

Article

Aluminum Stress of Oriental Melon (*Cucumis melo* L.) Is Linked to the Dehydrin *CmDHN3*

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Abstract: Dehydrins (DHNs; late embryogenesis-abundant D11 family) are a class of hydrophilic proteins involved in plant abiotic stress response. However, there is less information regarding DHN gene function in *cucurbit* crops. Herein, 34 DHN gene family members were identified and characterized in *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Benincasa hispida*, *Lagenaria siceraria*, and *Cucurbita maxima*. The DHN genes in the six *cucurbit* crops exhibited greater collinearity within subfamilies than between different subfamilies. Responses to stress (including low-temperature, salt, cadmium, and aluminum stress) varied among the DHN members, with a significant alteration in the expression of the acidic SnKn-type DHN gene *CmDHN3* in response to aluminum stress. Subcellular localization analysis confirmed that *CmDHN3* is expressed in the nucleus and cytoplasm. Virus-induced gene silencing (VIGS) revealed a remarkable decrease in *CmDHN3* expression, which markedly increased malondialdehyde content, relative conductivity, and proline content in the roots and leaves of plants under aluminum stress. Transcriptome analysis showed that the decreased *CmDHN3* expression reduced the expression of water channel protein-encoding genes. Interactions between *CmDHN3* and *CmAQP1* (MELO3C007188) and between *CmDHN3* and *CmAQP2* (MELO3C020774) were confirmed using yeast two-hybrid assays. These results clarify the pathway by which dehydrin genes are involved in the transcriptional-level response of melon to aluminum stress and provide a theoretical basis to comprehensively analyze the functions of this gene family in *cucurbit* crops.



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Keywords: *Cucurbitaceae*; dehydrin; oriental melon; aluminum stress; *CmDHN3*

1. Introduction

Dehydrins (DHNs) are a class of highly hydrophilic and intrinsically disordered proteins without distinct secondary or tertiary structures, which results in their functional diversity [1]. DHNs have crucial functions, particularly in defense against abiotic stress in higher plants. Previous reports have suggested that DHNs contribute to abiotic stress response by preventing protein aggregation or inactivation during dehydration by acting as protein-binding chaperones for cryoprotection and scavenging of free radicals [2–4]. However, along with their roles as molecular chaperones for protecting organellar structures from abiotic stress by stabilizing biological membranes and binding to reactive oxygen species (ROS) [5,6], DHNs participate in transcriptional regulation during stress response. They also indirectly participate in the modification of histones. During this epigenetic process, H3K4me3 modification shows a positive correlation with the expression of DHN and other drought response-related genes [7].

DHN proteins have evolutionarily conserved sequence motifs, including a lysine-rich (EKKGIMDKIKEKLPG) K segment located at the C-terminus, which forms an amphipathic α -helix structure that serves as the structural basis for the hydrophilicity of DHN [8,9]. Additionally, an S segment containing serine residues 3–13 can be phosphorylated by casein kinase 2 (CK2) and can enter the nucleus under the guidance of a signal peptide [10]. S segment deletion affects the nuclear localization of DHNs [11]. The Y segment (T/VDEYGNP) in the N-terminus is rich in tyrosine and typically contains 1–3 closely spaced copies. According to the number of motifs, DHN proteins are categorized as follows: YnSKn, YnKn, SKn, KnS, and Kn. YnSKn is the most common category; proteins in this category usually contain three or fewer conserved Y segments and several K segments, and they are neutral or alkaline. YnKn is a class of acidic DHNs containing Y and K segments, typically with no more than two segments each. Both SKn and KnS contain one S segment and two to three or one to two K segments and are mostly acidic. Most Kn proteins contain two to nine K segments and are acidic or neutral [11,12].

Different DHN proteins play varying roles in abiotic stress response by stabilizing cell membranes, scavenging ROS, binding metal ions, or acting as molecular chaperones. Silencing of the SKn-type DHN *CaDHN1* in *Capsicum annuum* L. exacerbated wilting under low-temperature stress and remarkably elevated the content of malondialdehyde (MDA) and conductivity [13]. The acidic SKn-type *MsDHN1* in *Medicago sativa* L. enhances tolerance to aluminum (Al) stress by increasing the exudation of oxalate from root tips through interactions with two aquaporin (AQP) water channel proteins. Moreover, an abscisic acid (ABA)-responsive element-binding transcription factor (*MsABF2*) may regulate the transcription of *MsDHN1* [14]. YnSKn-type DHNs have a role in the response of plants to stress due to low temperature and salinity. Silencing of the *CaDHN3* gene in *Capsicum annuum* L. reduces plant resistance to low-temperatures and saline stress [15]. *CaDHN4* can slow down the damage caused by low-temperature and saline stress by reducing sensitivity to ABA [16]. *CaDHN5* exerts a positive regulatory effect on the saline stress signaling pathway by affecting ROS accumulation [17]. Drought stress induces the expression of the *Oryza sativa* L. YnSKn-type DHN *OsDhn-Rab16D*, and overexpression of *OsDhn-Rab16D* maintains water balance and cellular homeostasis and improves the tolerance of plants to drought stress [18].

Under adverse conditions, some DHNs form complexes with regulatory factors and are involved in plant adaptation. *OsDhnRab16D* can interact with *OsFKBP* in the nucleus, and a novel FKBP- and YSK2-type DHN protein complex, *OsFKBP-OsDhn-Rab16D*, may be related to ABA signaling, thus enhancing the tolerance of *Oryza sativa* L. to drought [19]. Screening for freezing resistance genes in wild-type woodland strawberries revealed that the DHN protein *Xero2* functions as a key marker of freezing tolerance and binds to *CBF2* and *CBF4* to influence freezing tolerance in plants [20].

Acidic soils are widely distributed globally, and Al stress is considered an important factor that restricts crop growth in acidic soils [21,22]. Al stress hinders plant growth mainly by inhibiting root elongation [23,24]. Skn-type DHNs may affect oxalic acid secretion from plant roots by controlling water channel protein expression, which enhances Al tolerance [14]. However, it remains unclear whether Skn-type DHNs in plants of the *Cucurbitaceae* family have similar functions. In the present study, DHN proteins in cucurbit crops were identified and compared, and the functions of melon Skn-type *CmDHN3* under Al stress were explored. This study elucidates the regulatory mechanism of cucurbit DHN proteins in Al stress response and offers a theoretical basis for comprehensively analyzing the biological functions of DHNs in *Cucurbitaceae*.

2. Materials and Methods

2.1. Genome-Wide Identification of the Dehydrin Gene Family in Six Cucurbit Crops

Genome sequence data for six cucurbit crops were acquired from <http://cucurbitgenomics.org>. Furthermore, the DHN proteins of six *Cucurbit* crops (CsDHNs, CmDHNs, ClaDHNs, BhiDHNs, LsiDHNs, and CmaDHNs) were predicted with the HLH hidden Markov model (HMM) profile available in the Pfam database (<http://pfam.xfam.org>, PF00257).

Multiple sequence alignments of the full-length DHN domains of *Arabidopsis* (AtDHN), *Solanum lycopersicum* (SIDHN), *Cucumis sativus* (CsDHN), *Cucumis melo* (CmDHN), *Citrullus lanatus* (ClaDHN), *Benincasa hispida* (BhiDHN), *Lagenaria siceraria* (LsiDHN), and *Cucurbita maxima* (CmaDHN) were generated with ClustalW by utilizing default parameters [25]. By using multiple sequence alignments for all six *cucurbit* crops, *Arabidopsis thaliana*, and *Solanum lycopersicum*, we constructed a Neighbor-Joining (NJ) tree with MEGA v 7.0 and evaluated it through bootstrapping [26].

The conserved motifs in the protein sequences of candidate DHNs were analyzed using MEME 5.1.1 online software (Multiple EM for Motif Elicitation—<http://memesuite.org/tools/meme>) [27]. The GFF/GTF annotation files of cucurbit crop genomes (<http://cucurbitgenomics.org>) were utilized for examining the exon–intron organization and splicing phase of the predicted DHNs; the Gene Structure Display Server (GSDS—<http://gsds.cbi.pku.edu.cn>) was employed to visualize the results [28].

The *Cucurbit* crop genome sequences and GFF annotation files (FASTA suffix files) were retrieved from <http://cucurbitgenomics.org>. To perform synteny analysis, we obtained the gene duplication landscape by using MCScanX [29]. Next, we utilized TBtools (11 January 2024) to generate and visualize a syntenic map. Connector lines were used to highlight the putative duplicated genes.

2.2. Plant Materials and Stress Treatments

Cucumber (*Cucumis sativus* L. cv. Chinese long No. 9930), melon (*Cucumis melo* L. cv. balixiang), watermelon (*Citrullus lanatus* Thunb. cv. Qilin), wax gourd (*Benincasa hispida* L. cv. dafenpi), pumpkin (*Cucurbita maxima* L. cv. baby), and bottle gourd (*Lagenaria siceraria*) cultivars were obtained from the Liaoning Provincial Key Laboratory of Biological System Evolution and Agricultural Ecology, College of Life Sciences, Shenyang Normal University, Shenyang, China. Plant tissues, including roots, stems, leaves, flowers, and fruits, were rapidly frozen in liquid nitrogen and stored at -80°C until RNA extraction.

The *Cucumis melo* seedlings were cultured in Hoagland solution, and almost two-week-old seedlings (two true leaves fully unfolded) were used for abiotic stress treatment. The effects of cold stress (4°C), salt stress (100 mmol/L NaCl), cadmium stress (500 $\mu\text{mol/L}$ CdCl_2), and Al stress (500 $\mu\text{mol/L}$ AlCl_3) were evaluated. Root and leaf collection was performed at 0, 12, 24, and 48 h for cold-, NaCl-, cadmium-, and Al-treated seedlings. All samples were stored at -80°C by freezing them in liquid nitrogen before total RNA extraction.

2.3. Silencing of the CmDHN3 Gene in Melon

Cucumis melo L. cultivar “M1-23” was used for gene silencing. pTRV2:CmDHN3 and pTRV2:CmPDS were generated using two virus-induced gene silencing (VIGS) techniques, in accordance with a previously documented procedure [30]. Briefly, two 300 bp segments from CmDHN3 and CmPDS were inserted into a pTRV2 vector to develop the recombinant plasmids pTRV2:CmDHN3 and pTRV2:CmPDS, respectively. Subsequently, the freeze–thaw method was utilized for transforming the plasmids pTRV2:00 (negative control), pTRV2:CmDHN3, and pTRV2:CmPDS (positive control) in the *Agrobacterium tumefaciens*

GV3101 strain. All vectors were injected into melon cotyledons after GV3101 was combined with pTRV1, and the plants were cultured as reported earlier [30].

2.4. Gene Expression Analysis

Total RNA was isolated from tissues by using TRIzol reagent (Takara, Kusatsu, Japan). Real-time quantitative PCR (qRT-PCR) and data analyses were conducted by following previously described methods [31]. We employed the comparative $\Delta\Delta C_t$ method to calculate relative gene expression levels, using untreated samples or wild-type samples as the control group and 18S rRNA as the reference gene for normalization. Primer3 (<http://frodo.wi.mit.edu/>) was utilized for generating primers. All primers are shown in Table S1. A heatmap of expression profiles was created using the gene expression heatmap package in R.

After 2 weeks of AI stress, the roots of transgenic plants and control plants were subjected to total RNA extraction by using an Ultrapure RNA Kit (Kangwei, Beijing, China). RNA from each of the three biological replicates was reverse transcribed into cDNA by utilizing random primers. The obtained cDNA was sequenced at the Beijing Genomics Institute (BGI) (Guangzhou, China) with the Illumina HiSeq 2000 instrument. The raw reads were filtered using TopHat2 (version 2.0.3.12) to exclude adapters and low-quality reads, followed by alignment to the genome sequences (<http://melon-genome-hub.southgreen.fr/>) [32].

2.5. Malondialdehyde (MDA) Content, Proline Content, and Relative Electrolyte Leakage

Approximately 0.5 g of melon roots and leaves were used for determining MDA and proline contents and relative electrolyte leakage. MDA content was estimated by the thiobarbituric acid method as reported earlier [33]. The proline content was measured to assess membrane permeability based on a previously described method [34]. Relative electrolyte leakage was estimated using a previously described method [35]. All measurements were performed in triplicate (biological replicates).

2.6. Subcellular Localization

The positive control plasmids pBI221-GFP and pBI221-CmDHN3-GFP were mixed with gold powder after cleaning and disinfection. Gene gun technology was utilized to inject this gold powder into *Arabidopsis* protoplasts. The protoplasts were then cultured at 28 °C for 8–12 h in the dark. Confocal laser microscopy was used to observe green fluorescence [36].

2.7. Yeast Two-Hybrid Validation Assay

By using the yeast two-hybrid (Y2H) antibody optimization system (Y2H-AOS) constructed by ProNet Biotech (Nanjing, China), we simulated and analyzed the binding mode between antibodies and antigens and constructed a three-dimensional structural interaction model for CmDHN3-CmAQP1/CmAQP2 and CmDHN3-CmZIP proteins. To conduct the Y2H assay, CmDHN3, CmAQP1, or CmZIP coding sequences were amplified and cloned into the pGADT7 or pGBKT7 vector (Clontech, San Francisco, CA, USA). Y2H assays were performed using the Yeastmaker Yeast Transformation System 2 (Clontech).

2.8. Statistical Analysis

The experimental data were organized using Microsoft Excel 2010, and the physiological measurements were subjected to one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$) using SPSS 22.0 software. Data visualization was performed using Origin 8.5 (Microcal Software Inc., Northampton, MA, USA).

3. Results

3.1. DHN Gene Family Member Identification in Cucurbit Genomes

Thirty-four candidate DHN genes were identified by searching the whole genomes of six *Cucurbit* crops using the *hmmsearch* command, based on the HMM of the DHN (PF00257). The protein sequences of all candidate genes contained the conserved DHN structural domain (Figure S1). Therefore, 34 DHN members were identified in six *Cucurbit* crops, including four members in *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo*, and *Cucurbita maxima*; five members in *Lagenaria siceraria*; and thirteen members in *Benincasa hispida*.

3.2. Analyses of Phylogenetic Relationships, Conserved Structural Domains, and Physicochemical Properties

A phylogenetic tree was constructed with the protein sequences of thirty-four DHN members from *Cucurbit* crops, seven DHN members from *A. thaliana*, and four DHN members from *Solanum lycopersicum*. The DHN gene family of *Cucurbit* crops is classified into three DHN subfamilies: class I, class II, and class III; this is based on the number of K segments. Each subfamily contains varying numbers of DHN members, including YnSKn, YnKn, SnKn, YnKn, and Kn (Figure 1). The *A. thaliana* genome contains only one member of the subfamily YnSKn. Two members were detected in watermelons, cucumbers, and melons, and three members were detected in *Cucurbita maxima*, *Lagenaria siceraria*, and *Benincasa hispida*. In the subfamily YnKn, we detected one member in *Lagenaria siceraria*, *Cucumis sativus*, and *Benincasa hispida* and three members in *Solanum lycopersicum*. Two and eight members of the subfamily Kn were detected in *A. thaliana* and *Benincasa hispida*, respectively. In the SnKn subfamily, we detected one member in each of the six *cucurbit* crops and *Solanum lycopersicum* and four members in *A. thaliana*. Thus, in comparison with *A. thaliana*, the YnSKn-type subfamily in *cucurbit* crops expanded to a certain extent during evolution, whereas the Kn-type DHNs in *Benincasa hispida* expanded to a certain extent. Physicochemical analysis (Table 1) showed that the number of amino acids in the DHN family members of *Cucurbit* crops was 49–714, and the isoelectric points (pIs) ranged from 4.95 to 10.27. The number of amino acid residues varied substantially among the DHN members of different subfamilies.

3.3. Analyses of Gene Structure and Conserved Motifs

To determine the evolutionary relationships among the DHN gene family members in *Cucurbit* crops, the number and arrangement of exons and introns in 34 DHN members of the *Cucurbitaceae* family were analyzed. The number of exons and introns, as well as their arrangement, differed significantly among the subfamilies, whereas the intron–exon arrangement of DHN members within each subfamily was generally conserved (Figure 2).

By using the MEME tool, 15 conserved motifs were identified in the DHN members of *Cucurbit* crops. Each DHN member contains approximately five to nine conserved motifs. The number and distribution of the conserved motifs were generally conserved within the same subfamily (Figure 2), suggesting that DHNs of the same subfamily perform identical functions. Conserved motif distribution varied among DHN members in different subfamilies, which may contribute to functional differences.

3.4. Evolutionary Relationships of DHN Gene Family Members in Cucurbitaceae

To determine the evolutionary history of the DHN gene family within the *Cucurbitaceae* family, we conducted a pairwise collinearity analysis based on the phylogenetic tree. The closer the relationship, the higher the number of collinear pairs of DHN. Three collinear pairs were observed between *Cucumis sativus* and *Cucumis melo*, *Cucumis melo* and *Cucurbita*

maxima, and *Citrullus lanatus* and *Lagenaria siceraria*, while two collinear pairs were observed between *Cucurbita maxima* and *Cucumis melo*. A certain degree of collinearity was observed among members of the same DHN subfamily in different *Cucurbit* crops. The absence of collinearity between different DHN subfamilies suggests that an ancient duplication event occurred in the ancestral species of *Cucurbitaceae*, resulting in different types of DHNs (Figure 3).

3.5. Expression Patterns of DHN Gene Family Members in Different Plant Tissues

To determine the potential functions of DHN family members in *Cucurbitaceae*, DHNs of six common *Cucurbit* species were analyzed for their expression levels in the roots, stems, leaves, flowers, and fruits under normal physiological conditions. The different DHN genes showed remarkable variations in their expression patterns. Overall, the DHN genes were constitutively expressed in all tissues. High expression of most genes was observed in fruits. However, *CmDHN3* and *CmDHN4* expression levels in the vegetative organs (roots, stems, and leaves) were markedly higher than those in the reproductive organs (fruits and seeds). *CmDHN2* showed high expression in all tissues, except the roots (Figure 4).

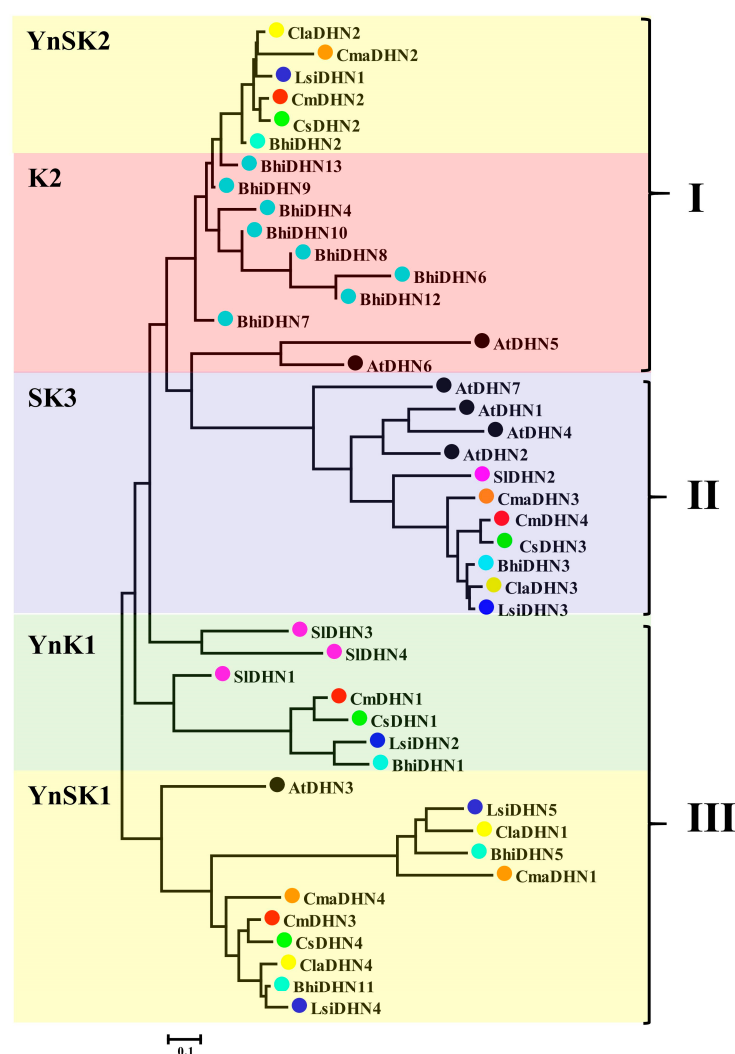


Figure 1. The phylogenetic tree of *Cucumis sativus*, watermelon, melon, wax gourd, bottle gourd, pumpkin, and *Arabidopsis* and *Solanum lycopersicum* DHN proteins was constructed from a complete alignment of DHN proteins with MEGA v 7.0 using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates. The groups are marked by colorful backgrounds. The protein names and corresponding accession numbers used to build the phylogenetic tree are listed in Table 1.

Table 1. Physicochemical properties of *DHN* gene family members in *Cucurbitaceae* crops.

Name	ID	Protein Length (bp)	CD Length (aa)	Molecular Weight (kDa)	PI	Chromosome Location	Predicted Location
<i>Citrullus lanatus</i>							
<i>ClaDHN1</i>	Cla97C02G031760	212	639	22.17	6.07	chr02:4613149 4615148 (−)	Nucleus
<i>ClaDHN2</i>	Cla97C07G135750	181	546	19.29	6.87	chr07:21540677 21541876 (+)	Nucleus
<i>ClaDHN3</i>	Cla97C08G152370	228	687	25.58	5.2	chr08:20736178 20737049 (+)	Nucleus
<i>ClaDHN4</i>	Cla97C11G210870	180	543	19.39	8.05	chr11:4212633 4215064 (−)	Nucleus
<i>Lagenaria siceraria</i>							
<i>LsiDHN1</i>	Lsi07G002410	165	498	18.04	9.00	chr07:2648912 2650391 (−)	Nucleus
<i>LsiDHN2</i>	Lsi07G002420	111	336	12.83	6.28	chr07:2652684 2653807 (−)	Peroxisome
<i>LsiDHN3</i>	Lsi08G008060	228	687	25.67	5.17	chr08:16443433 16444990 (+)	Nucleus
<i>LsiDHN4</i>	Lsi10G003970	171	516	18.21	6.8	chr10:6057706 6060658 (+)	Nucleus
<i>LsiDHN5</i>	Lsi11G011220	202	609	20.85	6.8	chr11:19658787 19661161 (+)	Nucleus
<i>Benincasa hispida</i>							
<i>BhiDHN1</i>	Bhi01G001874	105	318	12.12	6.43	chr1:58655060 58655377 (+)	Nucleus
<i>BhiDHN2</i>	Bhi01G001875	171	516	18.69	6.93	chr1:58672054 58673439 (+)	Nucleus
<i>BhiDHN3</i>	Bhi04G001222	228	687	25.81	5.43	chr4:39155443 39156748 (−)	Nucleus
<i>BhiDHN4</i>	Bhi04G001643	55	168	6.2	9.22	chr4:55256231 55256398 (−)	Nucleus
<i>BhiDHN5</i>	Bhi05G001121	172	519	18.38	6.06	chr5:45299333 45301282 (+)	Nucleus
<i>BhiDHN6</i>	Bhi06G000833	77	234	8.9	5.76	chr6:25580125 25580635 (+)	Cytoplasm
<i>BhiDHN7</i>	Bhi09G000580	69	210	7.9	8.76	chr9:15488834 15489043 (+)	Nucleus
<i>BhiDHN8</i>	Bhi09G000581	49	150	5.5	10.27	chr9:15569525 15569674 (−)	Nucleus
<i>BhiDHN9</i>	Bhi09G001205	71	213	7.8	9.72	chr9:37216318 37216530 (+)	Nucleus
<i>BhiDHN10</i>	Bhi10G001401	70	213	8.1	9.67	chr10:44590139 44590351 (+)	Nucleus
<i>BhiDHN11</i>	Bhi10G001578	154	465	16.54	7.21	chr10:49044633 49046400 (−)	Nucleus
<i>BhiDHN12</i>	BhiUN737G11	126	381	14.54	6.59	contig737:226811 227501(+)	Cytoplasm
<i>BhiDHN13</i>	BhiUN737G17	70	213	8.0	9.84	contig737:325991 326203 (+)	Nucleus
<i>Cucumis sativus</i>							
<i>CsaDHN1</i>	Csa016162	177	534	19.18	7.26	chr4:3543549 3544599 (−)	Nucleus
<i>CsaDHN2</i>	Csa016163	236	711	26.63	5.17	chr6:16107424 16109149 (+)	Nucleus
<i>CsaDHN3</i>	Csa013074	162	489	17.47	8.11	chr2:12194592 12196235 (+)	Nucleus
<i>CsaDHN4</i>	Csa020767	100	303	11.41	6.13	chr4:3546270 3546572 (−)	Nucleus
<i>Cucumis melo</i>							
<i>CmDHN1</i>	MELO3C016401.2	303	100	11.28	5.39	chr07:24180093 24180395 (+)	Nucleus
<i>CmDHN2</i>	MELO3C016402.2	534	177	18.95	7.28	chr07:24182063 24183504 (+)	Nucleus
<i>CmDHN3</i>	MELO3C023323.2	477	158	16.9	8.89	chr11:1435823 1437281 (+)	Nucleus
<i>CmDHN4</i>	MELO3C026550.2	714	237	26.43	4.95	chr03:24830447 24832398 (+)	Nucleus
<i>Cucurbita maxima</i>							
<i>CmaDHN1</i>	CmaCh00G004390	200	603	21.66	7.11	Chr00:33264719 33266319 (+)	Nucleus
<i>CmaDHN2</i>	CmaCh04G007640	172	519	18.5	7.28	Chr04:3882339 3883409 (−)	Nucleus
<i>CmaDHN3</i>	CmaCh05G009950	222	669	24.65	5.25	Chr05:7991924 7993668 (+)	Nucleus
<i>CmaDHN4</i>	CmaCh20G008280	204	615	21.51	6.84	Chr20:3941796 3943080 (−)	Nucleus

3.6. Expression Patterns of *CmDHN* Gene Family Members in Abiotic Stress Response

To determine whether *Cucumis melo* DHNs are involved in stress response, the expression levels of all *DHN* genes in *Cucumis melo* roots and leaves under various stresses were analyzed (Figure 5). The mRNA levels also exhibited variations under Cd stress. *CmDHN1* was the most affected gene, with a 16-fold increase in the number of roots, whereas *CmDHN3* expression was altered to a lesser extent. Under salt stress, the *CmDHN1* and *CmDHN2* expression levels in the roots increased 2000-fold and 2100-fold, respectively, whereas *CmDHN1* and *CmDHN2* expression levels in the leaves increased by more than 38-fold and 16-fold, respectively. Under Al stress, *CmDHN3* was upregulated 22-fold in the roots and 4.8-fold in the leaves, respectively, after 48 h. The mRNA levels increased following low-temperature treatment (4 °C) and increased with prolonged low-temperature stress. At 48 h, *CmDHN1* and *CmDHN2* levels were 1300-fold and 2100-fold higher than those in the control roots, respectively, and 1800-fold and 2600-fold higher in the leaves, respectively.

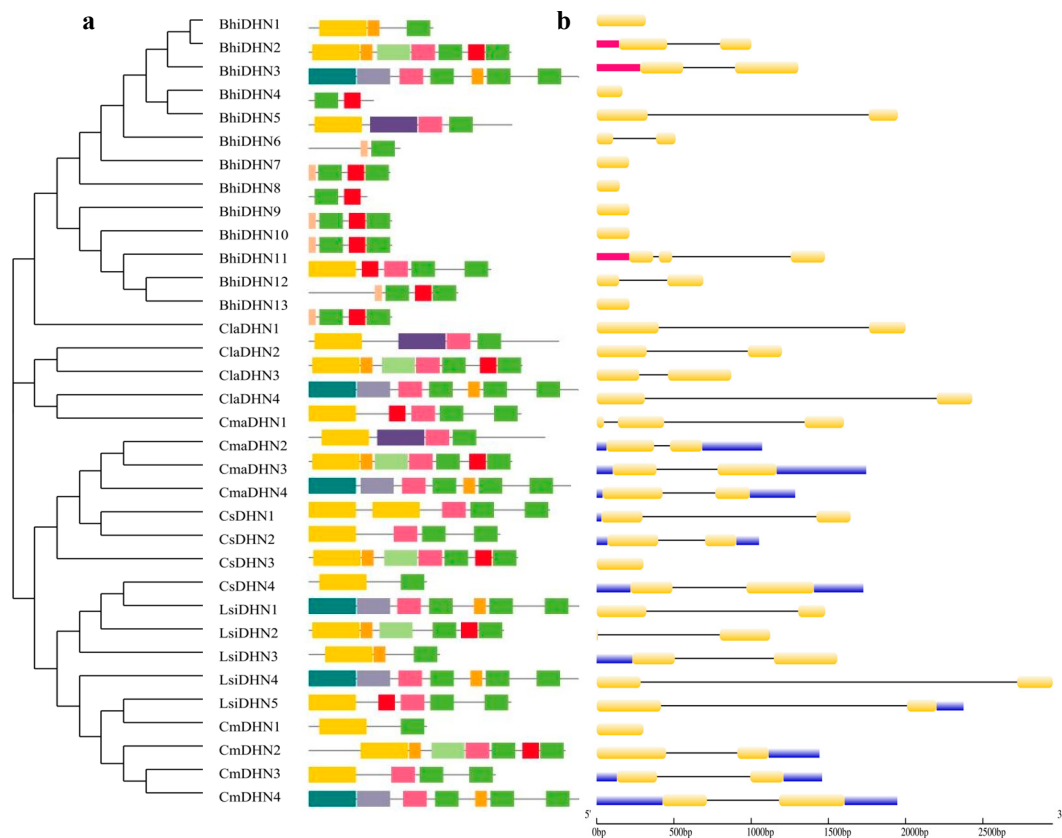


Figure 2. (a) Conserved motifs of DHN proteins in *Cucurbitaceae* crops; (b) exon–intron structures. Light blue boxes, yellow boxes, and black horizontal lines indicate exons, UTRs, and introns, respectively.

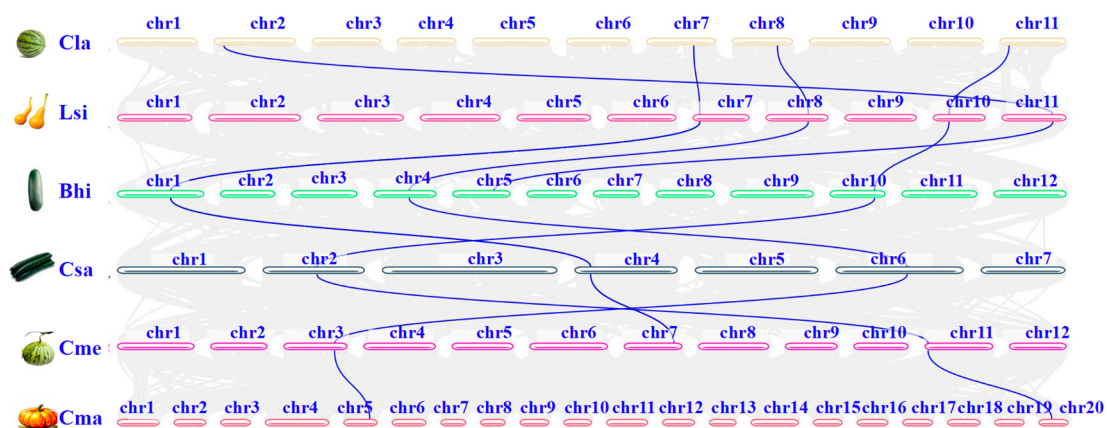


Figure 3. Synteny analysis of DHN genes among the six *cucurbit* crops. Light blue lines display the collinear DHN genes among the six *Cucurbit* crop genomes. The light blue lines denote collinear blocks.

3.7. Subcellular Localization of CmDHN3 in *Cucumis melo*

Gene function is often closely associated with the location of protein expression in cells. Therefore, these functions can be evaluated through subcellular localization analysis. To preliminarily investigate the location of CmDHN3 expression in *Cucumis melo* cells, a subcellular localization vector, pBI221-CmDHN3-GFP (Figure 6), was constructed using pBI221-GFP as a control. The localization and control vectors were jointly transformed

into protoplasts of *A. thaliana* by using the gene gun technique. After gene expression, the *CmDHN3* expression site was identified based on the green fluorescent protein signal (Figure 6). *CmDHN3* is expressed in both the nucleus and the cytoplasm.

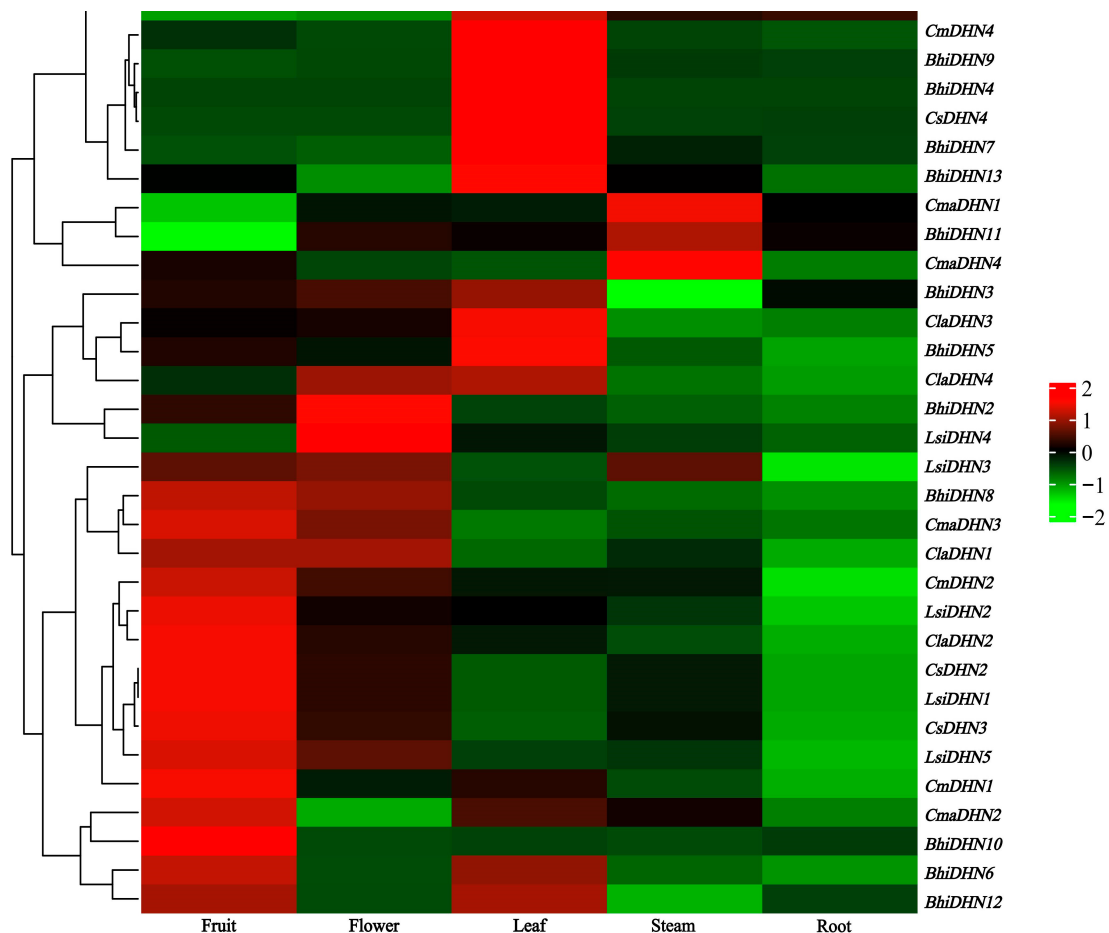


Figure 4. The DHN gene expression heatmap of the six cucurbit crops in different tissues.

3.8. Effect of *Cucumis melo* *CmDHN3* on Tolerance to Al Stress

VIGS is widely used to determine gene function. In a previous study, the authors utilized VIGS to determine the function of *CmDHN3* in Al stress response in *Cucumis melo* [28]. An approximately 300-base-pair (bp) sequence with high specificity for the *CmDHN3* gene was selected and inserted into the pTRV2 vector to prepare the recombinant plasmid pTRV2-*CmDHN3* (using pTRV2 as a negative control). pTRV2-*CmPDS* silences the *CmPDS* gene and induces leaf albinism (a phenotype observed in approximately 70% of plants after gene silencing). This gene is frequently used as a positive control for gene silencing. Two true leaves after induction by *Nicotiana tabacum* L. rattle virus (TRV)-mediated gene silencing, *Cucumis melo* plants injected with pTRV2-*CmDHN3*, and the control plants displayed no visible phenotypic differences. However, the *CmPDS*-silenced plants showed significant leaf albinism (Figure 7a). After two weeks of Al stress treatment, pTRV2-*CmDHN3* plants and negative control plants exhibited remarkable differences in their phenotypes. In particular, *CmDHN3*-silenced plants showed more prominent growth inhibition and significant suppression of root system growth (Figure 7b). Additionally, under normal growth conditions, *CmDHN3* expression in the roots and new leaves of gene-silenced melon plants was markedly lower than that in control plants, with no remarkable alterations in the expression of the remaining DHN genes; this finding suggests that *CmDHN3* silencing using VIGS was effective (Figure 7c).

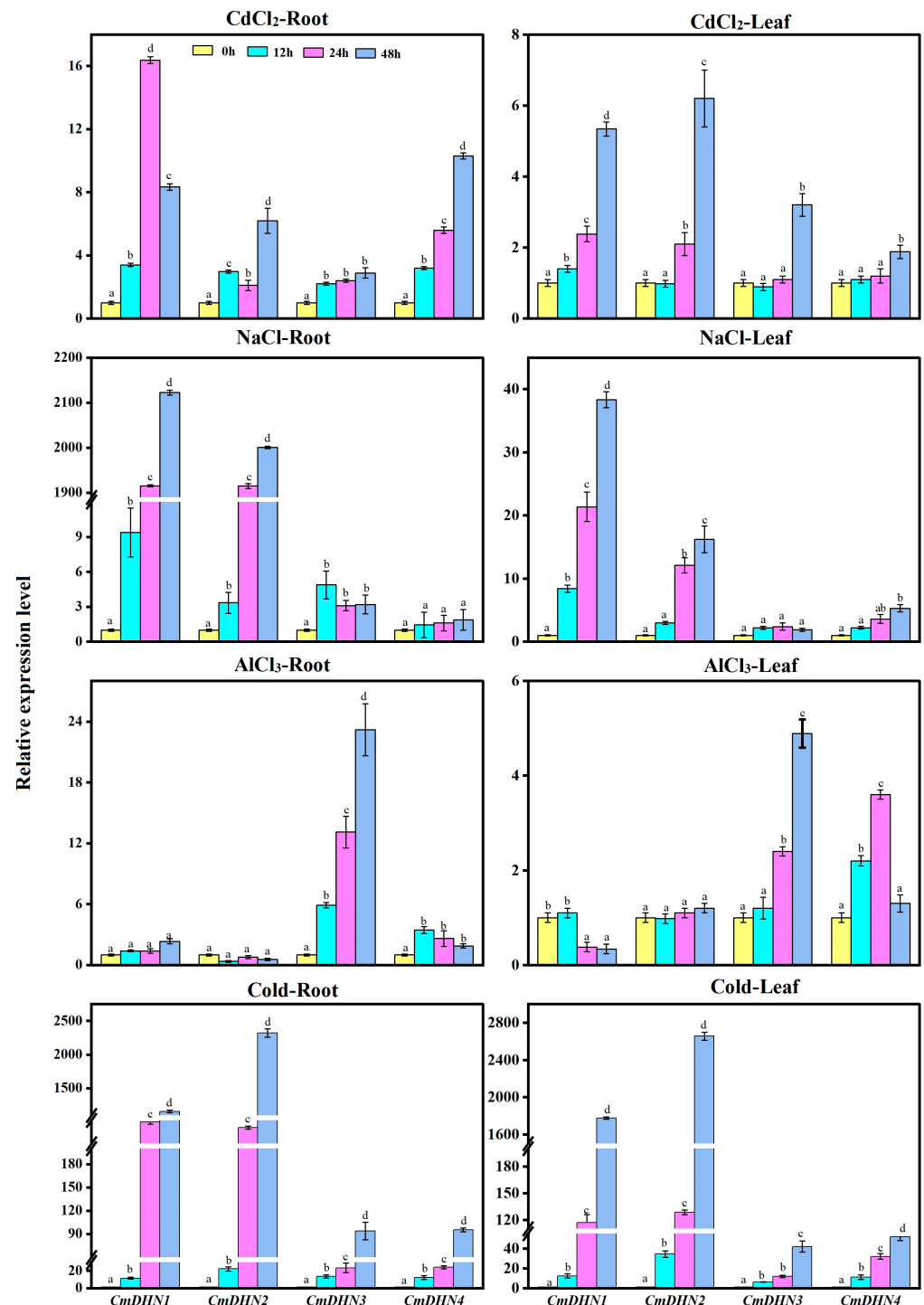


Figure 5. qRT-PCR analysis of *CmDHN* expression in the roots and leaves of oriental melon plants following abiotic stresses. The value for each sample is the mean $\pm$ standard error (SE), replicated thrice. The expression levels are relative to 0 h. Different lowercase letter (a–d) indicate significant differences (Student's test, $p < 0.05$).

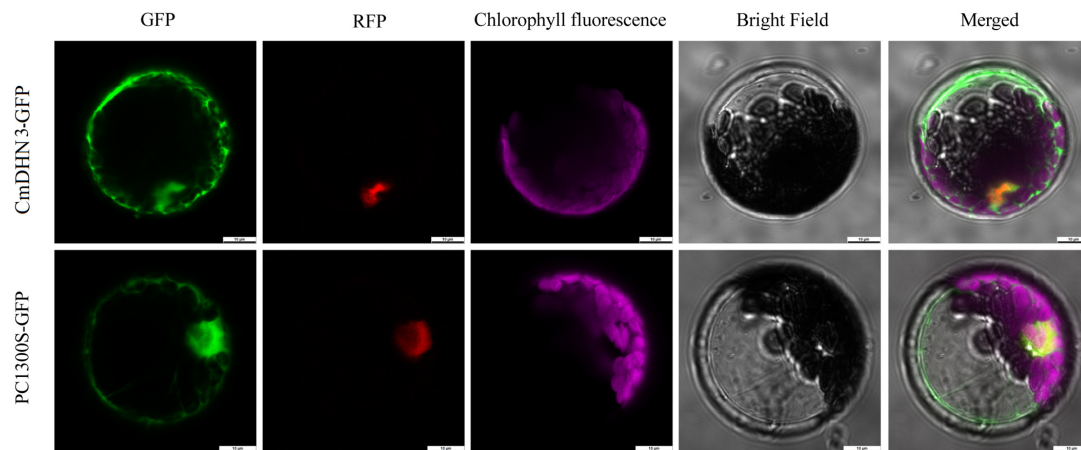


Figure 6. The subcellular localization of *Cucumis melo* CmDHN3 in *Arabidopsis* leaf protoplasts.

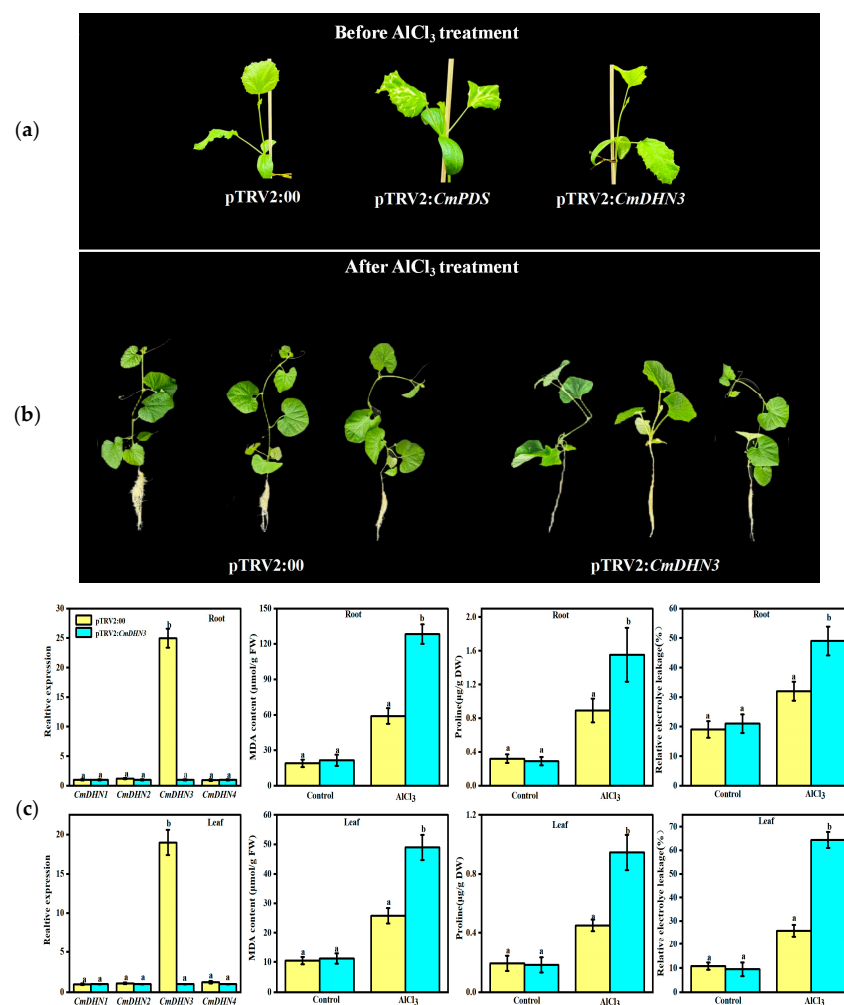


Figure 7. Effects of Al stress on plant phenotypes. (a) The phenotype empty vector (negative control), *CmPDS*-silenced (albino gene-positive control), and *CmDHN3*-silenced *Cucumis melo* plants when growing to two true leaves; (b) the phenotypes of the negative control and *CmDHN3*-silenced *Cucumis melo* plants under Al stress on day 14; (c) expression levels of the *DHN* gene, MDA content, proline levels, and relative electrolytic leakage in the roots and leaves of *CmDHN3*-silenced melon plants. Data that are significantly different are indicated with letters above the error bars ($\pm$ S.D.).

Physiological parameters were measured under Al stress. In normal physiological conditions, the control and silenced plants did not differ significantly in their MDA content.

However, after AlCl_3 treatment, both plants showed a remarkable increase in MDA levels. This increase in MDA content was 2.3-fold and 1.9-fold greater in the roots and leaves of silenced plants than in the roots and leaves of control plants. Following AlCl_3 treatment, the proline content increased by 1.4-fold and 2.1-fold in the roots and leaves of the silenced *Cucumis melo* plants, respectively, in comparison with that in the control plants. The relative conductivity values were 1.3-fold and 2.5-fold greater in the roots and leaves of the silenced plants, respectively, than those in the control plants (Figure 7c).

Following Al stress for two weeks, the root tips of the silenced and control plants were subjected to transcriptomic analyses. A total of 1284 differentially expressed genes (DEGs) were identified; of these genes, 575 were downregulated and 708 were upregulated under Al stress (Figure 8a). According to the KEGG enrichment analysis, the DEGs were enriched in various metabolic pathways, including mitogen-activated protein kinase (MAPK) signaling, hormone signaling, and glutathione metabolism (Figure 8b). Moreover, various genes, such as *CmAQP1*, *CmAQP2*, and *CmZIP*, were significantly downregulated in plants with silenced *CmDHN3* (Figure 8c,d).

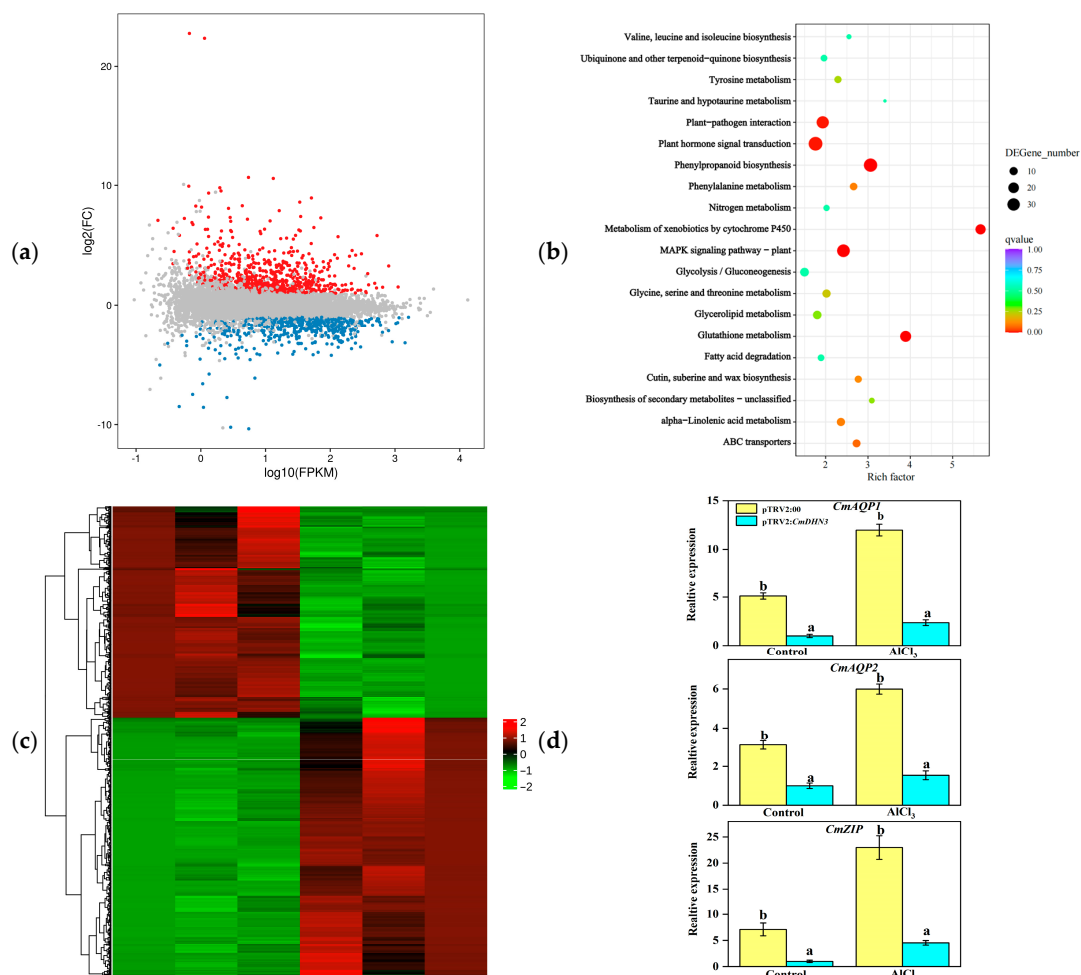


Figure 8. Overview of *CmDHN3*-silenced *Cucumis melo* plants transcriptomes of roots under Al stress on day 14. (a) Volcano diagram of gene expression. Red represents up-regulated genes, and blue represents down-regulated genes. (b) KEGG enrichment of genes. (c) Differential gene expression heatmap. (d) Expression levels of *CmAQP1*, *CmAQP2*, and *CmZIP* genes in the roots of *CmDHN3*-silenced *Cucumis melo* plants. The lowercase letters denote statistically significant differences ($p < 0.05$).

Three-dimensional protein structural interaction models of CmDHN3 with CmZIP, CmDHN3 with CmAQP1, and CmDHN3 with CmAQP2 were constructed using the Y2H-AOS three-dimensional protein structural simulation technique developed by Nanjing ProNet Biotech Co., Ltd. (Figure 9a). The confidence levels of these models were as high as 0.91, 0.97, and 0.80, respectively, thus indicating interactions between each group of proteins. Y2H assays further showed that CmDHN3 and CmZIP (MELO3C03794), CmDHN3 and CmAQP1 (MELO3C007188), and CmDHN3 and CmAQP2 (MELO3C020774) exhibited growth on the deficient culture medium, indicating the presence of interactions within all three groups of proteins (Figure 9b).

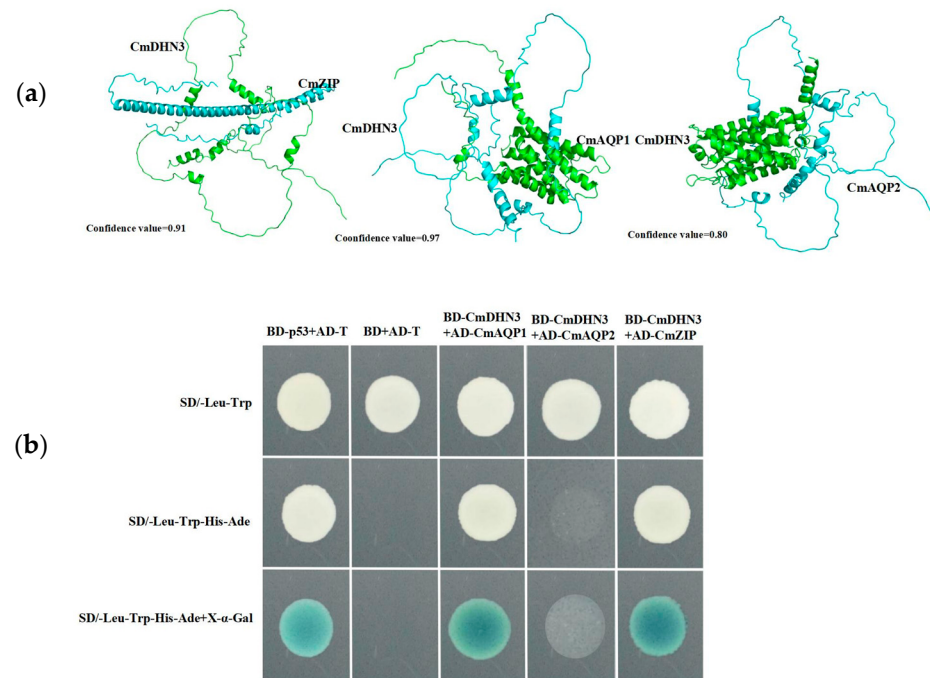


Figure 9. Validation of the protein that interacts with CmDHN3. (a) A three-dimensional model of the interaction protein between CmDHN3 vs. CmAQP1, CmDHN3 vs. CmAQP2, and CmDHN3 vs. CmZIP; (b) Interaction between CmDHN3 and CmAQP1, CmAQP2, and CmZIP determined in a yeast-two-hybrid (Y2H) assay. CmDHN3 was inserted into pGBKT7 and CmAQP1; CmAQP2 and CmZIP were inserted into pGADT7. Different co-transformed yeast cells were dropped onto synthetic dropout medium (SD/-Trp/-Leu/-Ade/-His) containing 20 mg mL⁻¹ X-α-gal.

4. Discussion

DHNs have pivotal roles in plant responses to different abiotic stressors [2,37,38]. The functions of DHN family members in many plants have been reported, such as *A. thaliana* [39], *Solanum lycopersicum* [40], barley [41], *Oryza sativa* L. [42], and chili pepper [13]. However, few studies have evaluated the functions of DHNs in *Cucurbit* crops under abiotic stress; to date, only four DHN members are known in *Cucurbits* [43]. In the study presented herein, we identified 34 DHN family members in *cucurbit* crops on the basis of the *Cucurbit* genome [44] (Table 1).

DHN protein sequences in the family *Cucurbitaceae* are disordered, and DHNs are highly hydrophilic but show significant variations in pI, kinases, and a number of conserved motifs. The pI values of YnSKn- and SKn-type DHNs are higher than those of SKn-type DHNs, suggesting that YnSKn-type DHNs bind more easily to negatively charged membranes [9]. Furthermore, DHN phosphorylation may be involved in the functional regulation of plant cells under stress and in the membrane binding of DHNs43. SKn-type DHNs of the *Cucurbitaceae* family contain more CK2 phosphorylation sites than YnSKn-

type DHNs (Table 1). These findings agree with those of studies on DHN gene families in *Capsicum annuum* and *Vitis vinifera*, thus showing that YnSKn-type DHNs and SKn-type DHNs are primarily phosphorylated by PKC and CK2, respectively [45–48].

We constructed a phylogenetic tree based on the DHN proteins of *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Benincasa hispida*, *Lagenaria siceraria*, *Cucurbita maxima*, *A. thaliana*, and *Solanum lycopersicum* (Figures 1 and 2). The *Cucurbit* crops had a similar number of DHN members (4–5), except for the 13 DHN members in the *Lagenaria siceraria*. However, the DHN families of *Cucumis sativus*, *Cucurbita maxima*, *Citrullus lanatus*, *Cucumis melo*, and *Benincasa hispida* included only five YnSKn-, YnKn-, and SKn-type DHNs, whereas *Cucurbita maxima* contained only Kn-type DHNs and lacked KS-type DHNs, suggesting that these DHN types were lost in *Cucurbit* crops. Moreover, in the phylogenetic tree, YnSK2-type DHNs constituted the largest fraction of total DHN members, accounting for a proportion of >37.78% (Figure 3). YnSK2-type DHN proteins are among the most abundant neutral and basic proteins found in nature [38]. DHN genes in *Cucurbit* crops showed largely distinct expression patterns, despite some overlaps (Figure 4). Rorat (2006) [38] showed that DHNs were expressed in almost all tissues under normal physiological conditions. *Cucurbit* DHN genes are also expressed in all tissues, suggesting that they participate in crop growth and development.

DHN genes are present in various plants of a multigene family and have been extensively investigated. As suggested previously, SKn- and Kn-type proteins are primarily involved in protection against cold stress, whereas YnSKn-type DHNs are primarily involved in plant adaptation to ABA, desiccation, and salt stresses [4]. Different expression patterns have been observed for different types of DHN family members. In *Capsicum annuum*, most DHN genes (including SKn and YnSKn) are upregulated under low temperature, NaCl, and mannitol stress. In particular, the SKn-type *CaDHN7* is highly upregulated, suggesting its critical function in response to abiotic stress [14]. YnSKn-type DHNs in *Hordeum vulgare* L. were almost entirely induced by ABA and drought stresses but not by low temperatures. In contrast, low temperatures upregulated the expression of DHN5 (K9) and DHN8 (Sk3). Furthermore, low temperatures induce DHN gene expression in *A. thaliana* and *Triticum aestivum* L. [49,50]. In *Cucumis melo*, almost all DHN genes displayed upregulated expression in stress due to low temperature, NaCl, and CdCl₂. In particular, *CmDHN1* (YnKn-type) and *CmDHN2* (YnSKn-type) were highly upregulated, which suggests their involvement in abiotic stress response. Notably, both YnSKn-type *CmDHN3* and SKn-type *CmDHN4* were upregulated under AlCl₃ stress, especially *CmDHN3*, which was upregulated to a greater extent, suggesting that *CmDHN3* has a crucial function in Al stress response (Figure 5).

In this study, *CmDHN3* silencing significantly reduced Al tolerance in *Cucumis melo* and led to substantial MDA and proline accumulation in the roots and leaves, as well as a significant elevation in relative conductivity (Figure 7). Transcriptome analysis showed that *CmDHN3* might contribute to Al tolerance by affecting the expression levels of Al stress-related genes, such as aquaporin (AQP) and aluminum-activated malate transporter (ALMT). The promoter of *CmDHN3* had an ABA-binding site, and *CmDHN3* expression levels showed a significant correlation with *CmZIP* levels (Figure 8). Therefore, interactions between these genes and *CmDHN3* were predicted using the yeast two-hybrid antibody optimization system (Y2H-AOS). Finally, Y2H assays confirmed that *CmDHN3*, *CmZIP*, *CmDHN3*, and *CmAQP1*, as well as *CmDHN3* and *CmAQP2*, interacted to varying degrees (Figure 9). These findings suggest that *CmDHN1* interacts with two AQP water channel proteins and is regulated by the ABA response factor *CmZIP* at the transcriptional level. The acidic SnKn-type *MsDHN1* in *Medicago sativa* increases the secretion of oxalic acid from the root tips to enhance plant tolerance to Al stress [14]. Whether *CmDHN3*, which is also

an acidic SnKn-type DHN, can increase the oxalic acid content in root tips to improve Al tolerance in plants requires further investigation. These findings suggest that SnKn-type DHNs have similar regulatory pathways in the Al stress response mechanism of plants.

5. Conclusions

We identified 34 *DHN* genes in the *Cucurbitaceae* genome database; these genes showed differential expression in various tissues. The *DHN* gene family members in melons exhibited varying expression patterns in response to different stresses. Al stress induces acidic SnKn-type DHN *CmDHN3* gene expression, and silencing of the *CmDHN3* gene reduces Al stress tolerance in melon. The knockout of the *CmDHN3* gene also suppressed the expression of the water channel proteins *CmAQP1* and *CmAQP2*, as well as the ABA signaling factor *CmZIP*. The Y2H assay verified the interactions between these groups of proteins. This study offers a theoretical basis for revealing the mechanism through which acidic SnKn-type *DHN* contributes to the response to Al stress in melons.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae11050480/s1>. Figure S1: Protein sequence alignment of DHN protein sequences from six *Cucurbitaceae* crops. Y, S, and K segments are boxed in pink, yellow, and blue, respectively; Table S1: The primers used in the study.

Author Contributions: C.Z.: Conceptualization, methodology, software, data curation, and writing—original draft; Q.C.: Analysis and interpretation of the data; X.G.: Conceptualization and methodology; H.P.: Funding; Y.Z.: Visualization, funding, investigation, supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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