

Article

Genome-Wide Identification and Expression Analysis of the Dirigent Gene Family in *Dendrobium lindleyi*

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Highlights

What are the main findings?

- Fifteen DIR genes were identified in *Dendrobium lindleyi*, classified into five subfamilies, with DIR-b/d containing the most members (9).
- *DIDIR8* expression was significantly downregulated by ABA, and the encoded protein localized to the nucleus.

What are the implications of the main findings?

- This work provides a genomic and transcriptomic resource for functional studies of DIR genes involved in hormone signaling in *D. lindleyi*.

Abstract

Dendrobium lindleyi is an orchid species valued for both ornamental and medicinal purposes; its secondary metabolites possess heat-clearing and fluid-generating properties. Dirigent (DIR) proteins play a key regulatory role in plant lignin polymerization and responses to abiotic and biotic stresses; however, systematic studies of DIR family genes in *D. lindleyi* are lacking. The objective of this study was to systematically characterize the DIR gene family in *D. lindleyi*. To achieve this, bioinformatics methods were used to identify DIR genes genome-wide, followed by analyses of their post-translational modifications, gene and protein structures, phylogeny, promoter cis-regulatory elements, expression patterns, and subcellular localization. The results show that 15 DIR family genes were identified in the *D. lindleyi* genome. The DIR family is divided into five subfamilies: DIR-a, DIR-b/d, DIR-c, DIR-e, and DIR-g, among which DIR-b/d has the most members (9), and *DIDIR2* and *DIDIR4* exhibit a fragment duplication event. The promoter regions are rich in light-responsive, ABA, MeJA, MYB, MYC, WRKY, and oxidative stress-related elements, suggesting that DIR genes in *D. lindleyi* may be involved in multiple signaling pathways. Transcriptomic and qRT-PCR analyses revealed that *DIDIR8* exhibits a strong response to ABA treatment, with ABA inducing its downregulation. Subcellular localization studies revealed that the *DIDIR8* protein is localized to the nucleus. These findings provide a foundation for the functional characterization of the DIR gene family in *D. lindleyi* and highlight *DIDIR8* as a candidate in the ABA-mediated stress response, offering a theoretical basis for the potential genetic improvement of this species.



Academic Editor: Jinzhi Zhang

Received: 16 May 2026

Revised: 23 June 2026

Accepted: 26 June 2026

Published: 28 June 2026

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Keywords: *Dendrobium lindleyi*; dirigent; phylogeny; transcriptome; qRT-PCR; subcellular localization

1. Introduction

Dendrobium lindleyi (*D. lindleyi*) is a perennial epiphytic herb belonging to the genus *Dendrobium* in the family Orchidaceae. It is primarily distributed in the tropical and warm subtropical regions of China, South Asia, and Southeast Asia, and is commonly found on forest edges or the trunks of sparse forests at elevations of 500–2000 m [1]. The plant is small and compact, with densely clustered pseudobulbs that are laterally compressed and segmented, each bearing a single leathery leaf at the apex. It is a spring-flowering *Dendrobium* species with typical tropical characteristics and serves as an important parental material for dwarfing breeding programs in *Dendrobium* orchids [2]. In traditional taxonomy, *D. lindleyi* is often classified within the Chrysotoxae section; however, recent molecular studies indicate that it is phylogenetically distant from some species within the same section, supporting the view that it should be established as a new section to reflect the species' phylogenetic uniqueness [3,4]. Medicinally, the whole plant of *D. lindleyi* possesses properties that nourish yin, clear heat, benefit the stomach, and promote fluid production; it is commonly used in folk medicine to treat conditions such as coughs and bronchitis [5,6]. Studies on its chemical constituents have revealed the presence of various secondary metabolites, including benzoquinones, phenanthrenes, and anthraquinones. Some of these compounds exhibit immunomodulatory and other bioactive properties, suggesting significant potential for medicinal development [7,8]. Currently, research on *D. lindleyi* has covered taxonomy, chemistry, and cultivation biology; however, understanding of its genetic background remains relatively limited and requires further systematic exploration. In particular, the regulatory mechanisms of hormone-responsive genes and their roles in secondary metabolite biosynthesis in this species remain poorly characterized. Identifying hormone-responsive gene families such as DIRs represents an essential first step toward filling this gap.

Dirigent (DIR) proteins are a class of key regulatory proteins widely distributed in vascular plants; their name derives from the Latin word “dirigere”, meaning “to guide”. Since its initial identification in *Forsythia intermedia*, research has revealed that this protein lacks enzymatic catalytic activity but precisely regulates the stereoselective polymerization of lignin monomers, thereby guiding the formation of polymers with specific spatial conformations [9,10]. DIR proteins are ubiquitous in ferns, gymnosperms, and angiosperms, and typically evolve as multigene families [11]. Based on phylogenetic relationships, the DIR family is primarily divided into several subfamilies, including DIR-a, DIR-b/d, DIR-c, DIR-e, DIR-f, and DIR-g. Among these, the DIR-a subfamily has been shown to be directly involved in the biosynthesis of lignin and lignans, while other subfamilies (such as DIR-b) have been found to potentially participate in the formation of secondary metabolites such as gossypol [12]. Extensive research indicates that DIR genes play a central role in plants' responses to biotic stress. By promoting cell wall lignification or accumulating disease-resistant lignans, they significantly enhance plant resistance to pathogens such as verticillium wilt and late blight [13–16]. Many DIR members can respond to various abiotic stresses, such as salinity and drought, as well as signaling molecules, and participate in the plant's stress adaptation process [17–19]. Consequently, the DIR gene family has become an important research focus for elucidating plant secondary metabolite regulatory networks and stress resistance mechanisms and holds potential application value in the genetic improvement of crop resistance [20].

Currently, there are few reports on DIR family genes in *D. lindleyi*. In light of this, this study identified DIR genes in the *D. lindleyi* genome using bioinformatics methods and analyzed their post-translational modifications, gene and protein structures, phylogeny, and promoter cis-acting regulatory elements. Furthermore, transcriptomic techniques and quantitative real-time PCR (qRT-PCR) were used to analyze their expression levels

following treatment with abscisic acid (ABA) and gibberellic acid (GA₃), and to examine their subcellular localization via transient expression in *Nicotiana benthamiana*. The aim is to investigate the response of DIR genes in *D. lindleyi* to hormones such as ABA and GA₃, thereby providing a theoretical foundation for understanding the biological functions of the DIR gene family and their roles in hormonal responses.

2. Materials and Methods

2.1. Materials and Treatment

Mature plants of the Thai ecotype of *D. lindleyi* (6–8 cm in height, 26 months after sprouting from basal buds) were selected as experimental material in this study. The experiment was conducted in a climate-controlled growth chamber (RXZ-380D, Ningbo Jiangnan Instrument Factory, Ningbo, China) at Lishui University, with a photoperiod of 14 h/10 h, LED (ODT518B, Zhongshan Odeer Electronics Lighting Co., Ltd., Zhongshan, China) light intensity of $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1} \pm 20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and day/night temperatures set at 28 °C/22 °C. To precisely control the dosage and timing, plants were treated by foliar spraying with $0.2 \mu\text{mol}\cdot\text{L}^{-1}$ ABA or $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ GA₃ [21,22]. At 0, 3, 6, and 12 h after treatment, stem segments were taken from the second internode below the shoot apex of *D. lindleyi*; the epidermis was removed, and only the inner flesh tissue of the stem was used. Subsequently, they were rapidly frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA Extraction, Transcriptome Sequencing, and Data Analysis

Powdered stem segments of *D. lindleyi*, ground after cryopreservation in liquid nitrogen, were used to extract total RNA using the RNeasy Pure Polysaccharide and Polyphenol Plant Total RNA Extraction Kit. mRNA was enriched from the total RNA using Oligo dT magnetic beads; after removing genomic DNA, first-strand cDNA was synthesized using random hexamer primers, followed by second-strand cDNA synthesis. Subsequent steps included end repair, A-tailing, adapter ligation, size selection, PCR amplification, and purification. After quality control, the library was sequenced using the Illumina platform (Illumina NovaSeq X Plus, Illumina, Inc., San Diego, USA). Additionally, first-strand cDNA for qRT-PCR was synthesized using the M5 Sprint qRT-PCR Kit with gDNA Remover from Mei5 Biotechnology®. RNA extraction, cDNA library construction, and high-throughput sequencing were performed by Beijing Novogene Technology Co., Ltd. The sequencing data have been submitted to NCBI under accession number: PRJNA1467120.

Two biological replicates per condition were used for RNA-seq. The raw transcriptomic sequencing data for *D. lindleyi* were quality-assessed with fastQC (v0.12.1) and filtered with fastP (v0.24.0). The transcriptomics quality metrics are summarized in Table S1. Subsequently, sequence alignment was performed using Hisat2 (v2.2.1), and gene expression quantification was conducted using featureCounts (v2.0.8) [23]. Differential expression analysis was performed using the DESeq2 package (v1.46.0) within the R software (v4.4.1) [24]. Differentially expressed genes (DEGs) were defined as those with $|\log_2(\text{fold change})| \geq 1$ and adjusted p -value < 0.05 (up-regulated if $\log_2\text{FC} \geq 1$, down-regulated if $\log_2\text{FC} \leq -1$) (listed in Table S2). Expression heatmaps were generated using the pheatmap package (v1.0.12).

2.3. Identification of the DIR Gene Family in *D. lindleyi*

Genomic data for *D. lindleyi*, *Dendrobium officinale*, *Phalaenopsis equestris*, *Vanilla planifolia*, and *Apostasia shenzhenica* were obtained from relevant literature [25–28]. The Dirigent domain model file (PF03018) was downloaded from the Pfam database (<https://www.ebi.ac.uk/interpro/>) (accessed on 4 February 2026). HMMER 3.4 was used to screen for DIR family candidate genes in the above Orchidaceae genomes (E-value $< 1 \times 10^{-10}$). Subsequently,

the NCBI CD-Search tool (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) (accessed on 6 February 2026) and the SMART database (<http://smart.embl-heidelberg.de/>) (accessed on 6 February 2026) were used to analyze conserved dirigent domain features in the aforementioned candidate DIR protein sequences, ultimately identifying members of the Orchidaceae DIR gene family. Each identified DIR family gene from each orchid species was renamed using a combination of the Latin name abbreviation, “DIR”, and a number; the results are shown in Table S3.

2.4. Sequence Alignment and Phylogenetic Analysis of DIR Proteins in *D. lindleyi*

Arabidopsis DIR protein sequences were obtained from TAIR (<https://www.arabidopsis.org/>) (accessed on 6 February 2026), rice DIR protein sequences were downloaded from the Rice Genome Annotation Project (<https://rice.uga.edu/>) (accessed on 6 February 2026), and spruce (*Picea* spp.) DIR genes were sourced from previous studies [12]. The DIR proteins from the three outgroup species mentioned above were combined with those from *D. lindleyi*, *D. officinale*, *P. equestris*, *V. planifolia*, and *A. shenzhenica*. Multiple sequence alignment was performed using the MUSCLE algorithm in MEGA 11 software, and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap repetitions [29], followed by visualization via iTOL (<https://itol.embl.de/>) (accessed on 9 June 2026) [30]. Classification statistics for DIR members were generated based on phylogenetic relationships (listed in Table S4). Additionally, to investigate the internal evolutionary relationships within the *D. lindleyi* DIR protein, a separate phylogenetic tree was constructed for its 15 DIR proteins using the same methods described above.

2.5. Chromosomal Localization of DIR Genes in *D. lindleyi*

Chromosomal localization information for DIR genes was extracted from the gene annotation files of *D. lindleyi*, and MapChat (v2.32) was used to plot the chromosomal distribution of these genes [31].

2.6. Analysis of DIR Gene Structure and Protein Structure in *D. lindleyi*

CDS, UTR, and intron structural information for DIR genes were obtained from gene annotation data of *D. lindleyi*. The local MEME software (v5.5.7) [32] was used to predict conserved protein motifs, with 10 motifs selected. The online protein domain prediction website (<https://www.ebi.ac.uk/interpro/search/sequence/>) (accessed on 7 May 2026) was used to predict the domains of DIR proteins in *D. lindleyi*, screening for signal peptide, dirigent, and jacalin domains. Visualization was performed using the BioVizSeq package (v1.0.4) in R software, integrating the phylogenetic tree, gene structure, conserved protein motifs, and protein domains of the DIR protein of *D. lindleyi* [33].

2.7. Post-Translational Modification of DIR Protein in *D. lindleyi*

The NetNGlyc online tool (<https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/>) (accessed on 9 May 2026) was used to predict potential N-glycosylation sites of the DIR protein sequence of *D. lindleyi* [34]. When the predicted “Potential” threshold was ≥ 0.5 , the site (N-X-S/T) was identified as an N-glycosylation site. The SignalP 6.0 software (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>) (accessed on 9 May 2026) was used to predict the protein signal peptide (SP) and its cleavage site [35]. The post-translational modifications and signal peptide cleavage sites of the DIR genes in *D. lindleyi* are summarized in Table S5.

2.8. Analysis of Cis-Acting Regulatory Elements of the DIR Gene Promoters in *D. lindleyi*

The promoter sequence upstream of the start codon of DIR genes (2000 bp) in *D. lindleyi* was extracted, and transcriptional regulatory elements were predicted using the Plant-

CARE database (<http://www.dna.affrc.go.jp/PLACE/>) (accessed on 8 May 2026) [36]. The distribution of promoter cis-acting regulatory elements was visualized using the R package BioVizSeq (v1.0.4) [34]. Cis-acting regulatory elements with similar functions were grouped (listed in Table S6).

2.9. Collinearity Analysis of DIR Genes in *D. lindleyi*

To visualize the genomic features of *D. lindleyi*, a collinearity analysis was first performed. The MCScanX software (v1.0.0) was used to perform BLAST (v2.16.0) alignments of genomic CDS sequences, identify collocated gene pairs, and extract collinearity associations for DIR genes in *D. lindleyi* [37]. Using the ‘sliding’ subcommand of Seqkit (v2.8.2), with a sliding window size of 1 Mb and a step size of 1 Mb, the average GC content within each chromosomal window was calculated in *D. lindleyi*. Bedtools (v2.27.1) was used to count gene frequencies in the genome annotation file using 1 Mb windows in *D. lindleyi*. Finally, a Circos diagram (v0.69-8) was generated. collinearity genes were connected by gray lines; collinearity among *D. lindleyi* DIR members was indicated by red lines; GC content was displayed as a heatmap; and gene density was presented as a scatter-plot [38].

2.10. Synteny Analysis of DIR Genes Between *D. lindleyi* and Other Plants

Using the JCVI software (v1.4.16), whole-genome alignments were performed between the CDS sequences of the *D. lindleyi* genome and those of *Arabidopsis thaliana*, *Oryza sativa*, *D. officinale*, and *V. planifolia*, respectively [39]. Homologous gene pairs were identified by setting a C-score threshold of 0.7, and colinearity blocks were identified by requiring a minimum gene interval of 10 genes. Homologous gene pairs associated with the DIR gene were extracted from the above pairwise alignments. The results were visualized using JCVI, with genomic colinearity blocks represented by gray lines and connections between DIR genes represented by dark lines.

2.11. Expression Analysis of DIR Genes by Using qRT-PCR in *D. lindleyi*

Primers of qRT-PCR were designed using Primer Premier 6 software (listed in Table S7). Three biological replicates were analyzed for each sample, and each biological replicate was assessed in duplicate technical replicates. The PCR program was as follows: an initial denaturation step of 30 s at 95 °C, followed by 40 cycles, each consisting of 10 s of denaturation at 95 °C and 30 s of annealing/extension at 60 °C; finally, a melting curve analysis was performed over a temperature range of 65 °C to 95 °C with a 0.5 °C ramp to assess amplification specificity. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [40]. Statistical analysis was performed for each gene by one-way ANOVA in R (v4.5.1), and bar charts were generated using the ggplot2 package in R (v4.5.1) [41].

2.12. Subcellular Localization of *DlDIR8*

The CDS sequence of the *DlDIR8* gene was synthesized by Qingke Biotechnology. Utilizing the NcoI and SpeI restriction sites on the plasmid, the aforementioned fragment was cloned into the pCambia1302 plasmid via homologous recombination, yielding the recombinant expression plasmid pCambia1302-35S::*DlDIR8*-GFP. The recombinant expression plasmid and the pCambia1302-35S::GFP empty vector plasmid (control) were transformed into *E. coli*, respectively. Positive clones were verified by sequencing and then transformed into Agrobacterium competent cells. Single colonies were picked for overnight shaking culture; after harvesting, the cells were resuspended in induction medium and induced for 4 h, then resuspended in permeabilization medium and adjusted to an OD₆₀₀ of approximately 0.5. Using a 1 mL syringe, the bacterial suspension was injected into 4-week-old tobacco leaves, which were then placed in a light-controlled incubator for dark treatment for 48 h. Subsequently, the lower epidermis of the tobacco leaves was peeled off

to prepare temporary slides, which were observed and photographed under a Zeiss LSM 900 (Carl Zeiss AG, Oberkochen, Germany) confocal laser scanning microscope.

3. Results

3.1. Phylogenetic Analysis of DIR Genes in *D. lindleyi*

To elucidate the evolutionary relationships within the Orchidaceae DIR gene family, we analyzed DIR protein sequences from five orchid species. These included *D. lindleyi*, *D. officinale*, *P. equestris*, *V. planifolia*, and *A. shenzhenica*, which were compared with those from three outgroup species (*A. thaliana*, *O. sativa*, and *Picea* spp.) to construct a phylogenetic tree (Figure 1). Based on this phylogenetic tree, the DIR genes were classified into distinct subfamilies, and the number of members in each subfamily was summarized for all species examined (Table S4). The results showed that *D. lindleyi* contains 15 DIR genes. This number is comparable to other Orchidaceae species, with *D. officinale* having 13, *P. equestris* 11, *V. planifolia* 13, and *A. shenzhenica* 10. In contrast, rice and Arabidopsis have significantly more DIR genes, with 49 and 25, respectively, indicating an overall trend of gene reduction in orchids. At the subfamily level, *D. lindleyi* and *D. officinale* are the only orchids that retain one copy each of DIR-e and DIR-c, whereas *P. equestris* and *A. shenzhenica* lack DIR-e, and *V. planifolia* and *P. equestris* lack DIR-c. The Orchidaceae generally lack the DIR-f subfamily, which has 11 members in spruce. The DIR-g subfamily shows interspecific variation: it is expanded in *A. shenzhenica* (7 members) and *V. planifolia* (4 members), but relatively scarce in *D. lindleyi* (2 members), *D. officinale* (3 members), and *P. equestris* (3 members). Notably, *D. lindleyi* has the largest DIR-b/d subfamily with 9 members, accounting for 60% of its total DIR genes, followed by *D. officinale* with 7 members (54%). These two species have the highest numbers of DIR-b/d members among all orchids examined. The genes in this branch are distributed alongside those of various seed plants and have not formed an orchid-specific clade. In summary, DIR genes in Orchidaceae have undergone overall contraction with differential subfamily loss and limited expansion.

3.2. Chromosomal Distribution of DIR Genes in *D. lindleyi*

The chromosomal distribution of the 15 DIR genes in *D. lindleyi* is shown in Figure 2. These genes are unevenly distributed across seven chromosomes (chr02, chr03, chr04, chr05, chr06, chr08, chr10, chr13) and an unchromosomally located scaffold (unchr364). Among these, chromosome chr04 hosts the largest number of genes (*DlinDIR3–DlinDIR8*, totaling 6), which are highly concentrated in a specific region (45.1–47.1 Mb) and exhibit a distinct tandem clustering pattern; the remaining chromosomes typically contain only 1–2 genes. The unlocalized scaffold (unchr364) contains only *DlinDIR15*. This distribution pattern suggests that the DIR genes on chr04 may have undergone a localized tandem duplication in *D. lindleyi*.

3.3. Prediction of DIR Gene and Protein Structure in *D. lindleyi*

Phylogenetic analysis (Figure 3A) and gene structure analysis (Figure 3B) of the DIR family in *D. lindleyi* revealed that 13 of the 15 DIR genes possess a relatively simple structure consisting of a single exon. *DIDIR10* contains two exons, while *DIDIR15*, a member of the DIR-c subfamily, has the most complex structure with five exons, suggesting that this gene may have undergone a more intricate evolutionary process. This structural variation may be related to gene function or regulatory mechanisms. Analysis of conserved motifs of DIR proteins in *D. lindleyi* (Figure 3C) indicates that these proteins contain 2 to 6 motifs, with motif 1 present in all 15 members. Except for *DIDIR15*, motif 2 is present in the other 14 members. Notably, motifs 9 and 10 are found exclusively in the *DIDIR15* protein of the DIR-c subfamily, indicating significant differences in conserved motifs among subfamilies. As shown in Figure 3D, all 15 DIR proteins in *D. lindleyi* conservatively contain a dirigent

domain, suggesting functional commonality. Furthermore, 12 members contain a signal peptide (SP), whereas DIDIR8, DIDIR13, and DIDIR15 lack one. Notably, the DIDIR15 protein contains both the dirigent and jacalin domains.

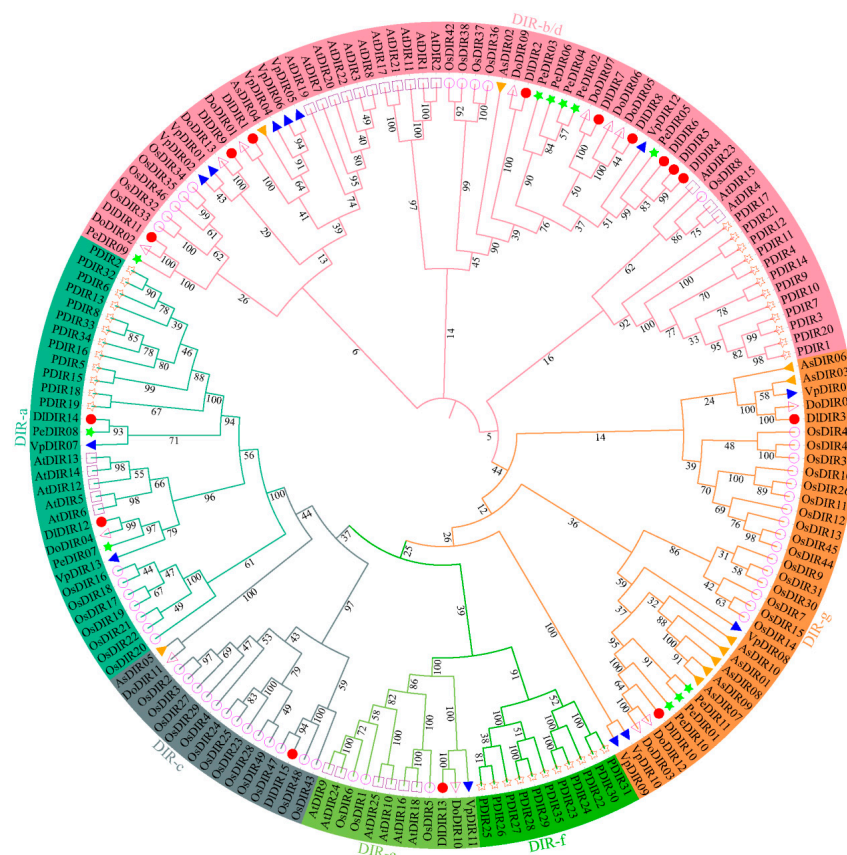


Figure 1. Phylogenetic analysis of dirigent (DIR) proteins from *Dendrobium lindleyi* (Dl), *Dendrobium officinale* (Do), *Phalaenopsis equestris* (Pe), *Vanilla planifolia* (Vp), and *Apostasia shenzhenica* (As), together with those from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), and *Picea* spp. (P). The branches and the colored ranges share the same color scheme for distinguishing DIR subfamilies, with DIR-a in teal, DIR-b/d in pink, DIR-c in gray, DIR-e in yellow-green, DIR-g in green, and DIR-f in orange. Leaf nodes are marked with different colors and shapes and combinations to distinguish species, with *D. lindleyi* as solid red circles, *D. officinale* as open triangles, *P. equestris* as solid bright green five-pointed stars, *V. planifolia* as solid blue triangles, *A. shenzhenica* as solid orange triangles, *A. thaliana* as open squares, *O. sativa* as open circles, and *Picea* spp. as open five-pointed stars.

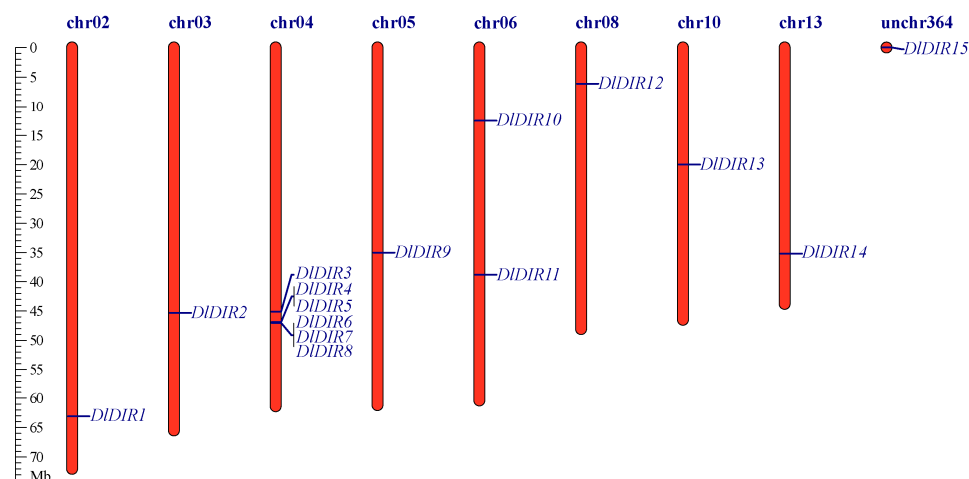


Figure 2. Chromosomal locations of DIR genes in *D. lindleyi*.

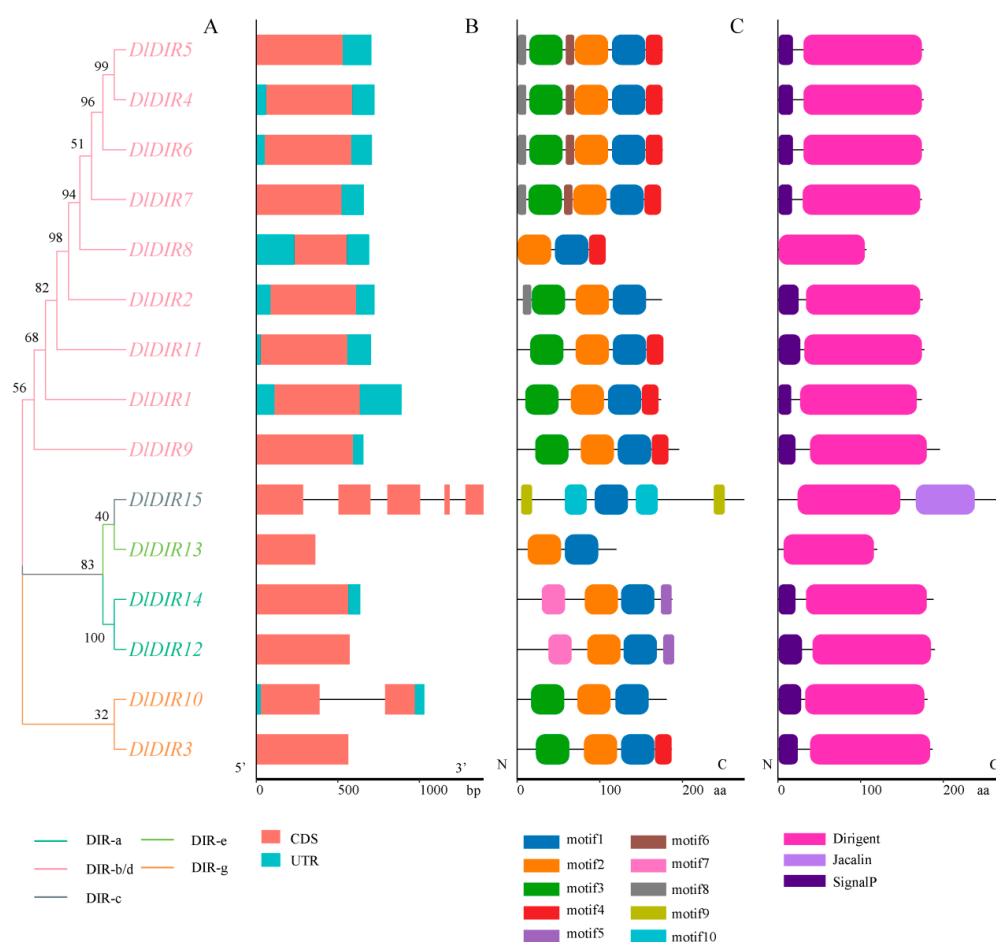


Figure 3. Structural features of DIR genes and proteins in *D. lindleyi*. (A) Phylogenetic tree of DIR protein of *D. lindleyi*; branches of the same color represent members of the same subfamily; (B) DIR gene structure of *D. lindleyi*; CDS and UTR are represented by differently colored blocks, and introns are indicated by black lines; (C) Conserved motifs distribution of DIR genes in *D. lindleyi*; different motifs are indicated by different colored blocks; (D) Conserved domains in DIR genes of *D. lindleyi*; different domains are indicated by different colored blocks.

3.4. Post-Translational Modifications Analysis of DIR Proteins in *D. lindleyi*

The results in Table S5 showed that all DIR proteins in *D. lindleyi* contain 1–3 N-glycosylation sites, with DIDIR3 containing only one site (Asn53). SignalP 6.0 signal peptide prediction results indicate that 12 members of this family contain signal peptides (SP). Among these 12 members, DIDIR10 has a predicted confidence score of 0.7286, while the remaining 11 members all have scores above 0.97, suggesting they may be localized to the endomembrane system. Conversely, members lacking a signal peptide (such as DIDIR8, DIDIR13, and DIDIR15) may localize to other subcellular regions. These differences in properties reflect the diverse biological functions that DIR genes in *D. lindleyi* may perform within the genus, and the signal peptide prediction results are consistent with those shown in Figure 3D.

3.5. Cis-Acting Regulatory Elements Analysis of DIR Genes in *D. lindleyi*

The distribution of transcriptional regulatory elements of DIR gene promoters in *D. lindleyi* (Figure 4) and the statistical analysis (Table S6) reveal that light-responsive elements are the most abundant (156) among the transcriptional regulatory elements of DIR gene in *D. lindleyi*, indicating that this family is generally regulated by light signals. Among hormone response elements, methyl jasmonate (MeJA) response elements were

the most abundant (64), followed by abscisic acid (ABA, 23) and ethylene (28), suggesting that DIR genes in *D. lindleyi* may be involved in jasmonate-mediated defense responses and ABA/ethylene-regulated stress adaptation. A diverse range of abiotic-stress-related elements was identified, including oxidative stress (32), drought response (22), wounding response (23), and anaerobic induction (17), reflecting the potential role of this family in adapting to multiple stress conditions. Among the transcription factor binding sites, MYB binding sites were the most abundant (112), covering the vast majority of genes, suggesting that MYB is the central upstream regulator of this family's expression; furthermore, MYC (59) and WRKY (26) sites were also widely present, further corroborating their integrative roles in hormone and stress signaling networks. The distribution of cis-acting regulatory elements varied significantly among genes in the DIR family of *D. lindleyi* (Table S6), with the total number of elements ranging from 31 to 64 across the 15 genes. *DIDIR8* had the highest number (64), followed by *DIDIR5* (62) and *DIDIR11* (58). *DIDIR8* contains a relatively high number of cis-acting elements associated with light response (17), MeJA response (10), MYB binding (8), oxidative stress (5), and ABA response (4), indicating its potential involvement in multiple signaling pathways, including light, hormone, and stress responses. *DIDIR5* is enriched in light response (12), MYB (12), and secondary xylem development (3) elements, suggesting a potential role in vascular tissue development. *DIDIR11*, on the other hand, possesses a higher number of light response (8), MYB (14), and wound response (3) elements. In contrast, genes such as *DIDIR1*, *DIDIR2*, *DIDIR3*, and *DIDIR10* have fewer total elements (31–40), suggesting relatively limited regulatory potential. In summary, the cis-acting regulatory element composition of the DIR genes in *D. lindleyi* exhibits both commonalities in light response and MYB regulation, as well as distinct intra-family specialization.

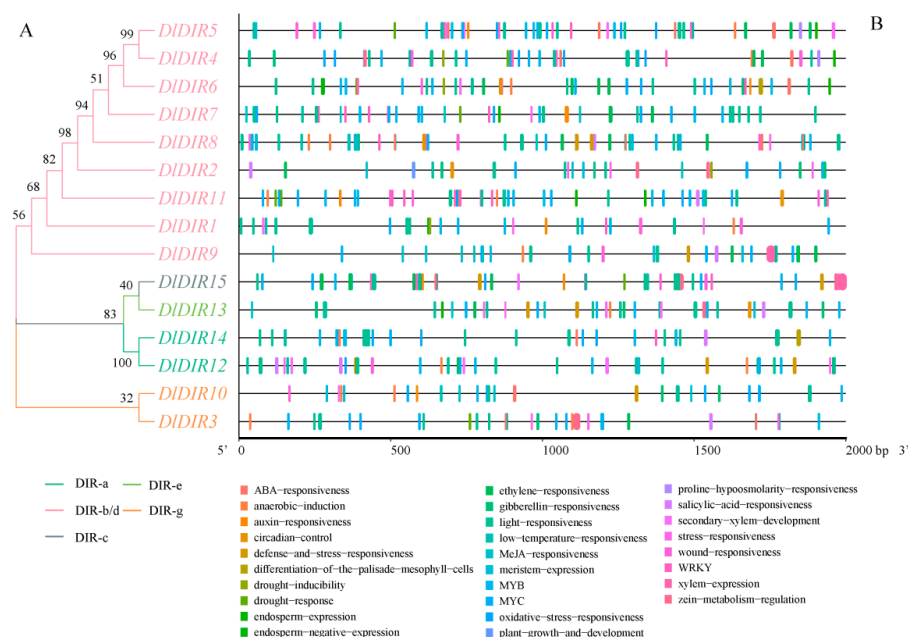


Figure 4. Phylogenetic tree of DIR protein and cis-acting regulatory elements in the DIR gene promoter sequence in *D. lindleyi*. (A) Phylogenetic tree of DIR protein of *D. lindleyi*; (B) Cis-acting regulatory elements in the DIR gene promoter sequence in *D. lindleyi*. Rectangles of different colors represent various cis-acting regulatory elements, and their positions are annotated based on their distribution within the promoter.

3.6. Gene Duplication Analysis of DIR in *D. lindleyi*

This study analyzed the GC content across the entire genome of *D. lindleyi*, with the results shown in Figure 5. The GC content of *D. lindleyi* is generally stable, with most

regions ranging between 34% and 37%, indicating a relatively uniform genomic base composition. However, locally anomalous high- or low-GC regions were identified across multiple chromosomes, suggesting heterogeneity in the genome's functional structure. The distribution of genes on chromosomes in *D. lindleyi* exhibits significant heterogeneity. Most chromosomes exhibit distinct gene-rich and gene-poor regions. Certain regions on chromosomes 2, 9, and 12 lack colinearity with other chromosomes, while the colinearity observed between *DIDIR2* and *DIDIR4* indicates that gene duplication has occurred. No gene duplication was detected in other DIR genes of *D. lindleyi*.

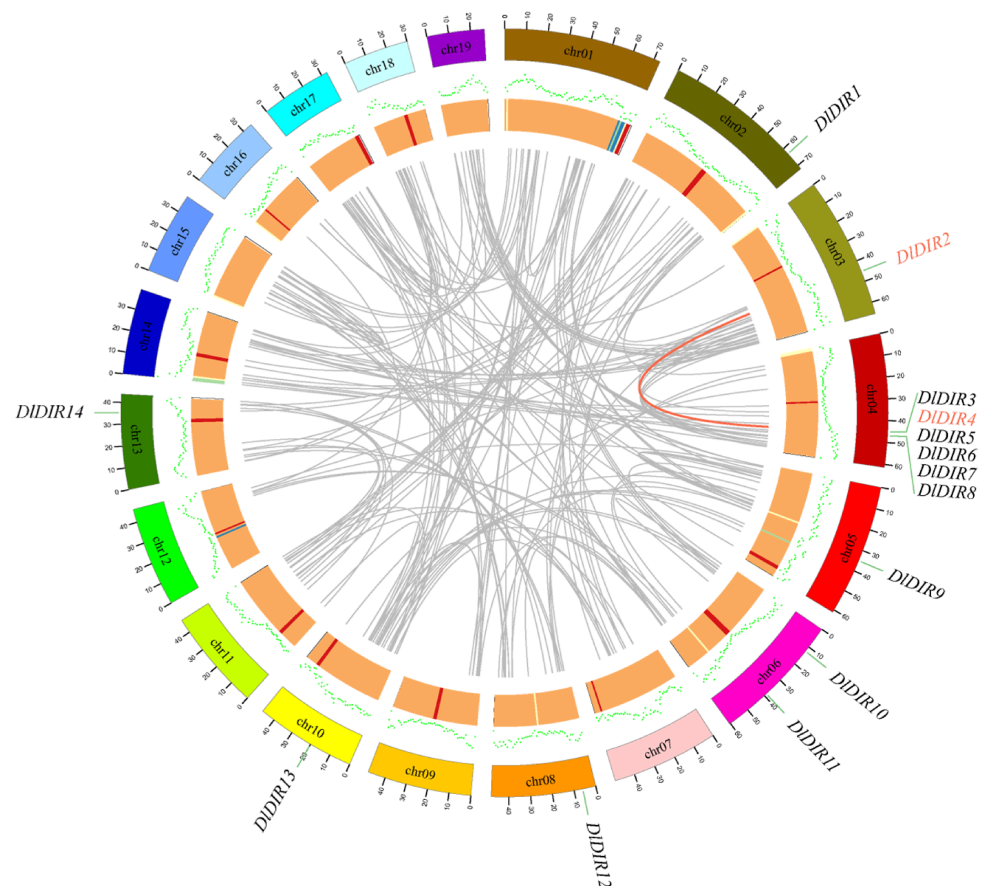


Figure 5. Intra-species colinearity analysis of genes in *D. lindleyi*. From outermost to innermost: DIR gene names of *D. lindleyi*, chromosome numbers, gene density (scatter-plot), GC content (heatmap), and gene duplication events highlighted in red.

To further investigate the evolution of DIR genes in *D. lindleyi*, we performed synteny analyses between DIR genes in *D. lindleyi* and the other DIR genes in *D. officinale*, *V. planifolia*, *A. thaliana*, *O. sativa*, respectively (Figure 6). The results revealed a large number of homologous genes between *D. lindleyi* and *D. officinale*, among which 8 pairs of DIR genes exhibited colinearity (*DIDIR1* and *DoDIR01*, *DIDIR2* and *DoDIR09*, *DIDIR3* and *DoDIR08*, *DIDIR7* and *DoDIR07*, *DIDIR10* and *DoDIR03*, *DIDIR11* and *DoDIR02*, *DIDIR12* and *DoDIR04*, *DIDIR13* and *DoDIR10*); Co-dominant genes were found to be sporadically distributed between the *D. lindleyi* and *V. planifolia* chromosomes, with one pair of DIR genes exhibiting colinearity (*DIDIR7* and *VpDIR12*); this indicates that the DIR genes of *D. lindleyi* share a closer evolutionary relationship with those of *D. officinale* than with those of *V. planifolia*.

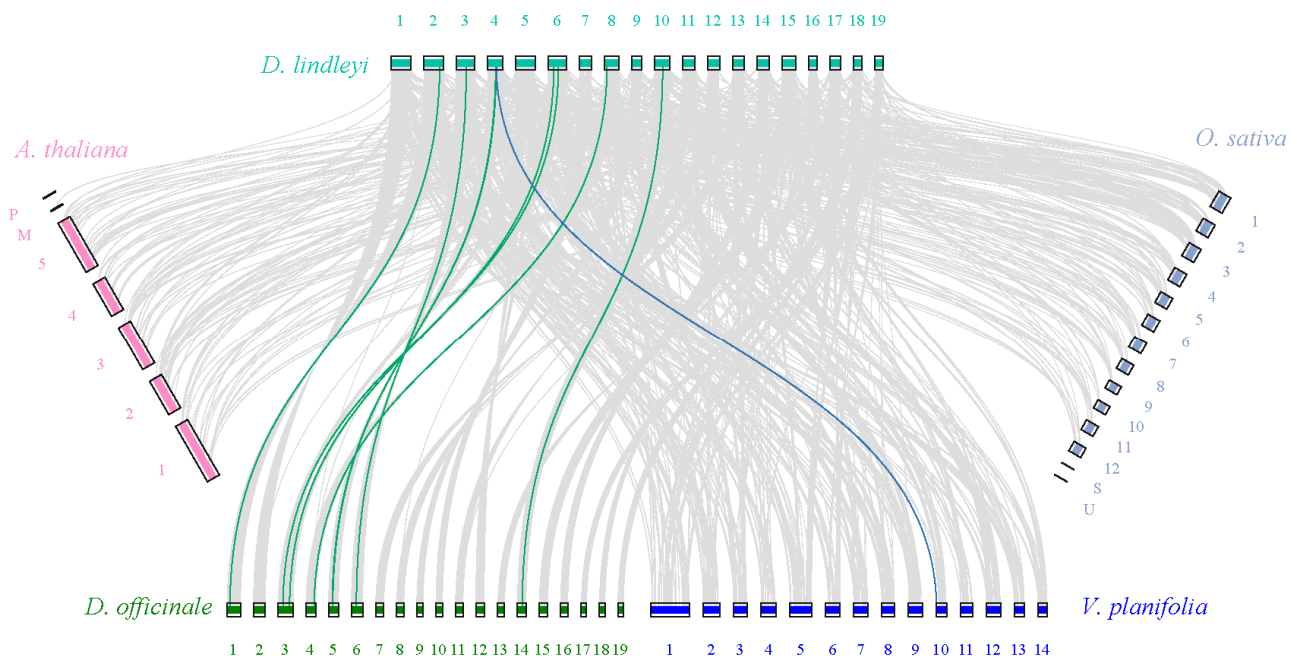


Figure 6. Analysis of DIR gene linkage pairs between *D. lindleyi* and *A. thaliana*, *D. lindleyi* and *O. sativa*, *D. lindleyi* and *D. officinale*, and *D. lindleyi* and *V. planifolia*. Chromosomes of *D. lindleyi*, *A. thaliana*, *D. officinale*, *V. planifolia*, and *O. sativa* are colored in light green, pink, green, blue, and light blue, respectively. Green lines represent collinear gene pairs between *D. lindleyi* and *D. officinale*. Blue lines represent collinear gene pairs between *D. lindleyi* and *V. planifolia*.

3.7. Transcriptomic Analysis of DIR Gene Expression in *D. lindleyi*

As shown in Figure 7 and Table S7, during ABA treatment, the expression levels of *DIDIR4* and *DIDIR5* in the stem increased, while the expression level of *DIDIR8* decreased significantly throughout the period, and the expression of *DIDIR11* increased significantly overall. In contrast, during GA₃ treatment, the expression levels of *DIDIR5* and *DIDIR8* first decreased, then increased, and then decreased again, whereas only *DIDIR8* expression decreased significantly. The expression level of *DIDIR11* gradually increased. Therefore, *DIDIR8* is a gene that responds simultaneously to ABA or GA₃ treatment. The highly expressed genes mentioned above all belong to the DIR-b/d subfamilies, whereas DIR-a, DIR-c, DIR-e, and DIR-g exhibit subfamily-specific expression patterns, with *DIDIR3* exhibiting extremely low expression levels.

3.8. Expression Analysis of the DIR Gene by Using qRT-PCR in *D. lindleyi*

The qRT-PCR validation included both an ABA-induced gene, *DIDIR5*, and an ABA-repressed gene, *DIDIR8* (Figure 8). The other genes that maintained consistently low expression across all samples were not prioritized due to the inherent unreliability of quantifying very low-abundance transcripts by qRT-PCR. The expression level of *DIDIR5* gradually increased during ABA treatment, reaching a level at 12 h that was significantly higher than that at 0 h ($p < 0.05$). In contrast, the expression level of *DIDIR8* gradually decreased during ABA treatment and was significantly lower at 12 h than that at 0 h ($p < 0.05$). Under GA₃ treatment, the expression level of *DIDIR5* increased significantly at 3 h ($p < 0.05$) and remained relatively stable at 6 and 12 h. In contrast, the expression level of *DIDIR8* decreased significantly at 3 h ($p < 0.05$) under GA₃ treatment and remained relatively stable at 6 and 12 h.

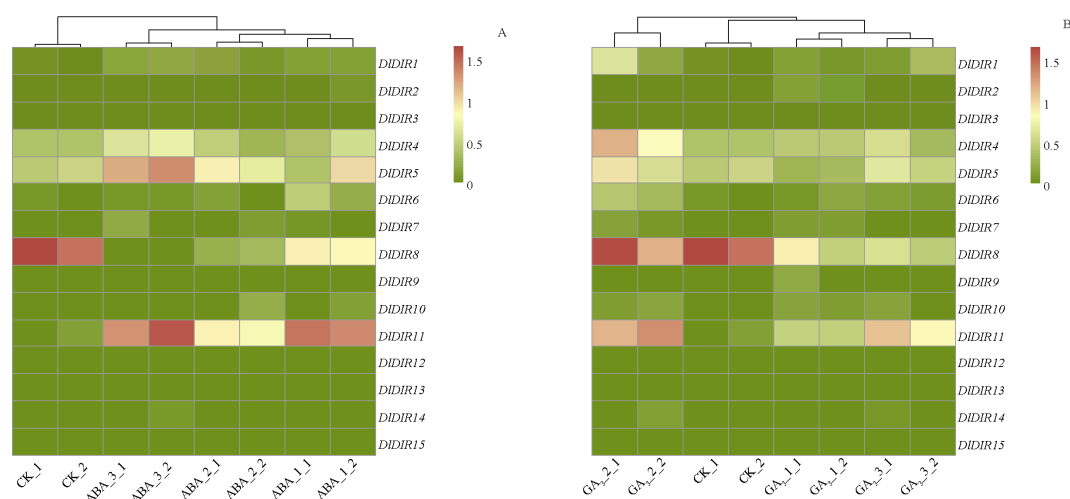


Figure 7. Expression profiles of DIR genes in stems of *D. lindleyi* under ABA and GA₃ treatment. **(A)** The expression patterns of DIR genes under ABA treatment in *D. lindleyi*; CK_1, CK_2 were two replicates of the untreated control collected at 0 h, ABA_1_1 and ABA_1_2 were two repetitions of ABA treatment for 3 h, ABA_2_1 and ABA_2_2 were two repetitions of ABA treatment for 6 h; ABA_3_1 and ABA_3_2 were two repetitions of ABA treatment for 12 h. **(B)** The expression patterns of DIR genes under GA₃ treatment in *D. lindleyi*; CK_1, CK_2 were two replicates of the untreated control collected at 0 h, GA₃_1_1 and GA₃_1_2 were two repetitions of GA₃ treatment for 3 h; GA₃_2_1 and GA₃_2_2 were two repetitions of GA₃ treatment for 6 h; GA₃_3_1 and GA₃_3_2 were two repetitions of GA₃ treatment for 12 h.

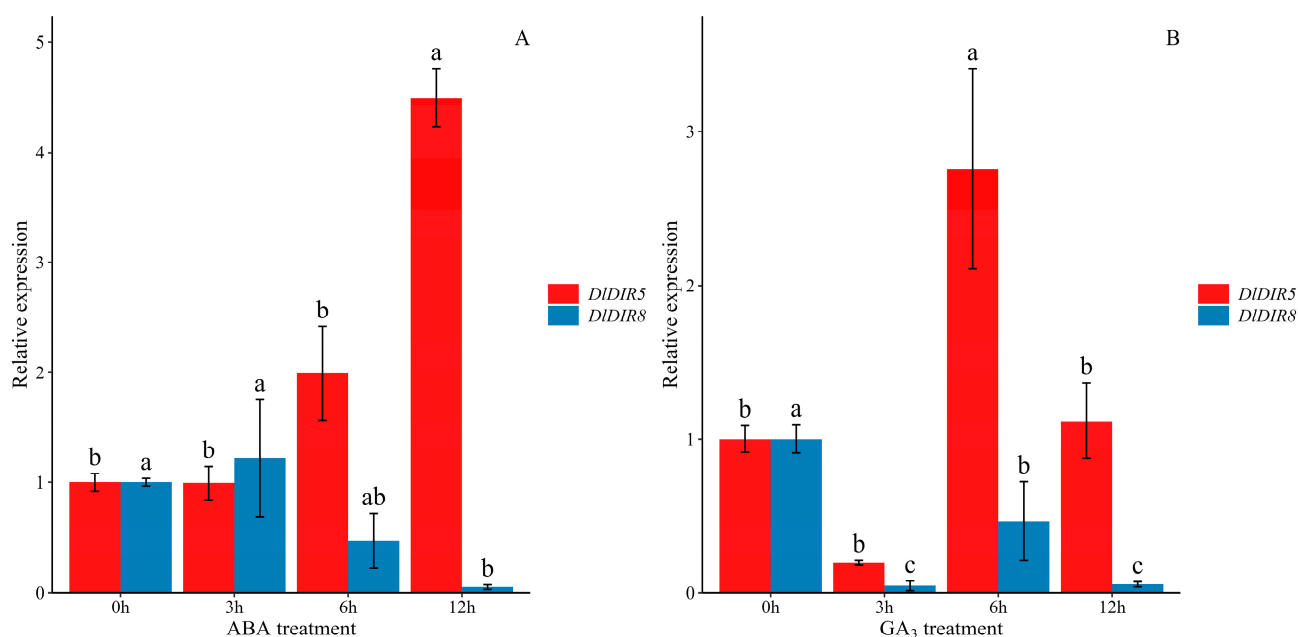


Figure 8. Relative expression levels of DIR genes in *D. lindleyi* under different durations of ABA and GA₃ treatment. **(A)** ABA treatment; **(B)** GA₃ treatment. A one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used to assess differences among treatment means at the 95% confidence level ($p < 0.05$). 0 h, 3 h, 6 h, and 12 h represent the four time points for ABA or GA₃ treatment, respectively. Letters on bars of the same color indicate significant differences within the same gene across different time points.

3.9. Subcellular Localization of DIDIR8 in *D. lindleyi*

Based on DIR gene expression patterns in *D. lindleyi* under ABA and GA₃ treatments, the *DIDIR8* gene, which showed the highest expression level and was differentially ex-

pressed, was selected for further analysis. Moreover, DDIR8 lacks a predicted canonical signal peptide (Table S5), unlike most DIR members, including DDIR5. This atypical feature raised the hypothesis that DDIR8 may not follow the classical secretory pathway and was therefore considered a more informative candidate for subcellular localization studies. A 35S promoter-driven DDIR8-green fluorescent protein (GFP) fusion expression vector was constructed, and transient expression was detected in tobacco leaves. As shown in Figure 9, the GFP fluorescence signal of the DDIR8 protein was distributed in the cell nucleus, indicating that DIR is expressed in the nucleus of a specific tobacco cell. But it may have other patterns in the other cell types.

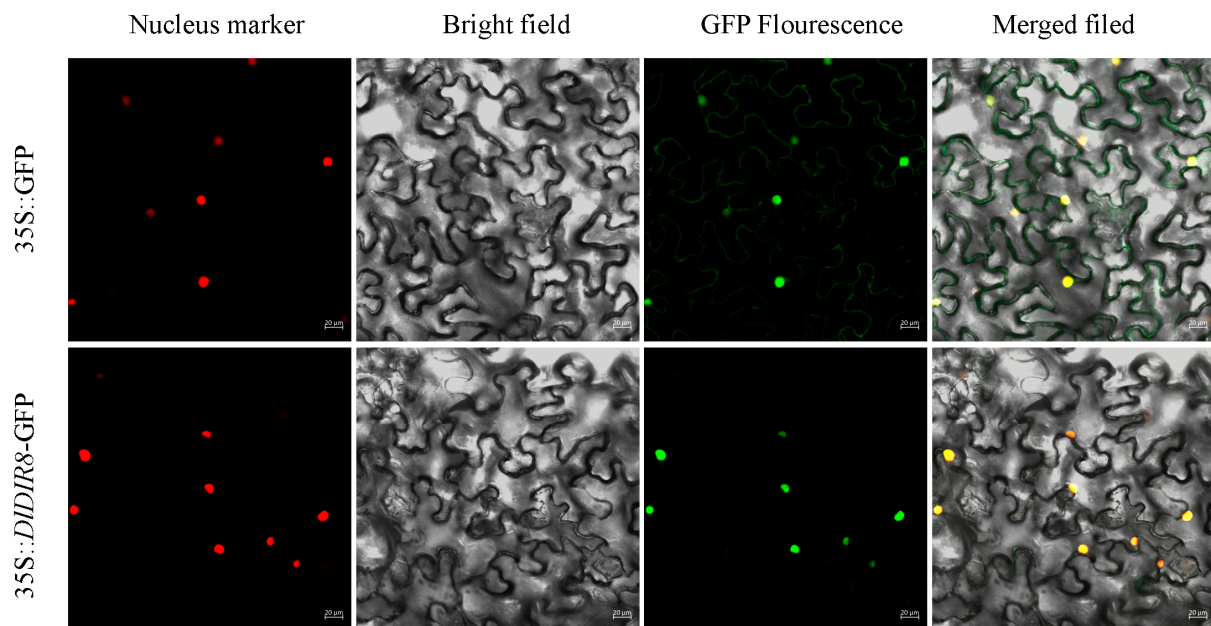


Figure 9. Subcellular localization of the DDIR8-GFP fusion protein. GFP was used as a control. Red fluorescence represents the mCherry protein, green fluorescence represents the GFP protein, and the yellow signal in the merged image arises from the colocalization of the red and green channels.

4. Discussion

The genes and protein structures of most DIR family members in *D. lindleyi* are relatively simple, consisting of a single exon and a single dirigent domain. Among them, DDIR15 has the most complex structure, comprising five exons and a dirigent-jacalin double domain, consistent with the double domain reported in rice, which is considered a chimeric protein unique to the Poaceae family [42–44]. Regarding post-translational modifications, all 15 DIR proteins in *D. lindleyi* contain varying numbers of N-glycosylation sites, whereas 3 of the 31 DIR proteins in tomato lack these sites [45], suggesting that DIR proteins in *D. lindleyi* may rely on significant post-translational modifications to regulate their activity or stability [46]. These characteristics are consistent with predictions for secreted proteins; despite the significant reduction in the number of DIR genes in *D. lindleyi*, all members retain the basic secreted protein functions. Most DIR members in *D. lindleyi* contain typical signal peptides, suggesting they are targeted to exosomes or the cell wall via the classical secretory pathway, which is highly consistent with the core functional model of DIR proteins directing the extracellular deposition of phenolic polymers such as lignin and lignans [13,47]. However, some members, such as DDIR8, lack a discernible signal peptide and localize to the nucleus [48], consistent with nuclear proteins not utilizing the secretory pathway. DDIR8 also lacks a predictable classical NLS, suggesting it may enter the nucleus

via non-canonical NLS sequences, passive diffusion, or protein–protein interactions. The precise mechanism remains to be determined experimentally.

From a phylogenetic perspective, the *D. lindleyi* (15 DIRs) and the *D. officinale* (13 DIRs) both contain the five subfamilies DIR-a, DIR-b/d, DIR-c, DIR-e, and DIR-g, whereas *P. equestris*, *A. shenzhenica*, and *V. planifolia* each lack 1–2 subfamilies. This indicates that the Orchidaceae ancestor already possessed a five-subfamily framework, with subsequent independent losses occurring within lineages. The fact that *D. lindleyi* and *D. officinale* retain all five subfamilies may be related to growth adaptations. The composition of these five subfamilies is consistent with that of rice (45 DIRs), suggesting that the monocot ancestor possessed these five subfamilies prior to the divergence of the Orchidaceae and Poaceae; however, *Sorghum bicolor* (53 DIRs) comprises DIR-a, DIR-b/d, DIR-c, DIR-e, and DIR-f (lacking DIR-g), and since DIR-f and DIR-g belong to the same evolutionary clade, it is inferred that a “DIR-g/ DIR-f replacement” occurred in the Poaceae. The rice lineage lost DIR-f and retained DIR-g, while the *S. bicolor* lineage lost DIR-g and retained DIR-f [49]; thus, the monocot ancestor likely possessed a complete six-subfamily pattern including DIR-f and DIR-g, and the current distribution results from loss at the family/genus level. Compared to eudicots, *Cajanus cajan* (25 DIRs) contains all six DIR subfamilies [50], supporting the hypothesis that the angiosperm ancestor possessed all six DIR subfamilies; however, phylogenetically specific losses have occurred across different groups. For example, Solanaceae species such as tobacco (57) and potato (33) retain only DIR-a, DIR-b/d, and DIR-e [51]; *Schima superba* retains DIR-a, DIR-b/d, DIR-c, and DIR-e [52]; *Fragaria vesca* retains DIR-a, DIR-b/d, DIR-e, and DIR-g [53], indicating that DIR families underwent multiple independent and differential losses during the radiation of core eudicots. Collinearity analysis reveals evolutionary drivers, extensive DIR collinearity exists between *D. lindleyi* and *D. officinale*, while only limited collinearity is observed with the more distantly related orchid *V. planifolia*, and no collinearity is found with *A. thaliana* or *O. sativa*; DIRs from tobacco and potato also lack collinearity with those in Arabidopsis [51], whereas *Sorghum bicolor* or *Citrullus lanatus* share 2–4 pairs of collinearity with Arabidopsis. These differences, combined with drastic variations in DIR gene numbers, reflect that different lineages have undergone gene expansion and contraction in a highly species-specific manner [49,54]. No collinearity was detected between the DIR-c subfamily of *D. lindleyi* and *D. officinale*, indicating that different subfamilies have undergone differential selective pressures; intraspecific collinearity analysis revealed a fragment duplication relationship between *DIDIR2* and *DIDIR4* within DIR-b/d, demonstrating that gene duplication is a key mechanism driving the increase in gene number and functional diversification. Overall, DIR-a and DIR-b/d are present in all species and constitute the most conserved core subfamily in angiosperms. Within the Orchidaceae, independent loss of DIR-c and DIR-e is characteristic, while DIR-b/d has expanded through duplication (9 in *D. lindleyi* and 7 in *D. officinale*). *D. lindleyi* and *D. officinale* retain relatively complete subfamily compositions and patterns of synteny, providing ideal samples for elucidating the evolution of cell walls in the Orchidaceae and the dual driving forces of “differential loss and specific duplication.” Further studies on physiological functions, including lignan and lignin biosynthesis, disease resistance, and abiotic stress tolerance, using gene expression analysis, are still needed to deepen our understanding of the specific roles of DIR genes in biotic and abiotic stress adaptation [55].

Analysis of promoter cis-acting regulatory elements revealed that the DIR promoter regions in *D. lindleyi* are enriched for various environmental and endogenous signal-response elements, with light-responsive elements being the most abundant. This suggests that light may be a key factor regulating DIR expression and phenolic metabolism in *D. lindleyi* [56]. Hormone treatment experiments showed that *DIDIR5* and *DIDIR8* respond to ABA and GA₃, respectively, with opposite patterns. This contrasting response may

be attributed to the complexity of their genetic backgrounds, particularly the differences in promoter cis-acting regulatory elements. The presence of multiple ABA-responsive elements in both promoters (five in *DIDIR5*, four in *DIDIR8*) suggests direct regulation, while ABA may also act indirectly via MYB, MYC, and WRKY transcription factors. Neither promoter contains canonical GA-responsive elements; thus, GA₃ likely regulates these genes indirectly via MYB and WRKY factors. This direct and indirect regulatory model remains hypothetical and requires experimental validation.

Furthermore, in *P. leptostachya*, MeJA, SA, and ethylene significantly induce *PIDIR1* expression, linking it to phenoxy radical metabolism and lignin accumulation [57], while in pear, *PbDIRs* respond to ABA, SA, and MeJA and may participate in sclereid formation and lignin polymerization [58]. In the resurrection plant *B. hygrometrica*, *BhDIR1* is strongly induced during dehydration and rehydration, likely through drought-triggered ABA accumulation, and may modulate lignin acid solubility to dynamically adjust cell wall properties [59]. These findings align with the ABA-induced expression of *DIDIR5* observed in this study. In contrast, the repression of *DIDIR8* by ABA may stem from structural or regulatory differences that remain to be elucidated. Notably, MeJA and ethylene response elements were also highly enriched in DIR promoters (64 MeJA elements, with 10 in *DIDIR8*), consistent with the involvement of DIR genes in defense responses reported in other species [54,60]; however, as no MeJA treatment was conducted here, this remains a hypothesis. DIR-mediated lignan metabolism may also be relevant to the edible quality of *D. lindleyi* stems. These hypotheses require further experimental investigation.

This study found that the DIR-a, DIR-c, DIR-e, and DIR-g subfamilies in *D. lindleyi* appear silent under normal conditions, similar to *GhDP1_D1* and *GhDP1_D2* in cotton [14], whereas the DIR-b/d subfamily is the largest and shows basal expression. These patterns suggest that the former subfamilies may require specific developmental or stress signals for activation, while the latter perform more conserved, fundamental functions. However, due to the technical limitations of bulk RNA-seq and qRT-PCR, we cannot exclude the possibility that opposite expression trends in distinct cell types within the stem may mask cell-type-specific regulation and contribute to the observed stable average values. Future studies using in situ spatial cell biology methods [61] and single-cell RNA-seq are needed to resolve cell-type-specific DIR expression and refine these functional inferences. Future studies should also examine how DIR-mediated lignification affects stem quality and the functional specialization of subfamilies in stress adaptation, with the aim of improving both quality and stress tolerance in *D. lindleyi*.

5. Conclusions

This study systematically identified 15 DIR genes in the *D. lindleyi* genome, which belong to five conserved evolutionary subfamilies and are phylogenetically closer to those of monocotyledons. These genes exhibit diversity in gene and protein structures, as well as in the composition of promoter cis-acting regulatory elements, including abundant light-responsive, ABA, MeJA, MYB, MYC, WRKY, and oxidative stress-related elements. These features suggest that DIR genes in *D. lindleyi* may participate in multiple signaling pathways. Hormone treatment experiments showed that *DIDIR5* was induced by both ABA and GA₃, whereas *DIDIR8* was repressed by both hormones. Subcellular localization analysis revealed that the *DIDIR8* protein is localized to the nucleus, consistent with its lack of a canonical signal peptide. These results provide a foundation for future functional studies of the DIR gene family in *D. lindleyi* and offer a theoretical basis for its potential genetic improvement.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070789/s1>, Table S1: Transcriptomic sequencing quality metrics of *D. lindleyi* after filtering with fastP; Table S2: DESeq2 differential expression analysis of DIR genes in *D. lindleyi* following separate treatments with ABA and GA₃; Table S3: Correlation table of DIR gene IDs and gene names in the Orchid family; Table S4: Number of DIR subfamily members in *D. lindleyi*, *D. officinale*, *P. equestris*, *V. planifolia*, and *A. shenzhenica*, with those from *A. thaliana*, *O. sativa*, and spruce (*Picea* spp.); Table S5: Post-translational modifications of the DIR gene in *D. lindleyi*; Table S6: Number of cis-acting regulatory elements in the DIR gene promoter sequence of *D. lindleyi*; Table S7: List of primer used for qRT-PCR.

Author Contributions: The authors confirm contribution to the paper as follows: Draft manuscript preparation and figure preparation: Y.Y. and L.Z. Data analysis: Y.Y. Study conception and design, and methodology supervision: L.Z. Manuscript revision and data collection: F.C. and Z.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (grant number 31801306) and the Lishui Key Research and Development Project (grant number 2023zdyf09).

Data Availability Statement: The data presented in this study are openly available in [NCBI Sequence Read Archive (SRA)] [<https://www.ncbi.nlm.nih.gov/sra>] (accessed on 15 May 2026) [PRJNA1467120].

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

Abbreviations

The following abbreviations are used in this manuscript:

qRT-PCR	quantitative real-time PCR
MeJA	methyl jasmonate
ABA	abscisic acid
GA	gibberellic acid
ETH	ethylene

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