

GENOME-WIDE IDENTIFICATION AND EXPRESSION PATTERN ANALYSIS OF *DHN* FAMILY GENES IN MAIZE

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Abstract

Plant dehydrin (DHN) is a hydrophilic protein widely present in organisms, and it is accumulated when plants encounter adverse environments. Based on the sequence information of *DHN* family genes in *Arabidopsis thaliana*, 13 *DHN* genes were found in maize using the homology sequence alignment method, in which five *DHN* genes were located on chromosome 8. The maize *DHN* gene family can be classified into five branches by evolutionary analysis. The DHN proteins contain five subclasses which are K_n, SK_n, K_nS, Y_nSK_n and FSK_n. Analysis of cis-acting elements showed that the promoters of *DHN* gene family harbor cis-acting elements mainly responsive to stress and plant hormones. Tissue expression patterns revealed that almost all *ZmDHN* genes are expressed in the embryo and aleurone layer. DHN proteins were mainly distributed in the nucleus and cytoplasm by subcellular localization prediction. The present findings allow insight into understanding maize *DHN* family genes' functions and facilitate the molecular breeding of *ZmDHNs* in coping with abiotic stress.

Key words: Maize, *DHN* gene family, Phylogenetic analysis, Expression patterns.

Introduction

As one of the world's three major grain crops, maize (*Zea mays* L.) was ranked first in the grain yield worldwide. Except as food and feed, maize could be processed into industrial products such as adhesives, industrial alcohol and fuel ethanol, which made it one of the most significant crops in the world (Jiao *et al.*, 2022). A range of environmental factors influence the yield and quality of maize, so mining and identifying stress-responsive genes are urgent and effective for molecular breeding to cope with increasingly severe environmental challenges. Late embryogenesis abundant proteins (LEA proteins) are vitally involved in plant stress which act as protective agents against cell dehydration. The LEA proteins keep the cells glassy and prevent excessive seed dehydration mainly by binding to intracellular sugars (Jia *et al.*, 2023). Secondly, LEA proteins can buffer cell ion concentration, regulate the osmotic pressure inside and outside cells, thus stabilize protein structure, and prevent other proteins and cell membranes from aggregating during drought and low-temperature stress (Magwanga *et al.*, 2018). LEA proteins are divided into seven subfamilies, and DHN proteins belong to Group 2 LEA family members (Wang *et al.*, 2021). They are also known as LEA II proteins and abscisic acid-responsive (RAB) proteins or water stress proteins (WSP) (Szlachtowska & Rurek, 2023). DHN proteins are hydrophilic proteins widely present in plants. Generally, they consist of 82-575 amino acids, and the molecular weight ranges from 9-200 kD. The primary structure of DHN proteins includes three highly conserved domains: K, S and Y segments. The K segment, an essential structure of DHN proteins, is capable of forming amphipathic α helices by binding to phospholipid vesicles, thereby maintaining the stability of cell membranes. The K segment is located at the C-terminus with abundant lysine (Jia *et al.*, 2022). The Y segment contains conserved sequences rich in tyrosine at the N-terminus. The S segment, mainly composed of serine, functions through phosphorylation by kinase. Besides, DHN proteins contain a poorly conserved ϕ segment with abundant glycine and polar amino acids. Based on the presence and distribution of K, S and Y fragments,

DHNs can be classified into five types: K_n, SK_n, K_nS, Y_nK_n, and Y_nSK_n. These types of structures vary among different plants (Chen *et al.*, 2023).

DHN was reported to protect cells from low-temperature and dehydration damage by preserving enzyme activity, reducing the phase transition temperature of the membrane, removing free radicals and reactive oxygen species from cells, and binding heavy metal ions (Shi *et al.*, 2020). During oxidative stress, the SbDHN1 protein, a YSK₂ dehydrating protein in *Sorghum bicolor*, protected transgenic tobacco from oxidative damage by directly eliminating free radicals and protecting reactive oxygen species scavenging enzymes. As a result, the plants reduced the accumulation of H₂O₂ significantly, increased proline content and other antioxidant levels (Halder *et al.*, 2018). The *AtHIRD11* gene, a KS-type *DHN* in *Arabidopsis thaliana*, bound to free Cu²⁺ and alleviated the physiological damage caused by heavy metal stress, and then restored the activity of lactate dehydrogenase that had been denatured by Cu²⁺ (Hara *et al.*, 2016). The KS-type DHN protein GmERD14 accumulated significantly under low temperature in soybeans (*Glycine max*) (Guo *et al.*, 2023). When barley (*Hordeum vulgare*) was subjected to low temperature and freezing, the expression level of DHN13, a KS-type DHN protein, increased significantly (Rodríguez *et al.*, 2005). When *Brassica napus* was subjected to cold stress, the content of three types of DHN proteins, which were SK_n (BnLEA10 and BnLEA18), Y_nK_n (BnLEA90), and Y_nSK_n (BnLEA104), increased significantly in cold tolerant varieties (Maryan *et al.*, 2019). Moreover, the homologous or heterologous expression of *DHN* genes could also improve the plant's ability to resist abiotic stress. The *AtLTI30* gene in *A. thaliana* positively regulated catalase activity and endogenous proline content, thereby reduced H₂O₂ accumulation caused by drought stress and improved the drought tolerance in transgenic *A. thaliana* (Shi *et al.*, 2015). The overexpression of *ZmDHN13* in tobacco, induced by H₂O₂, resulted in a reduction in the generation of free radicals under oxidative stress, thereby enhanced the oxidative tolerance of the

transgenic tobacco (Liu *et al.*, 2017). Overexpression of the soybean *GmDHN9* enhanced drought tolerance in *A. thaliana* by keeping the homeostasis of ROS (Fan *et al.*, 2024). Under drought stress, overexpression of the dehydrin gene *MtCAS31* in *Medicago truncatula* promoted autophagic degradation of the plasma membrane intrinsic protein 2;7 (PIP2;7), lessened root hydraulic conductivity and thereby decreased water loss and enhanced drought resistance (Li *et al.*, 2020). By knocking out two *DHN* genes in cotton, *GhDHN_03* and *GhDHN_04*, the antioxidant pathways were affected, resulted in a significantly increase in H₂O₂ and MDA content. Besides, the expression of stress response genes *GhLEA2*, *CDKF4* (cyclin-dependent kinase F-4), and *GPCR* (G protein-coupled receptor) were also considerably downregulated. As a result, the plant's tolerance to salt stress is significantly reduced, indicated that the *DHN* genes *GhDHN_03* and *GhDHN_04* were capable of enhancing the cotton's tolerance to salt stress (Kirungu *et al.*, 2020).

Maize is widely utilized in genetics and evolutionary research. Although some *DHN* genes have been identified in maize, a systematic analysis of *DHN* gene family at the whole genome level has not yet been conducted with the improvement and integration of genome databases. In this study, we used *AtDHN* family genes as a reference to identify 13 members of the maize *DHN* gene family in the Zm-B73-REFERENCE-NAM-5.0 genome. The physico-chemical properties, functional domains, phylogenetic relationships of homologous genes, chromosomal localization, promoter cis-acting elements (CREs), collinearity analysis, expression patterns and other aspects of *ZmDHNs* were studied. Our findings provided a foundation for function research in the maize *DHN* gene family.

Material and Methods

Identification of *DHN* genes in maize: The maize Zm-B73-REFERENCE-NAM-5.0 genome, CDS, protein sequence, and annotation files were acquired from the maizeGDB database (<https://www.maizegdb.org>), and the *A. thaliana* genome sequences and protein sequences from TAIR10 (<https://www.arabidopsis.org/>) database. Blast and Hmmer searches were performed by screening in maizeGDB and UniProt and using the *A. thaliana* *DHN* family protein as a reference (parameters evaluated <1e-5) in the maize protein sequence. After taking the intersection and removing duplicate genes, the selected maize *DHN* gene family members were analyzed using the reference genome's T001 transcript as a reference.

Analysis of physicochemical properties of *ZmDHN* family proteins: The basic protein characteristics of *ZmDHN* family members, such as the number of amino acids, molecular weight, theoretical isoelectric point, etc., were analyzed by using TBtools software (Chen *et al.*, 2020). The lengths of the coding region, exons/introns, were obtained by searching in the Ensembl database (<https://plants.ensembl.org/>). Subcellular localization was predicted by using Cell-PLoc online software (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>).

Prediction of secondary structure and subclass of *ZmDHN* family protein: The secondary structure of *ZmDHN* proteins was analyzed using SOPMA software online (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html). The subclass structure was classified according to the sequence features of K, Y (or F), and S domains based on the complete amino acid sequences.

Phylogenetic analysis of *ZmDHN* family proteins: Using ClustalX software, the *ZmDHN* protein sequences were aligned with the *DHN* protein sequences of *A. thaliana*, *Triticum aestivum*, and *Oryza sativa*. Next, the evolutionary tree of the four species was constructed using the Neighbor-Joining method of MEGAX (parameters: Bootstrap, 1000 iterations), and finally, the evolutionary tree was beautified using iTOL (<https://itol.embl.de/>).

Conserved motif, domain, and gene structure analysis of *ZmDHNs*: The *ZmDHN* protein sequences were aligned by using ClustalX software, and then an evolutionary tree was constructed using MEGAX's Neighbor-Joining method (Bootstrap 1000). The protein sequences of *ZmDHN* were submitted to the MEME Suite website to obtain information on conserved motifs. The *ZmDHN* protein sequences were submitted to the NCBI CDD website (<https://www.ncbi.nlm.nih.gov/Structure/cdd>) to obtain prediction information on protein domains. The gene structure information for the *ZmDHN* gene family was extracted from the gene annotation file downloaded from maizeGDB, and the data was visually analyzed using TBtools software.

Chromosome distribution and synteny analysis in the *ZmDHN* gene family: The *ZmDHN* genes' chromosomal location information was obtained from the TBtools software. The interspecies collinearity analysis of *DHN* gene families among maize, *A. thaliana*, rice, and within maize was performed using the TBtools software.

Cis-acting elements analysis in promoter region: Using TBtools software, the DNA sequence of the first 2000 bp of the initiation codon (ATG) of *DHN* gene family members was extracted. The cis-elements were predicted using PlantCare (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), and visualized (Lescot *et al.*, 2002).

Expression profiling of *ZmDHNs*: Based on the published transcriptome data (Walley *et al.*, 2016), the expression patterns were screened of the *ZmDHN* genes for 23 tissues and organs in different developmental stages. The fragments per kilobase of transcript per million mapped (FPKM) values were transformed and used to draw a heat map by TBtools. If the FPKM showed "NA" (not available), it was replaced by FPKM = 0, and all FPKM values were transformed into log₂ (FPKM+1) to make the heatmap.

Results and Analysis

Characterization of *DHN* genes in maize: By running Blast and Hmmer searches against the protein sequences of

10 *DHN* family genes in *A. thaliana*, a total of 13 *ZmDHN* genes were identified and named *ZmDHN1-ZmDHN7*, *ZmDHN13-ZmDHN18* in maize (Table 1).

Tables 1 and 2 show that the *DHN* gene family has 1-6 exons. The different number of exons in each phylogenetic group may be caused by alternative splicing, leading to structural differences in different genes. The length of the proteins encoded by the *DHN* gene family in maize ranges from 100 to 326 amino acids, with an isoelectric point between 5.51 and 9.52 and a protein molecular weight of 10423.37-31770.4 Da. Most are hydrophilic and stable, but *ZmDHN3* and *ZmDHN15* are less stable. The *DHN* gene family in maize is unevenly distributed on 7 of its 10 chromosomes, with most (five *DHN* genes) on chromosome 8. Chromosomes 3 and 5 have two genes each, and the remaining four chromosomes have one gene. This indicates the diversity of physicochemical properties of the proteins encoded by this family. Subcellular localization prediction found that 7 of the 13 *DHN* proteins have nuclear localization signals, and six have cytoplasmic localization signals. *ZmDHN6* and *ZmDHN7* proteins are also localized in chloroplasts, but *ZmDHN6* may also be localized in the cell membrane. *ZmDHN6* and *ZmDHN7* proteins have three transmembrane domains (Tables 1 and 2).

Secondary structure and subclass analysis of *ZmDHN* family proteins: Previous research has found that the K-segment rich in lysine, is consisted of 15 amino acids

(conserved motif: XKXGXX(D/E)KIK(D/E)KXPG) in all dehydrin proteins, located at the C-terminus, and contained highly conserved charged and nonpolar residues. The Y-segment, rich in T/VDEYGNP motifs and located at the N-terminus, is rich in tyrosine and typically has one to three closely spaced copies. The S-segment is a tandem repeat sequence of 3-13 serine residues (with a conserved sequence of SSSSSSED), often followed by a residue of D, E, or K (Chen *et al.*, 2023). Many SKn-type dehydrins possess a conserved sequence known as the F-segment, located at the N-terminus, which can form a short amphipathic helix and may have membrane or protein binding properties similar to the K-segment. They also typically contain a conserved motif of D(D/E/Q) (Y/H/F)GNP (Strimbeck G.R. 2017). In light of this, we observed that *ZmDHN*s encompass all types except for the YnKn-type motif, including Kn (*ZmDHN16* and *ZmDHN17*), SK (*ZmDHN6* and *ZmDHN7*), K2S (*ZmDHN13* and *ZmDHN14*), YnSKn (*ZmDHN2*, *ZmDHN5*, and *ZmDHN4*, *ZmDHN1*, and *ZmDHN18*), and FSKn (*ZmDHN3* and *ZmDHN15*). This is consistent with the phylogenetic relationships of *ZmDHN* proteins. Although the function of dehydrins is still to be determined, each structural type of *DHN* may be associated with specific functions. The secondary structure analysis displayed α helices, and irregular coils comprised the highest proportion: 11 *ZmDHN* proteins were irregular coils, while the *ZmDHN6* and *ZmDHN7* displayed α helices (Table 3).

Table 1. The sequence information and physicochemical properties of *ZmDHN*s.

Gene ID	Gene name	Location	Number of amino acid	CDS length	Molecular weight
Zm00001eb285360	<i>ZmDHN1</i>	chr6:148134643-148135795	168	504	17075.48
Zm00001eb376710	<i>ZmDHN2</i>	chr9:20829686-20831292	326	978	31690.09
Zm00001eb187010	<i>ZmDHN3</i>	chr4:159677441-159678859	289	867	31466.47
Zm00001eb348040	<i>ZmDHN5</i>	chr8:99203907-99204981	323	969	31588.08
Zm00001eb153940	<i>ZmDHN6</i>	chr3:205353689-205358204	257	771	28656.46
Zm00001eb371250	<i>ZmDHN7</i>	chr8:181883182-181920704	253	759	28296.99
Zm00001eb052790	<i>ZmDHN13</i>	chr1:265841055-265842318	100	300	11312.28
Zm00001eb218350	<i>ZmDHN14</i>	chr5:16294978-16295737	108	324	12199.09
Zm00001eb250120	<i>ZmDHN15</i>	chr5:198232923-198234455	290	870	31440.76
Zm00001eb348010	<i>ZmDHN16</i>	chr8:99135743-99136383	180	540	19058.54
Zm00001eb348190	<i>ZmDHN4</i>	chr8:99899524-99900604	325	975	31770.4
Zm00001eb347970	<i>ZmDHN17</i>	chr8:99044661-99044986	102	306	10423.37
Zm00001eb155620	<i>ZmDHN18</i>	chr3:210644563-210645769	236	708	24810.07

Table 2. The sequence information and physicochemical properties of *ZmDHN*s.

Gene name	Exon/intron	Theoretical isoelectric point	Instability index	Aliphatic index	Hydrophilic index	Subcellular localization
<i>ZmDHN1</i>	2/1	8.78	24.26	31.43	-1.144	Cytoplasm
<i>ZmDHN2</i>	2/1	7.37	24.17	36.04	-0.798	Cytoplasm
<i>ZmDHN3</i>	2/1	5.51	57.06	54.78	-1.3	Nucleus
<i>ZmDHN5</i>	2/1	7.38	24.08	39.38	-0.766	Cytoplasm. Nucleus
<i>ZmDHN6</i>	5/4	8.16	42.78	102.45	0.053	Cell membrane. Chloroplast
<i>ZmDHN7</i>	6/5	8.45	42.39	100.2	0.031	Chloroplast. Mitochondrion. Nucleus
<i>ZmDHN13</i>	2/1	6.59	49.45	31.2	-2.075	Nucleus
<i>ZmDHN14</i>	1/0	6.22	29.71	31.57	-2.158	Nucleus
<i>ZmDHN15</i>	2/1	6.05	54.2	55.93	-1.25	Nucleus
<i>ZmDHN16</i>	1/0	9.52	41.08	55.94	-0.711	Nucleus
<i>ZmDHN4</i>	2/1	8.85	22.61	41.23	-0.735	Cytoplasm
<i>ZmDHN17</i>	1/0	7.99	33.41	27.84	-1.138	Cytoplasm
<i>ZmDHN18</i>	2/1	5.99	44.47	44.75	-0.893	Cytoplasm

Table 3. Secondary structure and subclass of ZmDHN family proteins.

Gene name	Subclass	α helix (%)	Extended strand (%)	β -turn (%)	Random coil (%)
<i>ZmDHN1</i>	YSK ₂	12.5	14.29	17.86	55.36
<i>ZmDHN2</i>	YSK ₃	13.8	13.8	11.96	60.43
<i>ZmDHN3</i>	FSK ₂	41.52	6.92	4.5	47.06
<i>ZmDHN5</i>	YSK ₃	13.62	14.55	12.07	59.75
<i>ZmDHN6</i>	SK	66.15	8.17	2.72	22.96
<i>ZmDHN7</i>	SK	65.22	6.32	3.56	24.9
<i>ZmDHN13</i>	K ₂ S	36	4	6	54
<i>ZmDHN14</i>	K ₂ S	34.26	3.7	3.7	58.33
<i>ZmDHN15</i>	FSK ₃	43.79	3.1	5.17	47.93
<i>ZmDHN16</i>	K ₃	21.67	17.22	10	51.11
<i>ZmDHN4</i>	YSK ₃	13.85	15.69	16.62	53.85
<i>ZmDHN17</i>	K ₂	13.73	15.69	12.75	57.84
<i>ZmDHN18</i>	Y ₂ SK ₂	19.49	7.2	8.47	64.83

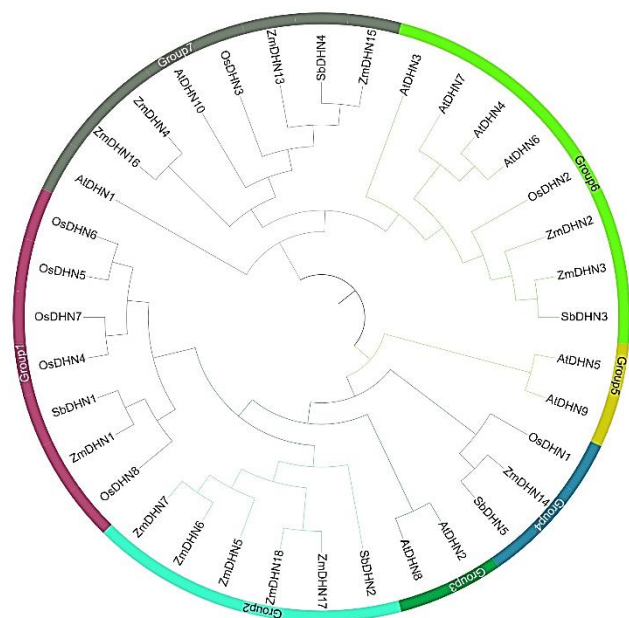


Fig. 1. Phylogenetic tree of DHNs in *Z. mays*, *A. thaliana*, *O. sativa*, and *S. bicolor*.

Phylogenetic analysis of ZmDHN family proteins: To gain a deeper understanding of the evolutionary patterns of the *DHN* gene across various species, we constructed a phylogenetic tree (Fig. 1) using the protein sequence data of the *DHN* family genes from *Zea mays*, *Arabidopsis thaliana*, *Oryza sativa*, and *Sorghum bicolor*. The phylogenetic tree divides the *DHN* family genes from different plants into seven groups. Closer branching of two genes indicates higher homology between them. Groups 3 and 5 contain only two *A. thaliana* *DHN* proteins, while no *DHN* proteins are found in the other three species. Group 2 includes five maize *DHN* genes and one *Sorghum bicolor* *DHN2* gene. In groups 1, 4, 6, and 7, maize and sorghum, as monocotyledonous plants, have the closest homology in their *DHN* proteins. This suggests that the domains of the *DHN* gene remain stable and conserved during plant evolution.

Conserved motifs, domain, and gene structure analysis of ZmDHNs: The conservative motifs,

domains, and gene structures of the amino acid sequences encoded by the corn *DHN* gene family were analyzed by using TBtools (Fig. 2). Among all ZmDHNs, a lysine-rich K segment, comprising 15 amino acids (EKKGIMDKIKEKLP), clusters at the C-terminus and contains highly conserved charged and nonpolar amino acid residues. The Y segment (T/VDEYGNP), located at the N-terminus, is rich in tyrosine and has one to three closely spaced copies. The S segment comprises a tandem sequence of 3-13 serine residues. Within the corn *DHN* gene family, the conserved motifs predicted within the same developmental group exhibit homogeneity. For example, *ZmDHN2*, *ZmDHN4*, and *ZmDHN5* contain eight conserved motifs. The conserved motifs in other developmental groups are consistent or similar. The domains of most *DHN* family genes comprise *DHN* proteins, but *ZmDHN6* and *ZmDHN7* each contain a reticulon domain, consistent with transmembrane three times and membrane or chloroplast localizations. The composition of introns and exons within genes is an essential basis for determining gene function. Having the same or a similar number of exons in the same developmental group indicates that the amino acid residue evolution of the *DHN* gene family is relatively conserved.

Chromosomal distributions and synteny analysis in *DHN* gene family: The gene density (per 1000 kb) is calculated using the starting and ending positions of genes in the gene annotation files. The maize *DHN* gene family is distributed on seven of the ten chromosomes, with five genes on chromosome 8. Except for chromosomes 2, 7, and 10, other chromosomes contain 1-2 genes (Fig. 3). During plant evolution, duplication events play a significant role in amplifying gene family members (Zhou *et al.*, 2022). If the spacing between two homologous genes is less than five genes, it is referred to as tandem duplication, and if the spacing is greater than five genes, segmental duplication. We observed that *ZmDHN17* is separated by only three genes (*Zm00001eb347980*, *Zm00001eb347990*, and *Zm00001eb348000*) from *ZmDHN16*, and *ZmDHN16* is separated by only two genes (*Zm00001eb348020* and *Zm00001eb348030*) from *ZmDHN5*, indicating that *ZmDHN17*, *ZmDHN16*, and *ZmDHN5* belong to tandemly duplicated genes.

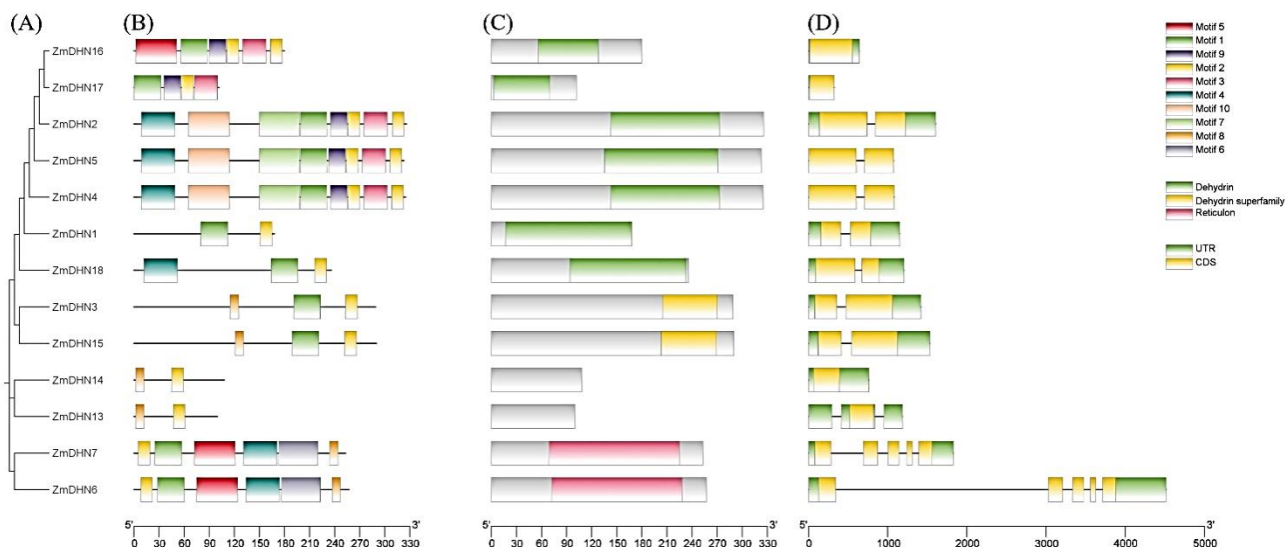


Fig. 2. The phylogenetics (A), conserved motifs (B), domains (C), and gene structure (D) of the *ZmDHN* gene family.

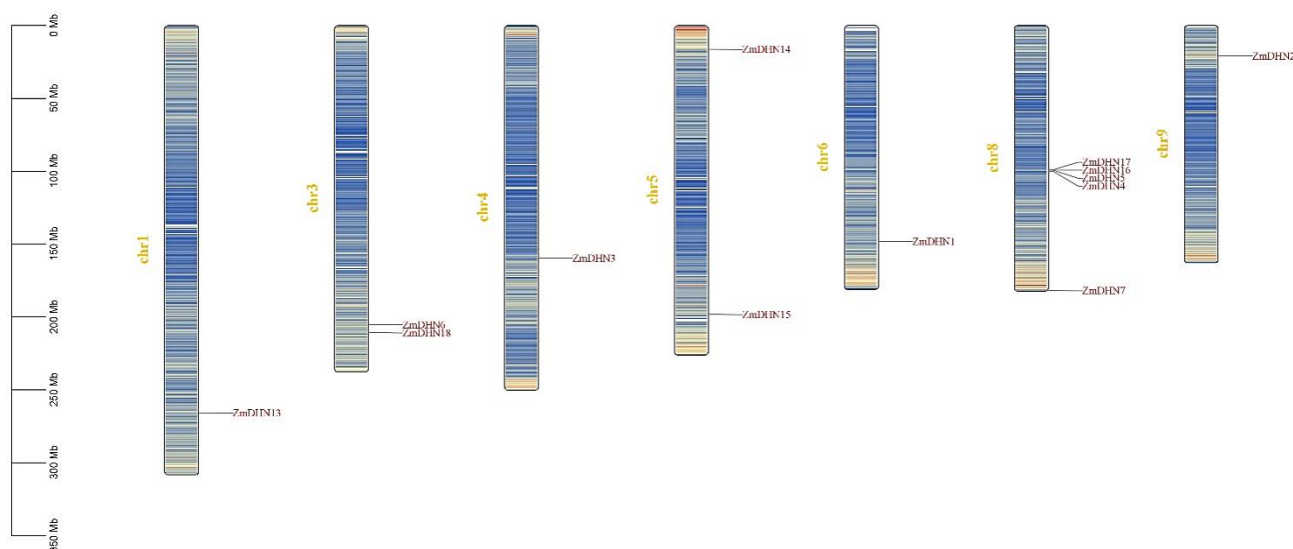


Fig. 3. The distribution of the *DHN* gene family on maize chromosomes.

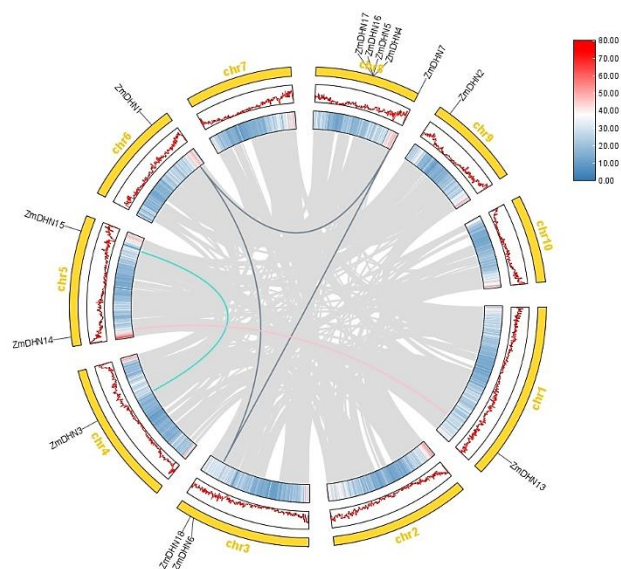


Fig. 4. Synteny analysis in *DHN* gene family of maize.

The collinearity of 13 genes within the *DHN* gene family was analyzed. The lines in the innermost loop in Fig. 4 represent the genes' collinearity, showing five segmental duplications. The first loop represents a heatmap of chromosome gene density, and the second is a line graph of chromosome gene density. The outermost loop displays the chromosome markers (Fig. 4). Through collinearity analysis of the *DHN* gene families in maize, rice, sorghum and *Arabidopsis*, no maize collinear *DHN* genes were detected in *Arabidopsis*, while eight pairs were detected in rice and six pairs in sorghum. This suggests that the *DHN* family is highly conserved during the evolution of Poaceae crops (Fig. 5).

Cis-acting element analysis in promoter region: Analysis of CREs within promoters provides a foundation for the functional study of genes. Previous studies indicated a certain correlation between the CREs and the specific response of genes (Abdullah *et al.*, 2018). Within the promoter regions of the *DHN* gene family, multiple CREs relating to stress, plant

hormones, and growth and development regulation were identified (Fig. 6). Elements responsive to stress include drought-responsive elements (DREs), light-responsive elements, anaerobic induction elements, low-temperature responsive elements (LTRs), and stress-responsive elements. The *DHN* genes may be involved in various stress responses. Among the promoter elements of the maize *DHN* gene family, light-responsive elements are the most numerous and are distributed among all the family members, with an average of 13.2 per gene. Elements responsive to plant hormones include methyl jasmonate (MeJA), gibberellic acid (GA), abscisic acid (ABA), salicylic acid (SA), and auxin (IAA). Some maize *DHN* genes also possess regulatory elements involved in plant growth and development, including seed-specific regulation, meristem expression, endosperm expression, and other elements.

Expression profiling of *ZmDHNs*: To investigate the functions of *ZmDHNs*, we analyzed the expression patterns of 13 *ZmDHNs* across 23 different tissues and organs using

publicly available transcriptomic data (Walley *et al.*, 2016). Based on the gene expression levels within the family, the 13 *ZmDHNs* can be divided into two groups. The first group consists of seven members (*ZmDHN2*, *ZmDHN18*, *ZmDHN17*, *ZmDHN16*, *ZmDHN4*, *ZmDHN5*, and *ZmDHN14*) that exhibit low expression levels in most tissues. The second group has relatively higher expression levels and includes *ZmDHN13*, *ZmDHN3*, *ZmDHN6*, *ZmDHN7*, *ZmDHN15*, and *ZmDHN1*. However, *ZmDHN14* is highly expressed only in maize B73 mature pollen, while four closely related genes (*ZmDHN4*, *ZmDHN5*, *ZmDHN16*, and *ZmDHN17*) are not expressed or exhibit very low expression levels across all stages of tissue. Interestingly, almost all *ZmDHNs* are expressed in the embryo (Embryo) and the pericarp/aleurone layer (Pericarp/Aleurone). Some *ZmDHNs* exhibit similar expression patterns, reflecting their close genetic relationship. This is particularly evident for *ZmDHN6* and *ZmDHN7*, suggesting that they may have similar functions in plant growth and development (Fig. 7).

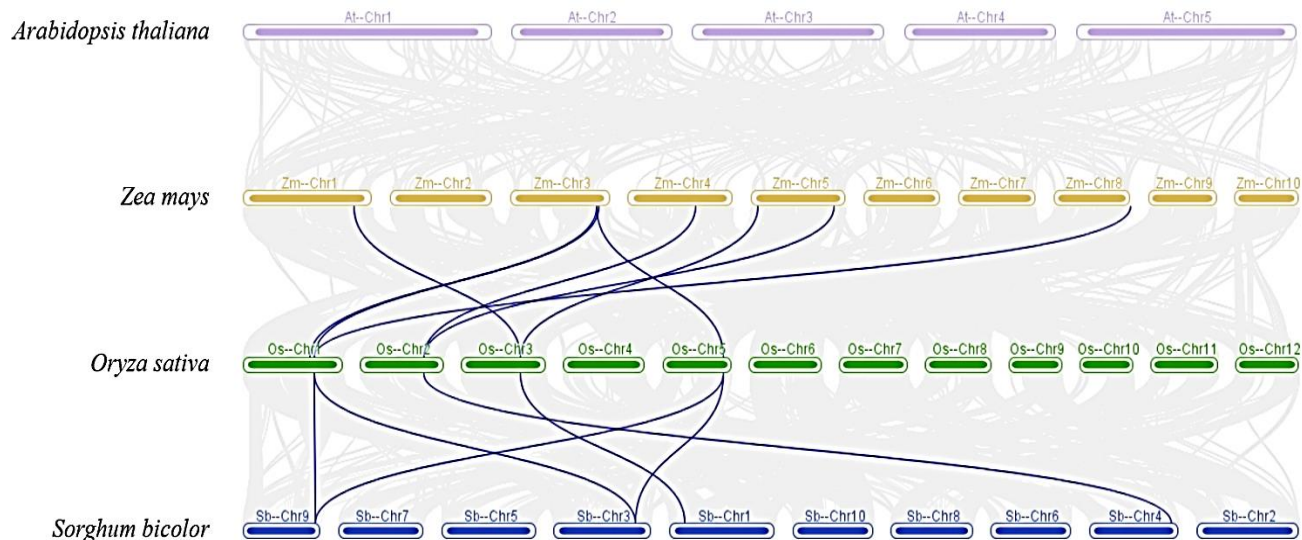


Fig. 5. Synteny analysis in the *DHN* gene family of *Z. mays*, *S. bicolor*, *A. thaliana* and *O. sativa*.

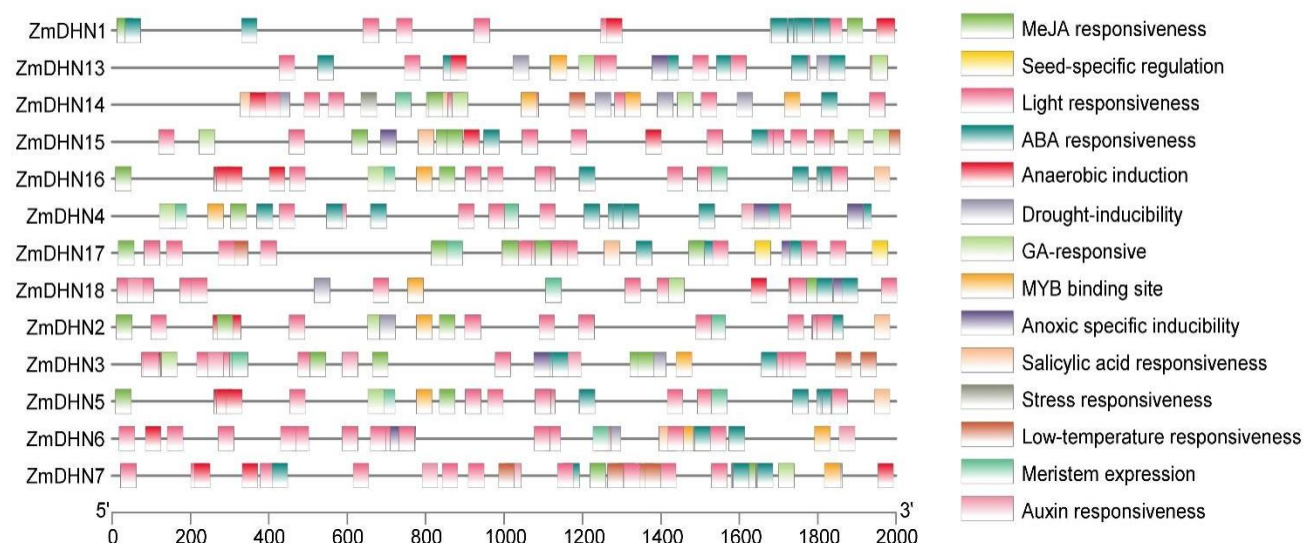


Fig. 6. Cis-acting elements analysis in the promoter region of the *ZmDHN* gene family.

Note: The horizontal axis represents the nucleotide length of the gene promoter; the bar's color represents the different cis-elements in the promoter region.

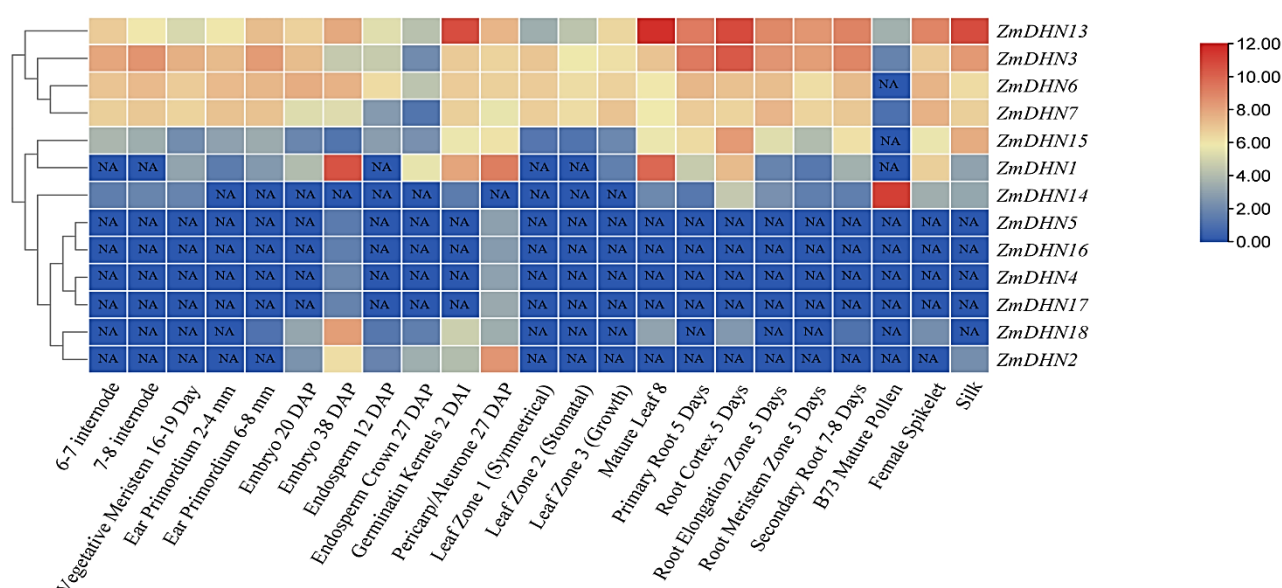


Fig. 7. Silicon expression pattern analysis of the *ZmDHN* genes in different tissues and organs.

Discussion

DHN proteins have characteristic irregular structures and can effectively resist adverse external environments. Under low water potential in cells, they adsorb water molecules and act as osmoregulators (Bao *et al.*, 2017). Studies have shown that *DHN* genes play a crucial role in maize in responding to different abiotic stress conditions. *ZmDHN13* reduces the formation of reactive oxygen species by binding to metal ions and positively regulating the tolerance of transgenic yeast and tobacco to copper stress (Liu *et al.*, 2019). Low temperature induces the expression of *ZmDHN2b* in maize, and overexpression of *ZmDHN2b* enhances tobacco’s cold tolerance (Zhang *et al.*, 2023). Overexpression of *ZmDHN11* could improve the tolerance of transgenic yeast and tobacco to osmotic stress (Ju *et al.*, 2021).

With the improvement of genomic data, some *DHN* genes have been discovered in maize, but a systematic analysis of this family is still to be conducted. Given the high accuracy and integrity of the B73 reference genome version 5.0, this study identified 13 maize *DHN* genes, which is similar to the numbers in other species (such as 10 in *Arabidopsis thaliana*, 8 in rice, and 5 in sorghum). *ZmDHNs* have high hydrophilicity, and the hydroxyl group within the molecule forms hydrogen bonds with the polar bonds in cell membrane phospholipid molecules, replacing the position of water, leaving the membrane lipids in a similar non-dehydrated state, thus maintaining the stability of the cell membrane (Jia *et al.*, 2023).

Previous studies have shown that different types of dehydrins respond differently to ABA signaling. The expression of KnS, Kn, and SKn type dehydrins is both ABA-dependent and ABA-independent, while the expression of YnSKn and YnKn type dehydrins is ABA-dependent only. The expression of ABA-dependent *DHN* genes is regulated by ABA signaling. These *DHN* genes have ABA-responsive elements (ABRE) or coupling elements (CE) in their promoters, and ABA targets these elements to regulate gene expression (Maruyama *et al.*, 2012). On this basis, we speculate that *ZmDHN2*, *ZmDHN5*, *ZmDHN4*, *ZmDHN1*, and *ZmDHN18* belong to ABA-dependent genes.

Constructing a multi-species phylogenetic tree allowed us to compare the phylogenetic evolution of *DHN* genes in maize, rice, sorghum, and *A. thaliana*. We found that the phylogenetic relationship between maize *DHN* genes and those in rice and sorghum was relatively close, indicating the conservation of monocots in evolution. Conservation domain analysis revealed that *ZmDHNs* contain typical conserved domains of the Y fragment (N-terminal of the protein sequence), S fragment, and K fragment (C-terminal) in the protein sequence. Cis-acting element analysis of promoter sequences revealed multiple functional elements, including LTR, DRE, MYB, G-box, and ABRE, which are associated with light, drought, cold, hormone, and other stress responses. Heterologous expression of *AmDHN200* localized to the cell membrane, *AmDHN154* to the nucleus, and *AmDHN132* to both the cytoplasm and nucleus in *A. thaliana* increased plant cold tolerance, with *AmDHN132* being the most significant (Cui *et al.*, 2020). At the same time, we observed diverse tissue expression patterns and subcellular localization of *ZmDHNs*. Previous studies have shown that *ZmDHNs* could maintain fresh weight and improve photosynthesis under drought stress by reducing stomatal density, increasing chlorophyll content, protecting photosystem II and the electron transport chain, maintaining the stability of thylakoid and chloroplast membranes, and protecting plant cells (Xie *et al.*, 2012). Therefore, different structural types of *ZmDHNs* suggest that they play specific functions. These findings lay an essential foundation for further studying the evolution of the *ZmDHN* gene family in maize and provide reference information for identifying key *ZmDHN* genes under abiotic stress.

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