



Article

Whole-Genome Analysis of ZF-HD Genes among Three *Dendrobium* Species and Expression Patterns in *Dendrobium chrysotoxum*

Xin He ^{1,2}, Xuwei Zhao ^{1,2}, Qinyao Zheng ² , Meng-Meng Zhang ^{1,2} , Ye Huang ² , Zhong-Jian Liu ^{1,2,*} and Siren Lan ^{1,2,*}

¹ College of Forestry, Fujian Agriculture and Forestry University, Fuzhou 350002, China; 5220422102@fafu.edu.cn (X.H.); zxxw6681@163.com (X.Z.); 1220428020@fafu.edu.cn (M.-M.Z.)

² Key Laboratory of National Forestry and Grassland Administration for Orchid Conservation and Utilization, College of Landscape Architecture and Art, Fujian Agriculture and Forestry University, Fuzhou 350002, China; qinyaozheng@fafu.edu.cn (Q.Z.); 5221726097@fafu.edu.cn (Y.H.)

* Correspondence: zjliu@fafu.edu.cn (Z.-J.L.); lkzx@fafu.edu.cn (S.L.)

Abstract: ZF-HD transcription factors, which are unique to land plants, are involved in the regulation of abiotic stress response and related signaling pathways, and play a crucial role in plant growth and development. *Dendrobium* is one of the largest genera of orchids, with a high ornamental and ecological value. However, the specific functions of the ZF-HDs in *Dendrobium* remain unknown. In this study, we identified a total of 53 ZF-HDs from *D. chrysotoxum* (17), *D. catenatum* (23), and *D. huoshanense* (13), and analyzed their physicochemical properties, phylogenetic relationships, chromosomal locations, protein structures, conserved motifs, and expression patterns. The phylogenetic relationships revealed that 53 ZF-HDs were classified into six subfamilies (ZHDI-V and MIF), and all ZF-HD proteins contained motif 1 and motif 4 conserved domains, while a minority of these proteins had exons. The analysis of *cis*-elements in the promoters of ZF-HDs from three *Dendrobium* species showed that growth- and development-related elements were the most prevalent, followed by hormone response and abiotic stress response elements. Through collinearity analysis, 14 *DchZF*-HDs were found to be collinear with *DhuZF*-HDs, and 12 *DchZF*-HDs were found to be collinear with *DcaZF*-HDs. Furthermore, RT-qPCR analysis revealed that *DchZF*-HDs play a regulatory role in the development of lateral organs during the flowering process. The results indicated that *DchZHDI2* plays a role in the unpigmented bud stage, while *DchMIF8* and *DchZHDI16* play significant roles during the pigmented bud and initial bloom stages. Hence, this study provides a crucial basis for further exploring ZF-HDs functions in regulating the floral organs of orchids.

Keywords: ZF-HD gene family; *Dendrobium*; genome-wide identification; flower organ development



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1. Introduction

Initially discovered as potential modulators of the C4 phosphoenolpyruvate carboxylase gene, known as PEP-Case, ZF-HD proteins have garnered attention within C4 species [1]. Zinc finger-homeodomain (ZF-HD) transcription factors have two main domains: the C2H2-type ZF domain situated at the N-terminal, and the HD domain located at the C-terminal [2]. According to the different conserved domains, the ZF-HD gene family can be classified into ZHD and MIF subfamilies [3,4], where the MIF subfamily exclusively consists of the ZF domain [5]. ZF-HD transcription factors have a significant impact on regulating seed germination [6], flower development [7], and stress reactions [8].

Recently, bioinformatics has been conducted on the ZF-HD transcription factors of many plants, revealing their expression patterns in various aspects of growth and development, such as *Arabidopsis thaliana* [2], *Oryza sativa* [9], *Brassica rapa* [10], and *Vitis vinifera* [11]. ZF-HD transcription factors have been shown to be important for the early

stages of flower development [12]. The 14 ZF-HDs detected in *A. thaliana* were all expressed in the flower, and 6 *AtZF-HDs* did not produce any noticeable floral phenotypes when expressed individually, indicating that *AtZF-HDs* probably perform convergent functions in controlling the development of flowers, thereby hinting at their potential overlapping roles in floral regulation [13]. *CsZF-HD5* is highly expressed in *Camellia sinensis* flower tissues, suggesting that it plays a key role in flower development [14]. In the presence of abiotic stress, ZF-HD genes play a pivotal regulatory role. Research on the *O. sativa* ZF-HD gene family has shown that the overexpression of *OsZHD1* affects the development of *O. sativa* leaves [15], and the four *OsZHDs* bound by *OsDREB1B* promoter can be induced by drought stress and low temperatures in *O. sativa* [16]. Moreover, the expression of ZF-HD transcription factors in *Triticum aestivum* and *Brassica rapa* was significantly upregulated under abiotic stress [10,17].

Orchidaceae rank among the largest and most geographically diverse groups within the realm of flowering plants, boasting an excess of 28,000 species [18]. *Dendrobium* is a large genus in the family Orchidaceae, with significant ornamental and medicinal values [19]. Flowers, which are considered an important ornamental trait, have been widely studied in *Dendrobium* [20]. As sequencing technology continues to advance, numerous *Dendrobium* genomes have been documented, including those of *D. chrysotoxum* [21], *D. nobile* [22], *D. huoshanense* [23], and *D. catenatum* [24]. These data have laid a solid foundation for exploring the key biological characteristics of *Dendrobium*. Previous studies have found that a variety of transcription factors occupy a crucial position in the floral development, growth, and adaptation to adversity of *Dendrobium* [25,26]. Despite extensive research on the ZF-HD gene family's role in other plants, there is still a scarcity of reports focusing on *Dendrobium*.

In this study, we analyzed ZF-HDs in *D. chrysotoxum*, *D. huoshanense*, and *D. catenatum*, encompassing physicochemical properties, gene structure, phylogenetic tree construction, collinearity analysis, protein structure, and *cis*-elements. Specifically, the study focused on the regulatory role of the ZF-HD transcription factors on the flower of *D. chrysotoxum*. Our research provides new insights on the molecular mechanisms and morphological diversity of *Dendrobium* flower organ development. The results of this study may also contribute to broadening our understanding of the role of ZF-HD transcription factors in regulating flower organ development.

2. Results

2.1. Identification and Analysis of ZF-HD Proteins

ZF-HD proteins from three *Dendrobiums* were initially obtained using Blast and HMMER searches. A total of 17 *DchZF-HDs*, 23 *DcaZF-HDs*, and 13 *DhuZF-HDs* were named consecutively from top to bottom utilizing their chromosomal locations. The amino acids (AAs) of ZF-HDs varied from 85 aa (*DchZHD9*, *DcaZHD8*) to 858 aa (*DhuZHD4*), with an average length of 250 aa. The grand average of hydrophilic values (GRAVY) of the ZF-HDs ranged from -0.977 (*DhuZHD9*) to -0.293 (*DhuZHD4*), and the whole ZF-HDs showed GRAVY values less than 0, suggesting that all ZF-HD proteins were hydrophilic. The theoretical isoelectric point (pI) ranged from 5.36 (*DhuZHD6*) to 10.17 (*DhuZHD9*). The isoelectric points (pI) of 4 ZF-HDs were less than 7.0, which was acidic, and the isoelectric points of the remaining 49 ZF-HDs were more than 7.0, which was alkaline. The molecular weight (MW) of 53 ZF-HDs ranged from 9.38 kDa (*DchZHD15*) to 92.47 kDa (*DhuZHD4*), with an average MW of 27.58 kDa. The aliphatic index (AI) ranged from 36.08 (*DcaMIF7*) to 80.65 (*DhuZHD4*). The instability index (II) ranged from 46.94 (*DhuZHD5*) to 84.44 (*DcaZHD1*), indicating that most ZF-HD proteins are unstable. In addition, subcellular localization prediction revealed that the ZF-HD proteins of the three *Dendrobiums* were predominantly localized to the nucleus, pointing to a possible alike with most transcription factors (Table 1). The protein sequence of ZF-HDs from three *Dendrobiums* are shown in Table S1.

Table 1. The physicochemical properties of ZF-HD proteins from three *Dendrobium* species.

Name	Gene ID	AA ¹	GRAVY ²	pI ³	Mw ⁴ (kDa)	AI ⁵	II ⁶	Subcellular Localization ⁷
<i>DchZHD1</i>	<i>Maker81525</i>	216	−0.593	8.73	23.70	57.55	60.85	Nucleus.
<i>DchZHD2</i>	<i>Maker28129</i>	216	−0.593	8.73	23.70	57.55	60.85	Nucleus.
<i>DchZHD3</i>	<i>Maker69363</i>	217	−0.767	8.96	24.50	57.60	83.88	Nucleus.
<i>DchZHD4</i>	<i>Maker78792</i>	314	−0.787	8.25	34.50	51.56	66.90	Nucleus.
<i>DchZHD5</i>	<i>Maker102972</i>	221	−0.871	8.68	25.19	48.55	69.86	Nucleus.
<i>DchZHD6</i>	<i>Maker82648</i>	297	−0.666	6.37	32.51	59.19	53.96	Nucleus.
<i>DchZHD7</i>	<i>Maker74870</i>	286	−0.598	9.07	31.28	59.34	57.57	Nucleus.
<i>DchMIF8</i>	<i>Maker100805</i>	126	−0.917	9.04	13.68	39.60	67.20	Mitochondrial.
<i>DchMIF9</i>	<i>Maker57367</i>	129	−0.788	7.61	14.24	46.20	64.53	Mitochondrial.
<i>DchZHD10</i>	<i>Maker92643</i>	249	−0.691	8.99	27.41	60.00	61.99	Nucleus.
<i>DchZHD11</i>	<i>Maker54517</i>	450	−0.471	8.87	49.31	67.62	48.02	Cytoplasmic.
<i>DchZHD12</i>	<i>Maker53183</i>	249	−0.623	8.99	27.72	59.16	61.03	Nucleus.
<i>DchZHD13</i>	<i>Maker57691</i>	232	−0.570	9.57	25.57	70.13	57.09	Nucleus.
<i>DchZHD14</i>	<i>Maker77088</i>	198	−0.622	9.24	22.43	60.10	70.26	Cytoplasmic.
<i>DchZHD15</i>	<i>Maker61228</i>	85	−0.326	8.70	9.38	55.18	60.76	Chloroplast.
<i>DchZHD16</i>	<i>Maker61267</i>	293	−0.670	7.63	31.52	60.00	57.07	Nucleus.
<i>DchZHD17</i>	<i>Maker58363</i>	273	−0.666	7.05	28.88	49.52	49.81	Nucleus.
<i>DhuZHD1</i>	<i>Dhu000007045</i>	198	−0.648	9.12	22.64	56.16	72.97	Nucleus.
<i>DhuZHD2</i>	<i>Dhu000019223</i>	198	−0.648	9.12	22.64	56.16	72.97	Nucleus.
<i>DhuZHD3</i>	<i>Dhu000028169</i>	146	−0.770	9.65	17.02	46.16	81.27	Nucleus.
<i>DhuZHD4</i>	<i>Dhu000024291</i>	858	13.510	9.64	92.47	80.65	47.66	Cytoplasmic.
<i>DhuZHD5</i>	<i>Dhu000009472</i>	280	−0.386	5.48	30.32	73.86	46.94	Nucleus.
<i>DhuZHD6</i>	<i>Dhu000013154</i>	390	−0.381	5.36	41.72	66.54	48.07	Cytoplasmic.
<i>DhuMIF7</i>	<i>Dhu000014526</i>	260	−0.955	5.39	28.60	45.12	68.88	Nucleus.
<i>DhuZHD8</i>	<i>Dhu000009060</i>	250	−0.638	8.79	27.01	57.80	54.54	Nucleus.
<i>DhuZHD9</i>	<i>Dhu000025178</i>	626	−0.977	10.17	70.34	57.68	65.98	Nucleus.
<i>DhuZHD10</i>	<i>Dhu000014745</i>	216	−0.584	8.73	23.75	58.01	62.13	Nucleus.
<i>DhuZHD11</i>	<i>Dhu000004498</i>	216	−0.581	8.73	23.74	58.01	62.27	Nucleus.
<i>DhuZHD12</i>	<i>Dhu000014369</i>	125	−0.942	9.04	13.54	37.60	70.65	Mitochondrial.
<i>DhuZHD13</i>	<i>Dhu000016048</i>	219	−0.762	8.54	24.84	52.10	59.08	Nucleus.
<i>DcaZHD1</i>	<i>rna-XM_020822518.2</i>	218	−0.855	8.77	24.54	56.01	84.44	Nucleus.
<i>DcaZHD2</i>	<i>rna-XM_020823161.2</i>	263	−0.719	8.92	29.55	58.56	66.36	Nucleus.
<i>DcaZHD3</i>	<i>rna-XM_020825047.1</i>	222	−0.725	8.55	25.09	50.99	59.33	Nucleus.
<i>DcaMIF4</i>	<i>rna-XM_020824991.2</i>	129	−0.736	7.60	14.16	49.22	65.82	Mitochondrial.
<i>DcaZHD5</i>	<i>rna-XM_020824023.2</i>	216	−0.595	8.73	23.72	57.13	62.13	Nucleus.
<i>DcaZHD6</i>	<i>rna-XM_020838304.2</i>	249	−0.646	8.92	26.89	57.67	50.52	Nucleus.
<i>DcaMIF7</i>	<i>rna-XM_020825579.2</i>	125	−0.962	9.04	13.51	36.08	70.65	Mitochondrial.
<i>DcaZHD8</i>	<i>rna-XM_020842089.1</i>	85	−0.420	8.10	9.68	52.82	51.73	Chloroplast.
<i>DcaZHD9</i>	<i>rna-XM_020842002.2</i>	293	−0.683	7.64	31.54	57.99	56.76	Nucleus.
<i>DcaZHD10</i>	<i>rna-XM_020831316.1</i>	200	−0.684	8.17	21.60	45.00	67.27	Nucleus.
<i>DcaZHD11</i>	<i>rna-XM_020837938.2</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD12</i>	<i>rna-XM_020837921.2</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD13</i>	<i>rna-XM_020837895.2</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD14</i>	<i>rna-XM_020837890.2</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD15</i>	<i>rna-XM_028698070.1</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD16</i>	<i>rna-XM_020837900.2</i>	249	−0.650	8.99	27.69	57.99	59.00	Nucleus.
<i>DcaZHD17</i>	<i>rna-XM_020825507.2</i>	258	−0.748	9.15	28.94	58.29	63.93	Nucleus.
<i>DcaZHD18</i>	<i>rna-XM_020846562.2</i>	199	−0.611	9.24	22.74	54.42	74.46	Nucleus.
<i>DcaZHD19</i>	<i>rna-XM_020841238.2</i>	285	−0.604	9.07	31.21	60.56	58.07	Nucleus.
<i>DcaZHD20</i>	<i>rna-XM_020831512.2</i>	264	−0.762	6.59	28.25	47.01	59.03	Nucleus.
<i>DcaZHD21</i>	<i>rna-XM_020848621.2</i>	273	−0.675	7.05	28.91	49.16	49.50	Nucleus.
<i>DcaZHD22</i>	<i>rna-XM_020845498.2</i>	312	−0.747	8.53	34.04	52.56	65.46	Nucleus.
<i>DcaZHD23</i>	<i>rna-XM_020823387.2</i>	147	−0.784	9.79	17.17	46.53	81.65	Nucleus.

¹ AA, amino acid; ² GRAVY, grand average of hydrophobicity; ³ pI, theoretical isoelectric point; ⁴ Mw, molecular weight; ⁵ AI, aliphatic index; ⁶ II, instability index; ⁷ subcellular localization predicted by WoLF PSORT [27].

2.2. Phylogeny and Classification of ZF-HDs

We used 70 ZF-HD protein sequences from *D. chrysotoxum*, *D. huoshanense*, *D. catenatum*, and *A. thaliana*, to construct the neighbor-joining (NJ) phylogenetic tree. Based on the *AtZF-HDs* classification, the 53 *Dendrobium* ZF-HDs were divided into ZHD and MIF subfamilies, including 17 *DchZF-HDs*, 13 *DhuZF-HDs*, and 23 *DcaZF-HDs*. The ZHD subfamilies could be divided into ZHDI, ZHDII, ZHDIII, ZHDIV, and ZHDV subfamilies. The ZHDI subfamily had the most members (17), while the ZHDV subfamily had the fewest members (5) (Figure 1).

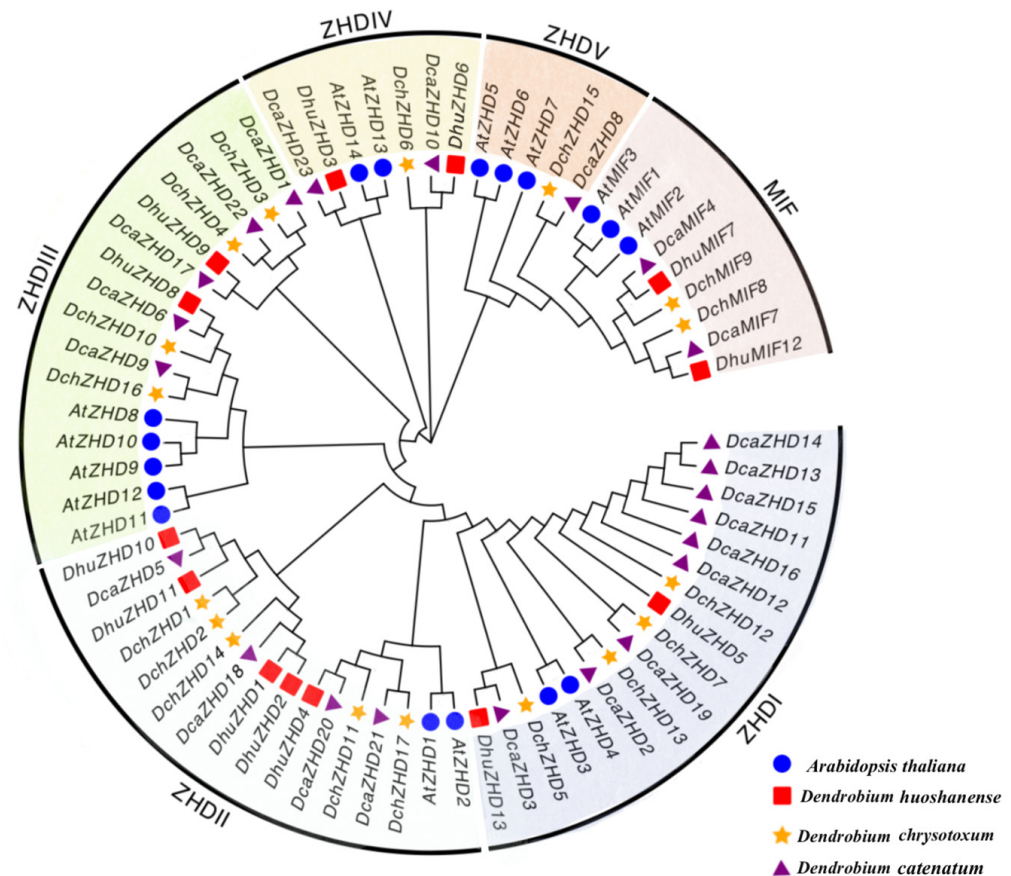


Figure 1. Phylogenetic tree generated for the 70 ZF-HDs retrieved from *D. chrysotoxum*, *D. catenatum*, *D. huoshanense*, and *A. thaliana*.

2.3. ZF-HD Structure and Motif Analysis

The MEME online tool was utilized to predict the conserved motifs of the ZF-HD proteins. The findings exhibited that the bulk of conserved motifs were located in the C-terminal domain, and the designated sequence of motifs began with 6, continued with 9, 1, 4, 10, 7, 5, 2, 3, and concluded with 8 (Figure 2B). In the ZF-HD domain, motif 1 and motif 4 were present in all ZF-HD proteins, which may be active regions for performing functions (Figure 2D). Conserved motifs from the same subfamily were similar, motif 5 was observed only in the ZHDI subfamily, and motif 10 only existed in the ZHDII subfamily. In addition, to delve deeper into the characteristics of ZF-HD genes, an intricate analysis of their intron–exon structure was executed, and the resulting structures were visualized by TBtools. Most of the 70 ZF-HDs did not contain introns, accounting for 71.7%, although a limited number of genes contained one to five introns (Figure 2C). A total of 13.2% of ZF-HDs had two exons and one intron.

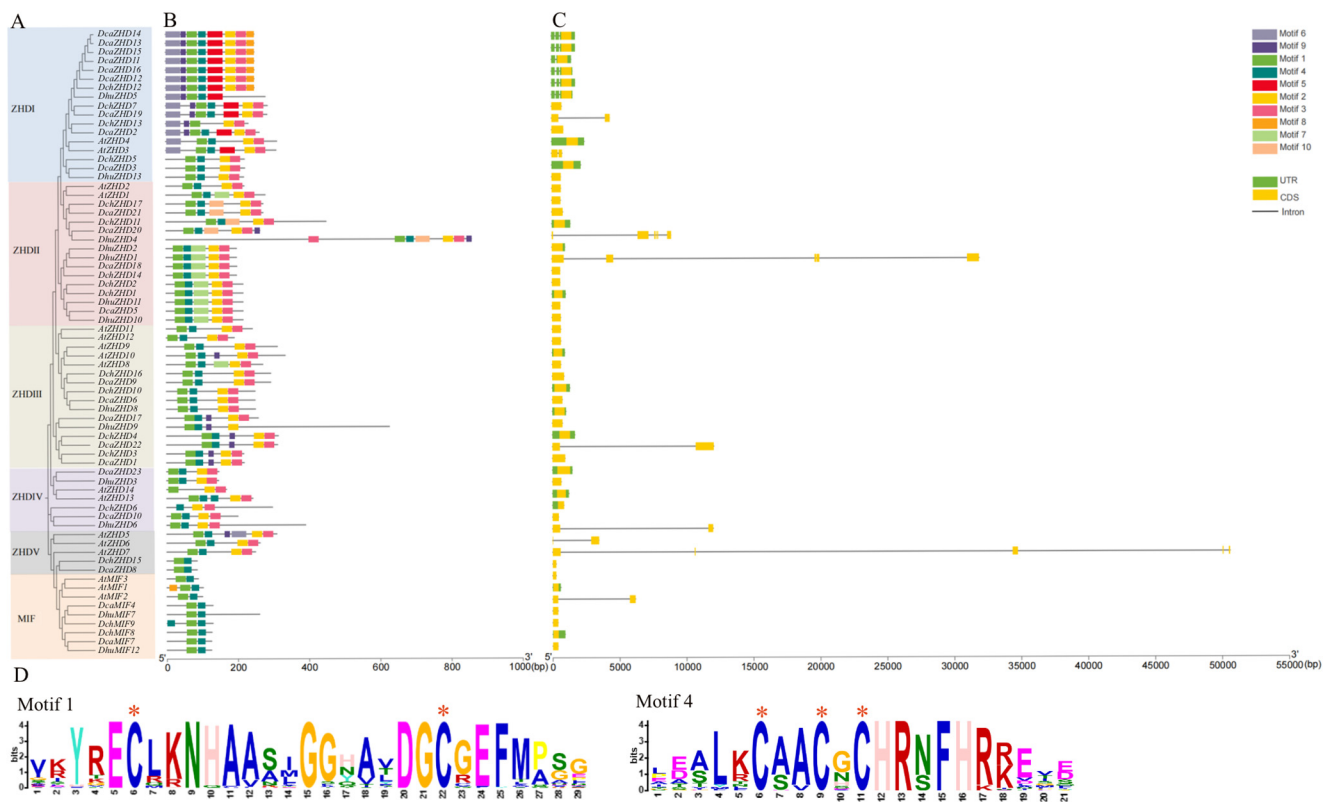


Figure 2. The motif and gene structure of ZF-HDs in *D. chrysotoxum*, *D. catenatum*, *D. huoshanense*, and *A. thaliana*. (A) Phylogenetic tree of 70 ZF-HDs constructed using MEGA7.0. (B) The conserved motif of ZF-HD proteins. (C) The ZF-HDs structure. (D) The sequence information for motif 1 and motif 4 (* indicates highly conserved cysteine residues).

2.4. Chromosomal Localization, Collinearity Analysis, and Promoter Analysis of ZF-HDs

In total, 17 *DchZF-HDs* and 13 *DhuZF-HDs* were distributed on chromosomes, and 23 *DcaZF-HDs* were localized to the unanchored scaffold. Seventeen *DchZF-HDs* were located on ten chromosomes, with Chr10 containing the greatest number of *DchZF-HDs* (5) (Figure 3A). Thirteen *DhuZF-HDs* were located on nine chromosomes, with Chr3 containing the greatest number of *DchZF-HDs* (3) (Figure 3B). In addition, Chr2 and Chr12 contained two *DhuZF-HDs*; Chr5, Chr7, Chr9, Chr10, Chr14, and Chr17 all contained only one *DhuZF-HD*. Twenty-three *DcaZF-HDs* were distributed on 17 scaffolds, with NW_021319500.1 containing the greatest number of *DcaZF-HDs*, specifically six genes in total (Figure 3C). All three *Dendrobium* species had gene tandem repeats, with *D. chrysotoxum* and *D. huoshanense* exhibiting two pairs of gene tandem repeats each. *D. catenatum* had six gene tandem repeats on NW_021319500.1 and a pair of gene tandem repeats on NW_021319360.1.

Collinearity analyses of ZF-HDs in three *Dendrobium* species were conducted. More collinear gene pairs were observed between the genomes of *D. chrysotoxum* and *D. huoshanense* than between those of *D. chrysotoxum* and *D. catenatum*. In total, 14 *DchZF-HDs* were collinear with *DhuZF-HDs*, and 12 *DchZF-HDs* were collinear with *DcaZF-HDs*. These results suggest that ZF-HD genes in *D. chrysotoxum* are more closely related to *DhuZF-HDs* than *DcaZF-HDs* (Figure 4).

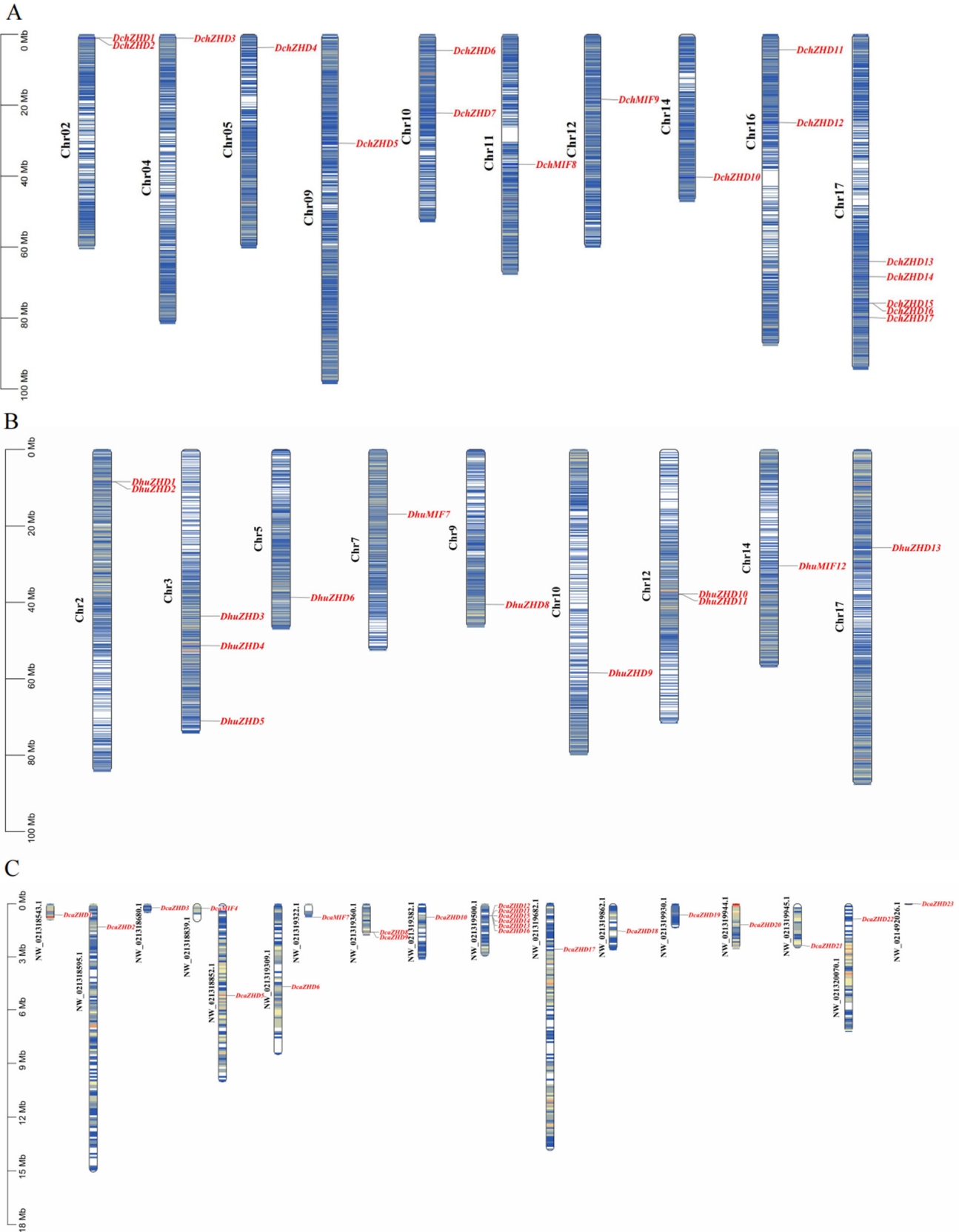


Figure 3. The ZF-HDs distribution on the chromosomes of three *Dendrobiums*. (A) *D. chrysotoxum*, (B) *D. catenatum*, and (C) *D. huoshanense*. Black designates the name of chromosomes, and red is employed to indicate the name of ZF-HDs.

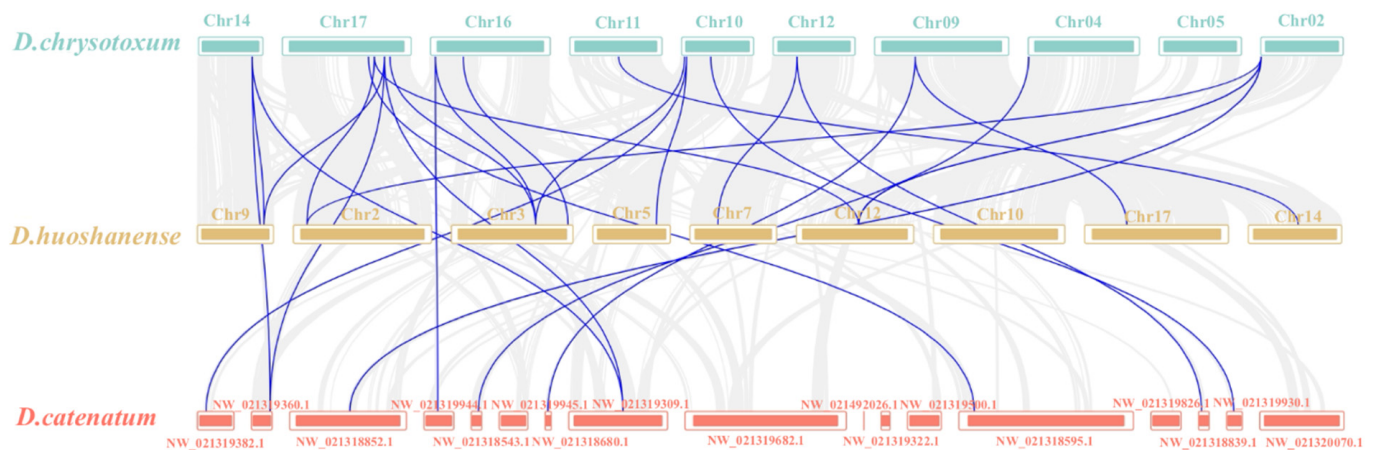


Figure 4. Collinearity analysis of ZF-HDs in three *Dendrobium*s.

To investigate the underlying functions of ZF-HDs in three *Dendrobium* species, an examination of *cis*-elements within the promoter regions of ZF-HDs was conducted using PlantCARE. *DchZF-HDs* contained 872 *cis*-elements, *DcaZF-HDs* harbored 1142 *cis*-elements, and *DhuZF-HDs* exhibited 785 *cis*-elements. A further analysis showed that, within the 17 *DchZF-HDs*, light-responsive elements accounted for 25.8%, growth and development-related elements made up 37.4%, plant hormone-responsive elements comprised 18.6%, and abiotic stress-responsive elements constituted 18.2% (Table S2). Notably, the AT~TATA-box element was the most abundant, with a total of 15 elements in *DchZHD17*. Among the 13 *DhuZF-HDs*, light-responsive elements accounted for 17.6%, growth and development-related elements for 35.9%, plant hormone-responsive elements for 24.3%, and abiotic stress-responsive elements for 17.6%. The AT~TATA element appeared most frequently, with a total of 62 elements in *DhuZHD9*. Among the 23 *DcaZF-HDs*, light-responsive elements constituted 21.2%, growth- and development-related elements 30.3%, plant hormone-responsive elements 24.4%, and abiotic stress-responsive elements 24.1%. The AT~TATA-box element was also the most numerous, with a total of 15 elements in *DcaZHD10* (Figure 5). To sum up, *cis*-elements related to plant growth response were the most frequently occurring in *Dendrobium*, followed by light-responsive elements, phytohormone-related elements, and abiotic stress-related elements, indicating that ZF-HDs are particularly important in regulating plant growth and development. In addition, the ZF-HDs of three *Dendrobium* species contain a large amount of MeJA-responsive elements, which is a hormone involved in plant signaling. Previous studies have shown that MeJA maybe be used against salt stress, drought stress, low temperature stress, heavy metal stress, and toxicities of other elements [28]. Therefore, ZF-HDs may play an important role in influencing stress tolerance in *Dendrobium* species. We also found meristem expression-related elements in three *Dendrobium* species, indicating that ZF-HDs are important for meristem development.

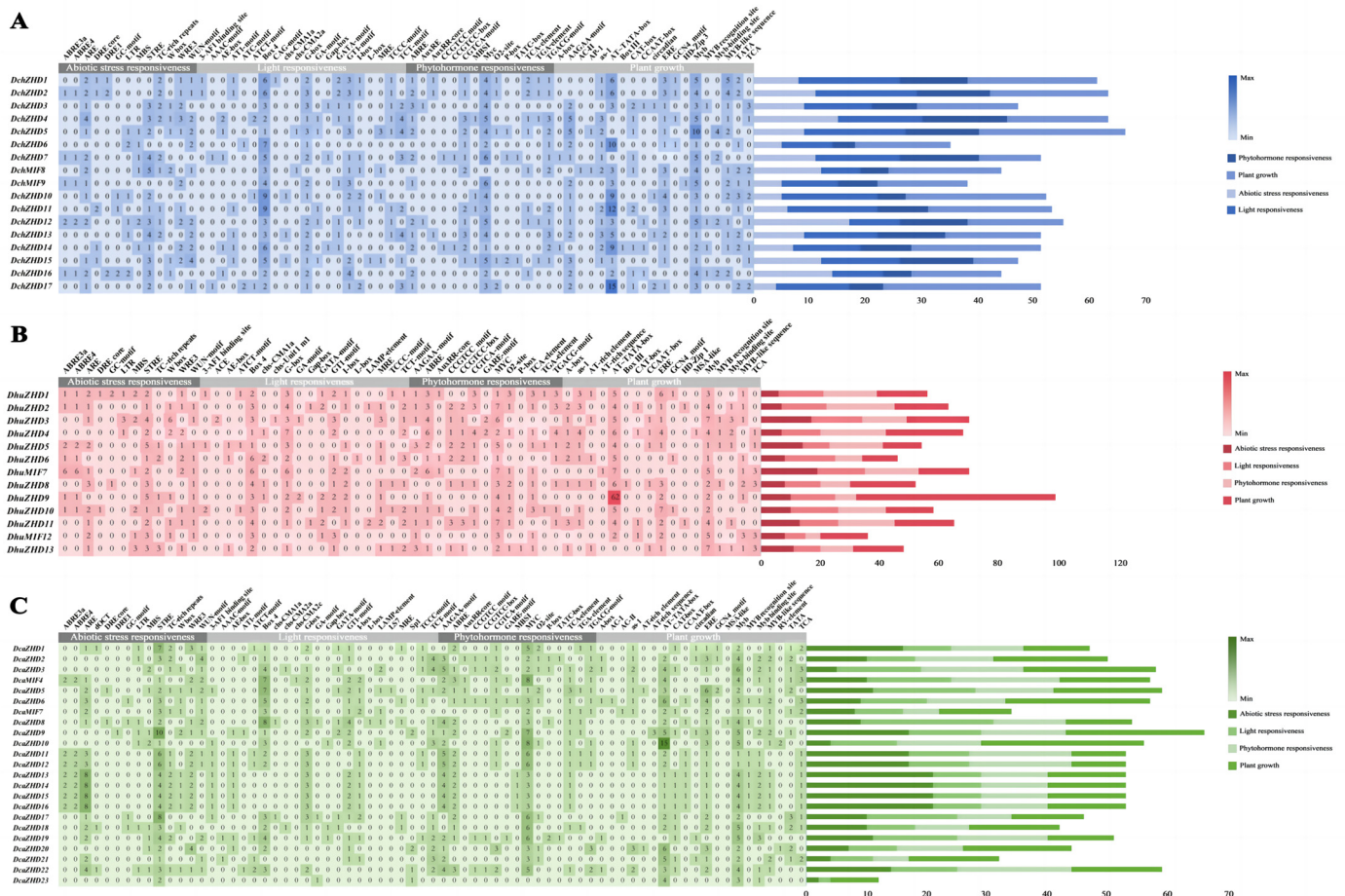


Figure 5. Analysis of *cis*-element components of the promoter of ZF-HDs across three *Dendrobium*s. (A) *D. chrysotoxum*, (B) *D. huoshanense*, and (C) *D. catenatum*. The left segment represents the count of *cis*-elements in ZF-HDs, while the right segment provides the statistical analysis of different categories of ZF-HDs. The types and numbers of ZF-HDs are listed in Table S2.

2.5. Prediction of Protein Structure

An analysis of ZF-HD proteins in three *Dendrobium* species revealed random coil as the main secondary structure, followed by α -helix, extended strand, and β -turn (Table S3). Using SWISS-MODEL for homology modeling, the tertiary structures of ZF-HD proteins from *D. chrysotoxum* (Figure S1), *D. catenatum* (Figure S2), and *D. huoshanense* (Figure S3) were predicted. The homology between the three *Dendrobium* species and the modeling templates exceeded 70%, indicating a strong structural similarity and high model reliability. Among SWISS-MODEL metrics, GMQE correlated positively with 3D model quality [29]. *D. chrysotoxum* and *D. catenatum* GMQE values exceeded 0.5, indicating good modeling, while four ZF-HD proteins in *D. huoshanense* had scores below 0.5 (*DhuZHD4*, *DhuZHD6*, *DhuMIF7*, and *DhuZHD9*), indicating high variability (Table S4).

2.6. Expression Pattern of ZF-HDs in *D. chrysotoxum*

Based on FPKM values, we drew a heatmap of ZF-HDs (Figure 6). The results showed that *DchZHD2* was highly expressed in the S1 stage, especially in the sepal. Conversely, *DchMIF8* and *DchZHD16* were highly expressed in the S2 and S3 stages, with *DchMIF8* exhibiting the highest expression in the sepal in the S2 stage and *DchZHD16* having the highest expression in the gynostemium in the S3 stage. *DchZHD6* is almost non-expressive, indicating that it may have lost its regulation of floral organ development. In addition, the study found that genes from the same subfamily have similar expression patterns. Consistent expression patterns were observed in *DchMIF8* and *DchMIF9* belonging to the

MIF subfamily, as well as *DchZHD1* and *DchZHD2* belonging to the ZHDII subfamily. Overall, most *DchZF-HDs* were generally highly expressed in the S1 stage, while their expression decreased in the later stages of floral organ development. The temporal and spatial specificity exhibited by these ZF-HDs may be the key to regulating the growth and development of various floral organs of *D. chrysotoxum* at different stages.

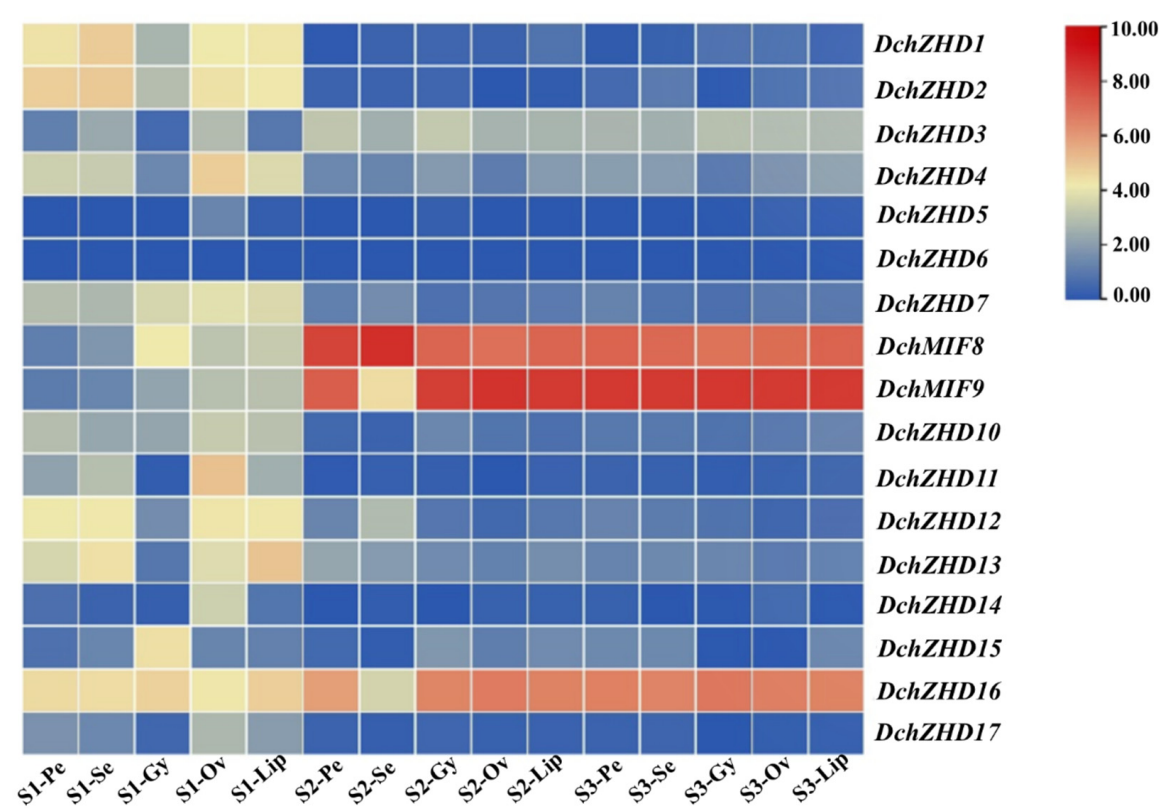


Figure 6. The expression levels of 17 ZF-HD genes during various developmental periods (S1: unpigmented bud stage; S2: pigmented bud stage; S3: initial bloom stage) and in distinct components (Pe: petals; Se: sepals; Ov: ovary, Lip: lip; Gy: gynostemium) of *D. chrysotoxum*. The FPKM values of ZF-HDs observed in *D. chrysotoxum* are detailed in Table S5.

2.7. qRT-PCR Analysis of ZF-HDs in *D. chrysotoxum*

The results revealed that the expression levels of the MIF subfamily genes were higher than those of the ZHD subfamily genes. To deeply understand the expression patterns of the two subfamilies in different flower organs at various developmental stages, *DchZHD2*, *DchMIF8*, and *DchZHD16* were selected for qRT-PCR analysis (Figure 7). *DchZHD2* exhibited expression across all flower organs except gynostemium, peaking in the lips during stage S1, which may have a significant promoting effect on early flower organ development. Meanwhile, *DchMIF8* showed the highest expression in the gynostemium during S1, and *DchZHD16* showed the highest expression in the petal during S3. The expression of *DchZHD16*, which may be involved in promoting the morphogenesis of *D. chrysotoxum*, showed an upregulated trend. *DchZHD2* is significantly expressed in the early stages of flower organ development and subsequently exhibits a downward trend with flower organ development. In contrast, as flowers develop, the expression of genes *DchMIF8* and *DchZHD16* shows an overall upward trend.

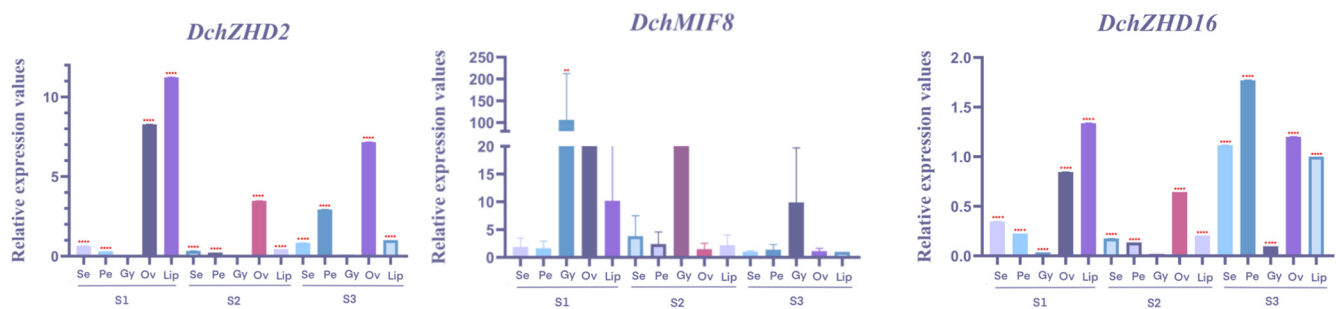


Figure 7. Real-time reverse transcription quantitative PCR (RT-qPCR) validation of *DchZF-HDs*. S1 represents the unpigmented bud stage, S2 represents the pigmented bud stage, and S3 represents the initial bloom stage. The Y-axis signifies the relative expression values ($2^{-\Delta\Delta CT}$). Additionally, the red asterisk serves as an indicator of the significance level of the P-value in the respective test (** $p < 0.01$, **** $p < 0.0001$). Primers and RT-qPCR analysis of *DchZF-HDs* are shown in Table S6.

3. Discussion

Transcription factors (TFs) are crucial proteins that play a regulatory role in various biological processes during plant growth, including flowering, fruiting, and fruit ripening [7,30,31]. As a transcriptional regulator, zinc finger-homeodomain (ZF-HD) has received increasing attention due to its pivotal roles in modulating plant growth, development, and responses to various biotic and abiotic stresses. Research has shown that ZF-HD transcription factors have been identified in various plants such as *A. thaliana* (14) [13], *Malus domestica* (19) [32], *Pisum sativum* (18) [33], *Gossypium hirsutum* (49), *Gossypium barbadense* (50), *Gossypium arboreum* (22), and *Gossypium raimondii* (32) [34]. Although ZF-HDs have been discovered in numerous plants, there remains a paucity of research regarding their specific functions within orchids. In our study, 17 ZF-HDs in *D. chrysotoxum*, 23 ZF-HDs in *D. catenatum*, and 13 ZF-HDs in *D. huoshanense* were identified, which represent a smaller number of genes compared to some of the aforementioned plants. The phenomenon indicates that these three *Dendrobiums* may have experienced genetic losses during the process of evolution.

ZF-HDs are typically classified into two major subfamilies, ZHD and MIF, based on their phylogenetic tree. Similarly, the ZF-HDs in three *Dendrobium* species also followed this classification. The ZHD subfamily includes I–V subfamilies, which is similar to the classification of *A. thaliana* and *Solanum lycopersicum* [5,7]. The number of ZF-HDs in the MIF subfamily was similar, but there was a significant difference in the number of ZF-HDs in the ZHD subfamily (15 *DchZHDs*, 11 *DcaZHDs*, and 21 *DhuZHDs*). Previous investigations have indicated that the distribution pattern of motifs within subfamilies is intricately linked to their functional attributes. We conducted an analysis of the conserved motifs present within ZF-HD family proteins. Notably, the conserved motifs exhibited significant differences between the ZHD and MIF subfamilies. However, within the same subfamilies, we observed similar conserved motifs, indicating a degree of conservation within each subfamily despite the overall variability [6,35]. Research has shown that genes with introns exhibit greater length and more diverse functions and have advantages in species evolution or recombination [14,32,36]. However, 71.7% of ZF-HDs in this study lacked introns (Figure 2C). This phenomenon is similar to earlier reports on conserved gene characteristics. Some studies have found that the number of introns is also related to the plant's adaptability to the environment, indicating that plant genes with fewer introns exhibit greater adaptability to the external environment and often respond quickly to stress [37]. Intron-free genes belong to large families that regulate physiological and biochemical processes and contribute to growth and development processes [38]. Therefore, many ZF-HD members in the three *Dendrobiums* may be able to respond quickly to environmental changes.

The process of gene duplication had a significant impact on the distinctive and varied characteristics of plants, emerging as a dominant force driving the expansion of gene

families. Gene duplication generally includes fragment replication and tandem replication, which is the key to promoting biological evolution [39]. Tandem duplications were discovered in three *Dendrobium* species. The expansion of tandemly duplicated gene families and the increase in gene dosage may have enhanced the genetic diversity and adaptability of *Dendrobium* in response to environmental changes [40]. The variation in the number of tandemly duplicated genes among *D. chrysotoxum*, *D. catenatum*, and *D. huoshanense* may be associated with their abilities to adapt to different environmental conditions, such as temperature, soil, and light. Further research will reveal the specific functions of these duplicated genes and their impacts on the biological characteristics of *Dendrobium*, providing new insights into plant evolution and adaptation mechanisms.

Investigating promoters is imperative for enhancing our fundamental comprehension of gene regulation [41]. We have identified a series of functional *cis*-elements in the ZF-HD promoter of three *Dendrobium* species, encompassing diverse functional categories such as factors responsive to light, phytohormones, stress, as well as components pertaining to plant growth and development. Many growth responsive *cis*-elements were found in the upstream 2000 bp promoter region of 53 ZF-HDs, accounting for 37.39% (326/872) of the total *Dch*ZF-HDs, 35.92% (282/785) of the *Dhu*ZF-HDs, and 30.30% (346/1142) of the *Dca*ZF-HDs. Among them, AT~TATA-box elements responsive to growth are the most abundant among the three *Dendrobium* species, with 15 in *Dch*ZHD17, 62 in *Dhu*ZHD9, and 15 in *Dca*ZHD10 (Figure 5). ZF-HDs in three *Dendrobium* species harbor many MeJA-responsive elements, indicating their potential significance in enhancing stress tolerance. In addition, we identified meristem expression-related elements, indicating that ZF-HDs are important for meristem development. The differences in the number of these elements may reflect their different roles and regulatory mechanisms in the growth and development of different *Dendrobium* species.

Exploring the genes associated with flower morphology significantly contributes to advancing our comprehension of functional genes and the evolutionary dynamics linked to orchid flower development [42]. Presently, investigations into orchid flower organs predominantly concentrate on elucidating the intricacies of the flowering process, floral morphology, color, and fragrance [43]. SPL [44], B-Box [45], and MADS-box gene families [46] have all been recognized for their involvement in the developmental processes of flowers. In addition, the ZF-HD gene family also plays a role in flower morphogenesis. Research showed that *AtZHD5* has an impact on both floral structure and leaf formation [13]. Similarly, Wang et al. [10] identified heightened expression levels in most *Bra*ZF-HDs within flowers, emphasizing their pivotal role in the regulation of flowering processes in *Brassica pekinensis*. Our work was based on transcriptome expression levels of *D. chrysotoxum* ZF-HDs in five flower organs of three stages. The findings revealed that members of the ZF-HD gene family exhibit varying transcript abundances at different stages within the flower buds, indicating that these genes possess distinct expression patterns and functions during the development of floral organs. There was a notable elevation in the expression levels of *Dch*MIF8, *Dch*MIF9, and *Dch*ZHD16 during the latter stages of flower organ development (Figure 6). The expression of *Dch*ZHD2 decreased with the development of flower organs, while the expression of genes *Dch*MIF8 and *Dch*ZHD16 generally showed an upward trend. This observation aligns with the identification of numerous ZF-HDs associated with flowering in *Malus pumila* [47]. The qRT-PCR analysis of key genes showed that the relative expression level of *Dch*ZHD2 was highest in the lip during the S1 stage, while the relative expression levels of *Dch*MIF8 and *Dch*ZHD16 peaked in the petal during the S3 stage. During early flower development, *Dch*ZHD2 expression was prominent but declined with flower organ development. Conversely, the expression of *Dch*MIF8 and *Dch*ZHD16 was on the rise as with flower organ development. This suggests that different genes may play distinct regulatory roles at various stages of flower organ development, potentially involved in processes such as flower morphogenesis, flower opening, or other processes related to floral development. In brief, the growth and development of three

Dendrobiums and the flower organ development of *D. chrysotoxum* may be regulated by members of the ZF-HD gene family.

4. Material and Methods

4.1. Experimental Materials and Data Sources

For this study, plant material sourced from wild-type plants cultivated in a greenhouse at Fujian Agriculture and Forestry University (26°05' N, 119°13' E) was utilized. Various parts of *D. chrysotoxum* flowers, specifically sepals (Se), petals (Pe), gynostemium (Gy), ovary (Ov), and lips (Lip), were collected across the unpigmented bud stage (S1), pigmented bud stage (S2), and initial bloom stage (S3). Subsequently, the collected samples were placed in liquid nitrogen for rapid freezing, followed by storage in a −80 °C degrees freezer for future use.

We accessed the National Center for Biotechnology Information database (NCBI, <https://www.ncbi.nlm.nih.gov/>, accessed on 18 September 2023) to acquire the genomic sequences and annotation files pertaining exclusively to three *Dendrobiums*. The BioProject accession number for *D. chrysotoxum* is PRJNA664445 (release date: 10 September 2021, accessed on 18 September 2023) [48], for *D. catenatum* it is PRJNA262478 (release date: 26 February 2016, accessed on 18 September 2023) [49], and for *D. huoshanense* it is PRJNA597621 (release date: 12 January 2021, accessed on 18 September 2023) [21]. Moreover, the protein sequence files corresponding to ZF-HD in *A. thaliana* were obtained from the Plant Transcription Factor Database (PlantTFDB, <http://planttfdb.gao-lab.org/index.php?sp=Ath>, accessed on 18 September 2023).

4.2. Identification and Physicochemical Properties of ZF-HD Genes

Using the protein sequence of *A. thaliana* ZF-HD proteins as a probe, local blast was performed using TBtools v1.120 software [50], and the obtained sequence was reverse-blasted using the Blast tool in NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 20 September 2023). To further identify ZF-HDs in the three *Dendrobiums*, we used the Hidden Markov Model (HMM) configuration profiles of ZF-HD (PF04770) from Pfam 34.0 database for analysis. Subsequently, protein sequences that were incomplete or redundant were manually excluded from the dataset. An assessment of protein attributes involving amino acid (aa) composition, isoelectric point (pI), molecular weight (MW), average hydrophilicity (GRAVY), instability index (II), and aliphatic index (AI) was conducted utilizing ExPASy 3.0 (<https://www.expasy.org/>, accessed on 20 September 2023) [51]. The value of an isoelectric point greater than 7 implied that the protein is alkaline, while a lower value indicates acidic properties [25]. The instability index, which serves as an experimental estimation of the stability of a protein, predicts that a protein is stable if its value is smaller than 40 [52]. And a deeper understanding of the potential subcellular localization of *Dendrobium* ZF-HD proteins through WoLF PSORT (<https://wolfpsort.hgc.jp/>, accessed on 21 September 2023) is crucial to elucidating the functional roles of these proteins in the cellular environment [27].

4.3. Phylogenetic Analysis of ZF-HDs

The MEGA7.0 software was employed to introduce 17 ZF-HD protein sequences of *A. thaliana* (14 AtZHDs and 3 AtMIFs), 17 ZF-HDs of *D. chrysotoxum*, 23 ZF-HDs of *D. catenatum*, and 13 ZF-HDs of *D. huoshanense*. We used the muscle program and default settings for multisequence alignment and the neighbor-joining (NJ) method to construct the phylogenetic tree [53]. The bootstrap method was executed with 500 replicates, setting partial deletion to 50%. The Evolview 3.0 online platform (<http://www.evolgenius.info/evolview/#/treeview>, accessed on 23 September 2023) beautifies phylogenetic trees, enhancing their clarity and interpretability [54].

4.4. Gene Structures and Conserved Motif Analysis

The NCBI conserved domain database (CDD) (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 23 September 2023) was employed to forecast the conserved domains inherent in *Dendrobium* ZF-HDs. Moreover, the MEME Suite 5.5.5 online software (<https://meme-suite.org/meme/tools/meme/>, accessed on 23 September 2023) was utilized to scrutinize the conserved motifs evident in the ZF-HDs of the three *Dendrobiums* and *A. thaliana*. The MEME analysis was conducted using default settings, with the adjustment of the maximum number of motifs to ten. The phylogeny analysis was conducted through the utilization of 1000 iterations of the bootstrap approach. Subsequently, the generated output was utilized to create a comprehensive comparative map encompassing the NJ phylogenetic tree (bootstrap method: 500). TBtools was used to integrate and visualize phylogenetic trees, conserved protein motifs, and gene structure.

4.5. Localization and Collinearity Analysis of ZF-HDs on Chromosomes

The genetic sequence and annotation data of *D. chrysotoxum*, *D. catenatum*, and *D. huoshanense*, TBtools were utilized to visualize the chromosomal positions of ZF-HDs within these species. To conduct the collinearity analysis of the ZF-HD gene family among the three *Dendrobium* species, genomic information for the respective species was retrieved from the NCBI database. The One Step MCScanX-Super Fast and Multiple Synteny Plot plugin in TBtools were used to perform collinearity analysis and visualize the results.

4.6. Analysis of ZF-HD Promoter

To further investigate the transcriptional regulation of *Dendrobium* ZF-HDs, TBtools extracted the upstream 2000 bp sequence of the ZF-HDs' promoter. Subsequently, PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 18 October 2023) was utilized to perform the analysis of the promoter regions of *Dendrobium* ZF-HDs, to predict the occurrence of *cis*-elements. The identification of these elements provided a reference basis for the potential transcriptional regulatory networks governing *Dendrobium* ZF-HDs expression. The resulting data were processed and organized using Excel, to facilitate further scientific exploration.

4.7. Protein Structure Prediction

The SWISS-MODEL software (<https://swissmodel.expasy.org/interactive>, accessed on 26 October 2023) is widely recognized in the field of bioinformatics for its accuracy and reliability in protein structure prediction [55]. By utilizing SWISS-MODEL, we were able to generate a three-dimensional representation of the *Dendrobium* ZF-HD proteins. Additionally, we employed the SOPMA program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20sopma.html, accessed on 26 October 2023) [56] for predicting the secondary structure of these proteins. SOPMA is a well-established method that helped us to predict the presence of various secondary structural elements, such as alpha-helices and beta-sheets, within the protein sequence. Through combining the results from both SWISS-MODEL and SOPMA, we obtained a comprehensive understanding of the structural characteristics of orchid ZF-HD proteins, which is crucial for further elucidating their functions in the cellular environment.

4.8. Analysis of Expression and RT-qPCR

We utilized the FastPure Plant Total RNA Isolation Kit (for polysaccharide- and polyphenol-rich tissues) (Vazyme Biotech Co., Ltd., Nanjing, China) to extract RNA from flower organs at different flowering stages. Leveraging the RSEM v1.2.8 tool, we accurately calculated the gene expression levels for each individual sample, thereby obtaining Fragments Per Kilobase of transcript per Million Fragments (FPKM) values. These computed FPKM values were then employed to generate a comprehensive heatmap using TBtools, visualizing gene expression levels.

Utilizing the PrimerScript® RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China), which was accompanied by gDNA Eraser, we performed reverse transcription. This methodology was employed to eliminate any potential genomic DNA contamination and enhance the process of cDNA synthesis. Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix was used for the qRT-PCR analysis on an ABI 7500 Real-Time System that was from Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China. In this study, a 96-well plate with a 20 µL reaction system in each well was utilized for the experimental setup. Primers for candidate and internal reference genes for RT-qPCR were designed using Primer Premier 5.0 software. A reference gene, *Maker75111*, was selected for use. Using the $2^{-\Delta\Delta CT}$ method and Graphpad prism 7.0 software, we evaluated the relative expression levels of the target genes.

5. Conclusions

For this study, we identified 17 *DchZF-HDs*, 23 *DcaZF-HDs*, and 13 *DhuZF-HDs* and classified them into ZHD (ZHDI–V) and MIF subfamilies based on their phylogenetic relationships. Members of the same subfamily had similar gene structures and conserved domains. Analyses of phylogenetics, gene structure, motif composition, collinearity, chromosomal localization, and *cis*-elements in the three *Dendrobium* species were carried out. In addition, the results of our expression and qRT-PCR analyses indicated that *DchMIF8* and *DchZHD16* play a significant role in the S2/S3 stage, and changes in the expression level of *DchZHD2* may affect the development of floral organs in the S1 stage. In summary, this study provides a reference for researchers to better understand how ZF-HDs are involved in regulating floral organ development and determination.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10060610/s1>. Figure S1. Prediction of the Tertiary Structure of *D. chrysotoxum*; Figure S2. Prediction of the Tertiary Structure of *D. catenatum*; Figure S3. Prediction of the Tertiary Structure of *D. huoshanense*. Table S1. Characteristics of ZF-HD proteins from three *Dendrobium* orchids; Table S2. Statistics of Cis-acting Element Quantity; Table S3. Secondary structure of ZF-HDs proteins in three *Dendrobiums*; Table S4. Detailed information of the predicted three-dimensional (3D) of ZF-HDs proteins in three *Dendrobiums*; Table S5. The FPKM values of ZF-HDs in *D. chrysotoxum*; Table S6. qRT-PCR primers.

Author Contributions: X.H. finalized the manuscript and integrated all other authors' comments; X.Z. and Q.Z. provided the data; M.-M.Z. and Y.H. analyzed the data; S.L. and Z.-J.L. conceived the study, coordinated with all coauthors, and supervised the whole project. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original data presented in the study are openly available in the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>, accessed on 18 September 2023) genome database. The BioProject number for *D. chrysotoxum* is PRJNA664445, for *D. catenatum* PRJNA262478, and for *D. huoshanense* PRJNA597621.

Conflicts of Interest: The authors declare no conflicts of interest.

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