



Article

Genome-Wide Identification of the ARF Gene Family in Three *Dendrobium* Species and Its Expression Pattern Analysis in *D. nobile* Flower

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Abstract: The ARF gene family is a representative transcription factor that plays a crucial role in the regulation of various growth and development processes in plants. Although the ARF gene family has been identified in five Orchidaceae species, limited research has been conducted on the ARF gene within *Dendrobium*. To explore ARF family genes in different *Dendrobium* species, we selected chromosome-level genomic data from *D. nobile*, *D. chrysotoxum*, and *D. huoshanense* for genome-wide identification, and to analyze expression patterns in the *D. nobile* flower. In this study, 13, 18, and 23 ARF genes were identified in the genomes of *D. chrysotoxum*, *D. huoshanense*, and *D. nobile*, respectively. These genes were then subsequently classified into four classes (Classes I, II, III, and IV) based on our phylogenetic analysis. Additional protein sequence analysis found that 30 ARF proteins with three classically conserved structural domains (BDB, MR, and RD) were present in the three *Dendrobium* species. Our gene structure comparative analysis also found the same evolutionary branch with similar intron-exon structural features. Specifically, Class I and Class III display longer introns that potentially constitute a distinctive characteristic of *Dendrobium*. Gene expression patterns analysis showed the potential involvement of DnoARF 5, 7, 10, 11, 12, 13, 16, 17, and 23 in initial differentiation and flower bud formation. Likewise, transcriptomic analysis and RT-qPCR expression profiles indicated flower-specific expression patterns for four ARF genes (*DnoARF5*, *DnoARF6*, *DnoARF16*, and *DnoARF22*), which suggest an important impact on flower development and regulation. Ultimately, this study provides comprehensive data to elucidate the potential functions of ARF genes in three *Dendrobium* species and suggests new insights for further exploration concerning the function and regulatory mechanisms in *Dendrobium* during flower development.

Keywords: Orchidaceae; ARF gene family; gene structure; *Dendrobium*; expression pattern



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1. Introduction

Auxin, a vital plant hormone, orchestrates a myriad of growth and developmental processes in terrestrial plants, and Auxin Response Factors (ARF) transcription factors (TFs) hold central positions within the auxin signaling pathway that are known to influence downstream gene expression and modulate the entire plant life cycle [1,2], including embryonic development, floral bud differentiation, apical dominance, lateral root formation, and flowering and fruiting [2,3].

In the auxin signaling pathway, transcription factors play a crucial role, notably ARFs. ARF is one of the key regulatory factors in response to auxin, and plays a crucial role in regulating auxin signaling transduction and controlling plant growth and development [4]. Structurally speaking, ARF gene family members typically exhibit three classically conserved structural domains: (1) the N-terminal B3 DNA-binding domain (DBD), (2) a variable middle region (MR) with dual functionality as an activation domain (AD) or an inhibition domain (RD), and (3) the carboxy-terminal dimerization domain (CTD) [1,4]. The CTD domain mediates the interaction between ARF proteins and Aux/IAA proteins in the auxin signaling pathway, thereby regulating the gene expression that influences plant growth and development [5,6]. Specifically, ARF modulates the transcription of auxin-responsive genes by controlling amino acid composition in the MR region. MR regions enriched with glutamine (Q) are typically associated with activation, while domains enriched with serine (S), proline (P), glycine (G), and leucine (L) are correlated with inhibition [7,8]. Additionally, the DBD domain can specifically recognize and bind auxin response elements (AuxREs), which further regulate gene expression [9,10]. They contain a TGTCTC sequence and its variations (TGTCCC, TGTCAC, and TGTCGG) [9,10]. Together, these findings underscore the pivotal role of ARF in the auxin signaling pathway and provide crucial clues for a deeper understanding of how auxin regulates plant growth and development.

Previously, whole genome-level studies on ARF genes have been conducted in various plants, including *Zea mays*, *Ginkgo biloba*, *Populus trichocarp*, *Apostasia shenzhenica*, *Phalaenopsis equestris*, *P. aphrodite*, and *Dendrobium catenatum* [11–14]. Although the ARF gene family has been identified in Orchidaceae, ARF genes among closely related species have not been studied of *Dendrobium*. In particular, some ARF genes have been demonstrated to play roles in the transport and regulation of auxin in various plant species, which highlights their functional diversity. For example, *RhARF7* in rose petals regulates sucrose transport and inhibits petal abscission by regulating *RhSUC2* gene expression [15], while the *Solanum lycopersicum* ARF gene (*SlARF*) regulates lateral root formation, and flower organ senescence [16,17].

Dendrobium, the second-largest genus of Orchidaceae, is notable for its aesthetic and medicinal value [18]. Flowers exhibit a rich array of colors, including yellow, white, and purple. However, the flowering period of yellow flowers is relatively short, lasting only 6–9 days, which impacts both harvest for medicinal purposes and aesthetic enjoyment [19]. Therefore, it is pertinent to understand the intricate processes of floral development influenced by transcription factors (TFs) [20]. With the recent availability of high-quality chromosomal-level genomes, orchid genome data provide an opportunity to systematically study the ARF family gene in *Dendrobium* and offer the unique ability to explore its expression dynamics during flower development. In this study, *D. nobile* (violet, opening time of a single flower 21 days), *D. chrysotoxum* (yellow, opening time of a single flower 9 days), and *D. huoshanense* (faint yellow, opening time of a single flower 7 days) were selected for genome-wide identification and comparative analysis of the ARF family gene at the chromosome level, as well as its expression pattern analysis in the *D. nobile* flower. Altogether, our study results provide comprehensive information to understand the potential functions of ARF genes in three *Dendrobium* species and will allow for researchers to have a deeper understanding of the auxin signaling pathway of the regulatory mechanisms of orchid plants' flowers' opening time.

2. Materials and Methods

2.1. Data Sources

Genomic data for *D. chrysotoxum* (PRJNA664445) and *D. nobile* (PRJNA725550) were downloaded from the genome library of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>, accessed on 12 August 2023), and the genomic data for *D. huoshanense* (CNA0014590) were obtained from the China Nucleotide Sequence Archive (CNSA, <https://ftp.cngb.org/>, accessed on 12 August 2023). The protein sequence

of ARF from *Arabidopsis thaliana* was obtained from TAIR2 (<https://www.arabidopsis.org/>, accessed on 15 August 2023), and four species (*Ap. shenzhenica*, *P. equestris*, *P. aphrodite*, *D. catenatum*) of ARF proteins sequence were downloaded from prior investigations [14].

2.2. Identification and Physicochemical Properties of ARF Gene Family

BLAST and Hidden Markov Model (HMM) methodologies were utilized for the identification of ARF protein sequences in the genomes of *D. nobile*, *D. huoshanense*, and *D. chrysotoxum*. ARF gene sequences from *A. thaliana* were used as the query, and a local BLAST search was performed, using TBtools software [21]. The HMM profile that corresponds to the B3 ARF domain (Pfam 02362) and Auxin_resp (Pfam 06507) was acquired from the InterPro (<https://www.ebi.ac.uk/interpro/>, accessed on 20 August 2023) protein family database [22]. These HMM profiles were applied to identify candidate protein sequences further using a Simple HMM Search within TBtools software [21]. BLAST and HMM results were combined to eliminate false-positive sequences, and any truncated or redundant proteins were manually removed. Subsequently, all ARF genes were subjected to analysis using the NCBI Batch-CDD (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 3 September 2023) and SMART (<http://smart.embl-heidelberg.de/>, accessed on 3 September 2023) databases to confirm the presence of the B3 and Auxin_resp conserved domain in the candidate orchid ARFs [14]. Additionally, several physicochemical properties that include protein molecule, aliphatic index (AI), theoretical isoelectric point (pI), and grand average of hydropathicity (GRAVY) within the ARF genes were predicted using the ExPASy online tool (<http://www.expasy.org>, accessed on 10 September 2023) [23]. Additionally, predictions of subcellular localization were performed using Plant-mPloc (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 10 September 2023) [24].

2.3. Phylogenetic Analysis of ARF Genes

Amino acid sequences of ARF from *A. thaliana*, *Ap. shenzhenica*, *P. equestris*, *P. aphrodite*, *D. catenatum*, *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* were aligned using the ClustalW algorithm in MEGA 7 [25]. We used the neighbor-joining (NJ) phylogenetic method with 1000 bootstrap iterations and a partial deletion threshold of 50% to construct an ARF phylogenetic tree. Additionally, an ARF phylogenetic tree was generated using online tools provided by Evolview 3.0 (<http://www.evolgenius.info/evolview/#/treeview>, accessed on 5 November 2023) for enhanced visualization.

2.4. Motif and Gene Structure Analysis

Predictions for conserved protein domains within the ARF gene sequences were executed using Batch CD-Search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 3 September 2023). Conserved motifs were also identified using MEME (<https://meme-suite.org/meme/tools/meme>, accessed on 5 September 2023) with default parameters [26]. Subsequently, the outcomes related to motifs and gene structures were graphically represented using TBtools with the Visualize MEME/MAST Motif Pattern functionality [21].

2.5. Prediction of Cis-Acting Elements

Sequences 2000-bp upstream of the three *Dendrobium* species ARF were extracted using TBtools based on their gene locations in the annotated file [21]. Cis-acting elements were identified and annotated using the software PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 5 September 2023) [27]. Subsequently, TBtools was used to visualize the number and annotations of cis-acting elements [21].

2.6. Collinearity and Chromosomal Localization of ARF Genes in Three *Dendrobium* Species

To determine the chromosomal localization information of the ARF genes, the genome files (fasta files) and annotation files (gff files) for *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* were subjected to TBtools. Comparative genome data analyses were then performed

between the three species for collinearity assessment using One Step MCScanx [18]. The collinearity results were visualized using TBtools with Dual Synteny Plot for MCScanX [21].

2.7. Expression Analysis

Transcriptional quantification was conducted using Expectation Maximized (RSEM) RNA-Seq [28]. Fragments Per Kilobase Per Million mapped read fragments (FPKM) values were calculated for each gene in the transcriptomic analysis. The FPKM matrix and heat map were generated using TBtools (v 2.096) [21]. Flower parts, including sepal (Se), petal (Pe), and labellum (Lip), from *D. nobile* (accessions) were cultivated in the National Orchid Germplasm Resources of Fujian Agriculture and Forestry University (E 119°23'41", N 26°08'75"). These materials were obtained from seed propagation and have been cultivated under the forest in the bionic habitat for 5–6 years. Flower development was divided into five stages according to the degree of flower opening, bud stage (S1), translucence stage (S2), first opening stage (S3), full bloom stage (S4), and decay stage (S5). Subsequently, all collected samples were gathered, placed in tubes, promptly frozen, and stored at -80°C .

RT-qPCR was utilized to evaluate the expression patterns of ARF genes in flowers. Total RNA was extracted from the tissues using the FastPure Universal Plant Total RNA Isolation Kit. Specific PCR primers were designed using online tools Primer3Plus (<http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi>) and DNAMAN v8 to ensure primer specificity and avoid secondary structure and repetitive sequences. Gene-specific primers for the four candidate genes (*DnoARF5*, *DnoARF6*, *DnoARF16*, and *DnoARF22*) are provided in Supplementary Table S1, along with the internal reference genes (*DnoGAPDH*). cDNA synthesis was performed using the TaKaRa PrimeScriptTM RT reagent Kit with gDNA Eraser (RR047Q) with the cDNA first-strand synthesis reaction system described in Supplementary Table S2. After obtaining the cDNA template, qRT-PCR reactions were performed using the TaKaRa TB Green[®] Premix Ex TaqTM II kit on the company's 7500 Real-Time PCR system (Supplementary Table S3), with three replicates for each sample. Gene expression levels were determined using the $2^{-\Delta\Delta\text{CT}}$ method.

3. Results

3.1. Identification and Protein Characterization of ARF Gene Family in Three *Dendrobium* Species

In this study, 13, 18, and 23 ARF genes were identified in the genomes of *D. chrysotoxum*, *D. huoshanense* and *D. nobile*, respectively. ARF proteins were characterized by the presence of the B3 and Auxin_resp conserved domains, which were typically distributed as follows, *D. chrysotoxum* (13 *DchARFs*), *D. huoshanense* (18 *DhuARFs*), and *D. nobile* (23 *DnoARFs*) (Supplementary Table S4). The terminology for these gene families was established based on their chromosomal distribution and resulted in their designations as *DchARF1-13*, *DhuARF1-18*, and *DnoARF1-23*. Notably, the ARF sequences exhibited significant diversity in the number of amino acids (AA) ranging from 243 to 1043, which corresponded to their molecular weights (MW) that ranged from 28.46 to 116.81 kDa. Furthermore, the GRAVY values for *Dendrobium* ARF proteins were consistently negative, which indicates a strong hydrophilic nature. The average isoelectric point (pI) was 6.63, with a range from 5.15 to 8.99. In addition, subcellular localization predictions revealed that all ARF proteins were predicted to localize in the nucleus (Table 1).

Table 1. Physicochemical properties and subcellular location of ARF genes' family in three *Dendrobium* species.

Gene Name	Gene ID	Theoretical pI	Molecular Weight MW (kDa)	Amino Acids (aa)	GRAVY	The Instability Index (II)	Subcellular Localization
DchARF1	Maker67368	5.19	111.83	1004	−0.419	56.94	Nucleus
DchARF2	Maker112730	8.38	57.70	522	−0.119	49.80	Nucleus
DchARF3	Maker78910	8.23	78.09	704	−0.401	46.56	Nucleus
DchARF4	Maker89068	7.50	87.43	787	−0.291	47.30	Nucleus
DchARF5	Maker67406	6.31	94.60	853	−0.453	52.27	Nucleus
DchARF6	Maker76677	8.09	69.22	622	−0.395	50.52	Nucleus
DchARF7	Maker68808	5.98	100.87	908	−0.433	58.92	Nucleus
DchARF8	Maker112343	6.34	94.09	843	−0.464	57.96	Nucleus
DchARF9	Maker65500	5.42	85.52	764	−0.399	62.47	Nucleus
DchARF10	Maker53290	5.72	74.75	669	−0.440	61.88	Nucleus
DchARF11	Maker90125	6.44	76.46	678	−0.473	43.63	Nucleus
DchARF12	Maker108475	7.64	80.97	721	−0.298	48.93	Nucleus
DchARF13	Maker115491	6.11	116.81	1043	−0.494	63.94	Nucleus
DhuARF1	Dhu000012013	5.35	57.59	516	−0.467	65.49	Nucleus
DhuARF2	Dhu000025273	6.20	28.46	248	−0.255	56.14	Nucleus
DhuARF3	Dhu000008258	6.19	68.49	601	−0.154	57.69	Nucleus
DhuARF4	Dhu000008039	8.09	68.69	615	−0.358	49.31	Nucleus
DhuARF5	Dhu000022632	6.19	78.10	694	−0.476	44.13	Nucleus
DhuARF6	Dhu000020562	5.44	61.27	542	−0.421	59.71	Nucleus
DhuARF7	Dhu000020741	6.51	63.38	561	−0.619	49.42	Nucleus
DhuARF8	Dhu000012552	6.55	96.10	867	−0.538	54.79	Nucleus
DhuARF9	Dhu000021532	6.34	77.78	700	−0.429	46.22	Nucleus
DhuARF10	Dhu000006783	8.06	77.75	702	−0.402	46.72	Nucleus
DhuARF11	Dhu000012252	8.12	68.80	616	−0.386	49.91	Nucleus
DhuARF12	Dhu000024660	5.94	84.48	762	−0.509	47.13	Nucleus
DhuARF13	Dhu000018860	6.10	104.93	947	−0.404	56.02	Nucleus
DhuARF14	Dhu000004687	5.87	95.96	864	−0.380	59.04	Nucleus
DhuARF15	Dhu000003428	5.78	94.91	850	−0.427	55.88	Nucleus
DhuARF16	Dhu000008590	5.67	36.28	324	−0.320	57.26	Nucleus
DhuARF17	Dhu000002910	8.61	57.65	523	−0.153	51.02	Nucleus
DhuARF18	Dhu000025920	5.5	41.19	366	−0.346	60.46	Nucleus
DnoARF1	cds-KAI0530553.1	5.15	109.41	982	−0.441	58.12	Nucleus
DnoARF2	cds-KAI0530789.1	6.18	70.90	634	−0.496	51.64	Nucleus
DnoARF3	cds-KAI0531046.1	8.37	57.63	523	−0.136	49.78	Nucleus
DnoARF4	cds-KAI0528813.1	8.06	77.70	702	−0.405	48.03	Nucleus
DnoARF5	cds-KAI0529214.1	6.00	90.82	809	−0.436	58.68	Nucleus
DnoARF6	cds-KAI0524417.1	6.61	76.33	685	−0.459	47.84	Nucleus
DnoARF7	cds-KAI0522726.1	6.60	94.12	846	−0.465	48.18	Nucleus
DnoARF8	cds-KAI0518880.1	8.33	94.27	845	−0.575	69.76	Nucleus
DnoARF9	cds-KAI0513561.1	8.12	68.73	616	−0.384	48.95	Nucleus
DnoARF10	cds-KAI0510213.1	6.01	101.42	912	−0.431	59.59	Nucleus
DnoARF11	cds-KAI0510575.1	6.32	94.41	843	−0.494	59.22	Nucleus
DnoARF12	cds-KAI0511442.1	5.72	88.99	803	−0.536	54.27	Nucleus
DnoARF13	cds-KAI0499593.1	5.76	88.69	791	−0.413	61.70	Nucleus
DnoARF14	cds-KAI0499914.1	8.99	58.20	508	−0.710	47.62	Nucleus
DnoARF15	cds-KAI0497955.1	6.36	81.25	724	−0.373	54.26	Nucleus
DnoARF16	cds-KAI0496169.1	5.87	71.76	640	−0.467	63.25	Nucleus
DnoARF17	cds-KAI0496703.1	6.52	74.64	662	−0.474	46.41	Nucleus
DnoARF18	cds-KAI0493458.1	6.71	75.35	674	−0.349	53.49	Nucleus
DnoARF19	cds-KAI0494314.1	5.64	74.52	672	−0.477	64.59	Nucleus
DnoARF20	cds-KAI0494588.1	6.28	108.38	962	−0.546	67.51	Nucleus
DnoARF21	cds-KAI0492193.1	6.42	78.40	718	−0.422	56.29	Nucleus
DnoARF22	cds-KAI0488159.1	7.66	68.69	605	−0.563	51.85	Nucleus
DnoARF23	cds-KAI0489348.1	6.54	93.72	843	−0.546	53.97	Nucleus

3.2. Phylogeny and Classification of ARF Genes in Three *Dendrobium* Species

To delve into the evolutionary relationships within the ARF gene family of *Dendrobium*, we employed amino acid sequences from a total of 149 ARF genes across eight species to build a phylogenetic tree. These species include *A. thaliana* (23), *Ap. shenzhenica* (17), *P. equestris* (16), *P. aphrodite* (18), *D. catenatum* (21), as well as *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* (Figure 1). Notably, members of the ARF genes family in *D. chrysotoxum*, *D. huoshanense*, and *D. nobile* were identified for the first time in this study. After eliminating

duplicate sequences, the *PeARF* gene family from *P. equestris* was determined to have 16 members. Based on branch nodes and previous research, the phylogenetic tree was constructed using the ClustalW algorithm in MEGA 7. From previous research, it was then further divided into four primary classes (I, II, III, and IV). Class II contains the largest number of ARF (63) genes that can be sub-divided into two sub-branches (class II A with 21 members, class II B with 42), followed by class III (39), class IV (34), and class I with the fewest members (13). Within these clades, we identified 21 sister pairs and seven triplets in the three *Dendrobium* species. Additionally, there are 10 sets of quadruplets (*DnoARF*, *DcaARF*, *DchARF*, and *DhuARF*). Moreover, we observed 11 sister pairs of *DcaARF* and *DhuARF*. These findings shed light on the evolutionary relationships and divergence patterns within the ARF family gene in three *Dendrobium* species.

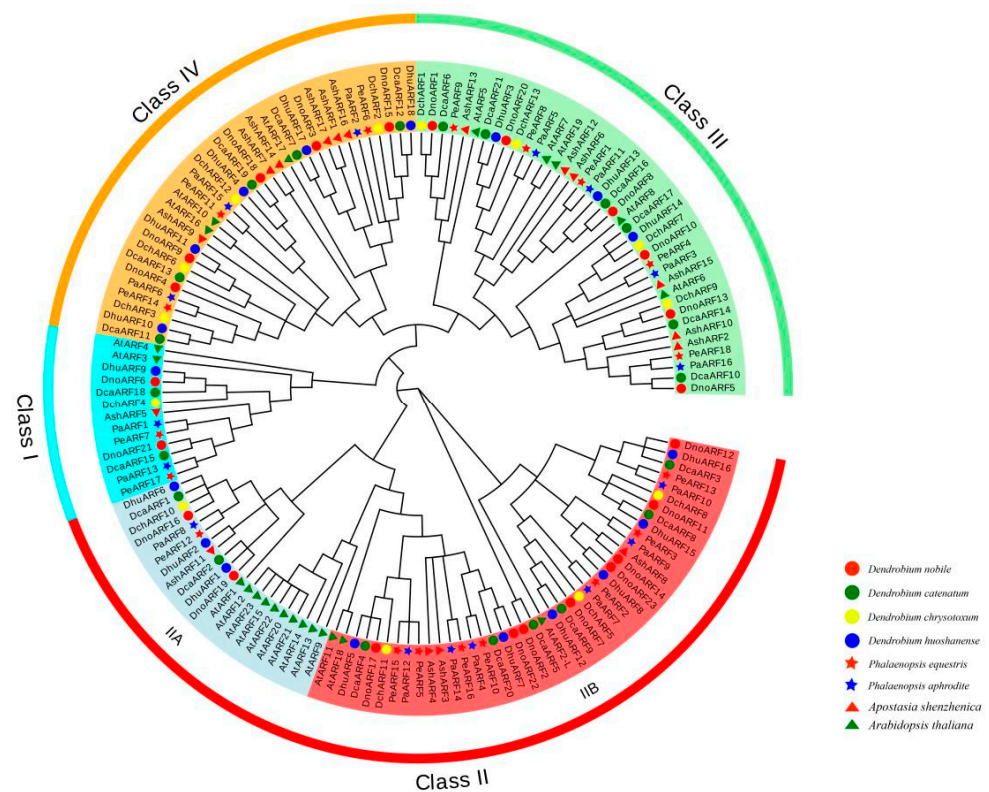


Figure 1. Phylogenetic tree of ARF protein sequences from *Dendrobium nobile*, *D. huoshanense*, *D. chrysotoxum*, *D. catenatum*, *Apostasia Shenzhenica*, *Phalaenopsis equestris*, *P. aphrodite*, and *Arabidopsis thaliana*. The ARF gene family was classified into four classes: Class I, Class II, Class III, and Class IV, with Class II divided into two subclades: IIA and IIB. ARF protein sequences of all species are available in Supplementary Table S4.

3.3. Chromosomal Localization and Collinearity Analysis of ARF Genes in Three *Dendrobium* Species

We then investigated gene locations within *Dendrobium* chromosomes (Figure 2). Chromosome mapping revealed the presence of 13 *DchARF* genes distributed across nine chromosomes in *D. chrysotoxum* (Figure 2A). Specifically, chromosomes 17, 16, 09, and 01 each contained two genes, while the remaining chromosomes each contained one gene. In total, 23 *DnoARF* genes exhibited non-uniform distribution across 13 chromosomes, with chromosomes CM039734.1, CM039726.1, and CM039718.1 each hosting three genes (Figure 2B). For *D. huoshanense*, chromosome mapping showed an uneven distribution of 18 *DhuARF* genes across 10 chromosomes and scaffold 1551 (Figure 2C). Additionally, chromosome 2 displayed four genes that featured tandem repeats of *DhuARF1* and *DhuARF2*.

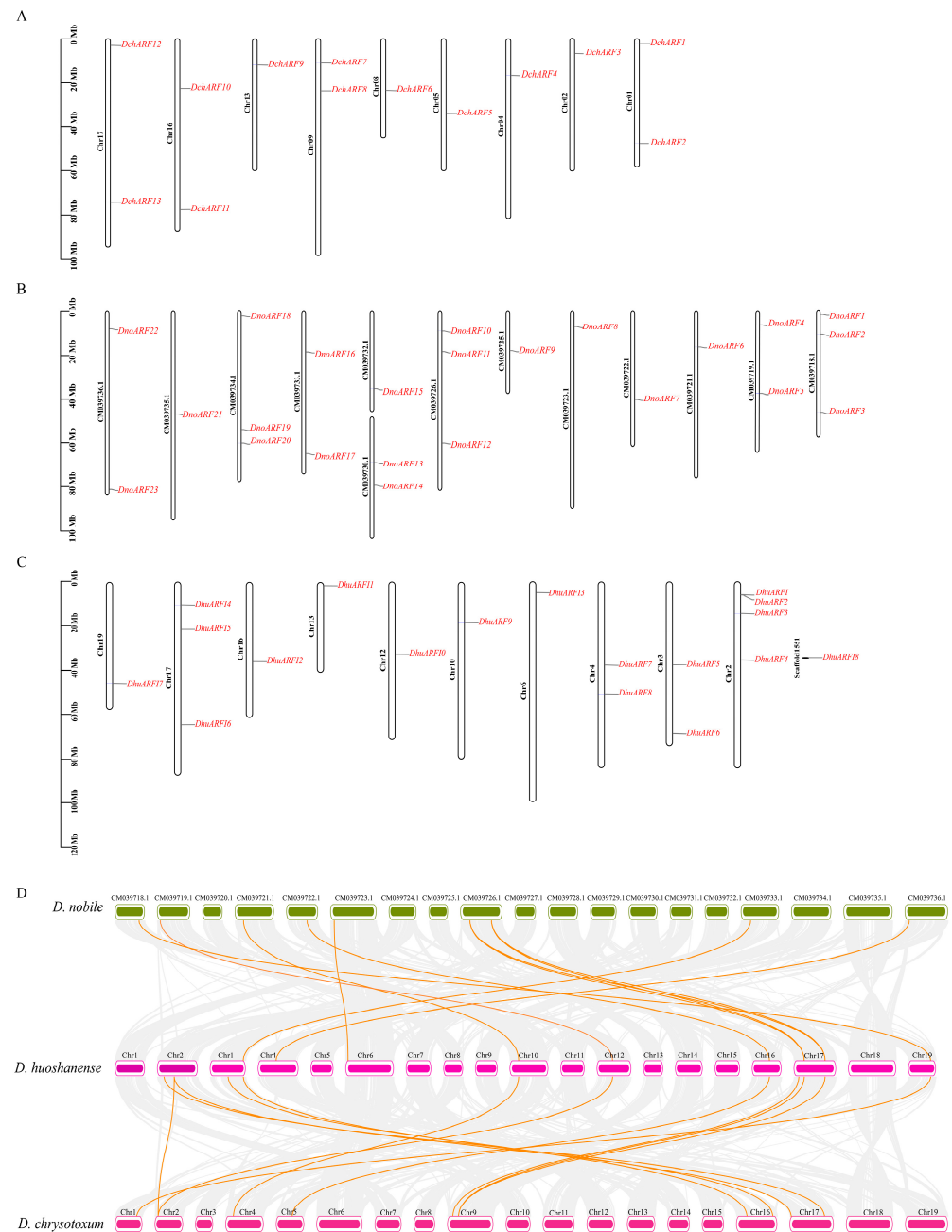


Figure 2. Collinearity of ARF genes between *D. chrysotoxum* and *D. nobile*, *D. nobile*, and *D. huoshanense*. (A) Chromosomal location of *DchARF* genes. (B) Chromosomal location of *DnoARF* genes. (C) Chromosomal location of *DhuARF* genes. (D) Collinear analysis of all ARF genes in three *Dendrobium* species.

We subsequently conducted a comprehensive analysis of collinear relationships among *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* (Figure 2D and Supplementary Table S5). The colored lines represent genes with collinearity among these species. Between them, 13 *DchARFs* genes and 12 *DhuARFs* and 10 *DnoARFs* genes had collinear gene pairs. Ten genes from *DnoARFs* co-occurred and showed genomic collinearity in three *Dendrobium* species (Supplementary Table S5). Together, our analysis indicates that *DchARF8* exhibits collinearity with four *DnoARF* genes in *D. nobile*, namely *DnoARF2*, *DnoARF7*, *DnoARF11*, and *DnoARF12*.

3.4. Gene Structure and Conserved Domains of ARF Genes in Three *Dendrobium* Species

To investigate the gene structure of ARF genes in these three *Dendrobium* species, we analyzed intron-exon arrangements and the distribution of upstream and downstream regulatory elements in *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* (Figure 3C). We found that the ARF gene family in *Dendrobium* possesses introns ranging from one to 22. Notably, the longest intron was identified in *DnoARF13*, followed by *DnoARF5* and *DchARF8*. Interestingly, *DhuARF8* had the highest number of introns, with a total of 22. In general, 26% of the total ARF genes (14 members) contained between 1–4 introns and 2–6 exons, while 72% (39 genes) had between 10–14 introns and 8–15 exons. Although we observed similar intron-exon structural features in gene structure within each clade, the introns for the ARF genes in clade I exhibited longer lengths compared to the other clades. However, the exon length of clade IV was also relatively long. Using the online tool MEME Suite, we further examined ARF amino acid sequence motifs from *D. nobile*, *D. huoshanense*, and *D. chrysotoxum*. Ten motifs were set as the upper bound (Figure 3B). Our analysis indicates that the number of ARF amino acid sequence motifs varied from 1–13 for *DnoARF14* and *DnoARF23*, respectively, and motifs 1, 2, and 10 encoded the B3 domain, while motif 6 belonged to the MR domains.

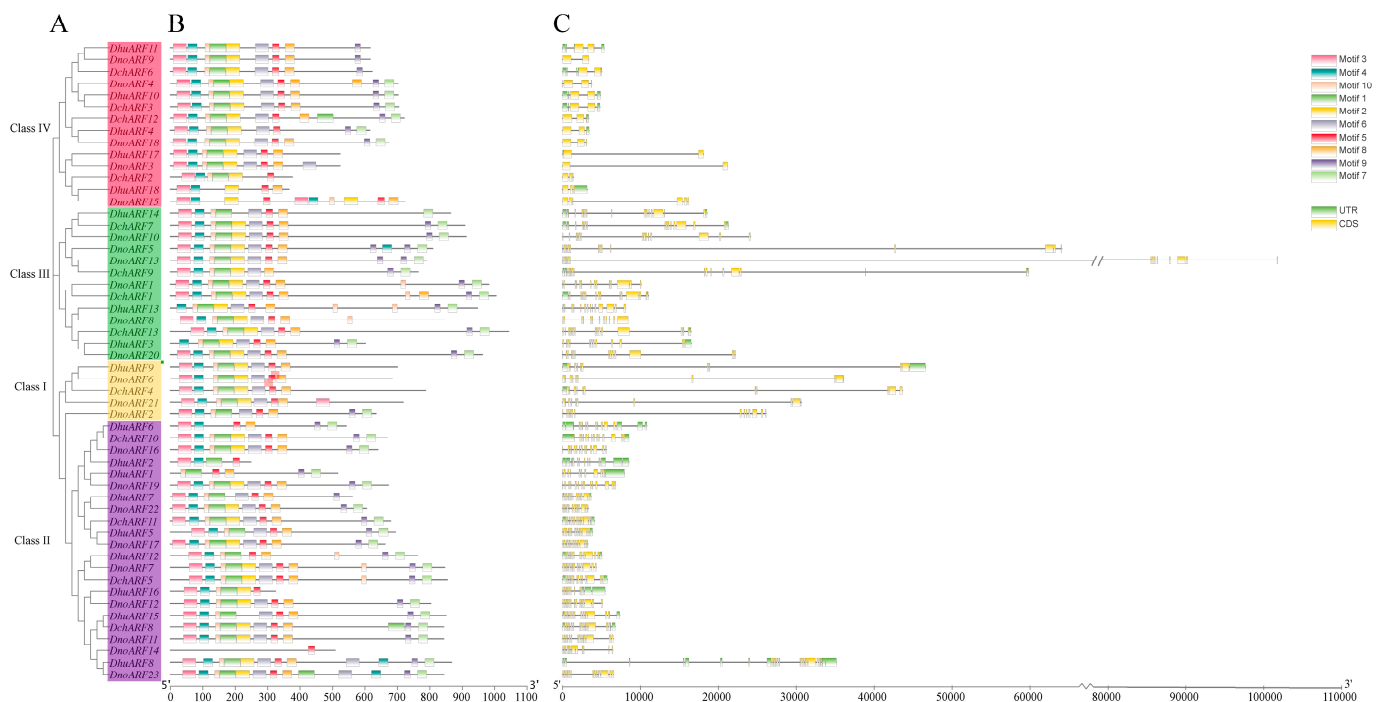


Figure 3. ARF gene structure and conserved motifs. (A) Predicted motifs from three *Dendrobium* species ARF phylogenetic tree. (B) Gene conservative motif analysis. (C) Gene structure of ARFs. Green blocks and yellow blocks represent upstream or downstream untranslated regions (UTR), and exons, respectively.

It is worth noting that, although *Dendrobium* ARF amino acid sequences contain the MR and B3 domains (Figure 3B), most genes in these subclades featured motif 1, motif 5, and motif 8. Only motif 5 is present in all ARF proteins, except *DchARF9*. In contrast, some ARF proteins lack specific motifs present in others: for instance, *DhuARF1* and *DnoARF14* lack motif 4, while *DchARF3* and *DhuARF3* lack motif 3 (Figures 3B and 4A). Nonetheless, most members within the same evolutionary branch share multiple motifs, and there were similarities or identical motif types and arrangements among the different evolutionary branches. These results showed that ARF proteins within specific evolutionary branches remain relatively conserved.

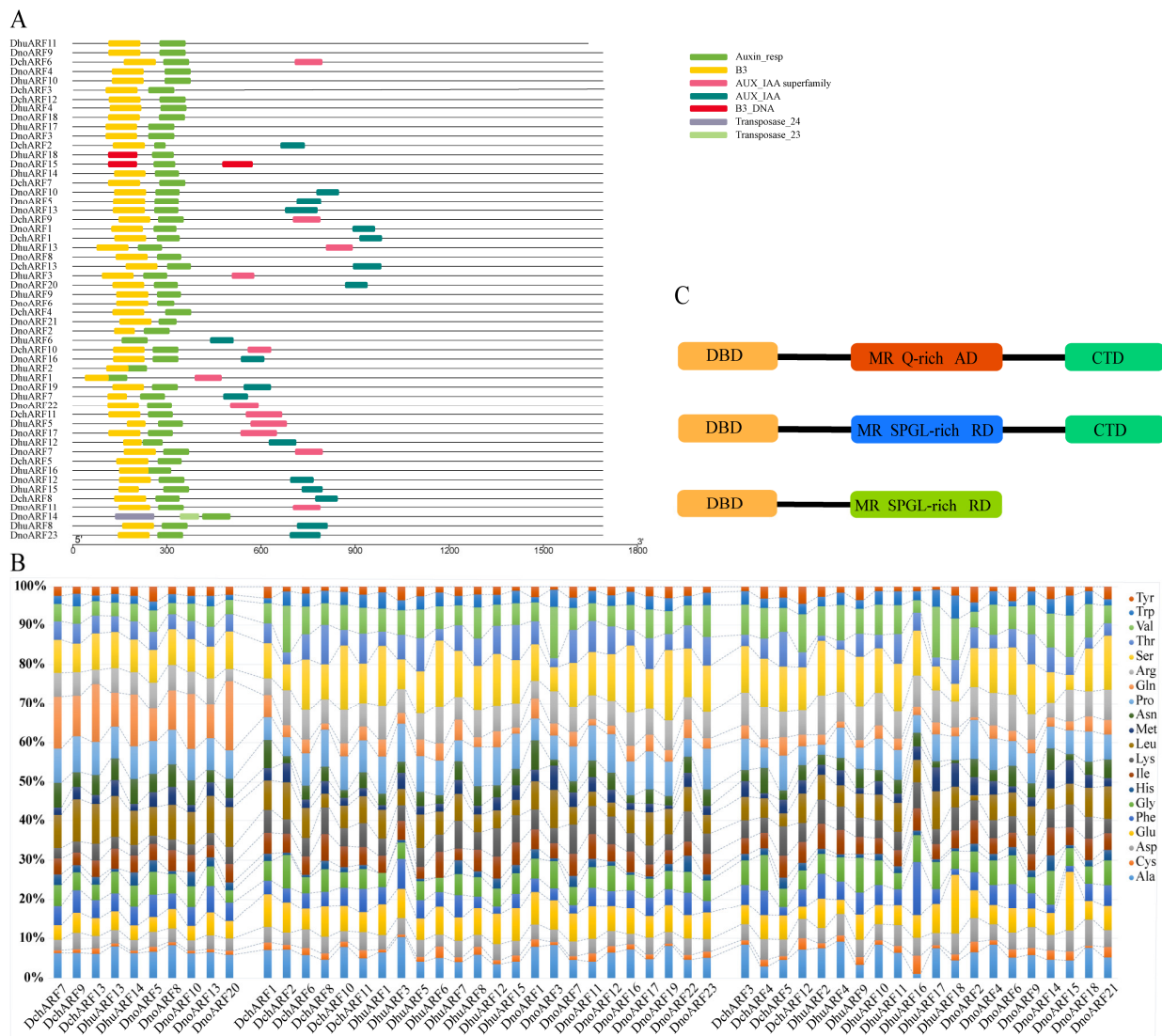


Figure 4. Conserved domains, amino acid compositions of middle regions (MR), and classification of ARF proteins. **(A)** ARF Protein conservative domains. **(B)** Amino acid contents of the MR for 54 ARF proteins. ARF proteins are represented in the horizontal axis, and corresponding amino acids are represented in the vertical axis. Colored bars represent different amino acids. **(C)** The protein structure of ARF proteins. DBD, DNA-binding domain, CTD, C-terminal dimerization domain, MR, middle region, RD, repression domain, AD, activation domain, Q, glutamine, S, serine, P, proline, L, leucine, G, glycine.

To characterize the function of the middle region of ARF in these *Dendrobium* species, we employed MEGA software to assess the amino acid composition of the MR domain in *DnoARF*, *DchARF*, and *DhuARF*. Fifty-four ARF proteins were classified into three groups based on their amino acid composition of the MR domain and the presence of CTD (Figure 4B). The first group consisted of 10 ARF genes, namely *DchARF* 7, 9, and 13, *DhuARF* 13 and 14, *DnoARF* 5, 8, 10, 13, and 20, which feature a DBD, MR abundant in glutamine (Q), and CTD.

According to previous studies, these genes were predicted to have a transcriptional activation role [29]. The second group of the other 20 ARF genes include DBD, CTD, and MR domains rich in serine (S), leucine (L), proline (P), and glycine (G) (Figure 4C). However, 23 ARF genes lack the CTD domain in the three *Dendrobium* species, and the percentages of truncated *DchARFs*, *DnoARFs*, and *DhuARFs* were 38.4%, 55.6%, and 34.7%, respectively. Interestingly, the *DnoARF14* gene was the only gene to possess the MR domains. Together,

these results suggest the sequences found in the 30 ARF proteins with the three classically conserved structural domains (BDB, MR, and RD) were present in three *Dendrobium* species.

3.5. Cis-Acting Regulatory Element Analysis of ARF Genes in Three *Dendrobium* Species

In this study, the 2000-bp promoter regions of the ARF family gene in three *Dendrobium* species were extracted to identify potential *cis*-elements and investigate the regulatory functions of the ARF family gene. Here, we identified 3126, 2309, and 1824 *cis*-acting elements attributed to 57, 51, and 49 types and 18, 20, and 14 responsive functions in *D. nobile*, *D. huoshanense*, and *D. chrysotoxum*, respectively (Figure 5 and Supplementary Table S6).

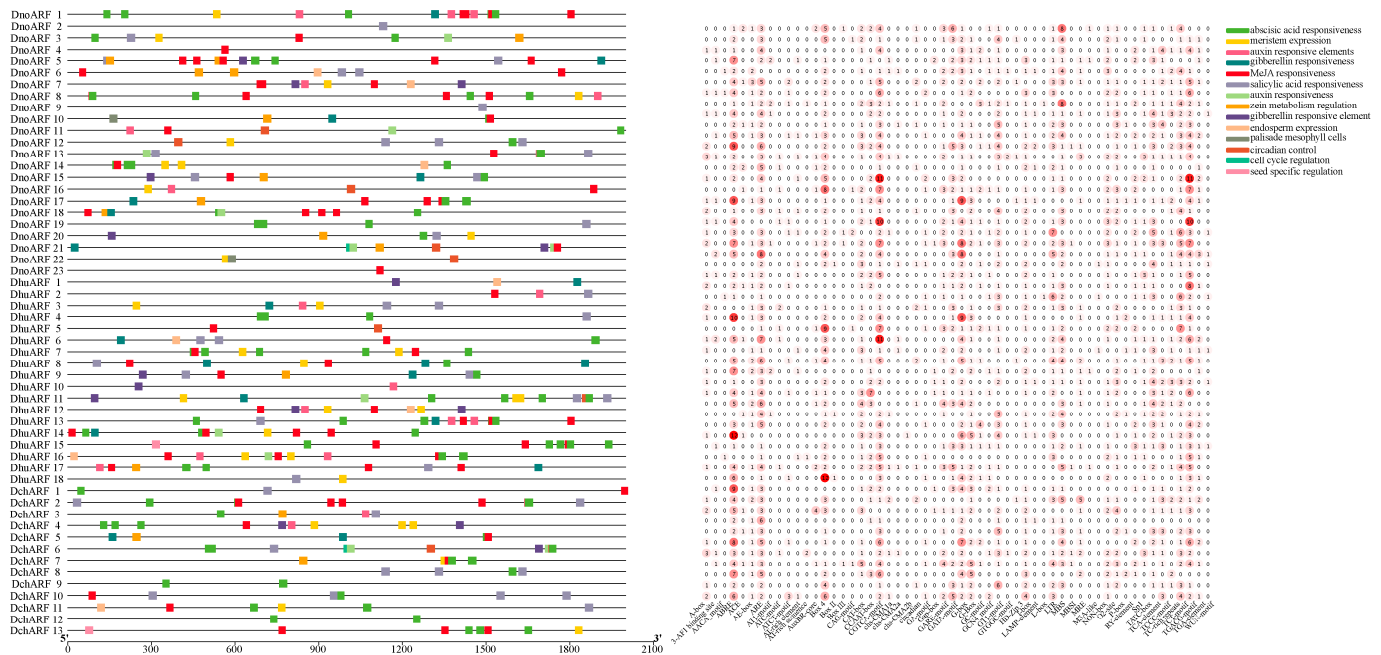


Figure 5. Cis-acting elements in the promoter regions of ARF genes. Elements with similar regulatory functions are displayed in the same color. Numbers of each type of element are shown on the right side.

Among these *cis*-acting elements, TATA-box accounted for the highest proportion in *D. nobile*, *D. huoshanense*, and *D. chrysotoxum* (40.9%, 42.9%, 44.4%, respectively), followed by the CAAT-box (21.7%, 21.4%, 20.6%, respectively). The functions of these *cis*-elements encompassed responses to phytohormones, such as gibberellic acid (GA), auxin, methyl jasmonate (MeJA), salicylic acid (SA), and 1H-Indole-3-acetic acid (ABA). They exhibited abiotic stress responsiveness to factors, such as low temperature, anaerobic conditions, drought, and anoxia. In addition, other elements were associated with plant growth and development, such as light response. (Figure 5). It is important to note that ARF genes contain a variety of elements, with light responsiveness functions being the most common element function. Additionally, MeJA-responsive elements and ABA-responsive elements were among the most prevalent types, which were also widely distributed in the ARF genes family from the three *Dendrobium* species.

3.6. Expression Analysis of ARF Genes in *D. nobile* and *D. chrysotoxum* Flower

D. nobile expression levels were analyzed based on the transcriptome data collected during different stages and parts from the flower (Figure 6A and Supplementary Table S7). Our heatmap and transcriptome data analysis showed that 10 genes exhibit sustained high expression across the five stages of flower development in *D. nobile*. This suggests that they may play important roles throughout the entire flower developmental process. However, 3 genes (*DnoARF* 2, 14, and 15) showed minimal expression, which indicates their likely

lack of involvement in *D. nobile* flower development. *DnoARF* 5, 7, 10, 11, 12, 13, 16, 17, and 23 also showed relatively higher expression levels during the early stages (S1–S2) of flower development, which suggests their potential involvement in initial differentiation and flower bud formation. Conversely, *DnoARF* 8, 6, and 19 exhibited higher expression levels during stages S3–S4, which implies their potentially crucial roles in the later stages of flower opening and maturation. Additionally, the expression level of *DnoARF*22 was elevated during the senescence phase (S5), which indicates its probable involvement in the aging process of flowers.

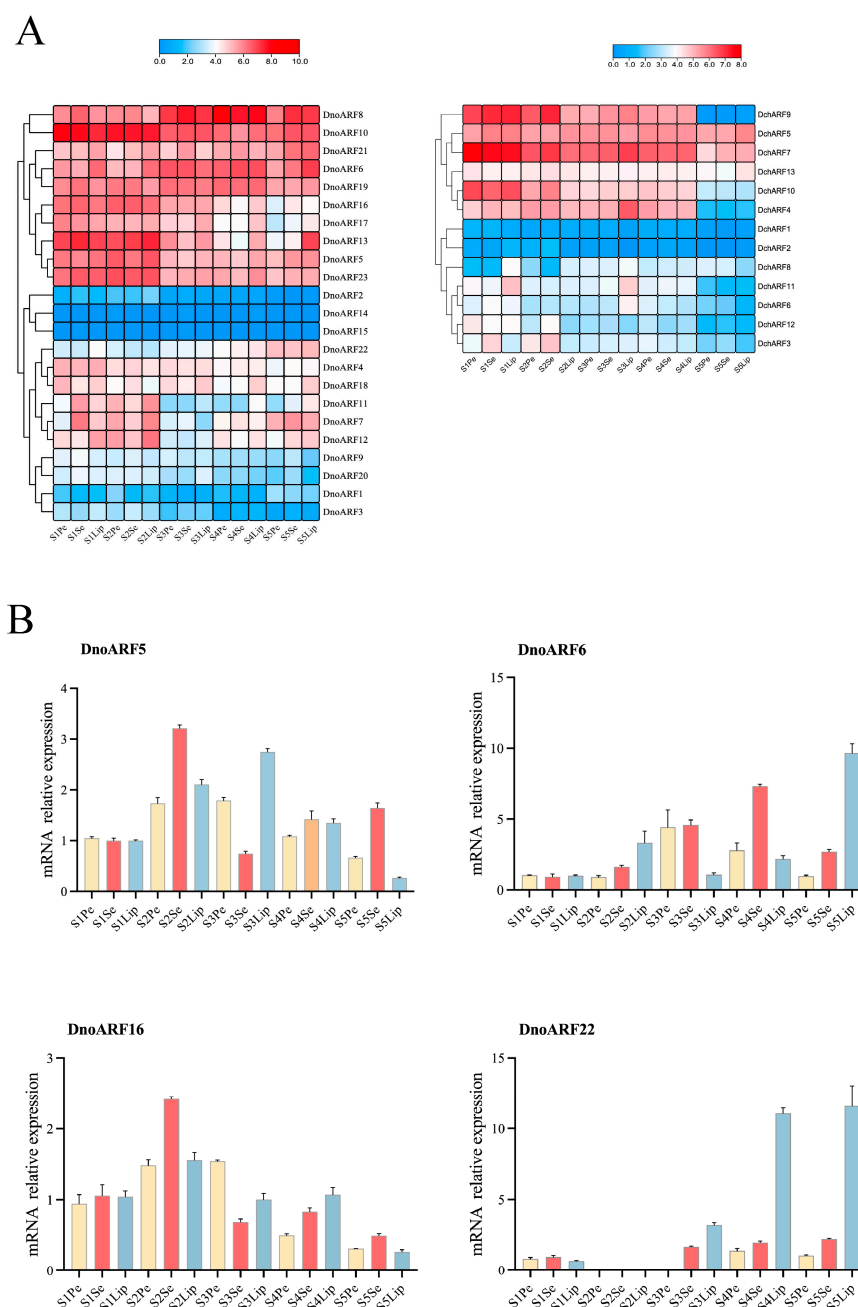


Figure 6. The expression pattern and RT–qPCR verification of ARF genes. (A) Expression heatmap of ARF genes in different tissues and flower developmental periods in *D. nobile* and *D. chrysotoxum*. S1: unpigmented bud stage, S2: pigmented bud stage, S3: initial opened flower, S4: fully opened flower, S5: withered flower. Left: *D. nobile*, right: *D. chrysotoxum*. (B) Expression patterns of *DnoARF*5, *DnoARF*6, *DnoARF*16, and *DnoARF*22 in five developmental periods of *D. nobile* by RT–qPCR. Yellow, red, and light blue represent petal, sepal, and labial flap, respectively.

Meanwhile, our heatmap analysis and transcriptome data also indicate distinct expression patterns for various *DchARF* genes during the development of *D. chrysotoxum* flowers (Figure 6A and Supplementary Table S7). *DchARF1* and *DchARF2* were minimally expressed throughout flower development, while *DchARF5* and *DchARF7* maintained consistently high expression levels across all stages. Conversely, *DchARF9*, *DchARF10*, and *DchARF14* demonstrate notably elevated expression during stages S1–S4 compared to stage S5. Notably, only three genes (*DchARF5*, *DchARF7*, and *DchARF13*) display relatively high expression levels at stage S5, which was different than the remaining genes that were expressed at very low levels, or were not expressed at all, during stage S5.

3.7. qRT-PCR Analysis of Four *DnoARF* Genes in *D. nobile* Flower

To understand the expression patterns of ARF family genes across the various subclades for the Pe, Se, and Lip during the floral development of *D. nobile* with single flowers open longer, we conducted a meticulous examination of four genes using qRT-PCR analysis: *DnoARF5* (Class III), *DnoARF6* (Class I), *DnoARF16* (Class IIA), and *DnoARF22* (Class IIB). Our comprehensive analysis revealed a remarkable coherence between the qRT-PCR results and the transcriptome data, which further validates the reliability and consistency of our findings (Figure 6B). Specifically, *DnoARF5* demonstrates relatively higher expression levels during the S2–S3 stages, while *DnoARF6* exhibits notably significant expression during the S4Se and S5Lip stages. Moreover, the expression trend of *DnoARF16* gradually declines as floral development progresses, whereas the expression level of the *DnoARF22* gene showed an ascending trend in the final developmental stage.

4. Discussion

The number of ARF family genes varies among different plant species. For example, ARF gene family members in *A. thaliana* (23) [4], *S. lycopersicum* (21) [30], *P. equestris* (16) [14], *Zea mays* (31) [11], and *Medicago sativa* (81) [31] are known to vary among these species. In this investigation, 13, 18, and 23 ARF genes were identified in the genomes of *D. chrysotoxum*, *D. huoshanense*, and *D. nobile*, respectively. Ultimately, we found the number of ARF gene families in *D. huoshanense* and *D. nobile* was similar to those in *P. aphrodite*, *S. lycopersicum*, and *A. thaliana*. However, the number of ARF gene families in *D. chrysotoxum* was quite different from other species. Here, the reduced number of ARF genes in *D. chrysotoxum* may be attributed to the early evolution of these plants, which contrasts with the known replication events in rice and *Arabidopsis* [32]. This ultimately may reflect the complexity and diversity of plant ARF transcription factors during evolution.

Here, we also constructed a phylogenetic tree to analyze the relationships among the ARF gene families in *D. huoshanense*, *D. chrysotoxum*, and *D. nobile*, which were subsequently classified into four classes (Classes I, II, III, and IV) based on their relationships that were aligned from earlier research from *Citrus sinensis* with the *CsiARF* genes [5]. These genes with similar or identical functions were grouped into the same subfamily, and the Class II clade contained the most ARF genes in the three *Dendrobium* species. This clade is closely related to 13 *Arabidopsis* genes, including *AtARF1*, *AtARF2*, and *AtARF12*. From this clade, our qRT-PCR analysis also suggests that the relatively high expression of *DnoARF16*, 17, and 19 in the whole process of flower development may hint that these genes are involved in the ABA biosynthesis pathway as auxin inhibitors [33].

In this work, 53 ARF genes containing both DBD and MR were identified and characterized, except for *DnoARF14*, which lacked the DBD domain (Figure 4B). The absence of the DBD domain suggests that the protein encoded by this gene was unable to recognize and bind auxin response elements (AuxRE) on the promoters of target genes. Our transcriptomic data analysis further showed minimal expression of the *DnoARF14* gene during the entire process of flower development in *D. nobile*. Previous research suggests that the *DnoARF14* gene may not be involved in the flower development of *D. nobile*, and the 23 ARF genes lack the CTD domain in the three *Dendrobium* species, which is comparable to *Pseudocymbidium sinensis* (36.4%) [34] and *S. lycopersicum* (28.6%) [30]. However, we also

found that the Class I genes exhibited a lack of CTD domains compared to ARF genes from other subfamilies, which acts as a structural feature consistent with the structure of *AtARF3* genes from the *Arabidopsis* subfamily and is similar to other results for *Orchidaceae* species [14].

Previous studies have also revealed that within MR domains, glutamine-rich residues tend to act as activators, in contrast to serine, leucine, proline, or glycine [1]. Subsequently, amino acid composition in the MR domain dictates whether ARF functions as a transcription activator or inhibitor. Previously reported findings indicated that the glutamine-rich activators in the ARF-MR domain were predominantly classified under subfamily Class III, which demonstrated significant activation potential for this subfamily [35]. In this study, the three *Dendrobium* species predicted that transcription activator factors, primarily found in Class III, were found to play pivotal roles in activating downstream target genes, particularly with *D. nobile* and *D. huoshanense*. In addition, our study further revealed a predicted ratio of transcription activators to inhibitors among identified ARF genes, with approximately 0.23 acting as activators, which closely aligns with previous predictions for ARF genes in *D. catenatum* [36]. Phylogenetically speaking, our study provides a comprehensive description of the structural characteristics of ARF family genes in three *Dendrobium* species. The ARF family genes within the evolutionary branch exhibit similar intron-exon structural features with previous studies that indicate the conservation of ARF structures. In particular, both Class I and Class III exhibit longer intron lengths (Figure 3), which is a trait that has been reported in nearly all sequenced orchids [37–39]. Additionally, the emphasis placed on longer introns in orchids during gene evolution suggests their potential to enhance natural selection efficiency by facilitating recombination between adjacent exons [40]. Ultimately, this phenomenon may contribute to the rich species diversity observed in orchids.

With gene expression, regulation occurs in the promoter region and is primarily controlled by *cis*-acting elements located upstream of the transcription start site [41]. Here, our study identified regulatory elements in the ARF gene promoter regions of *Dendrobium*, including plant hormone-responsive elements, developmental elements, and stress-responsive elements (Figure 4). These plant hormone elements are known to respond to Acrylonitrile-butadiene-acrylate (ABA), Methyl Jasmonate (MeJA), Gibberellic Acid (GA), and auxin. Recently, studies have also shown that the overexpression of *ARF3* and *ARF6* genes in bamboo increases 1H-Indole-3-acetic acid (IAA) content that regulates lignin biosynthesis [42]. Likewise, in durian fruits, *DzARF2A* plays a major part in fruit ripening by modulating ethylene biosynthesis [43]. In particular, *SlARF5* regulates the set and development of tomato fruit by modulating auxin and gibberellin signaling [34], while *SlARF6A* regulates the ripening of tomato fruits and the production of ethylene [44]. Overall, these studies confirm the important regulatory roles of ARF transcription factors in plant growth and development, and that diverse *cis*-acting elements may be associated with the multifaceted regulatory mechanisms of ARF genes.

5. Conclusions

In this study, a total of 54 *Dendrobium* ARF genes were identified and classified into four branches using phylogenetic analysis. In total, 13, 18, and 23 ARF genes were identified in the genomes of *D. chrysotoxum*, *D. huoshanense*, and *D. nobile*, respectively. Subsequently, they were classified into four classes (Classes I, II, III, and IV) based on their phylogenetic relationships. We also found a relatively higher number of members in Clade II (63) compared to the other clades. Additionally, motif, gene structure, and collinearity analyses indicated the conservation of ARF genes in three *Dendrobium* species. Protein sequence analysis also revealed that 30 ARF proteins with three classically conserved structural domains (BDB, MR, and RD) were present in the three *Dendrobium* species. *Cis*-acting element analysis also showed that *Dendrobium* ARF genes are regulated by light and various photoreceptors. Subsequently, our gene expression patterns analysis also indicates the potential involvement of *DnoARF* 5, 7, 10, 11, 12, 13, 16, 17, and 23 in the initial

differentiation and flower bud formation stages of flower development. Likewise, our RT-qPCR analysis suggests that *DnoARF5* and *DnoARF16* may have a significant impact on flower development and regulation. Together, these results improve our understanding of how ARF genes regulate flower growth and development in *Dendrobium*, while future research should investigate the functional mechanisms of ARF genes to characterize their additional roles in the evolution of more orchid plants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10060568/s1>.

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