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GENOME-WIDE IDENTIFICATION AND EXPRESSION ANALYSIS OF *EARLY FLOWERING* (*ELF*) GENE FAMILY IN *NICOTIANA TABACUM*

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Abstract

Early Flowering (*ELF*) genes are crucial in plant photoperiod pathways and are negative regulation genes of plant flowering. At present, the identification of ELFs in *Nicotiana tabacum* (*NtELF*s) has not been systematically carried out. Using the Arabidopsis *ELF* family genes as a reference, 28 *NtELF* family members were identified, which were divided into four subfamilies. The ELF4 subfamily exhibited the highest membership counts of 13 members and owned a unique motif 1; with the exception of *NtELF4-9*. All members of the ELF4 subfamily had only one CDS. The results of chromosome localization of the *ELF* family members showed that there were three sets of tandem repeat events in the *NtELF*s. Promoters of *NtELF*s contained four *cis*-acting element types, and the light response elements were significant abundance. The NtELF family proteins within the same subfamily exposed comparable 3D structures and demonstrated analogous gene expression patterns. GO enrichment analysis of *NtELF*s showed that terms related to stimulus, photoperiodism, and reproduction were significantly enriched. This study systematically identifies NtELF family members for the first time, thereby paves the way for further investigations into their biological functions and provides a valuable background information for gene editing breeding.

Key words: *Nicotiana tabacum*; Early flowering; Elf; Phylogenetic; Structure; Expression patterns

Introduction

In plant, flowering marks a significant milestone in the life cycle (Li *et al.*, 2023). It indicates the shift from vegetative growth into reproductive growth and the attainment of developmental maturity (Maple *et al.*, 2024). This critical phase initiates the reproductive stage, enabling population perpetuation through fruiting. The flowering process is precisely orchestrated by a myriad of genes, which collectively form a highly complex regulatory network to guarantee timely blooming (Putterill *et al.*, 2004; Bernier & Périlleux, 2005). Gene families associated with early flowering in plants play a crucial role in regulating flowering time (Liu *et al.*, 2018; Zhao *et al.*, 2022). Several gene families have been determined as playing a crucial role in the regulation of flowering time, such as the *FLOWERING LOCUS T* (*FT*) (Jin *et al.*, 2021), the *CONSTANS* (*CO*) (Song *et al.*, 2020) and the *EARLY FLOWERING* (*ELF*) (Zagotta *et al.*, 1996) gene families.

The *ELF* gene family is particularly significant in the regulation of plant flowering time (Zagotta *et al.*, 1992). They were recognized for their contribution as negative regulators of flowering, delaying the onset of flowering in response to various environmental cues and / or internal signals (Zhao *et al.*, 2021). These *ELF*s play a role in the photoperiodic pathway and interact with other key regulators of flowering time to slightly adjust the timing of floral initiation (Doyle *et al.*, 2002; Noh & Amasino, 2003; Yu *et al.*, 2008). The ELF family had been extensively studied in model plants like *Arabidopsis thaliana* (Doyle *et al.*, 2002; Noh & Amasino, 2003). ELFs were originally discovered in genetic screens of photoperiod mutants and were found to be able to regulate circadian rhythm (Zagotta *et al.*, 1992; Nusinow *et al.*, 2011). ELF3 and ELF4 were proved to affect the biological activity of GIGANTEA (GI) to affect the flowering time of various crops (Doyle *et al.*, 2002; Brandoli *et al.*, 2020), including rice (*Oryza sativa*) (Yang *et al.*, 2013), barley (*Hordeum vulgare*) (Boden *et al.*, 2014), and peas (*Pisum sativum*) (Rubenach *et al.*, 2017). In *A. thaliana*, ELF6 participates in brassinosteroids (BRs) signal transduction by responding to the recruitment of BES1 (brassinosteroid signaling positive regulator), thus affecting histone methylation of BRs-responsive genes and finally regulating flowering time (Yu *et al.*, 2008). By promoting *FLC* (*FLOWERING LOCUS C*) expression, PIE1 promotes FLC-mediated flowering delay, thus achieving the purpose of negatively regulating flowering time (Noh & Amasino, 2003).

Tobacco is both a significant economic crop and an ideal model plant for fundamental research. Its flowering period is closely related to the quality and yield of tobacco leaves. However, the comprehensive identification and analyses of tobacco flowering related gene families has not been reported yet. In this study, we identified the *Nicotiana tabacum ELF* (*NtELF*) gene family, including *ELF3*, *ELF4*, *ELF6* and *PIE1*. Furthermore, we scrutinized their conserved domains, gene structures, cis-acting elements, protein structures and interactions, gene expression patterns and functional enrichment. This study will establish a theoretical foundation for the functional investigation of flowering-related genes in tobacco and serve as a valuable reference for gene editing breeding.

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Materials and Methods

Identification of the *NtELF*s: The protein sequence and genome annotation information of common tobacco (*N. tabacum*) were obtained from the Chinese Tobacco Genome Database (CTGD). *A. thaliana* ELF family (AtELF) protein sequences were acquired byTAIR (https://www.arabidopsis.org/). AtELF family protein sequences were used as references, NtELF family members were identified by the Blastp program. The amino acid number, molecular weight, theoretical isoelectric point, aliphatic index and grand average of hydropathicity of NtELF were analyzed using the Protein Paramter Calc (ProtParam-based) functional module in TBtools (Chen *et al.*, 2023). The WoLF PSORT (https://wolfpsort.hgc.jp/) was operated to predict subcellular localization of NtELF proteins (Horton *et al.*, 2007). Chromosomal localization of each *NtELF* gene was also demonstrated using TBtools.

Phylogenetic analysis of NtELFs: The tobacco and Arabidopsis ELF protein sequences were compared using the ClustalW program in MEGA-X (https://www.megasoftware.net/) (Kumar *et al.*, 2018). The phylogenetic tree was then constructed using the inbuild Neighbor-Joining method, with the bootstrap-replications value set to 1,000. The tree was further embellished through the iTOL (https://itol.embl.de/) (Letunic & Bork, 2024).

Conserved motif and gene structure of the *NtELF*s: Conserved motifs of the *NtELF* gene family were analyzed using protein sequences via MEME (http://meme-suite.org) (Bailey *et al.*, 2009). The pattern of ELF proteins conserved motifs was demonstrated by TBtools. The genetic structure of the *NtELF* gene family was shown at the same time.

The *cis*-acting elements of the *NtELF*s promoters: The 2 kb upstream sequences of all *NtELF* genes were extracted from the CTGD to complete this analysis. The *NtELF*s *cis*-acting elements were predicted by PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.*, 2002). Then, the categorized *cis*-acting components were displayed by TBtools.

3D structure and interaction network analysis of NtELFs: The NtELF family protein structures were estimated through the SWISS-MODEL (https://swissmodel.expasy.org/) (Waterhouse *et al.*, 2018). Protein-protein interactions network between all NtELFs were performedvia STRING (https://cn.string-db.org/)(Szklarczyk *et al.*, 2023).

Expression patterns of the *NtELF*s: The *NtELF* gene expression values (FPKM) in roots, stems, leaves and flower buds were retrieved from the CTGD. The expression levels of tobacco ELF genes in these four tissues were standardized and displayed by the HeatMap module in TBtools. Genes with similar expression patterns were detected using row clustering.

GO enrichment analysis of NtELFs: Protein sequences of the NtELF family were used for gene function annotation by the eggNOG-mapper tool (http://eggnog-mapper.embl.de/) (Cantalapiedra *et al.*, 2021). The annotation result cleaning and GO enrichment analysis were executed via eggNOG-mapper Helper and GO Enrichment function modules of Tbtools. The significantly enriched GO terms were finally exhibited using chiplot (https://www.chiplot.online/).

Results

Identification and physicochemical properties of NtELFs: A total of 28 ELF family members were identified in tobacco by protein homology searching with BLASTP, and they were renamed according to their subfamily classification and chromosomal location. The physicochemical properties of NtELF family proteins were relatively different. To be specific, the amino acids number of the NtELFs proteins ranged from 105-2,080, with a molecular weight ranging from 11,721.95-234,661.64, the theoretical isoelectric point ranging from 4.88-9.51, and the aliphatic index ranging from 58.87-94.82. All proteins' grand average of hydropathicity (GRAVY) was less than 0, meaning all proteins were hydrophilic. Subcellular localization results revealed that all NtELF proteins were exclusively located in the nucleus.

Phylogenetic relationship between NtELFs and AtELFs: The phylogenetic tree was constructed using *N. tabacum* and *A. thaliana* ELF protein sequences. The results revealed that NtELFs were divided into four subfamilies, containing 10 ELF3 subfamily genes, 3 PIE1 subfamily genes, 2 ELF6 subfamily genes, and 13 ELF4 subfamily genes, respectively (Fig. 1). ELF4 was the largest subfamily. The genes of the same subfamily of tobacco and Arabidopsis were clustered in the same clade. The PIE1 subfamily had a close evolutionary relationship with the ELF6 and ELF3 subfamilies, but a relatively distant evolutionary relationship with the ELF4 subfamily (Fig. 1).

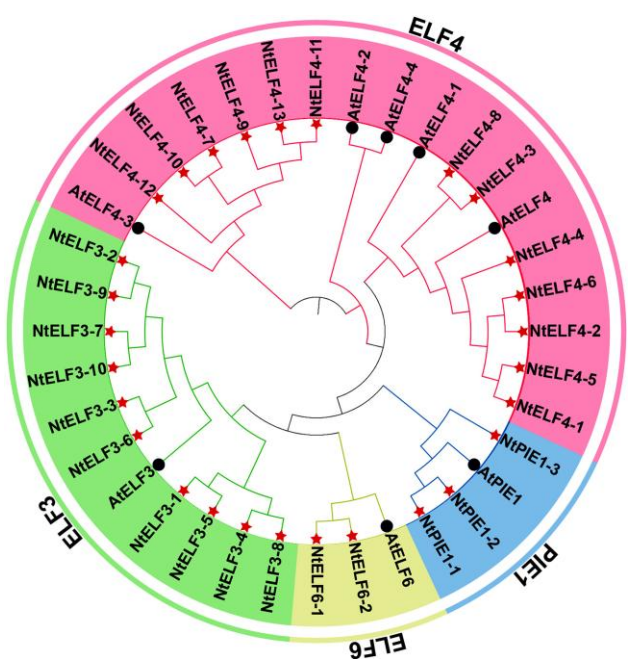

Fig. 1. Phylogenetic tree of ELF family in *Nicotiana tabacum* and *Arabidopsis thaliana* (Different colors on the rings indicated different subfamilies. Members of the *A. thaliana* family were represented by dots, and members of *N. tabacum* family were represented by five-pointed stars).

Chromosomal localization of *NtELF*s: Twenty-eight *NtELF*s genes were located on 11 chromosomes and 3 scaffolds (Fig. 2). The number of *NtELF* on each chromosome and scaffold varied from 1-5, with 5 *ELF* genes on Chr04, 4 on Chr01 and Chr02, 3 on Chr10, and 2 on Chr03 and Chr14. Other chromosomes and scaffolds had only one *NtELF*. The correlation between ELF family number and chromosome length was not found to be statistically significant. All this information indicated that *NtELF* genes were distributed unevenly and preferentially in the genome. In addition, there were three tandem duplication events in the tobacco genome of *NtELF*s (*NtELF4-1* and *NtELF4-2*; *NtELF4-4*, *NtELF4-5* and *NtELF4-6*; *NtPIE1-2* and *NtPIE1-3*). These closely related genes were closely distributed within 200 kb on the same chromosome, suggesting a high degree of homology and important biological functions of the *NtELF*s.

Conserved motif and gene structure of the *NtELF*s: The conserved motifs and gene structure were analyzed to gain an intensified understanding of the evolutionary relationships among *NtELF*s (Fig. 3A). Twenty motifs were detected in the *NtELF* gene family. The members belonging to the same subfamily exhibited comparable motif compositions. The ELF3 subfamily contained the most motif types, and the ELF4 subfamily contained the least motif types. Motif 1 was a unique type to the ELF4 subfamily and was shared by all of them (Fig. 3B). The CDS number of *NtELF*s ranged from 1 to 21. Genes within the same subfamily had comparable genetic structures. In the ELF4 subfamily, except for *NtELF4-9*, all the other genes have only one CDS and no intron. In the PIE1 subfamily, NtPIE1-1 has the most 21 CDS (Fig. 3C).

The *cis*-acting elements of the *NtELF*s promoters: The 2kb upstream nucleotide sequences of *NtELF* family genes were extracted as promoter sequences for the distribution patterns analysis of *cis*-acting element. The results revealed that the *cis*-acting elements of *NtELF*s were mainly divided into four categories (Fig. 4). The first category was light response elements, which were the predominant *cis*-acting elements of the *NtELF*s promoter sequences, including O2-site, ATCT-motif, TCT-motif, TCCC-motif and GT1-motif, etc. The second category was phytohormone response elements, including auxin response elements (TGA-motif and AuxRR-core), ABA response elements (ABRE), GA response elements (GARE-motif, P-box and TATC-box), salicylic acid response elements (TCA-element), MeJA response elements (CGTCA-motif and TGACG-motif). The third category was growth and development related elements, including circadian, CAT-box and GCN4_motif. The fourth category consisted of other elements, including low-temperature response elements (LTR), drought stress response elements (MBS), anaerobic stress elements (ARE), damage response elements (WUN-motif) and biological stress response elements (TC-rich repeats), etc.

3D structure of NtELFs: Protein structure directly determines the function of genes, and 3D protein structure analysis is very important to understand the function of the NtELF family. The findings demonstrated that the NtELFs contained in the same subfamily had comparable protein structures (Fig. 5). ELF4 subfamily proteins were mainly composed of α-helices, forming the simplest protein structure. ELF3, ELF6 and PIE1 subfamily proteins included a large number of β-sheets and random curls in addition to the α-helices. These basic structures were further coiled and folded to form more complex spatial structures. The highly conserved protein structure within the same subfamily implied that these genes exhibited similar functions in tobacco growth and development.

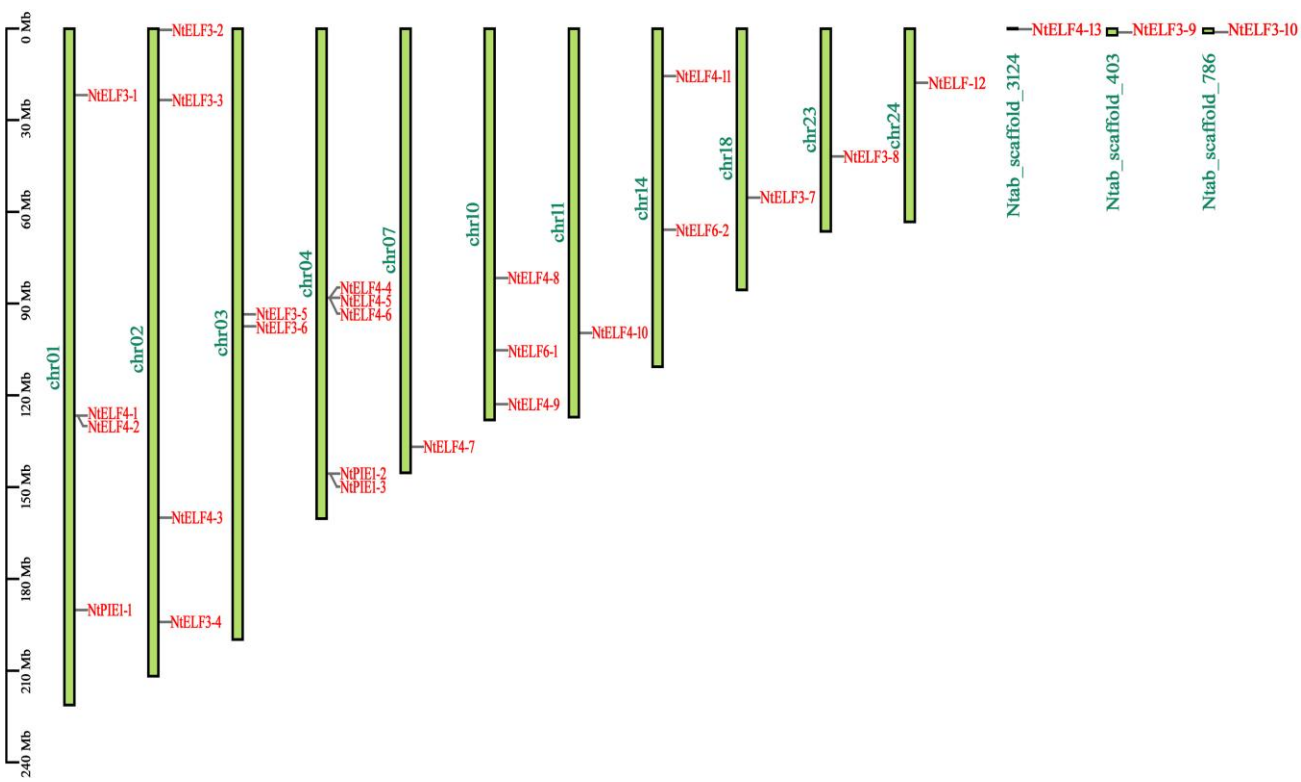

Fig. 2. The location of *NtELFs.* The green column indicated the chromosomes of *N. tabacum*, and its length indicated the chromosomes length).

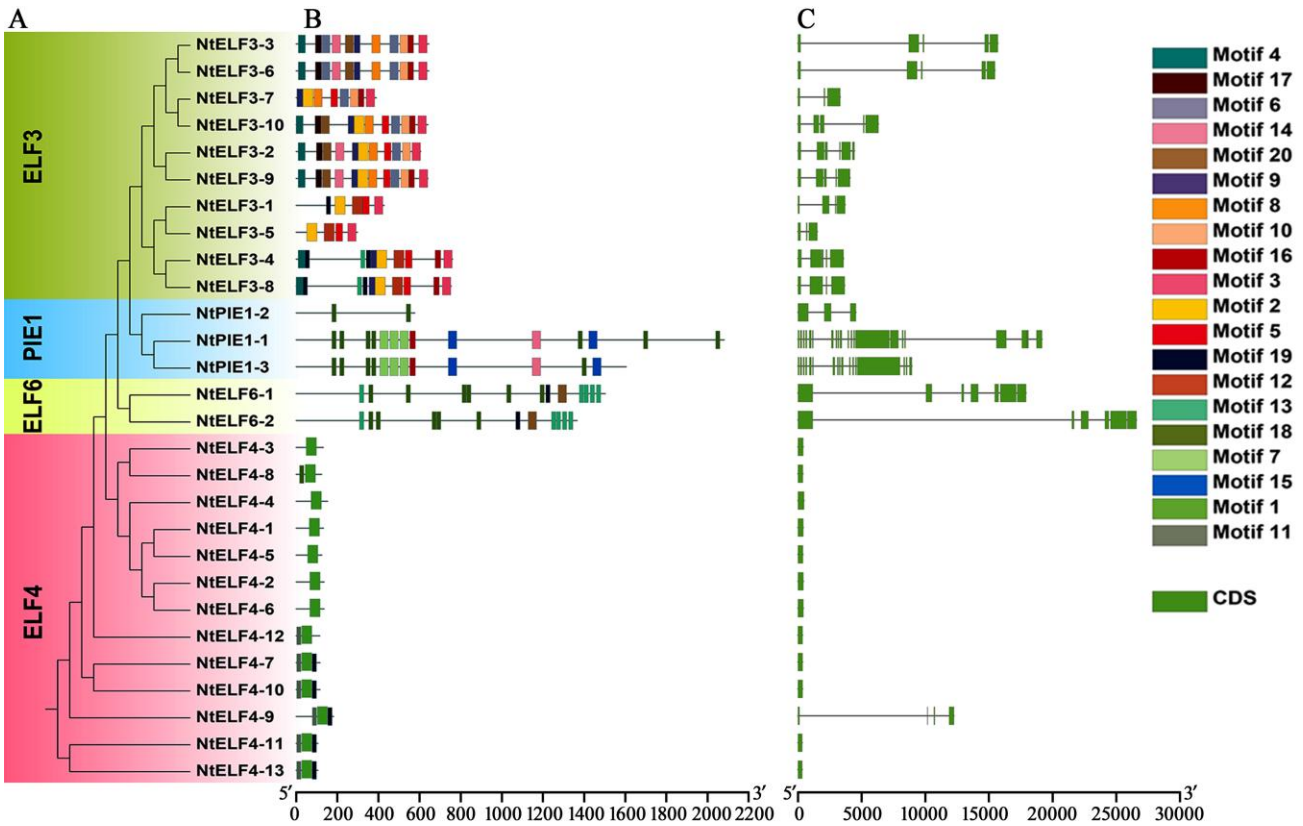

Fig. 3. Conserved motifs and gene structure of *NtELF* gene family: A, phylogenetic tree of ELF family in *N. tabacum*; B, motif structure of family proteins; C, gene structure of ELF family. (In Figure B, different color blocks represented different motifs, and the length of line segments represented the length of protein sequences. In Figure C, the green color block represented CDS, and the length of the line segment represented the length of the gene sequence).

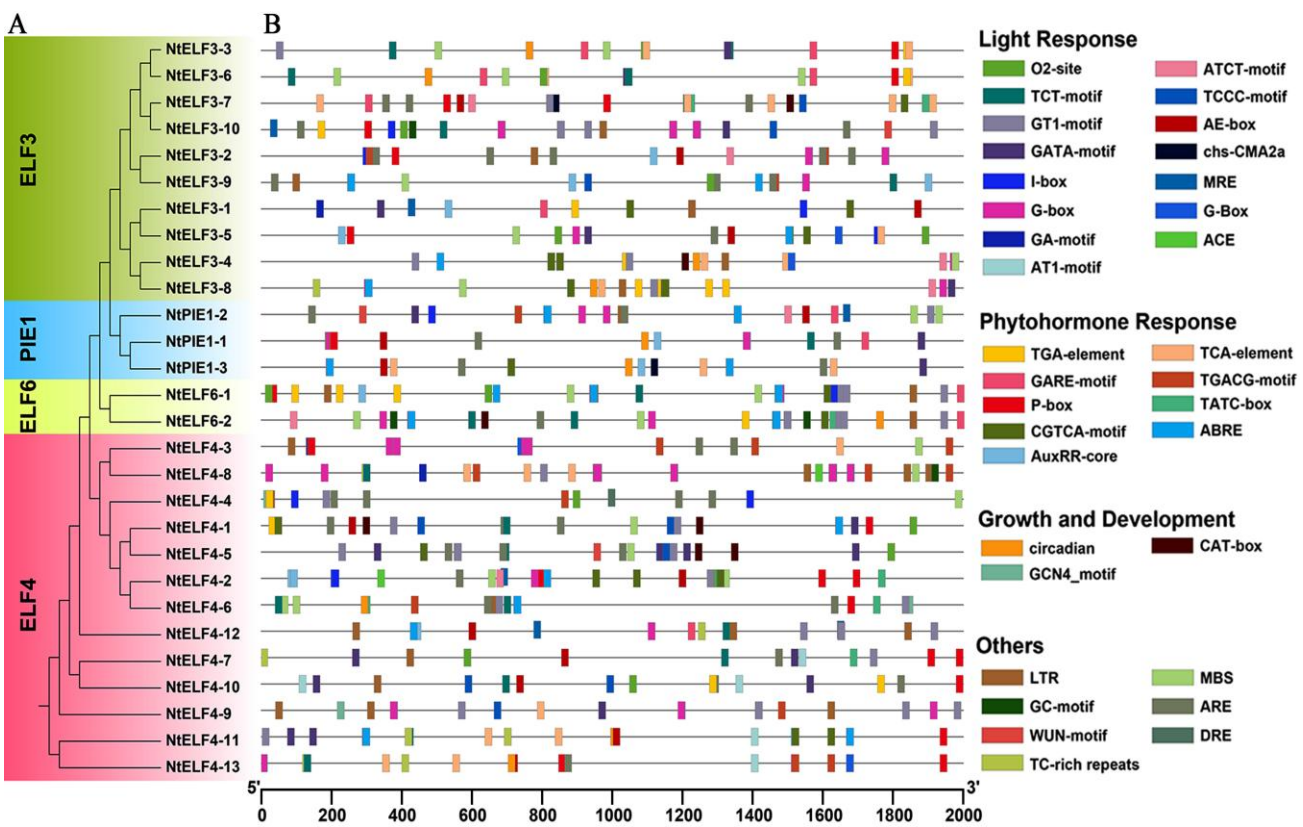

Fig. 4. The *cis*-acting elements of the *NtELF*s promoters. A: Phylogenetic tree of ELF family in *N. tabacum*, B: Distribution of the *cis*-acting elements. (Different color blocks represented different *cis*-acting elements with different functions, and the classification of elements was shown in the legend).

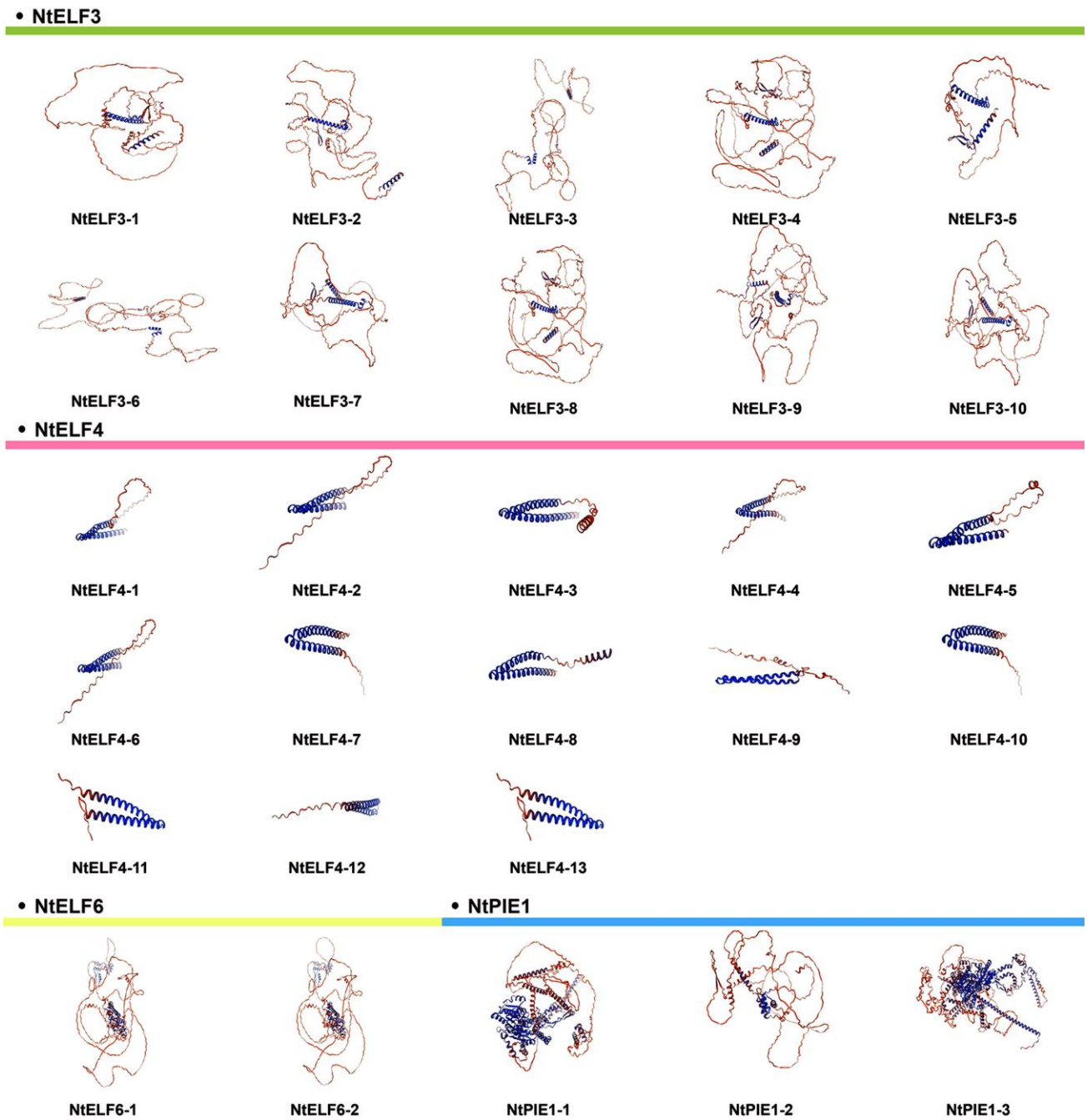

Fig. 5. 3D structure of NtELF family protein.

Protein-protein interaction network of NtELFs: Two protein-protein interaction (PPI) networks existed within the NtELF gene family (Fig. 6). A PPI network consisted of the ELF4 subfamily members and the ELF3 subfamily members. Another PPI network consisted of the ELF6 subfamily members and the PIE1 subfamily members. The biological function of the NtELF family heavily relies on the pivotal contributions of NtELF4-1, NtELF4-2, NtELF4-4, NtELF4-5 and NtELF4-6, among which the NtELF4-5 interacted strongly with other proteins except NtELF4-2.

Expression patterns of the *NtELF*s: The *NtELF*s genes were expressed to varying degrees in various tissues (Fig. 7). The expression of certain genes was found to show a high expression in roots, stems, leaves, and flower buds, including most ELF3 subfamily genes, all ELF6 subfamily genes, and all PIE1 subfamily genes. Genes from the same subfamily showed a similar expression pattern, such as *NtPIE1-1* and *NtPIE1-3*, *NtELF6-1* and *NtELF6-2*, and *NtELF3-2* and *NtELF3-10*. The expression of *NtELF3-10* was found to be the highest level across all four tissues. All ELF6 and PIE1 subfamilies members also showed similar expression patterns, but the relative expression amounts were slightly lower.

GO enrichment of NtELFs: GO enrichment analysis revealed significantly enriched GO terms of the *NtELF*s, which were classified into three categories, namely biological processes (BP), molecular functions (MF), and cell components (CC) (Fig. 8). All the enriched genes in the CC category were enriched in the organelle. In the category of BP, terms related to stimulus, photoperiodism, and reproduction

were significantly enriched, including “response to stimulus”; “response to external stimulus”; “response to light stimulus”; “photoperiodism”; “photoperiodism, flowering”; “vegetative to reproductive phase transition of meristem”; “regulation of reproductive process”, etc. In the MF, *NtELF*s were mainly enriched in terms related to transcription factor activity.

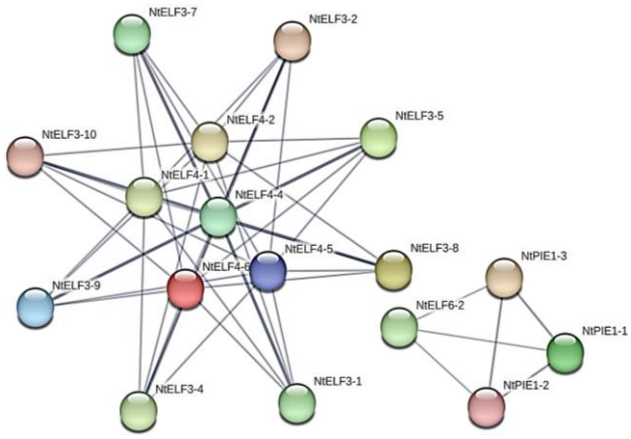

Fig. 6. Protein-protein interaction network of NtELF family proteins. (The thickness of the line segment represented the strength of the interaction).

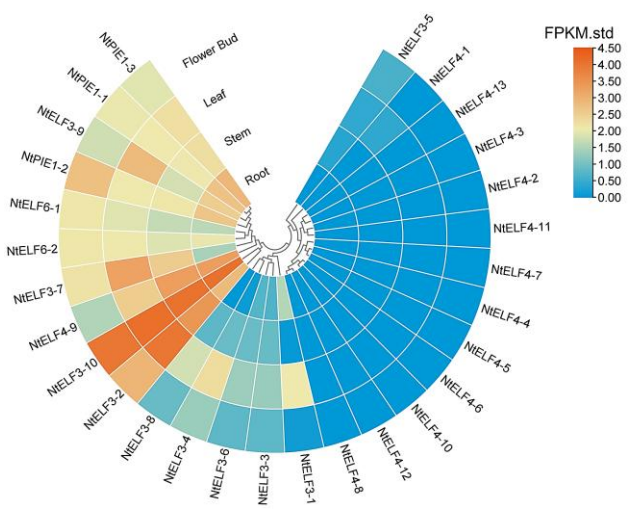

Fig. 7. Expression patterns of *NtELF*s in various tissues.

Discussion

ELF genes are involved in plant biological clock regulation, photoperiod pathway, flowering time control, hypocotyl elongation and shade response (Zhao *et al.*, 2021). In this study, the NtELF family was systematically identified, and a series of bioinformatic analyses were completed, including phylogenetic analysis, gene structure and localization, gene expression pattern and functional enrichment analysis, protein structure and physicochemical properties. Bioinformatics analyses revealed structural conservation and functional diversity of the *NtELF* gene family.

The tobacco genome contained a total of 28 ELF family members, which were divided into four subfamilies. The genes belonging to the same subfamily exhibited a remarkably conserved exon-intron structure as well as protein structure. The NtELF4 subfamily with 13 members was the largest subfamily. The number of NtELF4 subfamily members was greater than that in *A. thaliana*, *Glycine max* (Marcolino-Gomes *et al.*, 2017), *Diospyros deyangensis* (Fang *et al*., 2023) and *Gossypium arboretum* (Tian *et al.*, 2021), and their ELF4 subfamily consisted of 5, 9, 11, and 7 members, respectively. Although tobacco is an allotetraploid, the multiple of family members still cannot be fully explained. We hypothesized that a gene expansion event took place in the *N. tabacum* genome after the evolution of the two ancestor species *N. sylvestris* and *N. tomentosiformis* into the *N. tabacum*. In addition, we found three tandem replication events in the *NtELF* family, which provides favorable evidence for the expansion of the *NtELF* family and hints at the possibility of new biological functions (Hurles, 2004).

ELF family proteins participated in the plant photoperiod pathway and flowering time regulation by forming complexes with other proteins (Noh & Amasino, 2003; Nusinow *et al.*, 2011). The type and number of *cis*-acting elements within the promoter region exhibit a strong correlation with the functional attributes of the gene (Hernandez-Garcia & Finer, 2014). Analysis of *NtELF*s *cis*-acting elements in gene promoter region revealed that light response elements were the most elements, including O2-site, ATCT-motif, TCT-motif, TCCC-motif and GT1-motif, etc. In addition, the *NtELF*s were enriched into Photoperiodism-related pathways. The PPI network highlighted that NtELF4-1, NtELF4-2, NtELF4-5, NtELF4-5 and NtELF4-6 were relatively in the action centers of NtELF family proteins, and they were closely related to AtELF4. The role of AtELF4 in photoperiodic perception, circadian regulation and flowering was believed pivotal (Doyle *et al.*, 2002), thus we concluded that the five ELF4 subfamily genes had similar or even identical functions as previously reported. This can then be confirmed using molecular biological techniques, such as CRISPR/Cas9 or RNA interference (RNAi). By applying these methods to these NtELF4 genes, we can validate that these genes have similar or identical functions.

Another type *of cis*-acting element of *NtELF* family genes was related to phytohormone response, including auxin response elements, ABA response elements, GA response elements, salicylic acid response elements and MeJA response elements. This suggested that the NtELF family was also involved in growth and development mediated by plant hormones. This was similar to the results of previous research reports. In *Arabidopsis*, AtELF3 interacting with phytochrome B regulated plant development and flowering via a signal transduction pathway (Alvarez *et al.*, 2023). AtELF6 was involved in BRs signal transduction (Yu *et al.*, 2008). The mutation of AtPIE1 led to defects in petal growth (Noh & Amasino, 2003). The *NtELF3-10*, a homolog of *AtELF3* in tobacco, exhibited high expression levels in roots, stems, leaves, and flower buds. Similarly, all members of the ELF6 and PIE1 subfamilies displayed comparable expression patterns; however, their relative expression levels were slightly lower. These suggested their potential involvement in plant hormone responses. The molecular mechanism underlying the hormone response of NtELF3, NtELF6, and NtPIE1 subfamilies, as well as the associated downstream pathway, was likely to be intricate and warranted further investigation through a combination of physiological and biochemical approaches integrated with genetics or gene-editing techniques.

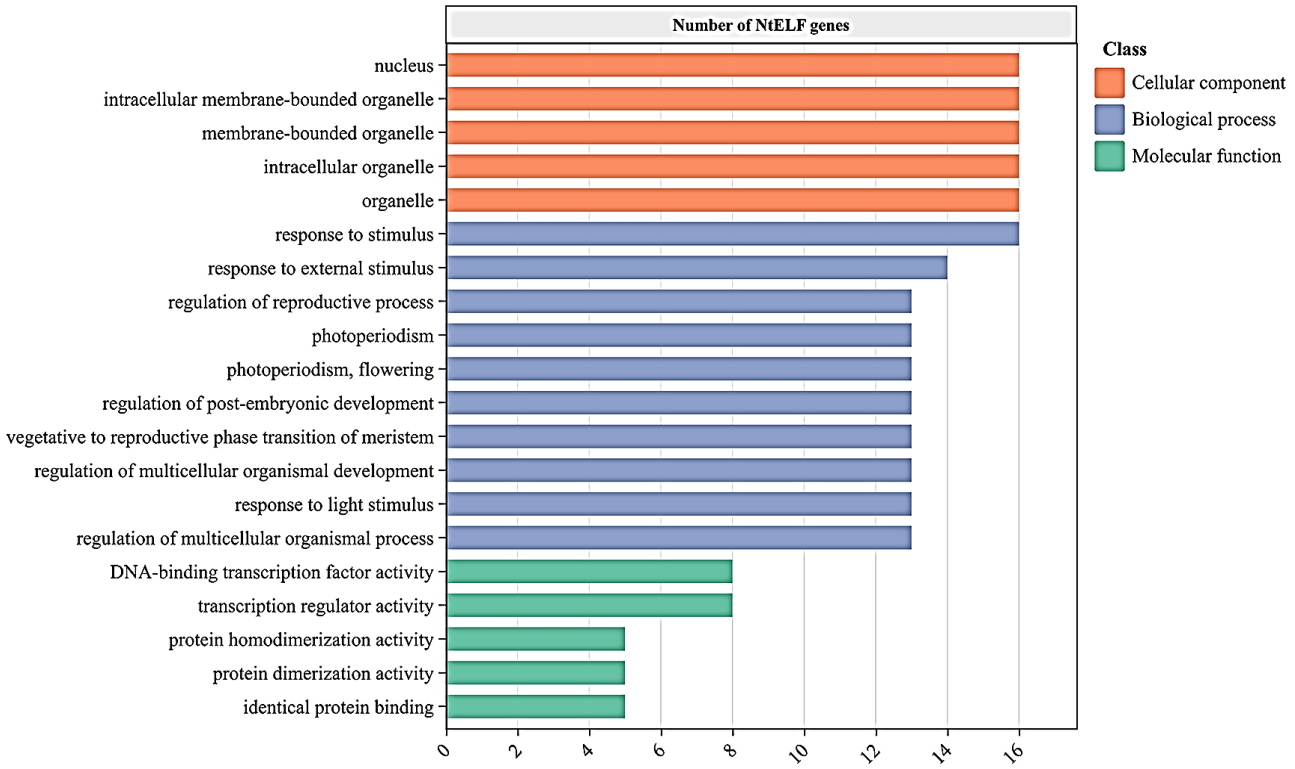

Fig. 8. GO enrichment of *NtELF*s.

Conclusion

EARLY FLOWERING (ELF) genes are important for plant photoperiod pathways and could negatively regulate genes of plant flowering. This study systematically identified 28 ELFs family members in *Nicotiana tabacum* (*NtELF*s) for the first time utilizing the Arabidopsis ELF family genes as a reference. At the same time, phylogenetic analysis, structural analysis, cis-acting element analysis and expression pattern analysis of NtELF family members were also completed. This study systematically answered the questions about the structures, evolution and expression patterns of the NtELF family, thereby establishing a theoretical groundwork for further investigations into the biological functionalities of its members. Additionally, it furnishes a comprehensive reference for gene editing breeding.

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Author Contributions: Yudong Chen: Conceptualization, Formal analysis, Investigation, Visualization, Writing - original draft, Writing –review & editing. Yanju Shuai: Data curation, Software, Writing –review & editing. Wenyuan Wang: Validation, Writing –review & editing. Lele Deng: Writing –review & editing. Haitao Hang: Writing –review & editing. Zhong Wang: Funding acquisition, Resources, Writing –review & editing. Wanli Zeng: Project administration, Supervision, Writing – review & editing. Kangkang Song: Formal analysis, Supervision, Writing –review & editing. Qian Gao: Funding acquisition, Methodology, Supervision, Writing – review & editing. All authors agree and approve the final version of the manuscript.

Conflict of Interest: The authors have not declared a conflict of interest.

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