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# Genome-wide identification and expression analysis of TaFDL gene family responded to vernalization in wheat (*Triticum aestivum* L.)

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## Abstract

**Background** FLOWERING LOCUS D (FD) is a basic leucine zipper (bZIP) transcription factor known to be crucial in vernalization, flowering, and stress response across a variety of plants, including biennial and winter annual species. The *TaFD*-like (*TaFDL*) gene in wheat is the functional homologue of *Arabidopsis FD*, yet research on the *TaFDL* gene family in wheat is still lacking.

**Results** In this study, a total of 62 *TaFDL* gene family members were identified and classified into 4 main subfamilies, and these genes were located on 21 chromosomes. A comprehensive analysis of the basic physicochemical properties, gene structure, conservation motif, conserved domain, and advanced protein structure of *TaFDL* gene family revealed the conservation among its individual subfamily. The family members underwent purifying selection. The segmental duplication events were the main driving force behind the expansion of the *TaFDL* gene family. The *TaFDL* gene family underwent differentiation in the evolution of *FD* genes. Additionally, the subcellular localization and transcriptional activation activities of five key *TaFDL* members were demonstrated. Gene Ontology (GO) annotations and promoter *cis*-regulatory element analysis indicated that the *TaFDL* members may play potential roles in regulating flowering, hormone response, low-temperature response, light response, and stress response, which were verified by transcriptome data analysis. Specifically, quantitative real-time PCR (qRT-PCR) analysis revealed that five *TaFDL* genes exhibited differential responses to different vernalization conditions in winter wheat seeding. Finally, the homologous genes of the five key *TaFDL* genes across nine different wheat cultivars highlight significant genetic diversity.

**Conclusion** These findings enrich the research on *FD* and its homologous genes, providing valuable insights into the *TaFDL* gene family's response to vernalization.

**Keywords** Wheat, *TaFD*-like, Vernalization, Transcriptome data analysis

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## Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops and is widely cultivated on the planet [1]. An appropriate flowering time in the life cycle of wheat is vital for adapting to the local environment and climatic conditions [2]. Wheat has developed sophisticated mechanisms to integrate diverse environmental signals, such as the vernalization requirement of winter wheat [3]. Plants with vernalization requirements, including winter wheat, must undergo an extended period of low temperature to initiate flowering [4]. Given that the vernalization process directly influences crop yield and quality, understanding this process and its molecular mechanisms is, therefore, crucial [5, 6, 7].

*VERNALIZATION* genes (*VRN*) are playing a critical role in the vernalization process [8, 9]. Vernalization requirements of winter wheat are determined by *VRN*s loci [10, 11], including *VRN1* [12], *VRN2* [13], *VRN3* [14], and *VRN4* [15]. *VRN3* gene in wheat encodes a polyethanolamine-binding protein and serves as an ortholog of the *Arabidopsis* *FLOWERING LOCUS T* (*FT*) gene [14]. In *Arabidopsis*, it acts as a mobile signal recognized as a crucial component of florigen, facilitating the flowering process [14]. In the vernalization process, *VRN3* can migrate to the stem apical meristem tissue (SAM) and interact with *FLOWERING LOCUS D-like 2* (*TaFDL2*) to enhance the expression of the flowering promoter *VRN1* [16, 17]. *TaFDL2*, the basic leucine zipper (bZIP) transcription factor (TF), has been identified as a functional homologue of *Arabidopsis*'s *FD* [5, 18, 19].

Several plant species have been reported to have homologues of the *FD* gene, underscoring their importance in regulating plant growth, vernalization, flowering, and response to stress [20, 21, 22]. In *Arabidopsis*, *FD* interacts with *FT* to activate expression of the *API* and *FRUITFULL* (*FUL*) which lead to promote flowering [18, 23, 24]. In addition, several studies conducted in chrysanthemums [18], loquat [25], pea [26], duckweeds [27] and tobacco [28] have also observed the interaction between *FT* homologs and *FD* homologs in regulating plant flowering. Interestingly, other studies have shown that the *FD* genes exhibit functional diversity, with *FD* genes within the same species potentially performing distinct roles. In rice, *OsFD1* promotes flowering by facilitating nuclear localization of flowering activator complex (FAC) [29, 30]. *OsFD2*, interacting with cytoplasmic 14-3-3 proteins (acting as intracellular receptors), participates in leaf development [20]. The reports on the functional diversification of three homologous *FD-like* genes in populus, namely *FDL2.2*, *FDL1*, *FDL3*, contributing to both regulatory and protein coding differences, further support this viewpoint. *FDL2.2* plays a crucial role in the flowering process of populus during the spring season [31]. *FDL1* and *FDL3*, on the other hand, were found to be the

highest vegetative expression at opposite season [31]. Furthermore, certain *FD* genes can exhibit different functions through alternative splicing (AS). In citrus, *CiFD* undergoes AS to generate the formation of two distinct proteins, namely *CiFD $\alpha$*  and *CiFD $\beta$* . In citrus, *CiFD $\alpha$*  induces flowering by forming the FAC, while *CiFD $\beta$*  independently regulates drought-induced flowering through a simplified pathway involving AS [22]. In London plane (*Platanus acerifolia*), similar results have been observed in the investigation of two closely related *FD* homologs, namely *PaFDL1* and *PaFDL2* [32]. The regulation of flowering may be achieved through two distinct pathways by AS, which has not been observed in model plant species. These results demonstrate the diversity of *FD* gene functions and its significant role in plants.

In 2008, *TaFDL*, predicted to be expressed in leaves, was first identified in wheat [3]. Among these, five *TaFDL* genes have been found to be transcribed in wheat leaves [3]. These genes are intricately linked with the regulatory mechanisms underlying flowering. For instance, *TaFDL2* and *TaFDL6* engage in interactions with *TaVrn3* (*TaFT*), and *TaVrn3*-*TaFDL2* complex upregulates *TaVrn1* to promote flowering [3, 17]. Additionally, *TaFDL13* has also been shown to interact with *TaFT2*, a paralogue of *TaVrn3* [3, 17, 33]. In another study, three homologues of *TaFDL2* encoding nuclear proteins, were isolated in wheat. These homologues were found to be induced by drought and abscisic acid (ABA) treatment. Further investigations reveal that *TaFDL2-1A* can interact with the *TabZIP8-7A* protein through the ABA signaling pathway, synergistically promoting drought stress tolerance [34]. These results suggest that the function of *FD* has diverged in wheat. However, research specifically focused on the *FD* gene family is currently limited [20]. Meanwhile, genome-wide identification and functional analysis of the total of *TaFDL* gene family members in wheat also remains lacking.

In this study, a total of 62 *TaFDL* gene family members were identified and divided into 4 main subfamilies. We conducted bioinformatics analysis on the physicochemical properties, chromosomal locations, conserved motifs, gene structures, conserved domain, evolutionary relationships, promoter *cis*-regulatory elements, Gene Ontology (GO) analysis, collinearity analysis, the advanced protein structures, and transcriptomics of all members of the *TaFDL* gene family. We also cloned five potential key genes that may play a role in the vernalization process in wheat seedlings. Furthermore, we performed subcellular localization analysis in tobacco leaf epidermal cells and verified their self-activation ability. We analyzed the transcriptome data of the *TaFDL* gene family under different conditions and assessed their relative expression levels under different vernalization conditions using quantitative real-time PCR (qRT-PCR). Finally, the homologous

genes of the five key *TaFDL* genes across nine different wheat cultivars highlight significant genetic diversity. The results of our study provide valuable insights for further studies on the gene and protein characteristics, evolution, and function related to the vernalization process of the *TaFDL* gene family in wheat.

Results

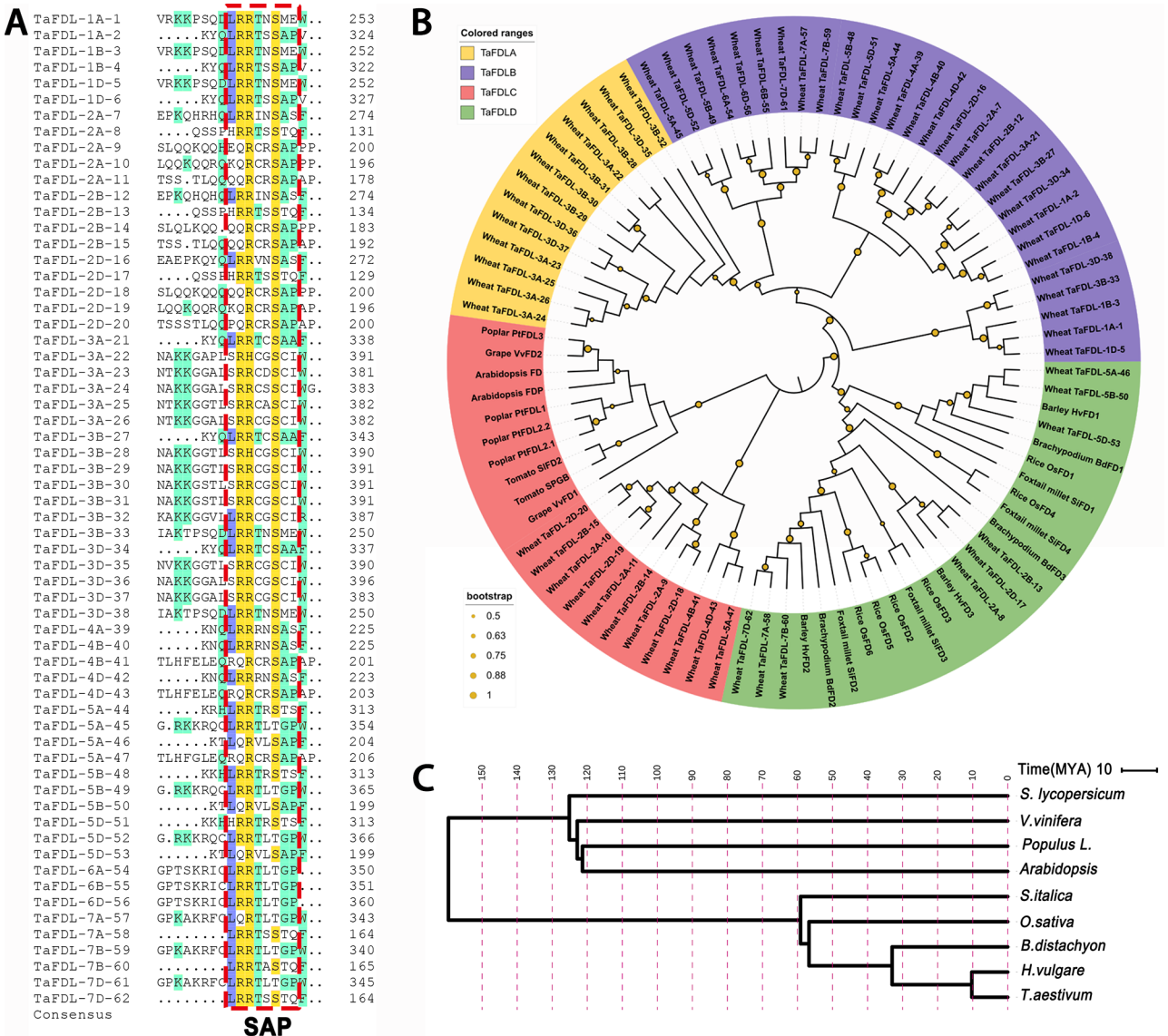
Genome-wide identification of the *TaFDL* gene family in wheat

After eliminating redundant transcripts and potential pseudogenes lacking the bZIP domain or the SAP motif (L[X]-R[QL]-R[H]-X-X-S[T]-A[GCTM]-P[ISQE] in

the C-terminal region (Fig. 1A), we identified a total of 62 members of the *TaFDL* gene family. Additionally, we assigned names to each family member based on their respective chromosomal locations (Table S1).

Physiochemical properties of the *TaFDL* members

Table S1 presents the predicted basic physicochemical properties of the *TaFDL* members. The predicted *TaFDL* proteins ranged from 129 to 396 amino acids in length. The relative molecular weights ranged from 14098.74 to 42724.46 with an average of 30594.33 Da. The theoretical isoelectric point (pI) between 5.22 and 10.2, and 43 and 19 *TaFDL* members were acidic (<7) and alkaline (>7),



**Fig. 1** The SAP motif in the C-terminal region of *TaFDL* genes and evolutionary relationship analysis of *TaFDL* genes. **(A)** The SAP motif in the C-terminal region of the *TaFDL* genes. **(B)** The phylogenetic analysis of *TaFDL* members and FD proteins comprising wheat, *Arabidopsis*, tomato, grape, rice, brachypodium, foxtail millet, barley, and poplar. **(C)** The whole-genome evolution timescale of wheat, *Arabidopsis*, tomato, grape, poplar, rice, brachypodium, foxtail millet, and barley

respectively. The grand average of hydropathicity results of TaFDL members were  $<0$ , indicating that all members were hydrophilic proteins with stable performance. All proteins within the TaFDL gene family were found to be unstable, as indicated by the instability index values ranging from 51.25 to 84.21. The aliphatic index values ranged from 76.36 to 54.03 among the identified members of the TaFDL family.

### Evolutionary relationship analysis of TaFDL members

To investigate the evolutionary relationship of TaFDL members, we constructed a phylogenetic tree using proteins from wheat along with *FD* homologous from *Arabidopsis*, tomato, grape, tomato, rice, brachypodium, foxtail millet, barley, and poplar (Fig. 1B).

According to the phylogenetic results, TaFDL family has been classified into 4 subfamilies with 13 members in TaFDLA, 29 members in TaFDLB, 11 members in TaFDLC and 9 members in TaFDLD respectively. During the analysis of the evolutionary relationship between TaFDL family members and *FD* homologous from 8 species, it was observed that different subfamily members of TaFDL clustered together with *FD* homologous from different species. The subfamilies of TaFDLA and TaFDLB did not include *FD* homologous from an additional eight species. However, TaFDLC subfamily contained 6 *FD* homologous from *Arabidopsis*, tomato, grape and poplar. The smallest subfamily, TaFDLD, contained all *FD* homologs from four closely related species (brachypodium, foxtail millet, barley and rice).

Further analysis of the whole-genome evolution timescale of these species revealed that the clustering

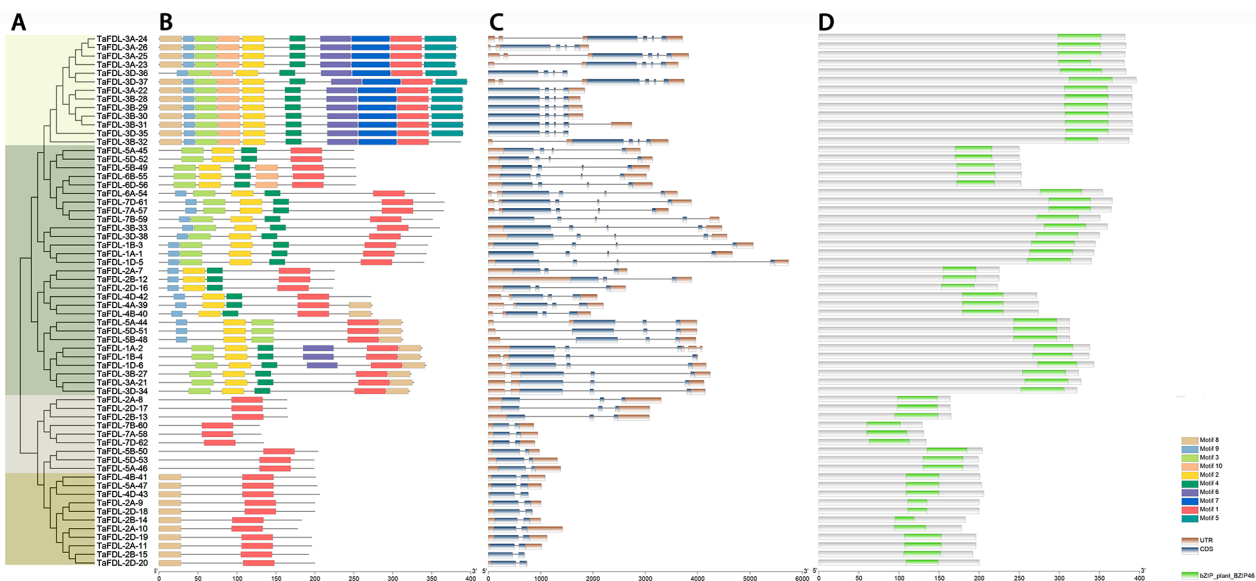
relationship of TaFDL subfamilies aligned with the overall genome evolution and species divergence timeline (Fig. 1C). The species *Arabidopsis*, grape, tomato, and poplar appeared to be more ancient and had a closer evolutionary relationship, with an estimated evolution timescale of approximately 130–120 million years ago (MYA). On the other hand, brachypodium, foxtail millet, rice, barley and wheat show a closer kinship and a divergence time of approximately 60–10 MYA.

### Conserved motifs, gene structure, conserved domain analysis and protein structure prediction

To gain a comprehensive understanding of the TaFDL gene family, we conducted a phylogenetic tree analysis of 62 TaFDL members (Fig. 2A). The results revealed a conservation in the distribution of conserved motifs, intron/exon structures, conserved domain and protein structure within the subfamilies.

#### Conserved motifs

Motif analysis revealed the presence of 10 conserved crucial motifs within the proteins encoded by the TaFDL gene family (Fig. 2B). In the TaFDLA subfamily, all ten identified motifs were found to be present. Notably, motif 7 and motif 5 exclusively existed in the TaFDLA subfamily, and all members of the TaFDLA subfamily contained motif 7, which serves as a characteristic motif for the subfamily. In the TaFDLB subfamily, all members were found to contain motif 2. In the TaFDLC subfamily, members were exclusively found to have motif 1 and motif 8. Motif 1 was predicted to be the only motif present in the TaFDLD subfamily.



**Fig. 2** Phylogenetic relationship, conserved motifs, gene structure, and conserved domain analysis of TaFDL members. (A) Phylogenetic relationship of TaFDL members. (B) Distribution of conserved motifs among TaFDL members. (C) Gene structure of TaFDL members. (D) Distribution of conserved bZIP domains among TaFDL members



### Gene structure

Gene structure analysis revealed that the number of exons in TaFDL members in wheat were 2, 3 and 4, respectively. There were notable commonalities in the distribution patterns of exons and introns within each subfamily (Fig. 2C). In the TaFDL gene family, *TaFDL-3A-24*, *TaFDL-3A-25*, and *TaFDL-3D-37*, identified as members of the TaFDLA subfamily, exhibited the highest number of introns and exons. Within the TaFDLB subfamily, 51.7% of the members had 3 exons while the remaining 48.3% had 4 exons. The TaFDLC subfamily displayed the lowest exon and intron count. In addition, 4 *TaFDL* genes have no terminal untranslated regions (UTRs) at the 3' end, and 9 genes have no UTR at the 5' end.

### Conserved domain

From the observed graph (Fig. 2D), it is evident that as a fundamental characteristic of the TaFDL family, all family members contain the bZIP conserved domain near the 3' end. Sequence logos provide valuable information regarding sequence similarities among all TaFDL members. The results indicate that the protein sequence logos of the bZIP domain are conserved across most sites within all TaFDL members, spanning the N and C terminals (Fig. S1A). The advanced predicted structure, generated by the highest consensus sequence, easily forms homo- and hetero-dimers, which aligns with the known requirement for bZIP transcription factors to form dimers to function and maintain stability [35] (Fig. S1B). The highest proportion of secondary structures observed in the TaFDL family proteins was random coil, followed by alpha helix (Fig. S1C). Conversely, the proportions of extended strand and beta turn were comparatively lower (Table S2, Fig. S2A).

### Protein structure prediction

Furthermore, the results demonstrated that within different TaFDL protein subfamilies, the members exhibited conserved secondary structures, suggesting the formation of similar higher-order structures to accomplish comparable biological functions (Table S2, Fig. S2A). To gain further insights into the mechanism of action of a protein, accurate prediction of its three-dimensional structure is crucial. The results revealed that the majority of FDL member exhibited a classic zipper three-dimensional conformation composed of two long  $\alpha$ -helices (Fig. S2B). However, in the TaFDLC subfamily, certain models of their three-dimensional structures displayed distinct conformations, characterized by individual long  $\alpha$ -helices. The existence of different secondary and three-dimensional protein structures suggests that different subfamily members may function through divergent pathways.

### Chromosome locations, duplication, synteny, and *Ka/Ks* analysis of *TaFDL* genes

#### Chromosome locations

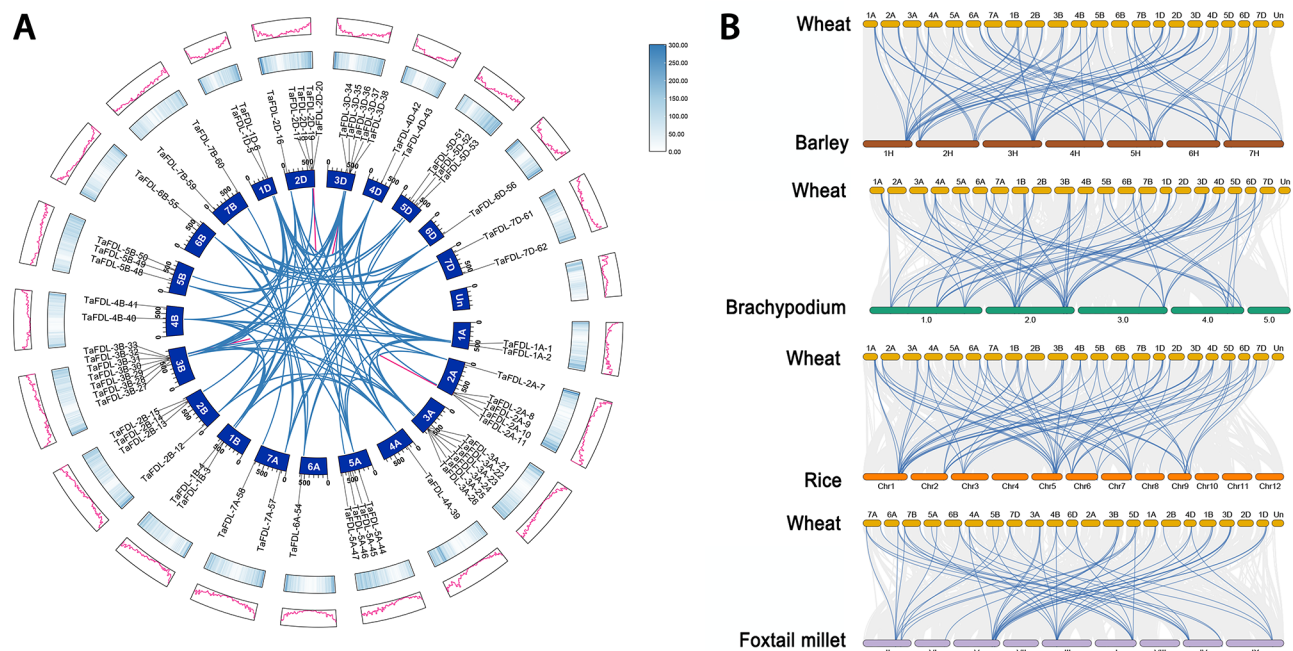
The chromosomal locations of the *TaFDL* genes in the wheat genome were comprehensively analyzed (Fig. 3A). The *TaFDL* genes exhibited a widespread distribution across all 21 chromosomes of wheat. The distribution of *TaFDL* genes on each chromosome group (AABBDD) of wheat was found to be relatively uniform. Notably, both the A and B sub-genome contained the highest number of *TaFDL* genes, with a total of 21 members, respectively. The D sub-genomes had 20 members. Furthermore, we meticulously analyzed gene duplication patterns, encompassing both tandem and segmental duplications. The TaFDL gene family was found to be affected by gene duplication events across all 21 chromosomes (Fig. 3A). Specifically, tandem duplications were observed on chromosomes 2A, 2D, 3B, and 3D, with a total of 7 pairs of such duplications identified.

#### Duplication

A total of 44 *TaFDL* genes were identified to be involved in the process of segmental duplications (Fig. 3A). All identified 95 pairs of segmental duplications were found to be located on distinct chromosomes, indicating that segmental duplications played a crucial role in the expansion of the TaFDL gene family in wheat. During the course of evolution, the TaFDL gene family experienced a limited number of tandem duplication events, whereas segmental duplication events emerged as the key driving force behind its evolution. To evaluate the presence of selection pressure on these *TaFDL* genes, we performed calculations of the synonymous mutation rate (*Ks*), non-synonymous mutation rate (*Ka*), and the ratio of non-synonymous mutation rate to synonymous mutation rate (*Ka/Ks*). The *Ka/Ks* ratios of the remaining duplicated pairs, except for the pair of *TaFDL-4B-41* and *TaFDL-4D-43*, were less than 1 (Fig. S3A), indicating that the TaFDL gene family underwent strong purifying selection (negative selection).

#### Synteny and *Ka/Ks* analysis

To comprehensively investigate the evolution of the TaFDL gene family, we conducted collinearity analysis between wheat and four other Poaceae species, namely barley, brachypodium, rice and foxtail millet, which have a closer evolutionary relationship as mentioned earlier (Fig. 3B). A total of 81, 80, 73 and 58 collinear pairs were identified between *TaFDL* genes and genes from barley, brachypodium, rice and foxtail millet respectively. The analysis identified a total of 45 *TaFDL* genes participating in collinear pairs. Consistent collinear gene pairs were observed across all collinearity analyses, with examples such as *TaFDL-4 A-39* and *TaFDL-5B-48*, underscoring



**Fig. 3** Synteny analysis of *TaFDL* genes. **(A)** Localization and synteny analysis of *TaFDL* genes in wheat genome. Collinear genes were highlighted with red curved lines (tandem replication) and blue curved lines (segmental duplication). The outermost two layer of the circle displays the gene abundance on the chromosome. **(B)** Syntenic relationships of *TaFDL* genes between wheat and barley, foxtail millet, brachypodium, and rice. Gray lines in the background represent collinear blocks within wheat and other plant genomes. Red lines connect syntenic gene pairs

their relative conservation throughout the evolution of the Poaceae family. Furthermore, we calculated the  $Ka/Ks$  ratios for these collinear pairs. The results showed that all  $Ka/Ks$  ratios were less than 1 (Fig. S3B), indicating that negative selection has eliminated deleterious mutations and maintained protein conservation during the evolution of gramineous species.

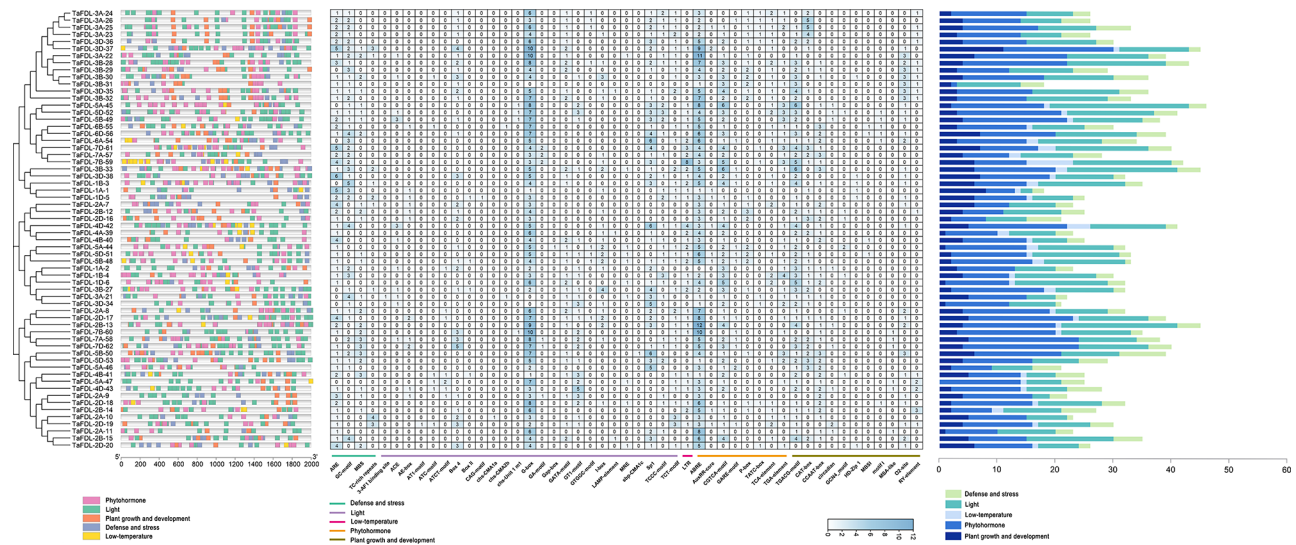
#### Cis-regulatory element analysis

The process of gene expression and transcription can be effectively elucidated through the analysis of *cis*-acting elements present in the promoter region of a specific gene. To investigate the role of the *TaFDL* gene family in vernalization, we categorized and quantified *cis*-acting elements in the promoter of *TaFDL* genes into five major separate groups: low-temperature, light, plant growth and development, phytohormone, and defense and stress (Fig. 4). We identified a total of 1943 predicted *cis*-regulatory elements within the promoter regions of *TaFDL* genes across five distinct groups using PlantCare. Among them, the most prevalent group of promoter *cis*-regulatory elements was the light group (with 746 elements), wherein the top three elements were G-box (302), Sp1 (102), and box 4 (55). Simultaneously, a substantial number of phytohormone group was identified (with 674 elements), including ABRE (270), CGTCA-motif (125), and TGACG-motif (125). We identified only one type element of low-temperature group, namely LTR. In the defense

and stress group, we identified four types of elements, with the ARE element being the most prevalent, occurring in approximately 85.5% of the promoter regions of *TaFDL* genes. The plant growth and development group, comprising 227 elements within 10 distinct categories, performs diverse functions, including the regulation of meristem expression and seed-specific regulatory activities. On average, *TaFDL* genes contain 31 *cis*-regulatory elements.

#### GO analysis of *TaFDL*

A total of 259 GO terms were enriched for the *TaFDL* gene family (Fig. 5). Specifically, 16 GO terms were identified for molecular function, 10 GO terms for cellular component, and 233 GO terms for biological process (Fig. 5A). The results revealed that some GO terms may be associated with the vernalization process, with a total of 42 terms identified, categorized as follows: Reproduction (14), Hormone (12), Light (6), Flower (5), and Temperature (5). These annotations were all derived from the category of biological process (Fig. 5B). Notably, genes linked to GO terms associated with temperature and hormone responses were predominantly found within the A and B subfamilies, whereas those implicated in reproduction, flowering, and light responses were mainly concentrated in the A, C, and D subfamilies. In conclusion, the results of the GO terms indicate the potentially crucial



**Fig. 4** The cis-regulatory element in the promoter region of *TaFDL* genes. The number represented the cis-acting element numbers of *TaFDL* genes in the promoter regions

role of *TaFDL* genes in various activities of wheat, including vernalization response.

## Expression pattern analysis of TaFDL gene family and molecular cloning of key *TaFDL* genes

The transcriptome datasets of the TaFDL gene family were analyzed across 70 different developmental stages or tissues of wheat. The expression patterns of *TaFDL* genes show a degree of conservation within different subfamilies (Fig. S4). Notably, compared to other developmental stages or tissues, 2 *TaFDLD* genes exhibited strong expression from the tillering stage to the flowering stage. Furthermore, 30 *TaFDL* genes demonstrated ubiquitous expression across all 70 growth stages and tissues surveyed.

To explore the low-temperature response of the TaFDL gene family, we gathered two transcriptome datasets of wheat under varying temperature conditions (4°C and 23°C, 12°C and 27°C) (Fig. 6A and B). In the 4°C-23°C response, a total of 51 *TaFDL* genes responded to the two temperature conditions in the environment. Analysis of the transcriptome data from the 4°C response revealed that 24 *TaFDL* genes showed upregulated expression. In another transcriptome analysis (12°C and 27°C), we noted that within the TaFDL gene family, 28 *TaFDL* genes were upregulated. In Fig. 6C, we observed the response of some *TaFDL* genes to exogenous Gibberellin 3 (GA<sub>3</sub>) treatment, indicating the potential hormone-responsive ability of family members. We collected transcriptome data on drought stress responses involving members of the TaFDL gene family. As depicted in Fig. 6D, we observed that 22 *TaFDL* genes displayed increased expression levels after 1 h of drought stress. These results indicate a strong induction in response to

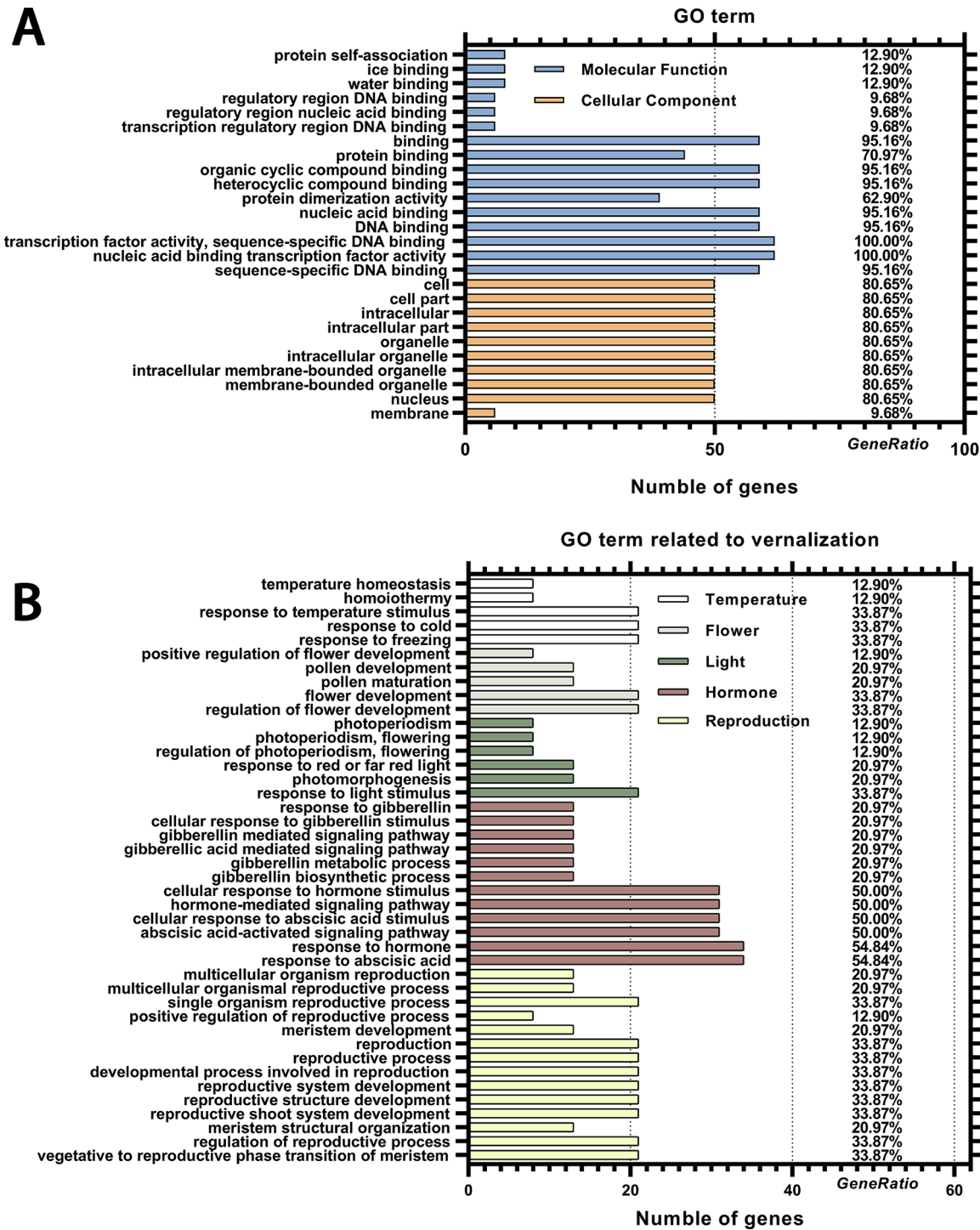
the stress. During 6 h of drought stress, 58 *TaFDL* genes showed decreased expression compared to 1 h, however, *TaFDL-6A-54*, *TaFDL-6B-55*, and *TaFDL-6D-56* maintained high expression levels.

Subsequently, based on the results of transcriptome data analysis, we selected five genes from the TaFDL gene family, namely *TaFDL-2A-8*, *TaFDL-2 A-11*, *TaFDL-5 A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56*, for cloning, aiming to further investigate the characteristics and functions of these family members.

### Subcellular localization analysis

Subcellular localization is a critical aspect of understanding the functional role of proteins. The DNA construct containing the chimeric pCambia1305.1-TaFDL gene was delivered into tobacco leaves by injecting it into *Agrobacterium tumefaciens* cells GV3101. The resulting epidermal cells were examined for a fluorescent signal using a turntable laser confocal live cell detection microscope system. The control imaging of the tobacco epidermal cells revealed that the green fluorescence was uniformly distributed across the entire cell, including the cell membrane, cytoplasm, and nucleus. As shown in Fig. 7A, the TaFDL-2 A-8-GFP fusion showed nuclear localization. Meanwhile, it was observed that TaFDL-2 A-11, TaFDL-5 A-44, TaFDL-5D-51, and TaFDL-6D-56 exhibited dual subcellular localization in the nucleus and the cell membrane. Notably, our experimental findings are in partial agreement with the predictions made by bioinformatics analysis. The online bioinformatics software WoLF PSORT predicted that all members of the TaFDL members localize to the nucleus, while our results indicated that some members are localized to both the nucleus and the cell membrane.





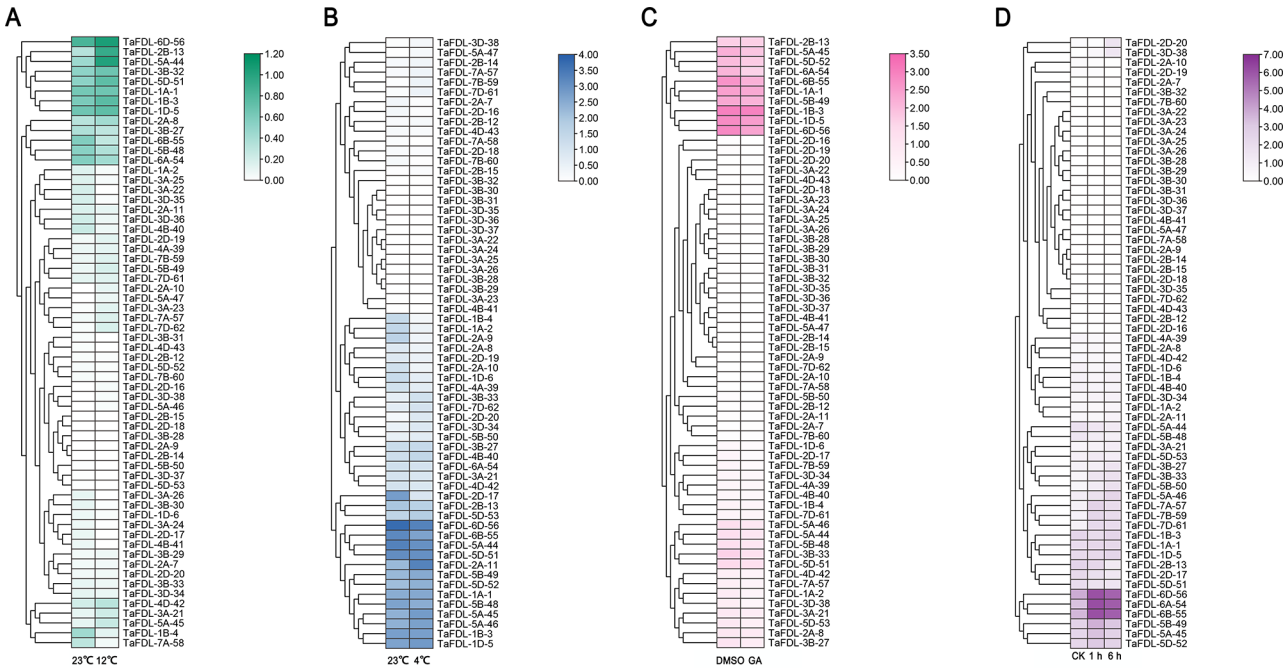
**Fig. 5** GO enrichment analysis of *TaFDL* genes

**Transcriptional activation assay**

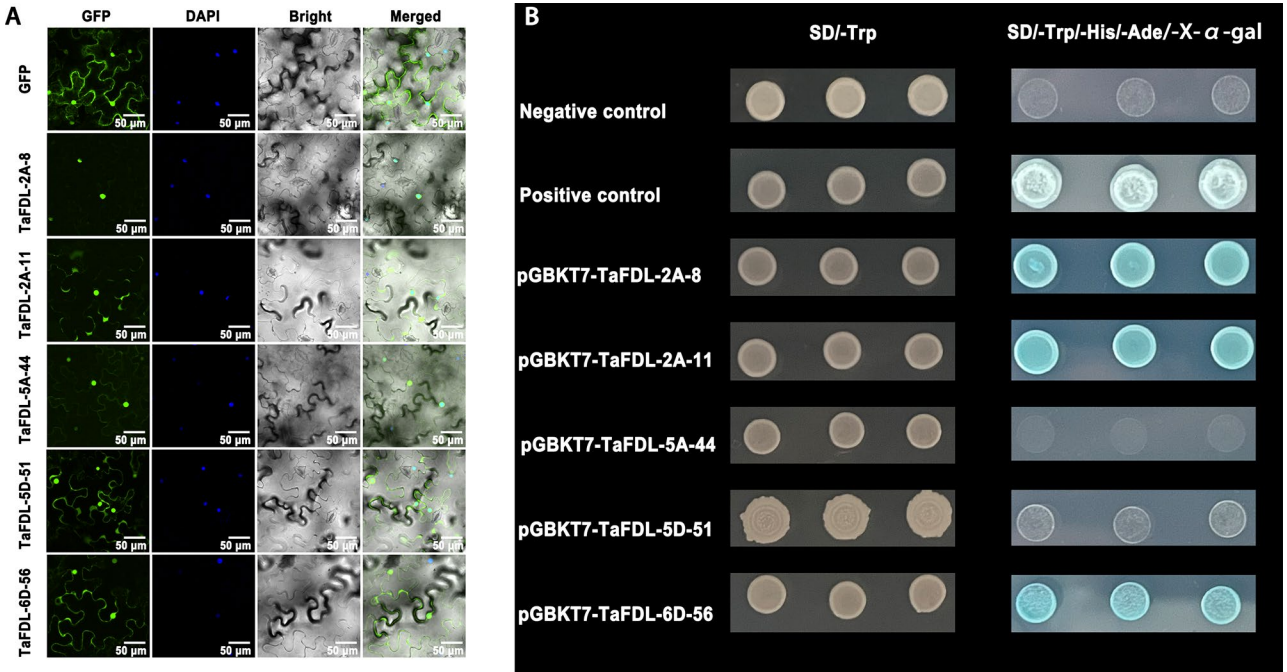
To investigate the transcriptional activity of *TaFDL*-2A-8, *TaFDL*-2A-11, *TaFDL*-5A-44, *TaFDL*-5D-51, and *TaFDL*-6D-56 genes in yeast, the complete open reading frames (ORFs) of these five proteins were fused with the GAL4 DNA binding domain of the pGBK17 vector. The fusion expression vectors, negative and

positive control were transferred into the yeast strain Y2HGold, respectively. The yeast transformants containing these vectors exhibited robust growth on selective SD/-Trp medium. The yeast transformants containing *TaFDL*-2A-8-pGBKT, *TaFDL*-2A-11-pGBKT, and *TaFDL*-6D-56-pGBKT, respectively, survived on SD/-Trp/-His/-Ade/-X- $\alpha$ -Gal medium, similar to the positive





**Fig. 6** Expression analysis of *TaFDL* genes in wheat under low-temperature stress (A and B), exogenous hormone supplementation (C), and drought stress (D)



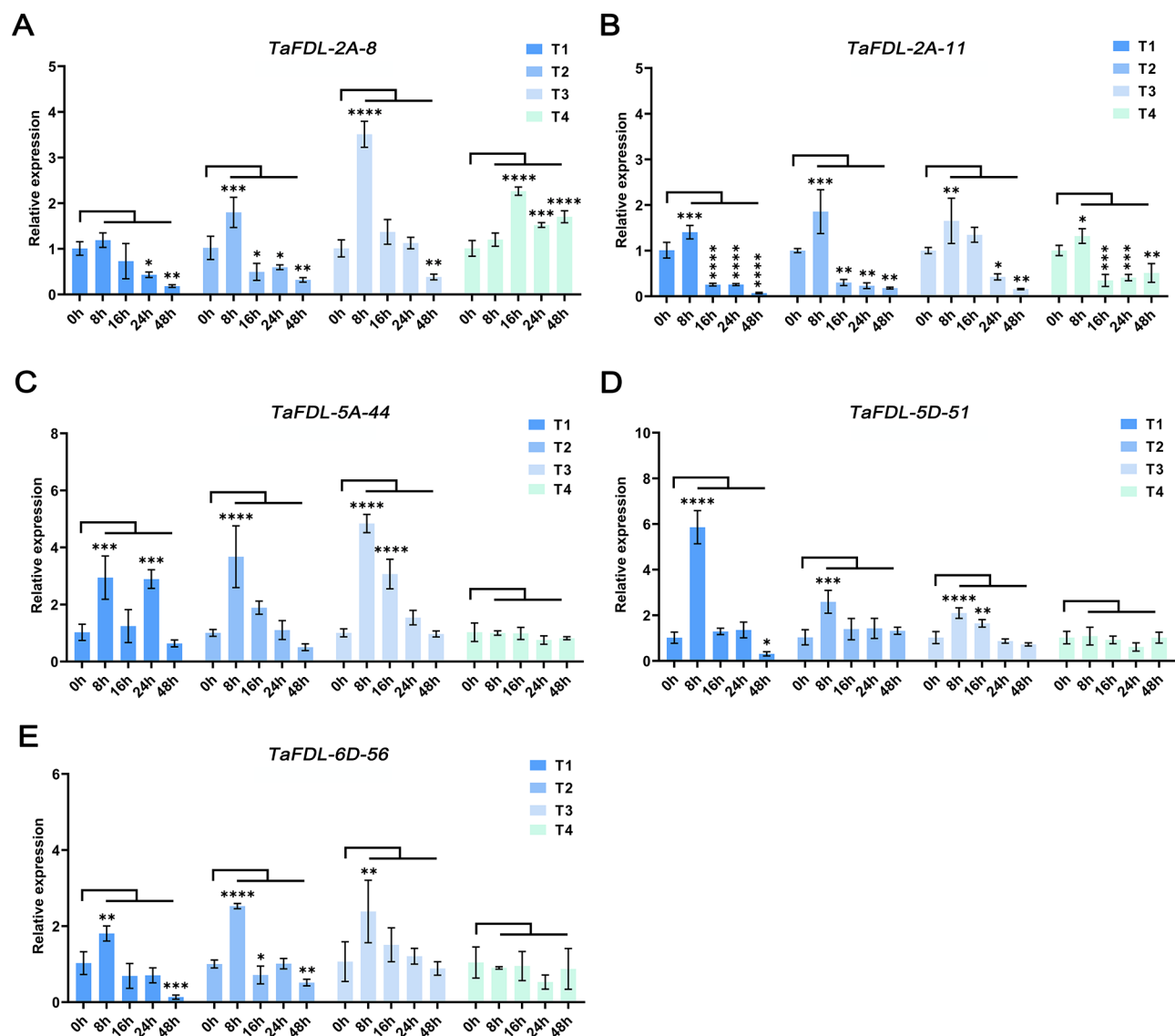
**Fig. 7** Subcellular localization analysis and transcriptional activation activity of *TaFDL* genes. (A) Transcriptional activation activity of *TaFDL*-2 A-8, *TaFDL*-2 A-11, *TaFDL*-5 A-44, *TaFDL*-5D-51, and *TaFDL*-6D-56. The empty vector pGBKT7 was used as a negative control. pGBKT7-53 + pGADT7-T co-transformation vector used as a positive control. (B) Subcellular localization analysis of *TaFDL*-2 A-8, *TaFDL*-2 A-11, *TaFDL*-5 A-44, *TaFDL*-5D-51, and *TaFDL*-6D-56. The image showed the location of GFP and aFDL-GFP proteins in the fluorescence channel, bright- field, and the merged diagram. Scale bar = 50  $\mu$ m

control yeast transformant, and generated blue biomass due to the activation of a yeast reporter gene (Fig. 7B). In contrast, the growth status of yeast transformants containing *TaFDL-5A-44*-pGBKT and *TaFDL-5D-51*-pGBKT resembled that of the negative control yeast transformant, as they were unable to survive on SD/-Trp/-His/-Ade/-X- $\alpha$ -Gal medium.

#### qRT-PCR analysis in different vernalization conditions

To further investigate the potential roles of *TaFDL* genes in the early response to vernalization, we established four different environmental conditions to explore the effects of temperature, photoperiod, and hormone addition on the expression of *TaFDL-2A-8*, *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51* and *TaFDL-6D-56* in wheat seedlings (Fig. 8). In the T1 treatment, the expression levels

of the five genes were highest at 8 h, with *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56* showing significant increases compared to 0 h. *TaFDL-5D-51* showed the highest response, with a 476.42% increase in expression compared to 0 h. Additionally, we observed a unique expression pattern in *TaFDL-5A-44*, where the relative expression between 0 and 48 h followed a decrease-increase-decrease trend, differing from the decrease-increase pattern observed in the other four *TaFDL* genes. In the T2 treatment, a similar expression pattern to T1 treatment was observed during the 0–8 h period, likely due to the consistent environmental conditions during the initial 8 h. Subsequent time points showed a decrease in the relative expression levels of *TaFDL* genes compared to 8 h, possibly as a result of prolonged dark cultivation of wheat seedlings under the



**Fig. 8** Expression analysis of the five *TaFDL* genes in four treatments

T2 treatment at these three time points. In the T3 treatment, exogenous GA<sub>3</sub> was applied to the roots, resulting in peak relative expression levels of the five genes at 8 h, significantly increasing by 247.51%, 65.06%, 381.20%, 105.17%, and 123.55% compared to 0 h. At 16 h, unlike the T1 treatment, the supplementation of exogenous GA<sub>3</sub> delayed the decrease in relative expression levels of *TaFDL* genes, which remained higher than at 0 h. The relative expression levels of *TaFDL* genes continued to decrease from 16 to 48 h, indicating that GA<sub>3</sub> can stimulate the expression of *TaFDL* genes in the short term, but the expression levels decrease after the depletion of exogenous hormones. Under the T4 treatment, there was no significant difference in the relative expression levels of *TaFDL-5 A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56* within 48 h compared to 0 h. In the T4 treatment, *TaFDL-2A-8* and *TaFDL-2A-11* reached their peak expression levels at 16 h and 8 h, respectively. The findings indicate that the responses of distinct *TaFDL* genes to low temperatures and reduced light intensities vary.

#### Variation analysis in five key *TaFDL* genes and their homologs across nine wheat cultivars

We conducted a comprehensive sequence analysis of five key *TaFDL* genes (*TaFDL-2A-8*, *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56*) and their homologs across nine wheat cultivars (Table S7). The analysis revealed significant genetic diversity in the CDS sequences of these genes, with various substitutions, insertions, and deletions identified in different cultivars (Table 8, Fig. S5). *TaFDL-2 A-8* exhibited multiple single-base substitutions and a 9-base insertion in five of the homologous genes, resulting in the lowest overall sequence identity of 96.35%. *TaFDL-5D-51* showed the highest overall sequence identity with its homologs, with only two homologous genes having a single-base substitution and all homologs having a deletion at the end of the sequence. Notably, these variations did not alter the translated amino acid sequence. *TaFDL-2A-11* and *TaFDL-5A-44* displayed similar patterns of single-base substitutions in their homologous genes. The variations in *TaFDL-5D-51* were primarily at the ends of the sequences, with the homologous genes in the cultivars being identical in the complete sequence.

#### Discussion

FD, a bZIP-type transcription factor, was initially discovered in *Arabidopsis* [19]. Subsequently, *FD* genes have been identified in multiple species and shown to play important roles in flowering, vernalization, growth and development, and responses to environmental stresses. Moreover, functional divergence of different *FD* homologs within the same species, including in wheat [3, 34], suggests potential differentiation in the functions and

mechanisms of action of *FD* [36, 37]. Based on these findings, new demands have been proposed for the study of *FD* and its homologous genes. The wheat bZIP-type transcription factor *TaFDL*, a homolog of *AtFD*, has been postulated to play a significant role in the vernalization process. Previous research primarily focuses on the overall study of the bZIP gene family, with some studies addressing specific subfamilies such as the *HY5* gene family [38] and *ABI5* gene family [39]. However, there is limited systematic research specifically targeting the *FD* gene family as part of the bZIP gene family. At least 7, 5, 2, 4, and 2 *FD* genes have been identified in rice [20, 40], wheat [3], poplar [37], *Arabidopsis* [19], and barley [17], respectively, however, the *FD* gene family has not been reported in these species. Identifying the *TaFDL* gene family is crucial for a deeper understanding of the vernalization molecular mechanism in wheat and the evolution of *FD* homologous genes.

A total of 62 *TaFDL* members were identified in the wheat genome, each possessing a bZIP structure and the characteristic C-terminal SAP motif of the *FD* gene family. The SAP motif is crucial for the functional role of *FD* genes, particularly in the formation of FAC [29, 41]. The presence of this conserved SAP motif in *FD* gene family suggests that the participation of *FD* in the FAC is conserved [20].

This study conducted a phylogenetic analysis of homologous *FD* genes from 8 different species and identified family members, revealing that the *FD* genes primarily comprise 4 subfamilies. It is interesting to note that the *TaFDLD* subfamily clusters with the *FD* genes of the Poaceae family, while the *TaFDLC* subfamily clusters with the *FD* genes of non-Poaceae plants. These results indicate differentiation in the evolutionary process of *FD* genes, highlighting unique evolutionary features in Poaceae plants. Based on the whole-genome evolution timescale of these species, we propose that this differentiation likely occurred between 120–60 MYA. Our results are in line with previous studies on the evolution of *FD* genes in plants, suggesting that the *FD* genes can be categorized into unique evolutionary aspects of *FD* genes in the Poaceae family and *FD* genes in the non-Poaceae [20]. This observation also suggests that members of different *TaFDL* subfamilies members may have distinct functional roles.

Further research revealed that the gene structure and conserved motifs of subfamily members are conserved, but there are differences in gene structure and conserved motifs among subfamilies. This suggests that *FD* genes within the subfamily may have similar functions, while the functions of *FD* genes may vary among subfamilies. The differences in gene structure and conserved motifs within subfamilies may be attributed to gene duplication events leading to intron loss. For instance, *TaFDL-3D-36*

and *TaFDL-3B-32* exhibited the absence of motif 8 and motif 5 compared to other members of the TaFDL family. Their positions on the chromosome and subsequent gene duplication analysis indicated that they had undergone duplication events. Meanwhile, it is worth noting that we identified 62 TaFDL members in wheat, which is significantly higher than the reported *FD* genes in rice (6) and *Arabidopsis* (6). Wheat, an allohexaploid with an extensive genome, originated from the hybridization of three progenitor species through two natural hybridization events [42, 43]. The wheat genome has also undergone multiple duplication events [44]. The TaFDL gene family may experience a higher degree of gene duplication events throughout its evolutionary history. Based on this assumption, we further explored whether gene family expansion occurred, leading us to investigate gene duplication events. Seven gene pairs underwent tandem duplication, while 95 pairs exhibited segmental duplication, indicating that segmental duplication is a major driving force in evolution. The *Ka/Ks* ratios were less than 1, indicating that *TaFDL* genes are under purifying selection (negative selection) [45]. This result suggests that the majority of members in the TaFDL family have been conserved during evolution. To comprehensively investigate the evolution of the TaFDL gene family, we conducted a collinearity analysis between wheat and four other gramineous species, namely barley, foxtail millet, brachypodium, and rice, which have a closer evolutionary relationship as mentioned earlier. The results showed that some *TaFDL* genes displayed collinearity with all four species, indicating the conservation of *FD* genes in the evolution of the Poaceae family. Additionally, all collinear pairs had *Ka/Ks* ratios less than 1, suggesting that the TaFDL proteins in gramineous plants exhibit a certain level of conservation during evolution. Based on the aforementioned results, some *FD* genes in Poaceae plants likely play essential roles and exhibit similar functions, such as forming the FAC with FT and other interacting proteins, as widely reported [29, 46].

The promoter region plays a crucial role in regulating the transcription of downstream genes by binding specifically to TF and RNA polymerase [47]. The family members responded to plant hormones such as GA, ABA, methyl jasmonate (MeJA), auxin, and salicylic acid (SA). Plant hormones are believed to play important roles in the flowering process and vernalization in plants [48]. GA promotes flowering by activating floral integrator genes [49] and shortens the vernalization period by accelerating the floral transition [50]. ABA and GA are a classic pair of plant hormones, exhibiting antagonistic effects in many biological processes [51]. ABA also plays a crucial role in regulating flowering [51] and adaptation to environmental changes during vernalization [52]. In sugar beet, vernalization promotes GA-mediated bolting initiation by

inhibiting ABA and JA biosynthesis [53]. The plant flower development is strictly regulated by auxin, and auxin is believed to respond to low temperatures in plants [54]. SA biosynthesis is enhanced under vernalization [55]. The competitive regulation of SAM development by the interacting proteins TFL1 and FT also involves multiple hormone pathways, with this process being crucial for the transition of plant development from vegetative growth to reproductive development [56, 57, 58, 59]. The analysis of promoter *cis*-elements revealed that all TaFDL members contain plant hormone response elements, indicating their involvement in plant hormone-mediated signaling processes related to flowering, vernalization, and stress responses. We also focused on light and low-temperature responsive elements related to vernalization. In the promoter regions of 34 *TaFDL* genes, we identified the low-temperature response element LTR, with the genes containing low-temperature elements mainly concentrated in the TaFDLB subfamily (64.7%). Light-responsive elements are the most diverse category among the five major classes identified, and all family members' promoter regions contain these elements. Photoperiod and light intensity are important factors influencing plant vernalization and flowering [60, 61]. Furthermore, the results indicate a potential role of *TaFDL* genes in response to abiotic stress. Two *FD* genes in wheat are believed to play crucial roles in drought resistance [34].

In the GO analysis, similar to the promoter *cis*-element analysis, the identified GO terms related to hormones, light, and low-temperatures further confirm the potential roles of TaFDL members in vernalization responses. Some GO terms were found to be related to reproductive processes and plant flowering, indicating the significant role of the TaFDL family in the transition from vegetative growth to reproductive growth. Interestingly, the conservation of the distribution of vernalization-related GO terms across subfamilies suggests that genes within different subfamilies may play distinct roles. The predicted functions of TaFDL gene family align with their characteristics as bZIP TFs. bZIP TFs have been reported to play crucial roles in plant responses to low temperature, light, hormones, growth and development, and stress [62, 63, 64]. However, there is limited documentation on the functional differentiation of *FD* genes and their homologs, with current research primarily focusing on the flowering promotion in plants. A few studies have mentioned the involvement of *FD* genes in leaf development [20], spikelet development [65], plant height regulation [37], and response to abiotic stress [34]. Our results enrich the potential research directions for the function of *FD* genes.

Subcellular localization experiments showed that the *TaFDL* gene is localized in the nucleus or both the nucleus and the cell membrane. This suggests that the



TaFDL family may be involved in plant signal regulation and transmembrane transport processes. Several FDs have been reported to be nuclear localization proteins [3, 19, 32], while some members of the bZIP family have been reported to be involved in transmembrane transport processes [66, 67]. In prior research, *OsFDL7*, expressed in the SAM, was found to translocate to the nucleus and become activated, mirroring our subcellular localization findings where *FD* gene homologs are implicated in nuclear functions associated with transmembrane transport mechanisms [40]. Meanwhile, bioinformatics predictions indicated that all members of the TaFDL gene family are localized in the nucleus. The underlying cause of these outcomes is attributed to the distinct expression patterns and regulatory factors of genes within the cellular contexts of different species. Additionally, due to the limitations of predictive models' algorithms, varying results may arise from predictions. These results of the transcriptional activation assay indicate that TaFDL-5 A-44 and TaFDL-5D-51 lack self-activation ability, while TaFDL-2 A-8, TaFDL-2 A-11, and TaFDL-6D-56 demonstrate transcriptional self-activation in yeast strains Y2HGold. The self-activation capacity of a transcription factor is a crucial aspect of its regulatory function, often signifying the ability to independently activate downstream gene transcription without additional activators.

To validate our previously predicted functional results and explore the expression patterns of TaFDL throughout plant growth and development, we analyzed multiple transcriptome datasets. Within the TaFDL gene family in wheat growth and development, TaFDLC members exhibited high expression levels during the reproductive stage, suggesting a potential important role in floral development. Interestingly, several members of the TaFDLA subfamily were found to be highly expressed in mature grains, indicating their potential role in plant maturation and dormancy. The bZIP TF has been found to play a role in seed maturation and dormancy in multiple species [68, 69, 70]. In prior research, the roles of multiple *FD* genes and their homologues in plant growth and development during the vegetative phase have also been established [20, 71, 72]. Two individual transcriptome data revealed that some members of the TaFDL gene family respond to low temperatures (including 12 °C and 4 °C), indicating a potential role in early cold stress and adaptation in wheat, in contrast to previous reports that the *TaFDL* gene functions primarily after vernalization [5]. Further qRT-PCR experiments confirmed this observation, with the expression levels of 5 genes significantly increasing upon exposure to 4 °C and decreasing within 48 h. To validate the hormone response of the TaFDL family, transcriptome analysis revealed a significant increase in the expression of some *TaFDL* genes

upon exogenous GA<sub>3</sub> treatment. qRT-PCR analysis demonstrated a significant upregulation of five *TaFDL* genes expression in the short-term following exposure to low temperatures and subsequent exogenous GA<sub>3</sub> application. Notably, the exogenous GA<sub>3</sub> treatment sustained elevated expression levels of five *TaFDL* genes at 16 h post-treatment compared to the initial time point of 0 h. Consistent with our findings, in field experiments with wheat and Chinese Radish Jumbo, exogenous GA<sub>3</sub> application during vernalization shortens the vernalization period and promotes flowering [73, 74]. The transcriptome data analysis also revealed potential functions of *TaFDL* genes in response to drought stress. In accordance with prior researches, the *FD* genes are implicated in the plant's response to drought stress [22, 34]. Some flowering-related genes have been widely reported to play significant roles in drought stress responses [75]. Interestingly, after vernalization in wheat and barley, drought stress promotes accelerated flowering in wheat. This suggests that drought stress can have a significant impact on the flowering time of wheat, potentially due to changes in gene expression and hormonal regulation associated with the vernalization process [76, 77]. Concurrently, drought stress induces an increase in ABA levels within the plant, which in turn enhances the expression of flowering genes [78]. These findings may elucidate the significant role that certain *TaFDL* genes play in the response to drought stress. The transcriptome data and qRT-PCR results were consistent with the bioinformatics predictions, indicating that the TaFDL family responds to light, temperature, hormones and stress to varying degrees. The variation analysis of five key *TaFDL* genes and their homologs across nine wheat cultivars revealed significant genetic diversity, including substitutions, insertions, and deletions. These variations suggest potential functional divergence among the TaFDL gene family members. For instance, *TaFDL-2A-8* and its homologs exhibited relatively lower sequence identity due to multiple single-base substitutions and a small insertion. In contrast, the other four key *TaFDL* genes and their homologs showed relatively higher sequence identity. The high sequence identity observed in most of the genes suggests a high degree of conservation, consistent with their essential role in wheat development and environmental adaptation. These findings highlight the genetic diversity within the TaFDL gene family and suggest that specific alleles of these genes could be targeted for breeding programs aimed at improving cold tolerance or flowering time regulation in wheat, thereby enhancing wheat productivity and adaptability under varying environmental conditions.

Our bioinformatics study of the TaFDL gene family serves as a valuable reference for further exploration of the potential functions and associated pathways of this gene family. However, the lack of functional validation for

key genes, such as transgenic overexpression and interaction analysis, limits the confirmation of their roles. Future studies should focus on functional validation and investigate the mechanisms of action of the TaFDL gene family in the early stages of vernalization, including responses to light and environmental temperature.

## Conclusion

This study provides novel insights into the TaFDL gene family in wheat by identifying 62 members, classified into four subfamilies, and elucidating their chromosomal distribution, physicochemical properties, and comprehensive structural information of genes and proteins. We focused on the evolutionary relationships of *TaFDL* genes with other *FD* genes and discovered that segmental duplication is a major driving force for gene expansion in the TaFDL gene family. Utilizing GO annotations and *cis*-regulatory element analysis, we predicted the functions of the TaFDL gene family and further confirmed through transcriptome data analysis that *TaFDL* genes respond to low temperature, drought stress, and hormones, with distinct expression patterns and functional conservation across different subfamilies. Based on these findings, we selected five potential key genes and determined their subcellular localization and transcriptional activation activities. qRT-PCR results indicated that five genes in the TaFDL gene family positively responded to early low-temperature exposure, light elements, and exogenous hormone supplementation. Finally, the homologous genes of the five key *TaFDL* genes across nine different wheat cultivars highlight significant genetic diversity. These findings expand the current understanding of the FD gene family and provide a foundation for future functional studies of the TaFDL gene family in wheat, thereby enriching the research on vernalization-related genes in wheat.

## Materials and methods

### Gene identification, sequence retrieval, and naming of *TaFDL* genes in wheat

A blastp method with a threshold of  $1e^{-5}$  was employed to search for FDL members in the wheat protein database using the amino acid sequences of 68 *FD* homologous genes from 22 species (Supplementary text 1). All the identified candidates of TaFDL gene family were further validated for the presence of the bZIP domain by utilizing the online tools, such as SMART (<https://smart.embl.de/>, accessed on March 8, 2022) and InterPro (<https://www.ebi.ac.uk/interpro/search/sequence>).

Additionally, the candidate genes were aligned using ClustalW in MEGA11 to determine if they contained the conserved and short SAP motif in the C-terminal region of the FD gene family.

Furthermore, all family members were named based on their respective chromosomal locations. The DNA library, coding sequences (CDS), amino acid sequences, gene sequences of wheat used in this study were downloaded from Ensembl Plants database (<https://plants.ensembl.org/info/data/ftp/index.html>, Chinese Spring wheat Genome assembly: IWGSC, iwgsc\_refseqv1.0). The ExPASy - ProtParam tool from the Expasy server ([www.expasy.org](http://www.expasy.org)) was employed to calculate the amino acid length, molecular weight (MW), isoelectric point (pI), and other physicochemical properties of the TaFDL members.

### Evolutionary relationships of *TaFDL* genes and *FD* homologous genes

Protein sequences alignment analysis of TaFDL members and FD homologs from 8 species (*Arabidopsis*, tomato, grape, rice, brachypodium, foxtail millet, barley, and poplar) was performed using the ClustalW in MEGA11. A phylogenetic tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replications, and the results were visualized using the iTOL tool.

Furthermore, the evolutionary timescale of life, including wheat and the other selected eight species, was obtained from the time-tree website (<http://timetree.org/>).

### Gene structure, conserved motifs, conserved domain analysis and protein structure prediction

The Gene sets GTF/GFF format files required for visualizing the gene structure of *TaFDL* genes were downloaded from the Ensembl Plants website (<https://plants.ensembl.org/info/data/ftp/index.html>). The files needed for visualizing the conserved motifs of TaFDL members were obtained from the MEME website (<https://meme-suite.org/meme/tools/meme>). The files required for visualizing the conserved domain of *TaFDL* genes were obtained from the SMART website ([http://smart.embl-heidelberg.de/smart/set\\_mode.cgi?NORMAL=1](http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1)) and organized accordingly. The methods employed to analyze the evolutionary rate of TaFDL members were consistent with those described above and produce an output file in the ".nwk" format for visualizing the phylogenetic tree in TBtools. Subsequently, the analysis and visualization of gene structure, conserved motifs, and conserved domain members were performed using the "Gene structure view (Advanced)" feature in TBtools. bZIP conserved amino acids were identified and characterized with Weblogo (<http://weblogo.berkeley.edu/>).

Prabi SOPMA SECONDARY STRUCTURE PREDICTION METHOD ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=npsa\\_sopma.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html)) and Swiss-Model (<https://swissmodel.expasy.org/>) was used to predict the secondary and tertiary protein structure.

**Table 1** Various growth conditions used in this study. In the T3 treatment, 100  $\mu$ m GA<sub>3</sub> (20 mL) was added to the root system twice weekly, from day 8 after germination (one leaf seedling)

| Treatment | Photoperiod            | Vernalization temperature | Light intensity | Exogenous administration of GA <sub>3</sub> |
|-----------|------------------------|---------------------------|-----------------|---------------------------------------------|
| T1        | 22 h light / 2 h night | 4 °C                      | 10,000 lux      | /                                           |
| T2        | 8 h light / 16 h night | 4 °C                      | 10,000 lux      | /                                           |
| T3        | 22 h light / 2 h night | 4 °C                      | 10,000 lux      | GA <sub>3</sub>                             |
| T4        | 22 h light / 2 h night | 4 °C                      | 1000 lux        | /                                           |

### Chromosome locations, duplication, synteny, and Ka/Ks analysis of *TaFDL* genes

The chromosome location information of *TaFDL* genes was obtained from the genome annotation data. Each *TaFDL* genes was mapped to its respective locus in the wheat genome, and the visualization was performed using TBtools. The intraspecific gene duplicates of wheat were analyzed using Advanced Circos feature in TBtools. Additionally, the interspecies genome collinearity analysis was conducted using the One Step MCScanx feature in TBtools. MEGA11 and TBtools was utilized to calculate Ks, Ka, and Ka/Ks.

### Promoter cis- regulatory element analysis

To extract the upstream 2000 bp regions of the *TaFDL* genes, we utilized TBtools. The extracted sequences were then submitted to the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) for the prediction of promoter cis-regulatory elements. The predicted elements were manually filtered and visualized using TBtools and GraphPad Prism 9.

### GO analysis

The GO annotations of *TaFDL* genes were obtained from the agriGO (<http://bioinfo.cau.edu.cn/agriGO/index.php>) and visualized using Graphpad Prism 9.

### Plant materials and growth conditions

Tissue-specific expression analysis was conducted utilizing the nationally recognized wheat variety “Bainong 207” from the Chinese National Catalogue of Promoted Excellent Crop Varieties ([http://www.zys.moa.gov.cn/gzdt/202303/t20230302\\_6422033.htm](http://www.zys.moa.gov.cn/gzdt/202303/t20230302_6422033.htm)). Wheat seedlings were cultivated to the one-leaf and one-tiller stage before being transferred to a vernalization chamber (Shanghai Bowen) for experimental procedures. A list of the various growth conditions used in this study is provided in Table 1. The photoperiod, light intensity settings and exogenous concentration of GA<sub>3</sub> were based on previous studies [79, 80, 81]. Leaves were sampled at 0, 8, 16, 24, and 48 h post-vernalization, with the 0-hour sample serving as the control. They were immediately placed in liquid nitrogen and stored at -80 °C.

### Expression pattern analysis and molecular cloning of key *TaFDL* genes

The transcriptome data of *TaFDL* gene family members under different temperatures, drought stress, drought and heat stress, as well as at different developmental stages and from different tissues, was downloaded from the WheatOmics 1.0 database (<http://wheatomics.sdau.edu.cn/>). The downloaded data were further analyzed using TBtools software to generate heatmaps.

The family members showing remarkable expression patterns in the analysis, *TaFDL-2A-8*, *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56*, were selected for cloning and further investigation. The cDNA used for cloning was derived from Chinese Spring wheat, and all cloned sequences were confirmed by sequencing after ligation into the Simple vector. Primer sequences are listed in Table S3.

### Transcriptional activation assay

The transcriptional activation assay vector used in this study was pGBKT7, as referenced in Liu’s research [82]. The CDS of *TaFDL-2A-8*, *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56* were individually integrated into the pGBKT7 vector using the double enzyme digestion technique and homologous recombination method. Subsequently, the *TaFDL*s-pGBKT fusion expression vectors, pGBKT7 empty vector (negative control), and pGBKT7-53 + pGADT7-T co-transformation vector (positive control) were transformed into the yeast strain Y2HGold, respectively. The transformed yeast cells were incubated at 30 °C for three days, and their growth status was observed. Primer sequences are listed in Table S4.

### Subcellular localization analysis

The subcellular localization analysis method was referenced from the study conducted by Liu with slight modifications [82]. The CDS of *TaFDL-2 A-8*, *TaFDL-2 A-11*, *TaFDL-5 A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56* genes was individually integrated into the binary expression vector pCAMBIA1305.1 using the double enzyme digestion technique and homologous recombination method. The pCAMBIA1305-GFP vector, which harbors the 35 S-driven green fluorescent protein (GFP) sequence, was used as the control in this study. The resulting fusion constructs, pCAMBIA1305.1-*TaFDL*s,

pCAMBIA1305.1-GFP and the negative control GFP were independently transformed into *Agrobacterium tumefaciens* cells GV3101. Subsequently, the transformed bacteria was infiltrated into *Nicotiana benthamiana* leaves for transient expression analysis. The infiltrated leaves were incubated in darkness for 40 h, followed by DAPI staining and sectioning. Finally, the injected leaves were observed using a turntable laser confocal live cell detection microscope system. In addition, we utilized a data model to predict the subcellular localization of all family members. The protein subcellular localization prediction analysis in this experiment was performed using the online bioinformatics software WoLF PSORT (<https://wolfpsort.hgc.jp/>). The organism type was set as “Plant” while other parameters were kept at the default settings provided by the website. Primer sequences are listed in Table S5.

#### qRT-PCR analysis in different vernalization conditions

RNA extraction from wheat seedling leaves was performed using the PolyATtract® M5 HiPer Plant microRNA Extraction Kit, following the instructions provided with the kit. The extracted RNA was then resolved on a 1% agarose gel to verify the quality of the extraction. The concentration of the extracted RNA was determined using an Analytikjena UV spectrophotometer.

The extracted RNA was used as a template for cDNA synthesis of wheat seedling samples. The cDNA synthesis was carried out using the Uni All-in-One First-Strand cDNA Synthesis SuperMix for qRT-PCR kit from Gene-seq Technology, following the instructions provided with the reverse transcription kit. The quality of the reverse transcription was validated by performing PCR using the reference gene TaGAPDH. The PCR was conducted using a thermal cycler purchased from Monad, USA. The primers for qRT-PCR were designed using Primer 6 software. The reference gene used for qRT-PCR was *TaGAPDH*. The qRT-PCR was performed using the QuantiTect SYBR® Green RT-PCR Kit with the Light Cycler® 96 instrument (Roche, Switzerland). The qRT-PCR results were initially analyzed using the Light Cycler® 96 System and further analyzed and visualized using appropriate software tools. Primer sequences are listed in Table S6.

#### Variation analysis in five key *TaFDL* genes and their homologs across nine wheat cultivars

To identify significant genetic diversity and potential allelic variation, we performed sequence alignment of five key *TaFDL* genes (*TaFDL-2A-8*, *TaFDL-2A-11*, *TaFDL-5A-44*, *TaFDL-5D-51*, and *TaFDL-6D-56*) and their homologs across nine wheat cultivars (AMN, BJ8, HD6172, JM22, MZM, XN6028, XY6, YM158, and ZM22) using DNAMAN software. The sequences of five key *TaFDL* gene homologs across nine wheat cultivars

were obtained via BLAST in WheatOmics 1.0 (<http://wheatomics.sdau.edu.cn>). The alignment focused on identifying substitutions, insertions, and deletions in the CDS sequences of these genes.

#### Statistical analysis

The experimental data obtained were subjected to a one-way analysis of variance (ANOVA) and using IBM SPSS Statistics 26. The experimental data is represented as the mean  $\pm$  standard error of the mean (SEM) of measured values for each treatment group. Significance analysis was performed using Dunnet's test.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-025-11436-w>.

Supplementary Material 1

Supplementary Material 2

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#### Author contributions

WJK and YMG designed the experiments; WJK analyzed the experimental data; YMG, YZ, ZQW, ZY, and YC performed gene cloning, qPCR, and transcriptional activation assays; YZ, ZQW, ZY, and DCW performed sample collection; WJK wrote first draft of the manuscript; YMG, CGT, and LFW revised the manuscript.

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#### Data availability

Plant genome data used in this study is available through the Ensemble Plants database (<https://plants.ensembl.org/index.html>), with Taxonomy IDs for wheat (4565), Arabidopsis (3702), tomato (4081), grape (29760), rice (39947), brachypodium (15368), foxtail millet (4555), barley (112509), and poplar (3694). Transcriptome data was obtained from the WheatOmics 1.0 database (<http://wheatomics.sdau.edu.cn/expression/index.html>). The transcriptome data for low-temperature stress and drought stress were sourced from the Wheat abiotic stress label, while transcriptome data for exogenous hormone supplementation were sourced from the Others label. Additionally, transcriptome data for different developmental stages or tissues of wheat was obtained from wheat development tissues.

#### Declarations

##### Ethics approval and consent to participate

Not applicable. The sampling of plant material was performed in compliance with institutional guidelines. The research conducted in this study required neither approval from an ethics committee, nor involved any human or animal subjects.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare no competing interests.



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