 30 s, 1 cycle; annealing at 95°C for 15 s, 60°C extension for 30 s, 40 cycles. The *ACT2* gene was used as reference⁴¹. Three biological and technical repetitions were conducted for each sample. The relative expression was calculated by $2^{-\Delta\Delta\text{Ct}}$. The SPSS 12.0 was used to analyze the statistical significance.

Data availability

The datasets of this article are included in the current article and its supplementary files, and they can be available from the corresponding author on reasonable request. The Sunflower Genome Sequence database (<http://www.sunflowergenome.org/>). The Pfam database (<http://pfam.xfam.org/>). The SMART online tool (<http://smart.embl-heidelberg.de/>). The ExPASy server (<https://web.expasy.org/protparam/>). The ProtComp v9.0 tool (<http://www.softberry.com>). The SOPMA web-server (<https://npsa.lyon.inserm.fr/>). The SWISS-MODEL server (<https://swissmodel.expasy.org/>). The *Arabidopsis thaliana* Information Resource (TAIR) database (<https://www.arabidopsis.org/>). The *Oryza sativa* Information Resource (TIGR) database (<http://rice.plantbiology.msu.edu/>). The GSDS 2.0 (<http://gsds.cbi.pku.edu.cn/>). The MEME Suite v5.4.1 (<https://meme-suite.org/meme/>). The Phytozome v13 database (<https://phytozome-next.jgi.doe.gov/pz/portal.html>). The PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

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Author contributions

Methodology, Y.Z. and J.Z.; Project administration, X.Z.; Software, Y.J. and J.S.; Validation, L.L.; Writing—original draft, K.Z.; Writing—review & editing, Y.Z. and J. G. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to X.Z.

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