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Systematic Investigations of the ZF-HD Gene Family in Tobacco Reveal Their Multiple Roles in Abiotic Stresses

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Abstract: Zinc finger homeodomain (ZF-HD) transcription factors play significant roles in plant growth and responses to environmental stresses. In this study, 32 *ZF-HD* genes identified in the tobacco (*Nicotiana tabacum* L.) genome were divided into six groups according to phylogenetic analysis with *Arabidopsis* and tomato ZF-HD members. An examination of gene structures and conserved motifs revealed the relatively conserved exon/intron structures and motif organization within each subgroup. In addition, various stress-related elements are found in the promoter region of these genes. The expression profiling analysis revealed that *NtZF-HD* genes expressed in different tissues and could be induced by several abiotic stresses. Notably, *NtZF-HD21* was highly expressed in response to the drought treatments. Subcellular localization analysis and a virus-induced gene silencing (VIGS) experiment were performed to investigate the potential functions of *NtZF-HD21*. The subcellular localization indicated that NtZF-HD21 is a nuclear protein. Furthermore, gene silencing of the *NtZF-HD21* gene reduced the drought resistance of tobacco. These findings provide insights for further biological functional analyses of the *NtZF-HD* genes in tobacco.

Keywords: *Nicotiana tabacum*; *ZF-HD* genes; genome-wide analysis; abiotic stresses

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1. Introduction

Transcription factors (TFs), as a special class of proteins with the activity of binding to specific regions of target gene promoters, play significant roles in plant growth and development, stress resistance, and signal transduction [1–3]. Zinc finger homeodomain (ZF-HD) TFs usually include the following two structural features: a cysteine-rich zinc finger motif (ZF) and a conserved homeodomain (HD) [3]. ZFs contain zinc ions and cysteine residues or histidine residues [4]. ZFs are widely found in diverse regulatory proteins, which can specifically bind to DNA/RNA sequences and be actively involved in protein–protein interactions [5,6]. The HD, as a DNA-binding domain (BD), consists of about 60 amino acids that fold into a recognition helix that is able to specifically bind DNA to activate or suppress the expression of target genes [7–11]. The proteins with an HD are classified into six subgroups according to the presence of structural differences: leucine zipper-associated HD (HD-ZIP), zinc finger motif-associated HD (ZF-HD), WUSCHEL-related homeobox (WOX), Bell-type HD, finger domain associated to a HD (PHD finger) and Knotted-related homeobox (KNOX) proteins [12].

Previous research studies revealed that *ZF-HD* genes participate in many essential biological processes in plants [13,14]. In *Arabidopsis*, a total of 17 *ZF-HD* genes were identified, and the contributions of a number of *ZF-HDs* to various developmental processes have been characterized [15–17]. For instance, overexpressing transgenic plants of *AtZF-HD5*

reportedly exhibit a larger size of leaves [18]. *AtZF-HD10* was detected highly expressed in the hypocotyl axis in *Arabidopsis* and induced the expression of the hypocotyl-elongation-related genes *HFR1* (*LONG HYPOCOTYL IN FAR-RED*) and *ATXTH17* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17*) [19]. *AtZF-HD8*, expressed highly in flower, revealed that they dominate significant roles in the development of flowering [7]. In other species, it was also found that *ZF-HD* genes have important roles in leaf development and flower bud development, such as *SlZF-HD7* of tomato and *FtZF-HD11* in Tartary buckwheat (*Fagopyrum tataricum*) [20,21].

Furthermore, a large number of *ZF-HD* genes were found to respond to abiotic stresses in plants [19,22–24]. *AtZF-HD1* encoded a positive transcriptional regulator that was reported to specifically bind to the *ERD1* (Early Response to Dehydration Stress 1) promoter region, whose expression was induced by salinity, dehydration, and ABA (abscisic acid) treatments [22]. *AtZF-HD4* could be induced under drought and salt treatments, implying that *AtZF-HD4* responds to abiotic stresses in *Arabidopsis* [25]. Previous studies indicated that AtZF-HD10 interacted with TANDEM ZINC-FINGER PLUS3 (TZP) to modulate hormone signaling in stress response [19]. More recently, a study showed that GmZF-HD1 and GmZF-HD2 can bound to the promoter of *GmCaM4* (Calmodulin Subtype 4) in soybean, and the expressions of *GmZF-HD1* and *GmZF-HD2* were up-regulated upon pathogen inoculation [26]. Additionally, the *ZF-HD* genes were involved in abiotic stress in tomato and Chinese cabbage [3,20].

Tobacco is a significant economic crop and a representative model plant widely cultivated all over the world. Biotic and abiotic stresses can severely decrease tobacco quality and yield. Although the ZF-HD gene family has been extensively studied in many species, such as *Arabidopsis* [11], tomato [20], rice [27], and apple [28], systematic and comprehensive identification had not been conducted in tobacco. Previous studies provided the necessary information for the data-predicted *NtZF-HD* gene family in tobacco due to the improved annotation of genome-wide sequencing of tobacco [29,30]. In this study, a comprehensive genome-wide investigation and expression analysis of the *NtZF-HD* gene family in tobacco were performed, including phylogenetic analyses, protein domain organization, gene structure, promoter analysis, expression pattern, and VIGS analysis. The results of this study indicate that the tobacco *ZF-HD* gene family members might play multiple roles in various biological processes, including development and responses to various stresses.

2. Materials and Methods

2.1. Identification of Tobacco ZF-HD Proteins

The genome data of the tobacco (*N. tabacum*), *N. sylvestris*, *N. tomentosiformis* and tomato (*Solanum lycopersicum*) were downloaded from the Sol Genomics Network (https://solgenomics.net/) (accessed on 21 October 2019) [31] and the genome data of the *Arabidopsis* were downloaded from TAIR (http://www.arabidopsis.org/) (Accessed 21 October 2019) [32]. The previously reported sequences of *Arabidopsis* and tomato ZF-HD full-length protein were used as queries to carry out BLASTP searches against the tobacco annotation database under the E-value cutoff of 0.01. Then, the resulting sequences were subjected to the Pfam (PF04770) [33] and SMART (http://smart.embl-heidelberg.de/) (accessed on 21 October 2019) [34] databases to determine the number of the ZH-FD domain. After removing the incomplete sequences and repeating manually, the remaining numbers were named based on their physical locations on the chromosome/scaffold and were submitted to the ProtParam online toolkits (http://au.expasy.org/tools/protparam.html) (accessed on 21 October 2019) for the calculation of Biochemical characteristics, including Mw (molecular weight), theoretical pI (isoelectricpoint), and subcellular localization [35].

2.2. Phylogenetic Analysis and Classification

The proteins of tobacco, *N. sylvestris*, *N. tomentosiformis*, *Arabidopsis*, and tomato ZF-HD members, as shown in the Table S1, were aligned using ClustalX 2.0. The phylogenetic tree

was constructed using the Neighbor-Joining (NJ) method in MEGA 7 with the p-distance method and pairwise deletion setting. A bootstrap statistical analysis was conducted with 1000 replicates to detect the reliability of each branch [36].

2.3. Gene Structure, Motif Analysis, and Cis-Elements Analyses

The gene structure of tobacco *ZF-HD* genes was analyzed by GSDS (http://gsds.cbi.pku.edu.cn) (accessed on 21 October 2019) using the coding sequence (CDS) [37] and genomic sequence. The online site Multiple Em for Motif Elicitation (MEME; http://meme-suite.org/) (accessed on 21 October 2019) was applied to identify conserved motifs of the ZF-HD full-length protein sequences [38]. The promoter region of tobacco *ZF-HD* genes was extracted by Promoter 2.0, and the *cis*-elements of promoters were analyzed by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 21 October 2019) [39].

2.4. Syntenic Analysis of NtZF-HD Genes

To explore the syntenic relationship of the orthologous genes obtained from tobacco and other tested plant species (*Arabidopsis*, tomato, grape, rice, and maize), syntenic analysis maps were investigated by the Systeny Plotter of Tbtools (https://github.com/CJChen/TBtools) (accessed on 21 October 2019) [40].

2.5. Plant Material and Stress Treatment

Cultivated tobacco (*Nicotiana tabacum* L. Cv. K326) plants were used in this study. Different organs, including the root, stem, leaf, and flower were used to analyze the tissue-specific expression patterns. The tobacco seeds were germinated on MS medium in a light incubator at 25 °C for two weeks. The tobacco seedlings were transferred and exposed to drought and salt stress treatment for 0, 3, and 6 h. These treatments were performed as previously described by Khatun et al. with some modification [41]. Drought stress treatment involved transferring seedlings to dry paper at 0, 3, and 6 h. Roots of seedlings were submerged in 150 mM NaCl during salt stress treatment at 0, 3, and 6 h. After different treatments, leaf samples from three biological replicates were collected and frozen in liquid nitrogen immediately and transferred to −80 °C for RNA extraction.

2.6. RNA Extraction and RT-qPCR Analysis

Total RNA was extracted using the method of Ren et al. [42] and the first-strand complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa). Using Primer3 software (http://bioinfo.ut.ee/primer3/) (accessed on 21 October 2019) [43], we designed the RT-qPCR primers (Table S2). RT-qPCR was performed on an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 2 μL template cDNA. The internal control used was the transcript of tobacco ribosomal protein gene *L25* (GenBank No. L18908). At least three biological replications and three technical repeats of all reactions were performed, and the relative expression levels of each gene was analyzed using the $2^{-\Delta\Delta CT}$ method [44].

2.7. Subcellular Localization

The subcellular localization of NtZF-HD proteins was predicted using the online Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (accessed on 21 October 2019) [45]. The coding regions of *NtZF-HD21* without the stop codon were amplified from tobacco cDNA and inserted into the PYG57 vector, generating the *35S::NtZF-HD21–GFP* fusion construct. According to the previous study [46], the recombinant *35S::NtZF-HD21-GFP* vector and the control vector were transformed into *Agrobacterium* competent cell GV3101 and then injected in the leaves of *N. benthamiana*. After the incubation, the confocal microscope (TCS-SP8 Leica, Wetzlar, Germany) was used to investigate Green Fluorescent Protein (GFP) signal under a 488 nm exciting light, and the nuclear localization signal was confirmed by 4,6-diamidino-2-phenylindole (DAPI) dye staining.

2.8. VIGS (Virus-Induced Gene Silencing)

The VIGS vector system including *TRV2* (empty vector), *TRV1* (auxiliary vector), and *TRV2::PDS* (positive control) was used in this experiment. The conserved sequences of *NtZF-HD21* gene were selected from the SGN-VIGS website (https://vigs.solgenomics.net/) (accessed on 21 October 2019) [47]. The conserved sequences were inserted into the *TRV2* vector with the KpnI site and EcoRI site. The new vector named as *TRV2::NtZF-HD21* was introduced into an *Agrobacterium* competent cell GV3101. The detailed VIGS operating methods were performed as previously described by Gao et al. (2013) [48].

3. Results

3.1. Identification of NtZHD Genes in Tobacco

To identify *NtZF-HD* family genes, we used the ZF-HD numbers of *Arabidopsis* as queries to the BLASTP search. A total of 32 *ZF-HD* genes were identified in tobacco and designated *NtZF-HD1* to *NtZF-HD32* based on their physical locations on the chromosomes (Table S3). The results showed that 12 *NtZF-HD*s were mapped on seven chromosomes and 20 *NtZF-HD*s were localized on scaffolds.

As shown in Table S3, the ORF (open reading frame) lengths of the *NtZF-HD* genes range from 417 bp (*NtZF-HD28*) to 3078 bp (*NtZF-HD2*). Their isoelectric points (pIs) range from 6.37 (NtZF-HD25) to 10.25 (NtZF-HD28), and their molecular weights (MWs) range from 15.59 (NtZF-HD28) to 113.63 kDa (NtZF-HD2).

3.2. Phylogenetic Analysis of the NtZF-HD Family Members

To further elucidate the phylogenetic relationships of the NtZF-HD family proteins, a neighbor-joining (NJ) tree was constructed based on the multiple sequence alignment of the 32 tobacco ZF-HD members and their homologs in *Arabidopsis thaliana*, *N. sylvestris*, *N. tomentosiformis*, rice, and tomato (Figure 1). The 32 *NtZF-HD* members were classified into eight groups (I–VIII), together with their *Arabidopsis* and tomato homologs based on previously reported [20,23]. Groups V and II contained the smallest (one) and largest (nine) number of proteins, respectively. Groups IV and VI both account for 19% of the total NtZF-HD proteins. Groups I and III both included three members of the NtZF-HD proteins. Only groups II, III, and IV contained rice members, suggesting that the member of these groups in the *ZF-HD* gene family occurred before the divergence of dicotyledon and monocotyledon. Notably, groups VII and VIII only contained tobacco and tomato members, suggesting that these ZF-HD numbers might occur after the divergence of *Arabidopsis* and were unique to *Solanaceae* plants. Interestingly, a large number of NtZF-HD members from different groups could be clustered with NtomZF-HD and NsylZF-HD members, implying that these species had a closer evolutionary relationship. However, in group V, NtZF-HD1 was clustered only with NsylZF-HD13 and NsylZF-HD17, suggesting that the NtomZF-HD from *N. tomentosiformis* may be lost after interspecific hybridization.

3.3. Structural Analysis and Motif Composition of the NtZF-HD Family

The multigene family analysis of genetic structural diversity can provide important insights into the evolutionary information of *NtZF-HD* genes. To further explore the evolution of the *ZF-HD* genes in tobacco, the number and arrangement of their exon–intron structures were identified (Figure S2). The members of different groups exhibited different exon–intron organization. Among the 32 NtZF-HD genes, there were 15 NtZF-HD genes with intronless and clustered into groups III, IV, and V. The remaining 17 NtZF-HD genes coding sequences were interrupted by introns, and the number of exons varied from one to eight. The remaining NtZF-HD genes belonged to groups I, II, and VI (Figure S2B). The introns of the *NtZF-HD* gene were obtained by evolutionary inheritance or by mutations in the evolutionary process, and whether the emergence of introns affects the function of these genes is a worthy question for further research.

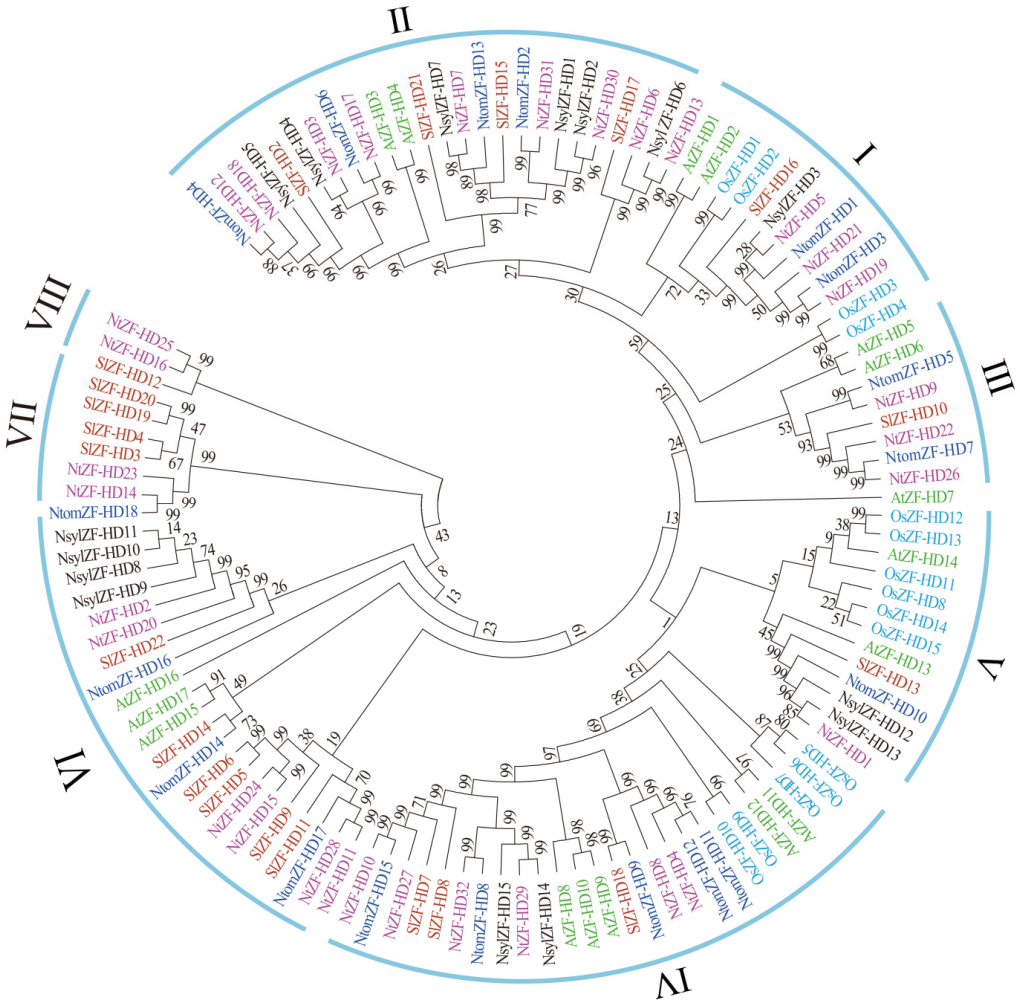

Figure 1. Phylogenetic relationship of *Arabidopsis* (AtZF-HD), tomato (SlZF-HD), rice (OsZF-HD), *N. sylvestris* (NsylZF-HD), *N. tomentosiformis* (NtomZF-HD), and tobacco (NtZF-HD) ZF-HD members, divided into six groups. The phylogenetic tree was generated using MEGA7 with the neighbor-joining method. The number of bootstrap values was set to 1000 replications. ZF-HD: Zinc finger homeodomain.

To further investigate the structural characteristics of the ZF-HD proteins in tobacco of the ZF-HD proteins in tobacco, the conserved motifs of the 32 NtZF-HD, 17 AtZF-HD, and 22 SlZF-HD proteins were analyzed using the online MEME tool. A total of 10 conserved motifs were identified and named as motifs 1 to 10 (Figure S2C). These motifs contained 20 to 49 amino acids, and their amino acid sequences are shown in the Supplementary Table S4. The NtZF-HD proteins were clustered into the same group that usually has a similar motif distribution, which is consistent with that of the SlZF-HD proteins. Notably, all NtZF-HD proteins have motif 3, and all proteins except for NtZF-HD12 have motif 1. Interestingly, similar conditions exist in SlZF-HD proteins, with only one protein, SlZF-HD1, without motif 1. Moreover, some conserved motifs existed only in specific groups. For example, motif 5 and motif 6 were only presented in group IV with a high specificity, while they could only be found in groups II and III. The specific distribution of conserved motifs in each group may reflect the specific functions of NtZF-HD genes in tobacco.

3.4. Syntenic Analysis of NtZF-HD Genes

To further understand the evolutionary relationship among the tobacco *ZF-HD* genes, the syntenic analysis was carried out for tobacco and five other plant species, including dicotyledonous plants (*Arabidopsis*, tomato, and grape) and monocotyledonous plants (rice and maize) (Figure 2A). The results showed that there was a syntenic relationship between

six of the *NtZF-HD* genes with *ZF-HD* genes in *Arabidopsis*, six *ZF-HD* genes in grape, four *ZF-HD* genes in tomato, two *ZF-HD* genes in maize, and two *ZF-HD* genes in rice. The numbers of predicted collinear pairs between tobacco and *Arabidopsis*, tomato, grape, rice, and maize were seven, seven, six, three, and two, respectively. Meanwhile, only one *ZF-HD* gene (*NtZF-HD5*) was predicted to form collinear pairs with *ZF-HD* genes of all the other five species, suggesting that this *ZF-HD* gene may exist before the differentiation of these species and has maintained a collinear relationship since then. Interestingly, one collinear gene pair was identified between tobacco and tomato/grape/*Arabidopsis* species, but no collinear gene pairs were found in the rice and maize genome, indicating that this pair may have appeared after the divergence of dicot and monocot (Figure 2B, Table S5).

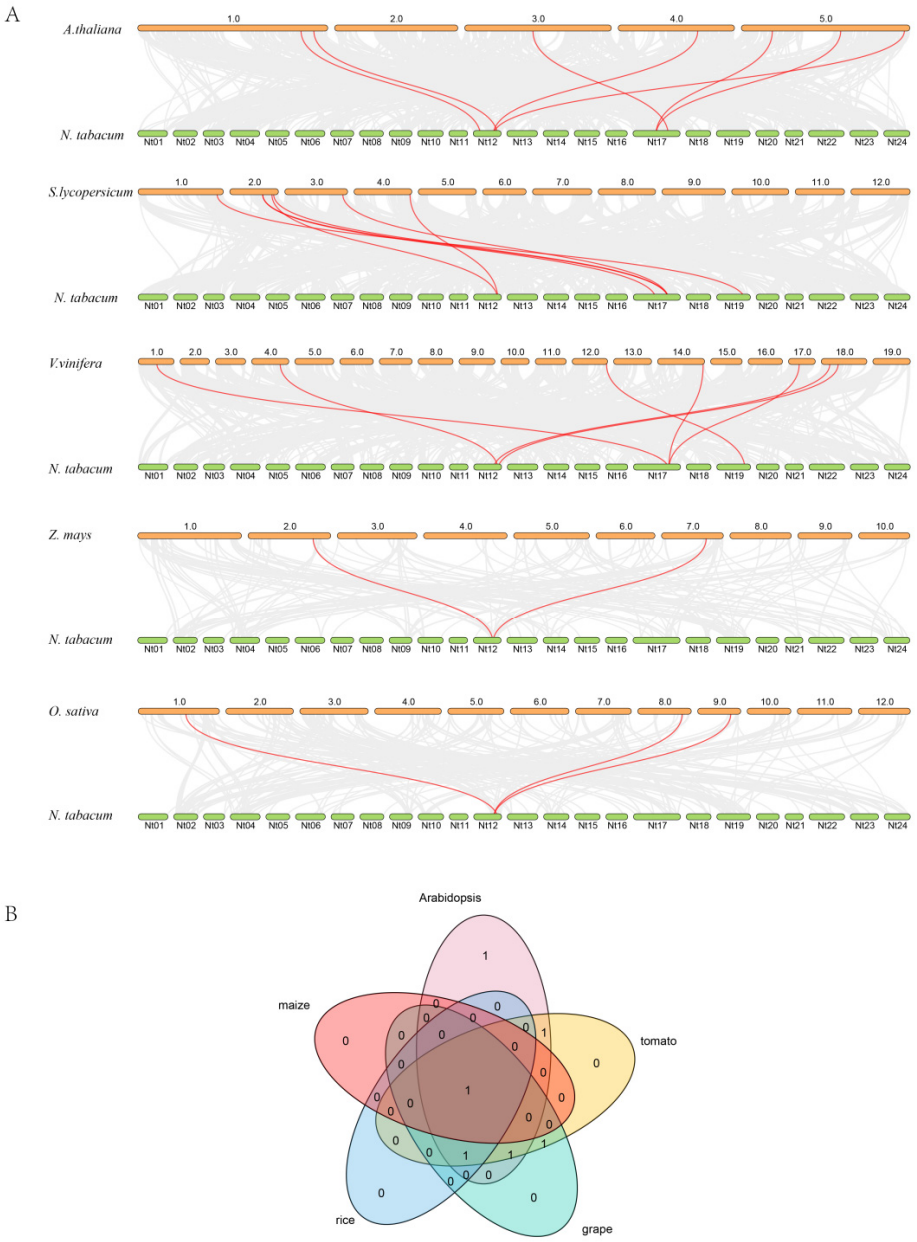

Figure 2. Syntenic analysis of *ZF-HD* genes between tobacco and five other representative species. (**A**) The gray line in the background represents the colinear blocks between tobacco and five other representative species, while the red line represents the syntenic *ZF-HD* gene pairs. (**B**) The numbers of *ZF-HD* genes that formed syntenic pairs between *ZF-HD* and the other four selected species, which was visualized by the Venn diagram.

3.5. Promoter Analysis of NtZF-HD Family Genes

To further investigate the potential response patterns of *ZF-HD* genes, *cis*-elements in promoter regions of 32 *NtZF-HD* gene were analyzed by the online PlantCARE tool (Figure 3, Table S6). One or more MYB binding sites (v-myb avian myeloblastosis viral oncogene homolog) were identified in promoters of all the *NtZF-HD* genes, except *NtZF-HD5* and *NtZF-HD11*. In addition, the hormone-response elements were also identified in promoter regions, including estrogen response element (ERE), abscisic acid response element (ABRE), TCA-element and CGTCA-motif. Furthermore, 17 of the *NtZF-HD* genes contain at least one wound-responsive element (WUN-motif), 15 of the *NtZF-HD* genes contain at least one anaerobic induction element (ARE), and 11 of the *NtZF-HD* genes contain at least one stress-responsive element (TC-rich repeats), implying that these *NtZF-HD* genes may be involved in various stress responses.

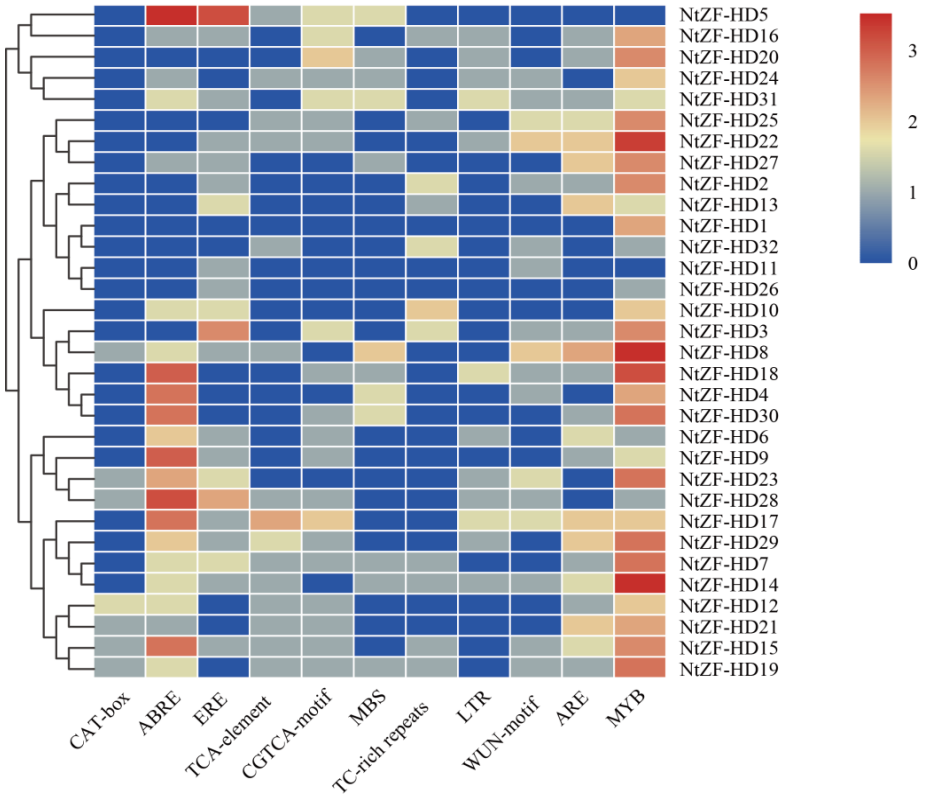

Figure 3. Statistics diagram of *cis*-elements in the promoter region of NtZF-HD genes. The colors represent the numbers of every *cis*-element in NtZF-HD family genes.

3.6. Expression Profiles of NtZF-HD Family Genes

To preliminarily elucidate the roles of *NtZF-HD* genes in tobacco growth and development, the relative expression levels of *NtZF-HD* genes in six different tissues including young leaves, mature leaves, aging leaves, roots, stems, and flowers were analyzed using RT-qPCR (Figure S2). A number of the *NtZF-HD* genes were expressed in a tissue-specific manner; for example, in group I, *NtZF-HD19* was highly expressed in young leaves and flowers. In group II, *NtZF-HD7* was only expressed in root, and the expression of *NtZF-HD30* was detected in the stems exclusively. *NtZF-HD3*, *NtZF-HD13*, and *NtZF-HD16* were mainly expressed in all leaf stages. Similarly, *NtZF-HD22* of group III was found to be highly expressed in aging leaves. Other tissue-specific expression patterns were also detected; in group VI, *NtZF-HD11* and *NtZF-HD25* were highly expressed in young leaves and flowers. The tissue-specific expression pattern of these genes may be involved in the mediation of various developmental processes of tobacco.

In addition, to further investigate the expression patterns of tobacco *NtZF-HD* genes under abiotic stress, 16 *NtZF-HD* genes were detected to determine their expression levels under drought and NaCl treatment (Figure 4A,B). Genes in group I (*NtZF-HD21*) and IV (*NtZF-HD4* and *NtZF-HD27*) could be induced and up-regulated significantly under drought treatment. Furthermore, *NtZF-HD12* and *NtZF-HD18* in group II had up-regulations of the transcription levels under salt and drought treatment. Moreover, in group VIII, *NtZF-HD16* and *NtZF-HD25* were up-regulated under salt and drought treatments, whereas *NtZF-HD24* in group VI was down-regulated by salt and drought treatments. These expression analyses would provide a valuable resource for researching the roles of *NtZF-HD* genes in control of the stress responses.

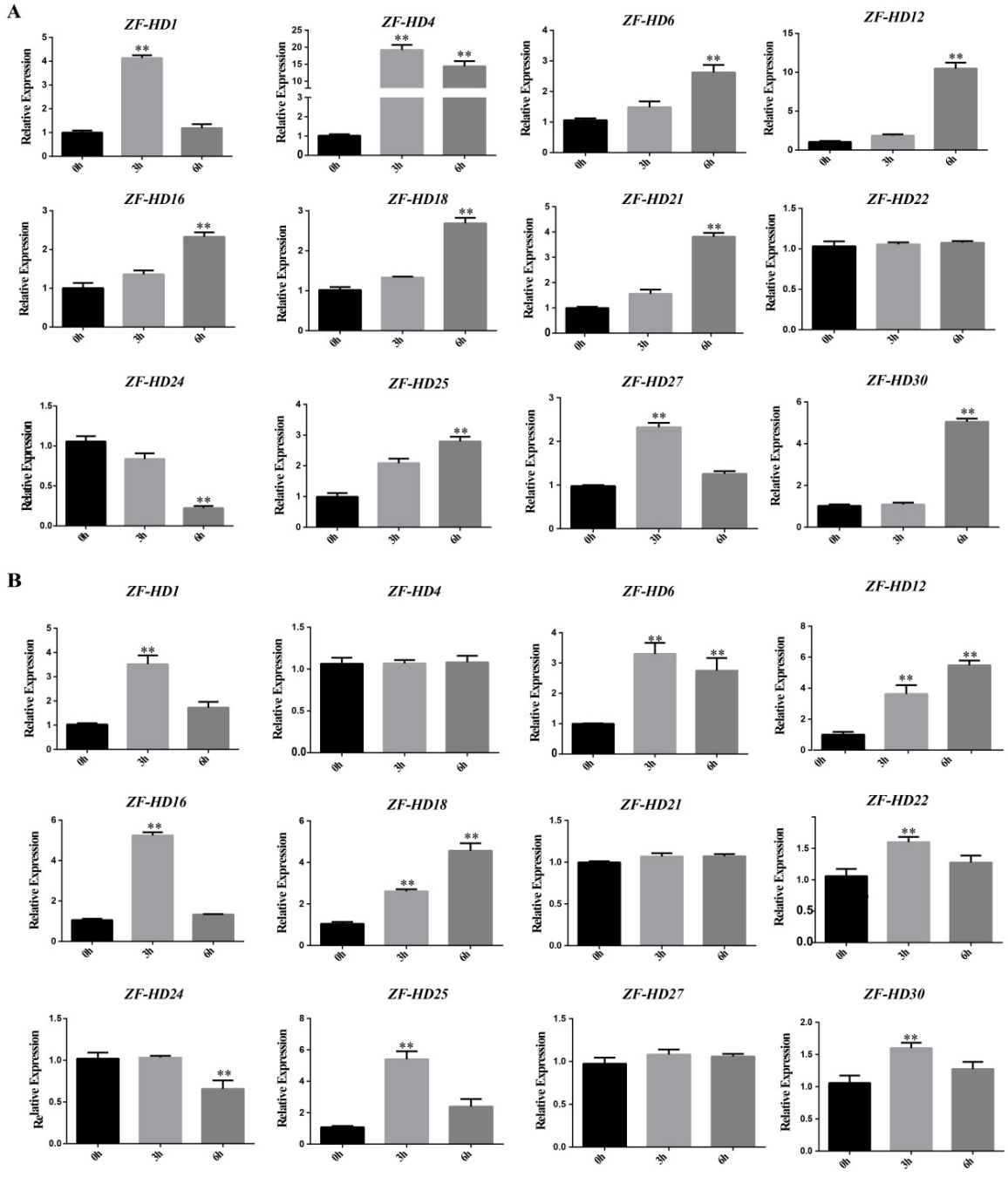

Figure 4. The expression patterns of selected *NtZF-HD* genes under (**A**) drought and (**B**) NaCl. The asterisks indicate significant statistical differences from the 0 h using the Student's *t* test ($p < 0.005$).

3.7. Subcellular Localization of NtZF-HD Proteins

According to previous reports, the ZF-HD protein, as a transcriptional activator, was located in the nucleus [18]. The prediction of subcellular localization indicated that almost all NtZF-HD proteins are located in the nucleus except for NtZF-HD14, which is located in the chloroplast and nucleus (Table S3).

To further investigate the subcellular localization of NtZF-HD proteins, NtZF-HD21 was selected to examine the subcellular localization. We constructed an NtZF-HD21-GFP fusion protein to detect the transient expression of NtZF-HD21 in the leaf of *Nicotiana benthamiana*. The NtZF-HD21-GFP fusion protein was predominantly observed in the nucleus and was able to overlap the staining signal of the dye 4,6-diamidino-2-phenylindole (DAPI) staining signal (Figure 5A). This result indicated that the NtZF-HD21 protein is a nuclear localization protein, which is consistent with its essential characteristics as a transcription factor.

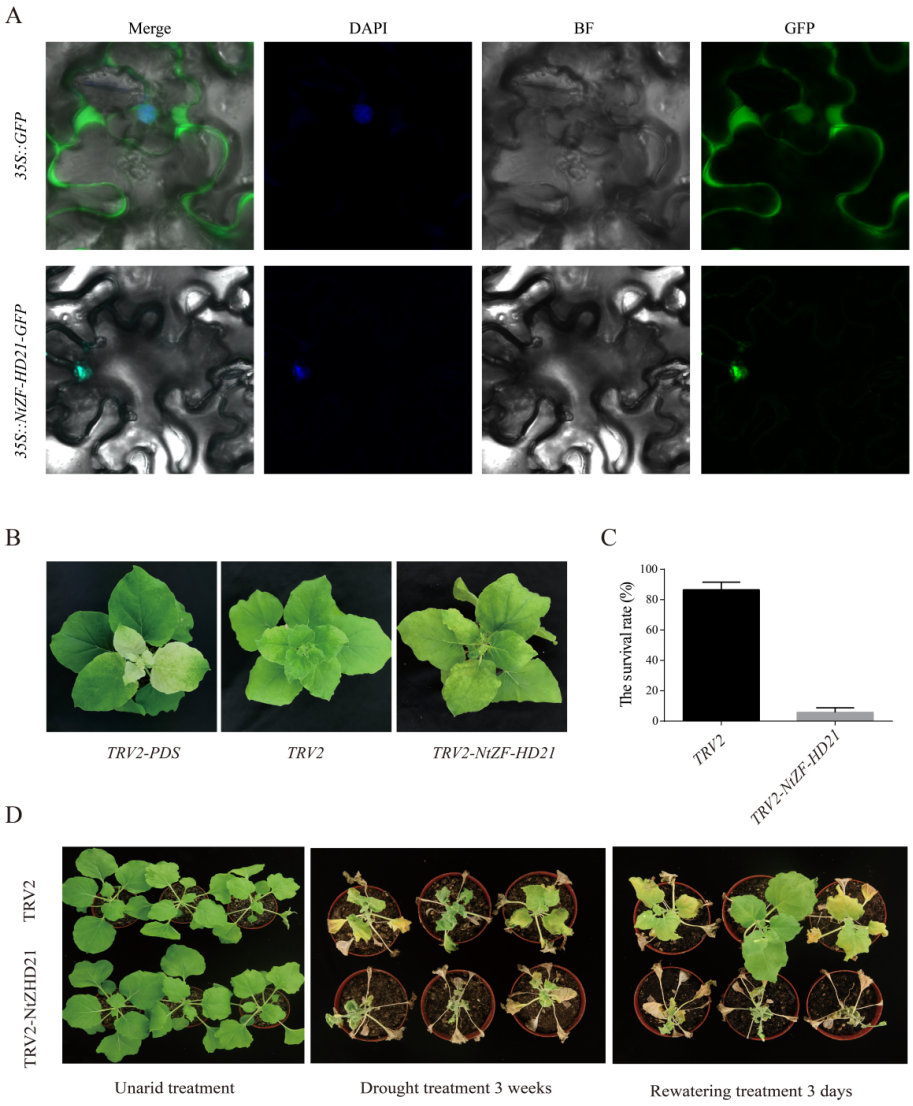

Figure 5. The subcellular localization of NtZF-HD21 and virus-induced gene silencing (VIGS) of the *NtZF-HD21* gene in *Nicotiana benthamiana*. (**A**) The subcellular localization of NtZF-HD21. The NtZF-HD21–GFP fusion construct and GFP gene driven by the CaMV-35S promoter were transiently expressed in *Nicotiana benthamiana* separately. 4,6-diamidino-2-phenylindole (DAPI) staining indicates the nucleus. (**B**) The phenotype of VIGS of the *NtZF-HD21* gene in *Nicotiana benthamiana.* (**C**) The survival rate of silent plants and negative controlled plants. (**D**) The silence of *NtZF-HD21* gene conferred the drought tolerance of tobacco.

3.8. Effect of NtZF-HD21 Gene Silencing on Drought Resistance

Virus-induced gene silencing (VIGS) is a natural mechanism of plants' defense to virus invasion at post-transcriptional gene silencing [48]. VIGS is widely used in the study of gene function related to physiological pathways such as plant stress resistance, growth and development, and metabolic regulation [49]. To further investigate the functions of NtZF-HD numbers, *NtZF-HD21* was selected to verify the function in response to drought stress by the VIGS system. The albino phenomenon of plants infected with *TRV2-PDS* as a positive control indicated that the recombinant plasmid was expressed in the plant (Figure 5B). There was no obvious difference in the phenotype between plants infected with *TRV2-NtZF-HD21* silent plants and TRV2 negative-controlled plants. Subsequently, the silent plants and negative controlled plants were treated under drought stress for three weeks before re-watering treatment for three days. The results showed that the survival rate of silent plants was significantly lower compared with that of negative-controlled plants (Figure 5C,D), suggesting that the *NtZF-HD21* gene was able to enhance the drought tolerance of tobacco.

4. Discussion

Previous research showed that ZF-HD transcription factors are only found in plants and play significant roles in plant developmental processes and various environmental stress responses [50]. A total of 32 members of the *NtZF-HD* genes were identified in the tobacco genome using BLASTP searches. The *NtZF-HD* genes were studied using phylogeny, gene structure, motif organization, synteny, cis-elements, and expression profiles.

The 32 NtZF-HD members were clustered into eight groups according to the phylogeny analysis of *Arabidopsis* and tomato NtZF-HD members. *NtZF-HD* genes of the same group contain similar gene structures and motif organization, suggesting that the previous evolutionary relationship and classification analysis of *NtZF-HD* genes were reliable. In addition, some *NtZF-HD* genes of the same group contained similar *cis*-elements types, implying that they may reflect similar functions in plant development and abiotic stress responses.

Increasing evidence indicates that *ZF-HD* genes regulate the development of plants. The *AtZF-HD5* in group III was reported to specifically regulate the size of leaves. *NtZF-HD22* and *NtZF-HD26* were clustered with *AtZF-HD5* and highly expressed in the leaves (Figures 1 and 4), suggesting its possible contribution to the growth and development of leaves. In addition, the *AtZF-HD8* in group IV was reported to be involved in flower and leaf development. The *NtZF-HD10* formed a collinear pair with *AtZF-HD8*, which expressed highly in flower and leaves, suggesting that they might have similar biological functions in flower and leaf development. Whereas, in the same group, *AtZF-HD10* was reported to regulate the growth of root [19] and clustered with *NtZF-HD4,* which highly expressed in the roots, implying that *NtZF-HD4* may be involved in root development.

Previous studies have indicated that a number of *ZF-HD* genes mediated responses to drought and salinity stresses. *AtZF-HD4* in group II, which has been reported to be involved in regulating drought and salt stress responses [25], was clustered together with *NtZF-HD6* and *NtZF-HD12.* Interestingly, the promoter regions of *NtZF-HD6* and *NtZF-HD12* were found to harbor the cis-elements of the MYB binding site and TC-rich repeats element, and these genes could be induced under drought and salt treatments. These findings suggested that these genes may confer stress responses in tobacco. Notably, the overexpression of *AtZF-HD1* altered ABA sensitivity, dehydration tolerance, and the expression levels of ABA/stress-regulated genes [22]. In group I, *NtZF-HD5*, *NtZF-HD19*, and *NtZF-HD21* were clustered with *AtZF-HD1.* In the present study, these genes were found to harbor the ABRE element (Figure 1 and Table S6), suggesting that they might participate in drought response and ABA signaling. Moreover, *NtZF-HD21* was up-regulated significantly under drought stress treatment. Furthermore, the VIGS experiment demonstrated that *NtZF-HD21* can regulate the drought tolerance of tobacco plants (Figure 5).

Overall, most *NtZF-HD* genes were expressed in different tissues and induced by drought and salinity abiotic stresses treatment in tobacco. Hence, we further suggested that *NtZF-HD* genes may play significant roles in various developmental processes and environmental stress responses in tobacco.

5. Conclusions

In this study, 32 *ZF-HD* genes were identified in *Nicotiana tabacum*. These genes were grouped into six groups based on the evolutionary analysis. Genes divided into the same group tend to have a similar exon–intron organization, motifs, and cis-acting elements. We also conducted expression patterns analysis of *NtZF-HD* genes, suggesting that they may be important for regulating plant responses to abiotic stresses and development. Notably, *NtZF-HD21* was significantly induced by drought treatments, and it was able to enhance the drought tolerance of tobacco. Overall, the results from this study will be helpful to the further investigations of the function of tobacco *NtZF-HD* genes.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/3/406/s1, Figure S1. Gene structure and motif organizations of NtZF-HD members in tobacco. Figure S2. The expression pattern of the selected *NtZF-HD* genes in different tissues. Table S1. The ZF-HD protein sequences of *Arabidopsis*, tomato, and tobacco, Table S2. The RT-qPCR primers of *NtZF-HD* genes, Table S3. The detailed information about *NtZF-HD* genes and NtZF-HD proteins in tobacco, Table S4. The amino acid sequences of motifs, Table S5. The syntenic analysis of *NtZF-HD* genes, Table S6. The predicted cis-acting elements in the promoter region of *NtZF-HD* genes.

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