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Evolution and Regulatory Roles of the KNOX Gene Family in *Eucommia ulmoides* in Response to Phytohormones and Abiotic Stresses

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Abstract:

Background: KNOX (KNOTTED1-like HOMEOBOX) belongs to a class of important homeobox genes, which encode the homeodomain proteins binding to the specific element of target genes, and widely participate in plant growth and development, secondary metabolite synthesis, biotic and abiotic stresses responses. However, genome-wide identification of the KNOX gene family has not been reported in *E. ulmoides*. **Result:** In this study, 8 KNOX proteins were identified based on genomic database of *E. ulmoides*. Phylogenetic analysis showed that the EuKNOX proteins were distributed in five subgroups based on homology with KNOX proteins in *Arabidopsis*, these genes were unevenly distributed across six chromosomes. Gene structure analysis revealed that *EuKNOX* genes contained between three and four introns. The number of introns within members of the same evolutionary branch was generally consistent. Prediction of cis-regulatory elements indicated that the *EuKNOX* genes were involved in hormone response, light response and stress response. RNA-seq data revealed tissue-specific expression patterns, among which five *EuKNOXs* were highly expressed in the xylem. The expression levels of most *EuKNOXs* were higher in female flowers than in male flowers. Functional analysis suggested that *EuKNOXs* contribute to bark, fruit and leaf development, Eu-rubber biosynthesis and leaf-color formation. qRT-PCR analysis demonstrated that *EuKNOXs* may act as positive regulators of NaCl and GA_3 treatments, however, *EuKNOXs* may act as negative regulators of MeJA treatments, *EuKNOX*s showed a trend of first increasing, then decreasing and then increasing under drought and ABA treatment, especially, *EuKNOX5* was induced 343-fold at 24 h under NaCl stress, suggesting that *KNOX* genes in *E. ulmoides* regulate plant growth and respond to different stresses by following different hormone signaling pathways, which laid a valuable foundation for further understanding the function of *KNOX* genes in multiple stress responses and phytohormone crosstalk in *E. ulmoides.* **Conclusion:** This study systematically identified and characterized the KNOX superfamily genes in *E. ulmoides*. Abiotic stress-responsive candidate genes were identified via qRT-PCR analysis under multiple stress conditions. The findings furnish essential insights into subsequent functional investigations of *E. ulmoides* KNOX genes and the genetic improvement of *E. ulmoides* abiotic stress resistance.

Keywords: *Eucommia ulmoides*; KNOX gene family; Growth and development; Abiotic stress; Hormone treatment; Expression pattern analysis

1. Introduction

Eucommia ulmoides Oliver (*E. ulmoides*), a single species of *Eucommia* genus belonging to the Eucommiaceae family, a highly valued tree species in China. Known for its attractive tree shape, this

species also holds considerable economic importance[1]. *E. ulmoides*, a valuable medicinal, industrial and food-related plant (leaves) in the Eucommiaceae family and the genus Eucommia, is commonly known as "plant gold". Its bark, leaves, and male flowers are utilized for medicinal purposes. Research has demonstrated that *E. ulmoides* is rich in beneficial active compounds, including chlorogenic acid, geniposide, periplocoside, eucommiflavone, and polysaccharides[2-5]. These compounds exhibit anti-inflammatory, antihistamine, resist allergies, liver and kidney-tonifying, and antioxidant effects, treating Atherosclerosis[6-10]. *E. ulmoides* exhibits broad environmental adaptability and high resistance to pests and diseases. Furthermore, its leaves, bark, and pericarp are abundant in trans-polyisoprene, endowing it with significant economic value. Owing to these superior traits, *E. ulmoides* is recognized as an ideal alternative or complementary tree species to Hevea brasiliensis in rubber production[11-12]. Research on *E. ulmoides* has primarily concentrated on breeding, economic value development, and pharmacology. In contrast, studies exploring its growth and development, as well as the regulatory mechanisms involved in abiotic stress adaptation, are still insufficient.

Knotted1-like homeodomain (KNOX) has been demonstrated to play a key role in plant growth and development and in the response to abiotic stress[13-15]. The first *KNOTTED1* gene to be identified in plants was *KNOTTED1* (*kn1*) in maize[16]. Following this discovery, several studies of KNOTTED1 proteins have been carried out in model and non-model plants, named KNOTTED1-like. The classification of the KNOX gene family into Class I, Class II, and Class M KNOX subfamilies was made based on structural characteristics, phylogenetic relationships, and expression patterns[17]. Class I KNOX genes that regulate lateral organ formation and growth are expressed in apical meristematic tissues and determine the fate of meristematic cells[18-19]. Class II KNOX genes regulate the secondary cell wall, xylem, root development, and fruit ripening[20-21]. KNATM, which is primarily expressed at the edges of mature organs and in the proximal domains of plant organ primordia, may be used to detect or generate signals related to flowering[22].

The functions of KNOX genes have been studied in various plants, including gymnosperms like *Pinus sylvestris*, monocots such as rice and orchids, and dicots like *Arabidopsis*, poplars, pears and cotton[15, 23-25]. The functions of class I *KNOX* genes have been extensively studied. In *Arabidopsis*, there are four class I genes: *SHOOTMERISTMELESS* (*STM*), *KNAT1*(*BP*), *KNAT2*, and *KNAT6*. The gene *STM* is essential for the formation and maintenance of the shoot apical meristem (SAM)[26]. Furthermore, *STM* has been identified as a positive regulator of related *KNOX* genes. The Class I genes play a vital role in maintaining floral meristem and carpel formation, influencing floral architecture[27]. Loss-of-function mutants of *bp/knat1* exhibit enhanced lignin accumulation in inflorescence stems, and the BP transcription factor modulates lignification patterns through regulating the expression of *KNAT2* and *KNAT6*[28-29]. Additionally, other Class I KNOX family proteins participate in various plant developmental processes: they regulate flower patterning, promote the outgrowth of primordia, contribute to leaf tip morphogenesis, and establish basipetal auxin streams that propagate from the tip through internal tissues, thereby inducing the differentiation of vascular strands[20, 30]. In contrast, the functional mechanisms of Class II genes (e.g., *KNAT3*, *KNAT4*, *KNAT5*, and *KNAT7*) are still not fully elucidated. It is worth noting that these Class II genes exhibit more extensive expression patterns in differentiating and mature tissues, as documented in previous studies[20, 30]. Specifically, among Class II

members, *MdKNOX19* in apple serves as a positive regulator of *ABI5* transcription, and the evolutionarily conserved MdKNOX19-MdABI5-ABA module may contribute to the regulation of organ development[31].

In angiosperms, the Class I subfamily genes are expressed primarily in the apical meristem and play essential roles in the growth and maintenance of meristematic tissue[18-19]. The Class I genes regulate plant organ development and secondary cell wall biosynthesis. They are also expressed in various plant organs, including roots, stems, leaves, and flowers[32]. In *Prunus mume*, overexpression of *PmKNAT2/6-a*, a class I *KNOTTED1-like homeobox* (*KNOX*) member, in *Arabidopsis* resulted in a multi-pistil phenotype, further analysis found that AGAMOUS-like 24 (PmAGL24) could directly bind to the *PmKNAT2/6-a* promoter and regulate its expression. PmAGL24 also interacted with Like Heterochromatin Protein 1 (PmLHP1) to recruit lysine trimethylation at position 27 on histone H3 (H3K27me3) to regulate *PmKNAT2/6-a* expression, which is indirectly involved in multiple pistils formation in Japanese apricot flowers[33-34]. In poplar, *PagKNAT2/6b* is an ortholog of *Arabidopsis KNAT2* and *KNAT6* that is highly expressed in phloem and xylem tissues. Compared with nontransgenic control plants, transgenic poplar plants with constitutive overexpression of *PagKNAT2/6b* present obvious alterations in vascular tissue patterning. These alterations are characterized by a reduction in secondary xylem biomass, accompanied by thinner cell walls with decreased accumulations of cellulose, xylose, and lignin. At the molecular level, *PagKNAT2/6b* can inhibit cell differentiation and secondary wall deposition during wood formation in poplar by modulating the expression of NAC domain transcription factors. Furthermore, the direct activation of PagXND1a by PagKNAT2/6b acts as a key regulatory node in the negative regulatory network of xylem differentiation by KNOXs[35]. In tomato, knockout mutants of *SlKN5* which is the KNOX II member display increased leaf complexity, furthermore, SlKN5 physically interacts with three transcriptional repressors from the BLH1-clade of BEL1-like homeodomain (BLH) protein family, SlBLH4, SlBLH5, and SlBLH7. Transient assays further demonstrate that the SlKN5-SlBLH7 interaction enhances binding capacity to regulatory regions of key chloroplast- and chlorophyll-related genes, including *SlAPRR2-like1*, *SlCAB-1C*, and *SlGUN4*. indicating that the KNOX II SlKN5-SlBLH regulatory modules serve to inhibit fruit greening and subsequently promote lycopene accumulation, thereby fine-tuning the color transition from immature green fruit to mature red fruit[36].

Eucommia ulmoides is one of the most widely planted economic tree species in China, with significant ecological and economic value. Although KNOX family genes have been widely studied in many species, there is a lack of systematic reports on the identification and comprehensive analysis of *KNOX* genes in *E. ulmoides*. To address this research gap, we conducted the present study, in which 8 *KNOX* genes were identified from *E. ulmoides* and divided them into five subgroups. In addition, a series of in-depth analyses were performed, including investigations into gene structure, motif composition, chromosomal distribution, phylogenetic relationships, cis-acting elements within promoters, and interspecific syntenic relationships. Furthermore, the expression profiles of the KNOX gene families in different tissues and developmental stages of *E. ulmoides* were analyzed using RNA-seq. In addition, the expression patterns of *EuKNOX* genes under various abiotic stress conditions were examined via quantitative real-time PCR (qRT-PCR). The outcomes of this study will not only

provide a solid theoretical basis and technical platform for subsequent studies on the biological functions of *EuKNOX* genes but also offer crucial support for further deciphering the regulatory mechanisms of *EuKNOX* genes in *E. ulmoides* growth and development, thereby facilitating the genetic improvement of this valuable tree species.

2. Materials and methods

2.1 Identification of *EuKNOX* genes in *E. ulmoides*

The KNOX protein sequences of *Arabidopsis* and rice were obtained from The *Arabidopsis* Information Resource (https://www.arabidopsis. org/) and Rice Data (http://www.ricedata.cn/gene/), respectively. The protein sequence was obtained from the *E. ulmoides* genome. The genome accession number is PRJNA599775 in NCBI database (https://www.ncbi.nlm.nih.gov/). To identify the non-redundant KNOX genes in the *E. ulmoides* genome, we using the Hidden Markov Model (HMM) profile of the KNOX1 domain (PF03790) with the threshold e-value of 10^{-5}. All redundant sequences were discarded from further analysis based on Cluster W alignment results[37]. To confirm the KNOX domain in identified proteins, domain analysis was performed using Interproscan tool (http://www.ebi.ac.uk/Tools/pfa/ iprscan5/)[38]. The resulting sequences were further subjected to NCBI Conserved Domain Search Service (CD Search) (http://forestry.fafu.edu.cn/db/PhePacBio/blast/blast_cs.php), Pfam database (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/)[39] online tools were used to determine whether the candidate sequence contained the KNOX1, was identified. Using pI/Mw tool from ExPASy (http://www.expasy.org/tools/) to estimate the physicochemical parameters, including the molecular weight (MW) and isoelectric point (pI) of each gene product, and the parameter (resolution) was set to average[40]. The 3D structures of the EuKNOX proteins were modeled using SWISS-MODEL (https://swissmodel.expasy.org/)[41].

2.2 Phylogenetic analysis, gene structural, and conserved motif of KNOX in *E. ulmoides*

A total of 8 KNOX protein sequences from *E. ulmoides* were aligned using Clustal W with the default parameters, and then a phylogenetic unrooted tree was constructed using MEGA6.0 software by using the Neighbor-joining (NJ) algorithm, and the support of the tree topology was assessed by bootstrap analysis with 1000 replicates[42]. For the phylogenetic analysis of KNOX protein from *E. ulmoides*, *Arabidopsis*, *Triticum aestivum*, *Pyrus bretschneideri*, *Malus domestica*, and rice were used to construct the neighbor-joining phylogenetic tree.

To assess the exon-intron organization of the *KNOX* genes, the entire *E. ulmoides* genome and coding sequences were obtained from the Genome Warehouse Database. Gene Structure Display Server (GSDS) was used to illustrate the exon/intron structure for individual *EuKNOX* genes by comparing their cDNAs and corresponding genomic DNA sequences[43]. To identify the conserved motif structures shared by the *EuKNOX* proteins, the online MEME (Multiple Expectation Maximization for Motif Elicitation) program (http://memesuite.org/tools/meme) was used with the following parameters: number of repetitions = any, maximum number of motifs = 20, minimum width ≥ 6, maximum width ≤ 200, and only motifs with an e-value < 1e-20 were retained for further analysis[44].

2.3 Cis-regulatory element analysis in the promoter regions of *KNOX* genes in *E. ulmoides*

To identify the cis-elements in the promoter region of each *KNOX* gene in *E. ulmoides*, the 2000

bp upstream sequences of the KNOX-encoding DNA sequences were extracted from the the available *E. ulmoides* genome data using the TBtools program, and possible cis-acting elements were analyzed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/index.html). A total of 22 cis-elements were selected by removing cis-elements of functional ambiguity as well as core promoter elements. The cis-element distributions were visualized using TBtools[45].

2.4 Chromosomes location, and synteny analysis with several plant species

The chromosome position of *KNOX* genes was obtained from the *E. ulmoides* genome annotations using TBtools. Dual Synteny Plotter software (https://github.com/CJ-Chen/TBtools/) was used to analyze the homology of the *KNOX* gene between *E. ulmoides*, Populus, *Arabidopsis* and Soybean. TBtools software was used to visualize the obtained results, the obtained *KNOX* homologous pairs are highlighted[45].

2.5 Expression patterns of *EuKNOXs* in different tissues and different developmental stages based on the public RNA-seq data sets

Transcriptome data of *E. ulmoides* across different leaf developmental stages (leaf buds, growing leaves, young leaves and mature leaves) were obtained from the NCBI Sequence Read Archive (SRA) database was SRP218063[46]. Different tissues (leaves, seeds, marginal pericarp, central pericarp, and xylem) with SRA accessions SRX7525252-54, SRX7532003-05, SRX7531725-27, and SRX7533248-50, leaves of different colors (green leaves and purple leaves) under accession SRP327902. Different developmental stages of floral with accessions SRR2170964 and SRR2170970)[47]. In addition, RNA-seq data from two *E. ulmoides* cultivars, 'Xiaoye' and 'Qinzhong 2' under accessions SRP158357[48]. These datasets were used to investigate the expression patterns of *EuKNOX* genes during *E. ulmoides* rubber biosynthesis. The expression abundance of *EuKNOX* genes was determined using their Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values. Notably, some *EuKNOX* genes exhibited an FPKM value of 0, which likely reflects expression levels below the detection limit of the current transcriptomic sequencing technology rather than absolute absence of expression. A heatmap of *EuKNOXs* expression was generated using TBtools, with expression values represented as $\log_2$(FPKM) to standardize the data. The FPKM values were shown in supplementary Tables.

2.6 Plant materials and abiotic stress treatments

The *E. ulmoides* seedling were cultured in a growth chamber with a 16-h light/8-h dark cycle at 22°C/18°C, 80% humidity, and watered with Hoagland nutrient solution every week. In the drought stress treatment, a 20% PEG 6000 solution (and in the salt stress treatment, a 200 mM NaCl solution) was poured into the medium vermiculite with *E. ulmoides* seedlings. To examine the effect of hormone s on the expression of *EuKNOX* genes, 2-month-old seedling leaves of *E. ulmoides* were sprayed with 200 mM GA_3 and ABA, until there is liquid dripping. Untreated samples were used as the control treatment. The leaves were sampled at 0, 6, 12, 24, 36, 48, and 72 h after hormone treatments, frozen in liquid nitrogen, and finally stored at -80 °C for RNA extraction. There were three independent replicates for each treatment.

2.7 RNA extraction, cDNA synthesis, and qRT-PCR gene expression analysis

The RNA extraction kit (TIANGEN Biotechnologies, DP441, China) was used to extract total

RNA according to the manufacturer's instructions. A NanoDrop ND-2000 (Thermo Scientific, USA) spectrophotometer and 1% agarose gel electrophoresis were used to detect the RNA quality of all samples. For qRT-PCR, Primer 3 web (http://primer3.ut.ee/) was used to design the specific primers based on the coding sequence of *EuKNOX* genes[49]. The primers of all the *EuKNOX* genes were listed in supplementary table S1. *Ubiquitin-conjugating enzyme S* (*UBC*) was used as the internal reference gene[50]. The qRT-PCR reactions were conducted on a Quant Studio 5 (ABI, USA) using the 2×Universal SYBR Green Fast qPCP Mix. The PCR reaction mixture (20 μL) contained 7.2 μL of cDNA, 10 μL of SYBR Advantage Premix, 0.4 μL of each primer (10 nmol mL^{-1}). The following amplification reactions were performed for qRT-PCR: 95 °C for 5 s, 60 °C for 30 s, 40 cycles. All reactions were performed in triplicate, both technically and biologically. The ΔCT and ΔΔCT values were calculated using the $2^{-\Delta\Delta CT}$ method. To ensure the accuracy of the data, we performed 3 biological replicates and 3 technical replicates.

3 Results

3.1 Identification of the *EuKNOX* genes in *E. ulmoides*

Based on the genomic information of *E. ulmoides*, 11 putative *KNOX* genes were initially obtained using the HMM. To verify the authenticity of these candidate genes, we analyzed their conserved domains via the CDD and SMART databases. Three genes that failed to encode the core KNOX I, KNOX II, ELK and Homeobox KN domains were eliminated, ultimately identifying 8 bona fide *EuKNOX* genes, which were sequentially named *EuKNOX1* to *EuKNOX8*. A comprehensive analysis of their molecular characteristics was conducted, and the detailed parameters—including CDS length, protein sequence length, protein MW, pI, GRAVY value, aliphatic index, instability index, and predicted subcellular localization—are presented in Table 1. The results showed that the amino acid sequence lengths of EuKNOX proteins ranged from 212 (EuKNOX8) to 426 (EuKNOX5). Their MWs were between 24,696.76 Da (EuKNOX8) and 47,353.5 Da (EuKNOX5), and the pI values varied from 5.03 (EuKNOX6) to 6.19 (EuKNOX4). Notably, all EuKNOX proteins had a pI <7, suggesting an acidic nature. Regarding protein stability, the instability indices of EuKNOXs ranged from 39.90 (EuKNOX6) to 62.53 (EuKNOX4). Specifically, EuKNOX6 exhibited an instability index below 40, categorizing it as a stable protein, whereas the other EuKNOX members were classified as unstable.

The aliphatic index of the EuKNOX protein family varied from 60.27 (EuKNOX3) to 82.26 (EuKNOX1), suggesting that these predicted EuKNOX proteins contained rich in aliphatic amino acids. Additionally, the GRAVY values of all EuKNOX proteins were negative, which is indicative of their hydrophobic nature. Moreover, subcellular localization predictions showed that all 8 EuKNOX proteins were targeted to the nuclear region. Detailed information regarding the nucleotide and amino acid sequences of these 8 *EuKNOX* genes is documented in Table 1.

We analyzed the secondary structures of EuKNOX proteins using the SOPMA tool, and the results revealed that all EuKNOX secondary structures comprise four components: α-helix, extension strand, β-angle, and random coil (Table S2). Among these components, α-helix was the most abundant secondary structure in the amino acid sequences of *E. ulmoides* KNOX proteins, with a content ranging from 37.74% (EuKNOX8) to 57.68% (EuKNOX4). This was followed by random coil from 27.99% (EuKNOX4) to 40.09% (EuKNOX8), extended strand (6.77% in EuKNOX6 to 12.26% in EuKNOX8),

and β-angle from 4.76% (EuKNOX7) to 10.97% (EuKNOX6). For example, the secondary structure of the EuKNOX4 protein consists of 57.68% alpha helix, 27.99% random coil, 6.83% extended strand, and 7.51% β-angle. Notably, the order of component abundance differed across EuKNOX members: five proteins followed the order α-helix > random coil > extended strand > β-turn. EuKNOX4 and EuKNOX6 displayed α-helix > random coil > β-turn > extended strand. Meanwhile, EuKNOX8 showed a unique order of random coil > α-helix > extended strand > β-turn (Supplementary Table S2). To further explore the structural characteristics of EuKNOX proteins, we also constructed their 3D models. The results demonstrated that all 3D structures share a similar structural composition to their respective secondary structures (Fig. 1).

Table 1 Sequence characteristics and predicted subcellular location of *E. ulmoides* KNOX proteins

Gene ID	Gene name	Homologous genes in Arabidopsis thaliana	ORF length (bp)	Protein size(aa)	Molecular weight(Da)	Isoelectric point	Subcellular location	Aliphatic index	GRAVY	Instability index
IMPTEUL1N40804_1	*EuKNOX1*	AT1G62990.1	867	288	32955.40	6.15	Nucleus	82.26	-0.598	59.41
IMPTEUL1N122712_1	*EuKNOX2*	AT1G62360.1	1002	333	37122.67	6.08	Nucleus	62.46	-0.629	49.86
IMPTEUL1N109939_1	*EuKNOX3*	AT4G08150.1	660	219	24858.77	5.4	Nucleus	60.27	-740	54.34
IMPTEUL1N119462_1	*EuKNOX4*	AT1G62990.1	882	293	33351.92	6.19	Nucleus	80.58	-0.623	62.53
IMPTEUL1N113602_1	*EuKNOX5*	AT5G25220.1	1281	426	47353.5	5.72	Nucleus	75.07	-0.706	56..61
IMPTEUL1N84155_1	*EuKNOX6*	AT1G23380.2	933	310	35308.71	5.03	Nucleus	72.45	-0.648	39.90
IMPTEUL1N63682_1	*EuKNOX7*	AT5G25220.1	1200	399	44774.55	5.71	Nucleus	66.52	-0.808	54.33
IMPTEUL1N109554_1	*EuKNOX8*	AT4G08150.1	639	212	24696.76	6.07	Nucleus	62.12	-0.916	46.87

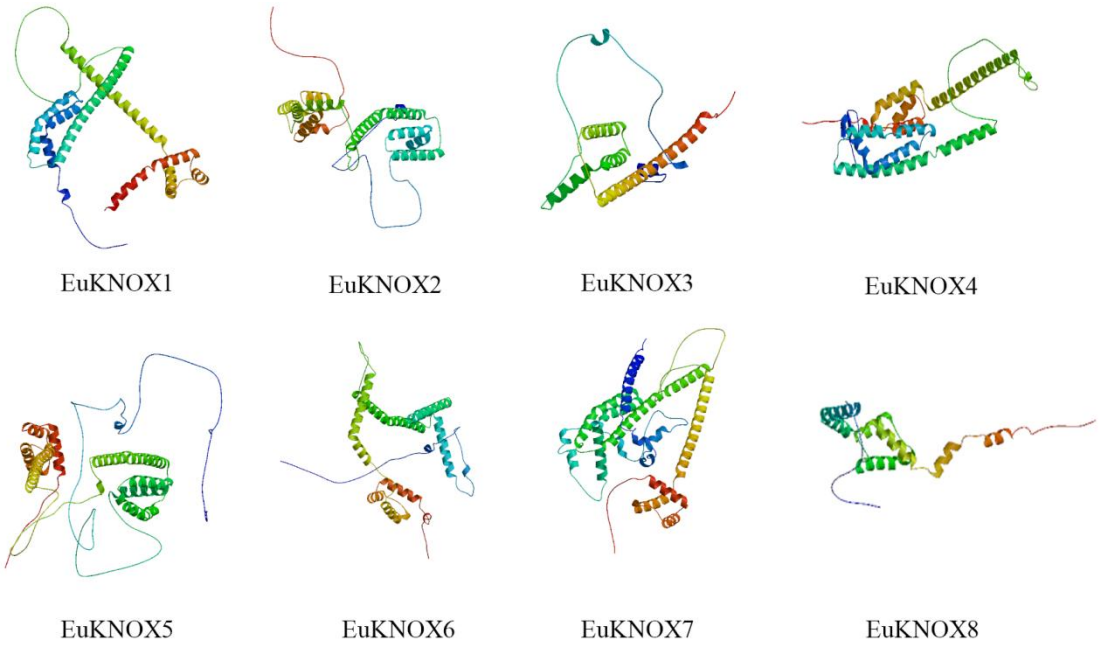


Fig.1 The tertiary structure prediction of EuKNOX proteins in *E. ulmoides*

3.2 Phylogenetic reconstruction to delineate evolutionary relationships among *EuKNOX* genes

Given that homologous genes often exhibit conserved structures and functions, we explored the evolutionary relationships of the KNOX gene family in *E. ulmoides* by constructing an unrooted neighbor-joining (NJ) phylogenetic tree with 1000 bootstrap replicates. This tree was based on amino acid sequence alignments of KNOX proteins from six species: *E. ulmoides* (8 proteins), *Arabidopsis* (9), pear (19), *Triticum aestivum* (36), *Malus domestica* (21), and rice (1). The 95 KNOX proteins included in the analysis were clustered into three major subfamilies (Class I, Class II, and Class KNATM), which were further subdivided into six distinct branches (Fig. 2). Within this framework, the 8 EuKNOX proteins were assigned to five subgroups: KNAT7-like, KNAT3-5-like, KNAT2/6-like, STM-like, and BP-like. Notably, no members of the KNATM-like subgroup were detected in *E. ulmoides*. Among the classified EuKNOX proteins, the KNAT3-5-like, KNAT7-like, and BP-like subgroups each contained 2 members; the KNAT2/6-like subgroup included only EuKNOX6; and EuKNOX2 was clustered in the STM-like subgroup. Notably, *Triticum aestivum*, as a monocotyledonous plant, exhibited a distinct evolutionary relationship compared to *Arabidopsis* and *E. ulmoides*. This divergence might be attributed to the potential gene loss and degradation that monocotyledonous can undergo during evolution. Consequently, the phylogenetic analysis underscored that the homology of EuKNOX proteins with *Triticum aestivum* was considerably lower than their similarity to *Arabidopsis* KNOX proteins.

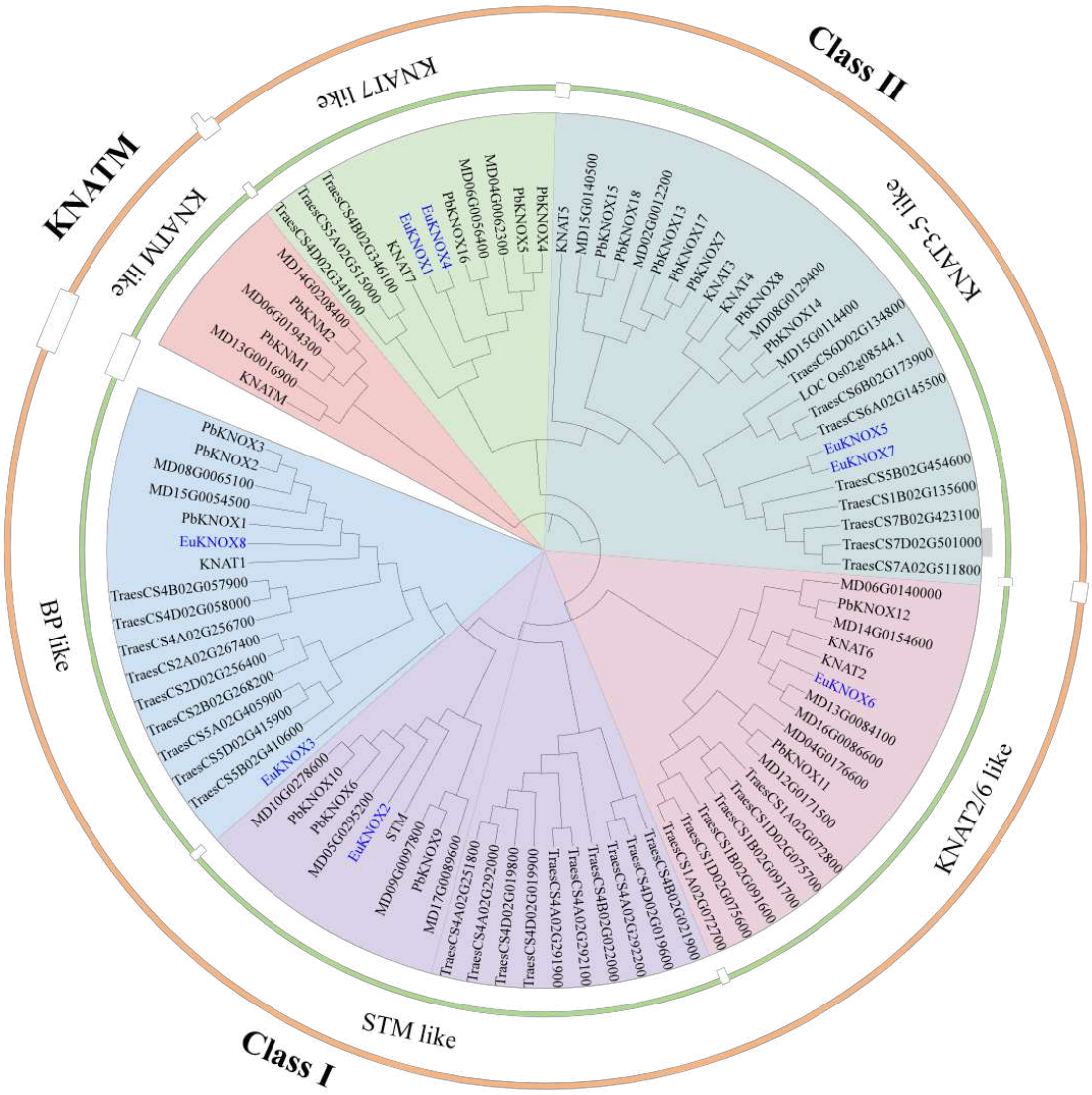


Fig. 2 Phylogeny of the KNOX TFs of *E. ulmoides*, *Arabidopsis*, *Triticum aestivum*, *Pyrus bretschneideri*, *Malus domestica*, and rice. Note: The phylogenetic tree was prepared using the neighborjoining (NJ) method with 1000 bootstrap replicates using MEGA 6.0. The tree branched the KNOX proteins into different groups illustrated by different colored clusters within the clade. The *E. ulmoides* KNOXs proteins were marked in blue.

3.3 Multiple sequence alignment, gene structures, domain, and putative motifs characterization of *EuKNOX* genes

We performed multiple sequence alignment to analyze the conserved domains of EuKNOX proteins, and the results identified four relatively conserved domains (Fig. 3). Specifically, the KNOX1 and KNOX2 domains are located at the N-terminus of the proteins, whereas the ELK and HOX domains are positioned at the C-terminus. Notably, the HOX domain displays the highest degree of conservation and exhibits the canonical structural characteristics of the TALE homeobox protein superfamily. In addition, the KNOX1, KNOX2, and ELK domains also show significant conservation. For instance, the KNOX2 domain contains a highly conserved E-L-D amino acid sequence, while the ELK domain features two highly conserved amino acid sequences: E-L-K and G-K-L-P.

We conducted a comprehensive gene structure analysis of *EuKNOX* genes, and the results showed that all *EuKNOX* genes were characterized by the presence of exons, which is a typical structural

feature of protein-coding genes (Fig. 4A). Specifically, *EuKNOX2*, *EuKNOX3*, and *EuKNOX8* each harbor three introns and four exons, whereas the other five *EuKNOX* genes (*EuKNOX1*, *EuKNOX4*, *EuKNOX5*, *EuKNOX6*, and *EuKNOX7*) possess four introns and five exons. Furthermore, most genes belonging to the same subgroup display a conserved exon/intron organization pattern. For instance, all members of the Class II subgroup contain five exons, reflecting the structural conservation of genes within the same evolutionary branch.

To reveal the protein structural diversification of *EuKNOX* proteins, 10 conserved motifs were identified by MEME (Fig. 4C). The lengths of these conserved motifs ranged from 9 to 40 amino acids and were highly diverse (Fig. 4D). Motifs 1-4 were the most conserved motifs in EuKNOX proteins, Motif 3 and Motif 5 only appeared in Class I. Motif 4, Motif 6 and Motif 7 only appeared in Class IIA. Among 8 EuKNOX proteins, EuKNOX1 only had one type of motif, whereas EuKNOX7 contain the largest number of motifs (seven types). The motifs of EuKNOX members within the same subgroups show similar patterns, indicating that KNOX proteins placed in the same group probably have similar functions.

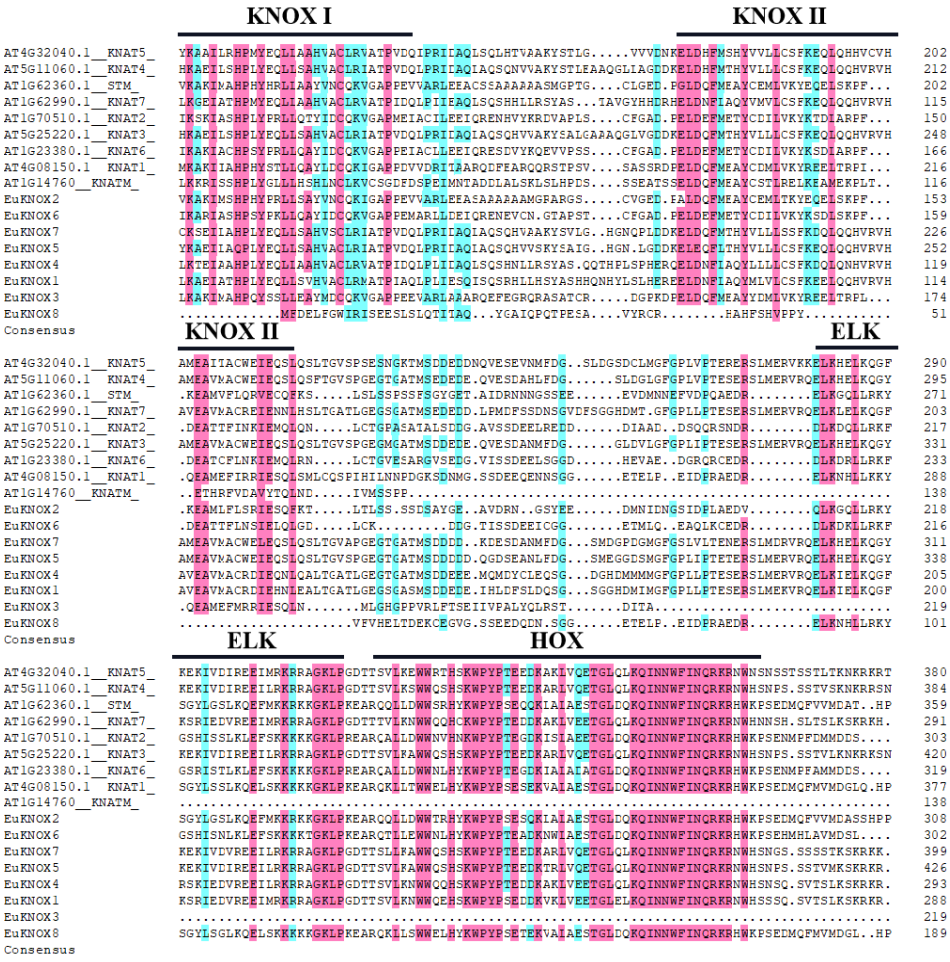


Fig. 3 Multiple sequence alignment of *E. ulmoides* KNOX sequences. Typical domains KNOX1, KNOX2, ELK, and HD of KNOX protein were marked by black line.

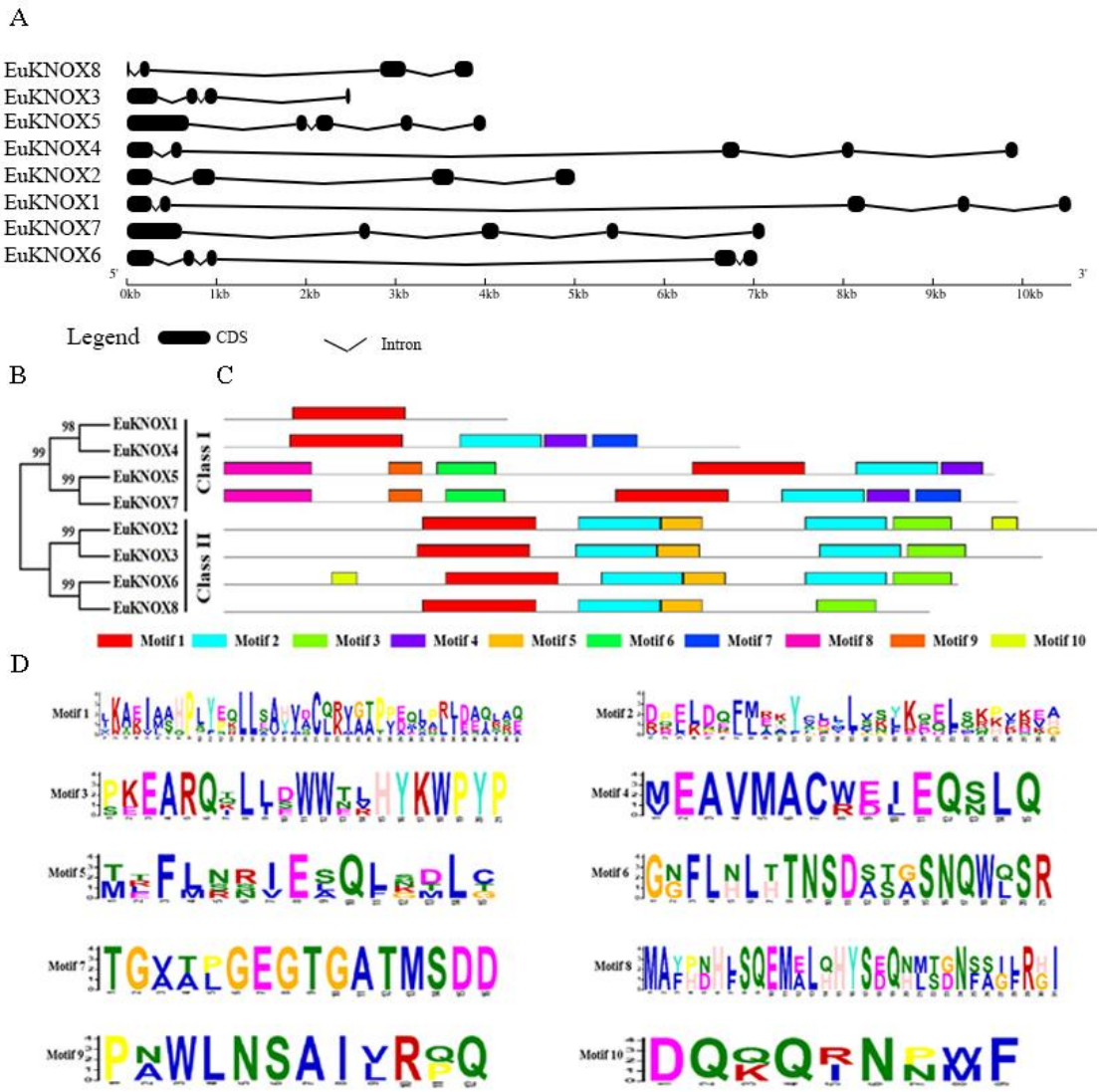


Fig. 4. Gene structure and conservative motif analysis of *E. ulmoides* KNOX proteins. Note: A: Gene structure of *EuKNOXs*. The black boxes, and black lines represented the exons, and introns, respectively. B: The phylogenetic tree of EuKNOX gene family was generated with MEGA 6.0 software using the neighbour-joining method. C: The gene structure analysis of EuKNOXs. D.Motifs amino acid distribution.

3.4 Systematic analysis of chromosomal arrangement and collinearity of *EuKNOX* genes

To systematically investigate the chromosomal distribution of EuKNOX genes in *E. ulmoides*, TBtools software was utilized to map the chromosomal locations of all identified EuKNOX members in this study (Fig. 5A). A total of 8 *EuKNOX* genes were unevenly distributed across six chromosomes. Notably, chromosomes 6 and 10 each contained two EuKNOX members, while each of the remaining chromosomes carried only one. This asymmetric distribution pattern implies potential functional or evolutionary significance associated with the clustering of *EuKNOX* genes on specific chromosomes.

To further elucidate the evolutionary relationships of *EuKNOX* family genes across various species, a comparative analysis was conducted on the collinear maps of KNOX family genes in *E. ulmoides* with *Populus*, *Arabidopsis* and soybean. The results showed varying numbers of gene pairings between *KNOX* genes in *E. ulmoides* and the other species: sixteen with Populus (Fig. 5B), three with *Arabidopsis* (Fig. 5C), and fourteen with soybean. The result implies a particularly close evolutionary relationship between the *E. ulmoides* and *Populus.*

The total number of KNOX family genes varied greatly among different species (Table 2). The classification results showed that the Class M subfamily had the smallest number of genes. Notably, no KNATM family gene was identified in tobacco, rice, maize, wheat and bamboo, while the remaining members of the Class M subfamily across other species ranged from 1 to 4 genes. Except for pear, *Brassica napus*, *Dendrobium huoshanense*, and moso bamboo, all other species harbored fewer Class II subfamily genes than Class I subfamily genes. For the majority of species, the Class I subfamily had the highest number of genes, with a variation range of 4 (in *Arabidopsis*, *E. ulmoides*, and *Dendrobium huoshanense*) to 25 (in wheat). Wheat and *Brassica napus* possessed the maximum number of *KNOX* genes (36 each), and the specific counts of KNOX genes in other species are detailed in Table 2.

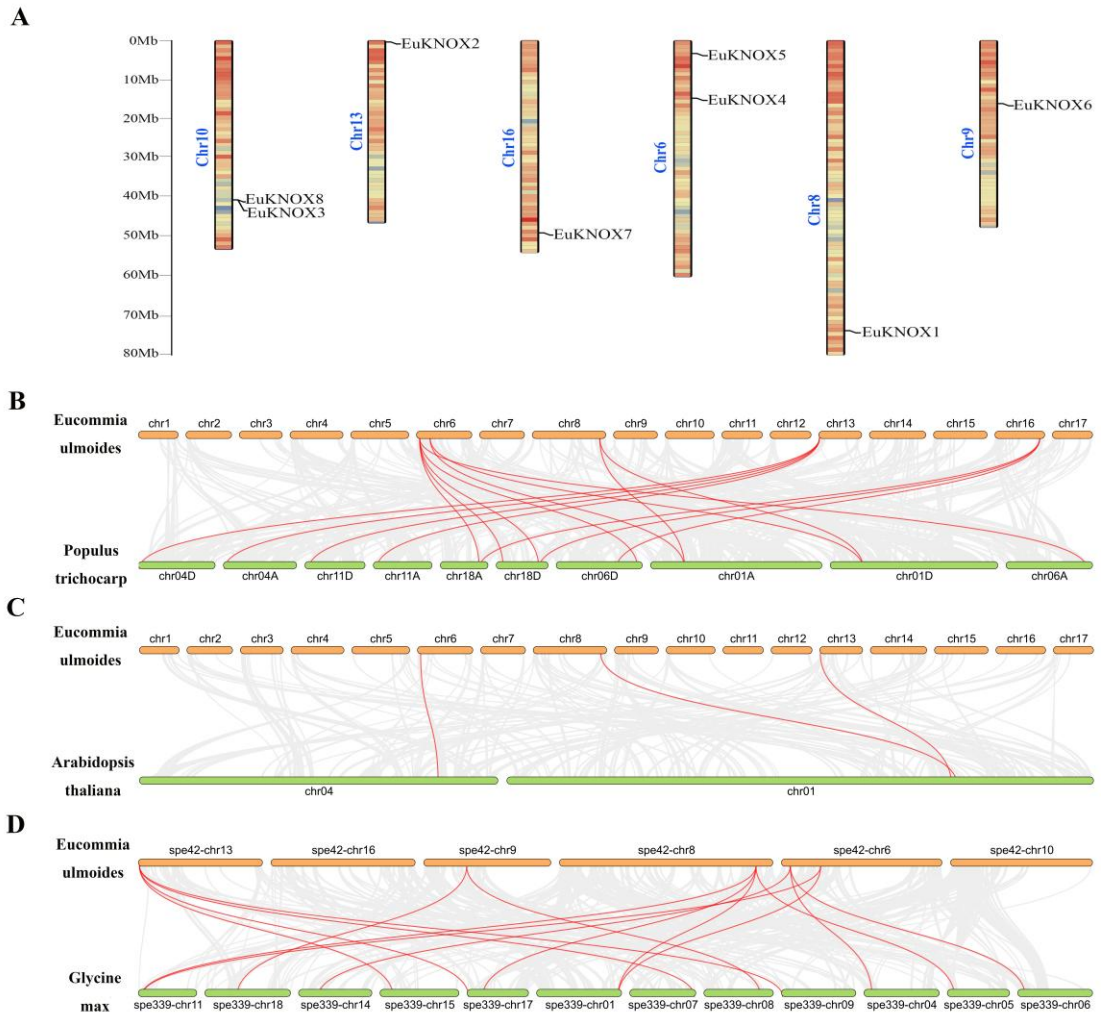


Fig. 5 Chromosomal distribution (A) and collinearity analysis of KNOX gene family of *E. ulmoides*, populus (B), *Arabidopsis* (C) and soybean (D). Note: Vertical bars represent the chromosomal of *E. ulmoides*. The chromosome name is located on the left side of each chromosome. The scale on the left represents chromosomal length. Gray lines indicate all synteny blocks between populus, *Arabidopsis*, soybean and *E. ulmoides*; red lines highlight the syntenic *KNOX* gene pairs.

Table 2 Comparison of the number of KNOX gene families in different species

Species	Total	Class I	Class II	Class M	Reference
Arabidopsis thaliana	9	4	4	1	[51]
Camellia sinensis	11	5	5	1	[52]
Prunus mume Sieb. et Zucc.	11	6	3	2	[34]

Brassica napus	36	14	18	4	[53]
Nicotiana tabacum	18	11	7	0	[54]
Populustrichocarpa	15	9	5	1	[51]
Pyrus bretschneideri Rehd	19	7	10	2	[55]
Eucommia ulmoides	8	4	4	0	
Dendrobium huoshanense	19	4	13	2	[56]
Oryza sativa	13	9	4	0	[51]
Zea mays	22	17	5	0	[57]
Triticum aestivum	36	25	11	0	[58]
Phyllostachys edulis	12	5	7	0	[59]

3.5 Characterization of promoter regions of *EuKNOX* genes

To investigate the potential functions of *EuKNOXs*, we employed PlantCARE to predict the cis-acting elements within the 2.0 kb sequence upstream of the initiation codon (ATG) of *EuKNOXs*. As expected, both TATA and CAAT boxes with good characteristics were found in the results; we also found several other cis-regulators. They were mined in the promoter region of *EuKNOXs*. We divided the homeopathic elements into four categories according to their functions, which were related to phytohormone responsive, stressresponsive, light responsiveness, and plant growth and development (Fig. 6).

We found that 76 elements were involved in light responsiveness, including AE-box, ATCT-motif, Box 4, G-Box, TCT-motif, GATA-motif, G-box, GT1-motif, and MRE. In addition, 20 elements, including CAT-box, GCN4_motif and O2-site, were related to plant growth and development. The promoter regions of many *EuKNOX* genes contain multiple binding sites involved in stress response. A total of 15 elements were involved in stress response, such as ARE, LTR and MBS motif. A total of 1458 elements were predicted in the promoter regions of *EuKNOX* genes. Among them, 49 elements were involved in response to plant hormones, such as ABA (ABRE), auxin (TGA-element), GA_3 (P-box, TATC-box, GARE-motif), and methyl jasmonate (CGTCA-motif, TGACG-motif). Except for *EuKNOX6*, all *EuKNOX* genes contained MeJA-responsive elements. In addition, 5 *EuKNOX* genes (*EuKNOX3/5/6/7/8*) contained ABA-responsive elements, accounting for 24% of the total number of hormones cis-acting elements in the EuKNOX family. Based on the above results, *EuKNOXs* may be involved in stress response, light, hormones, and growth pathways.

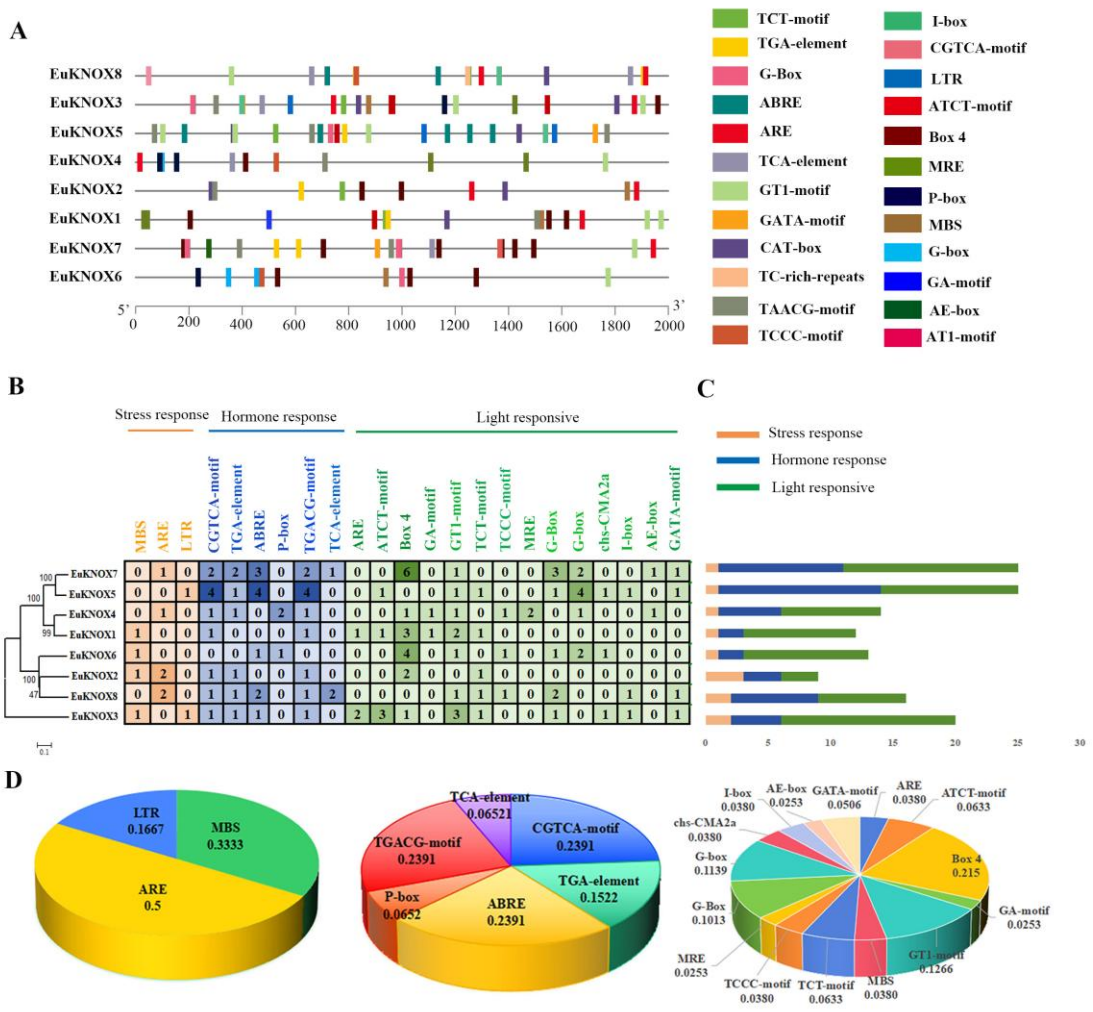


Fig. 6 Cis-elements analysis in the promoters of *EuKNOX* genes. A: location map of promoter elements of *EuKNOXs* genes. B: number map of each elements in *EuKNOXs* genes, the gradient colors in the grid correspond to the quantity of cis-acting elements in *EuKNOXs*. Total number map of each type of elements in *EuKNOX* genes. The multicolored histogram indicates the cis-elements in each category. These elements are divided into three categories in terms of the functional notes: abiotic stress response element, plant hormone response element, and light stress response element. Proportion map of each element in three categories of elements (from left to right are abiotic stress response element, hormone response element, light stress response element).

3.6 Comprehensive expression profiling of *EuKNOX* genes in multiple tissues

To evaluate the transcriptional expression patterns of *EuKNOX* genes in various tissues of *E. ulmoides*, including leaves, seeds, edges of peels, central of peels, and xylems, we utilized 15 distinct transcriptome datasets of *E. ulmoides* downloaded from the Short Read Archive (SRA) available on the NCBI database. Following data normalization, a heatmap was generated to visualize the differential expression profiles of these genes(Fig. 7; Table S4).

The transcript levels of different *EuKNOX* genes varied significantly across these samples. For instance, the FPKM of *EuKNOX2* genes was zero in leaves examined organizations, reflecting that it was not expressed during the sampled stages. In contrast, *EuKNOX4* was expressed at high levels in all examined tissues. Notably, four *EuKNOXs* members (*EuKNOX2*, *EuKNOX3*, *EuKNOX6*, and *EuKNOX8*) exhibited low expression in leaves but the highest transcript abundances in xylem, suggesting their potential involvement in xylem formation. Furthermore, *EuKNOX4*, *EuKNOX5*, and *EuKNOX7* exhibited the highest expression levels in the edges of peels, whereas *EuKNOX3* showed the

lowest transcriptional activity in this tissue. Additionally, *EuKNOX1* was highly expressed in the leaves. Collectively, these results demonstrated that *EuKNOXs* exhibit tissue-specific expression patterns and play distinct functional roles in the development of different tissues in *E. ulmoides*.

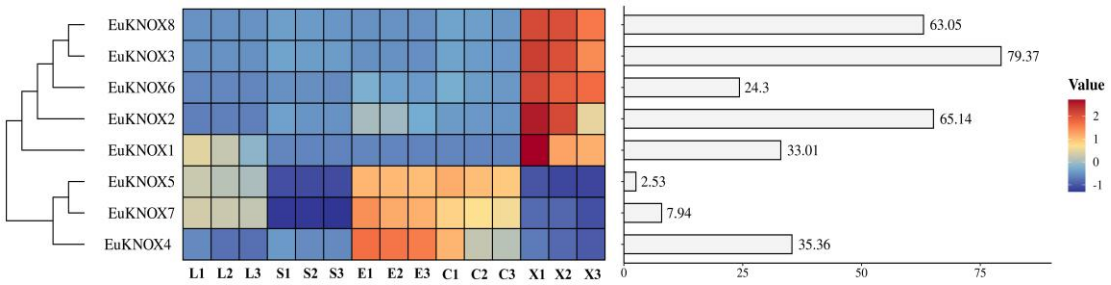


Fig. 7 Expression patterns of *E. ulmoides* KNOX gene family in different tissues. Note: L: Leaf; S: Seeds; E: Edges of peels; C: Central of peels; X: Xylems. The color key on the right represents the relative expression level in FPKM values, the red and blue bars (FPKM) indicate high and low expression, respectively.

3.7 Expression dynamics of *EuKNOX* genes during leaf, bark and fruit development

To compare the expression levels of *EuKNOX* genes across different samples and to identify potential KNOX transcription factors involved in leaf growth of *E. ulmoides*, we extracted the expression patterns of eight *EuKNOX* genes from transcriptome data. These data were obtained from two-year-old saplings at four distinct leaf developmental stages: leaf bud (T01–T03), growing leaves (T04–T06), young leaves (T07–T09), and old leaves (T10–T12). The result showed that most *EuKNOX* genes had a higher expression level in leaf bud than other stages (Fig. 8A; Table S5). Notably, the expression of several *EuKNOX* genes exhibited distinct and significant trends across different leaf developmental stages. For instance, *EuKNOX1*, *EuKNOX2*, *EuKNOX3*, *EuKNOX6* and *EuKNOX8* showed preferential expression in leaf bud. In contrast, two members of the gene family—*EuKNOX4* and *EuKNOX5*—maintained consistently high expression levels throughout the four leaf developmental stages, with a particularly prominent enrichment in senescent leaves, suggesting that *EuKNOX4* and *EuKNOX5* may play significant roles in the later stages of leaf development. On the contrary, *EuKNOX2*, *EuKNOX3*, and *EuKNOX6* were not expressed in old leaves, with their FPKM values reaching zero, suggesting that these three genes may not be involved in the developmental processes of mature leaves. Furthermore, among the eight *EuKNOX* genes, *EuKNOX1*, *EuKNOX6*, *EuKNOX7* and *EuKNOX8* displayed low expression level across the four examined leaf stages, with average FPKM value below 7, indicating that these genes are relatively weakly expressed in *E. ulmoides* leaves. These findings underscored the stage-specific functional roles of *EuKNOXs* during leaf development in *E. ulmoides*, providing insights into their regulatory contributions at different developmental phases, laying the theoretical foundation for the subsequent functional studies of *EuKNOXs* in the development of *E. ulmoides* leaves.

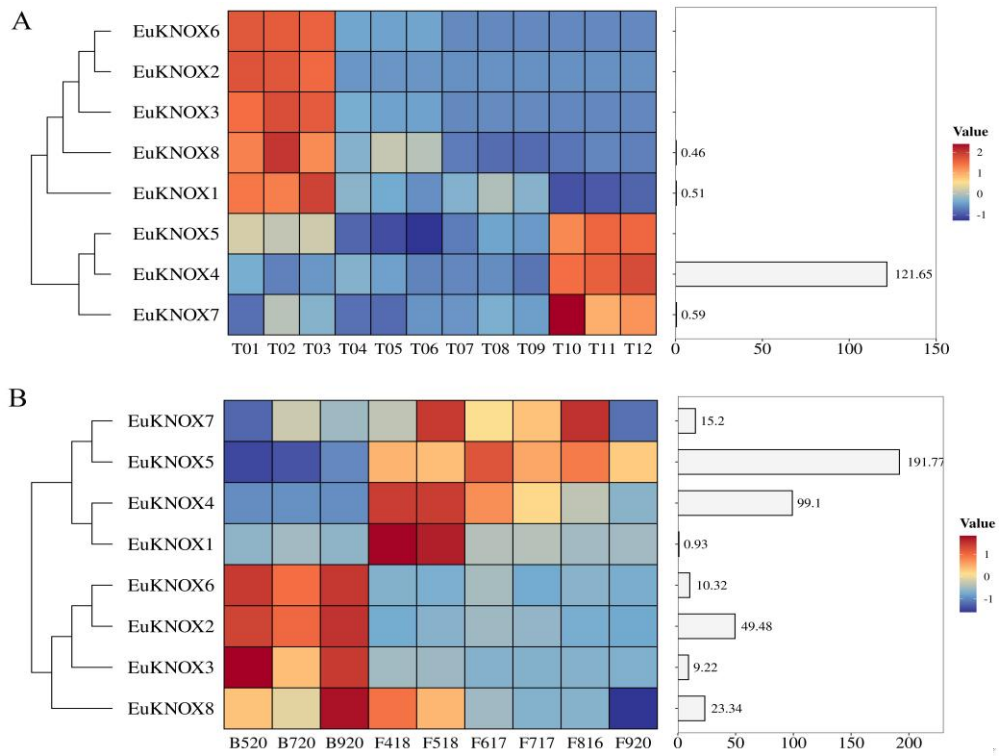


Fig. 8 Expression patterns of *E. ulmoides* KNOX gene family in different developmental stages of leaves. T01~T03: leaf buds; T04~T06: growing leaves; T07~T09: young leaves; T10~T12: mature leaves, B: bark; F: fruit, three replicates per group. The color key on the right represents the relative expression level in FPKM values, the red and blue bars (FPKM) indicate high and low expression, respectively.

During bark development, both *EuKNOX2* and *EuKNOX3* exhibited high expression levels. Notably, *EuKNOX2* showed the most prominent expression, with an average FPKM value exceeding 1300 and peaking in September (Fig. 8B). In contrast, *EuKNOX3* displayed an expression pattern of initial decrease followed by subsequent increase. Additionally, the transcript levels of *EuKNOX5* and *EuKNOX8* increased significantly at the late stage of bark development (September), with FPKM values reaching 70.53 and 100.28, respectively, while *EuKNOX6* maintained a relatively stable expression level throughout the process. Collectively, these findings suggest that *EuKNOX2* and *EuKNOX3* may play critical regulatory roles in the secondary growth of *E. ulmoides* bark and in the biosynthesis of Eu-rubber.

During fruit development (Fig. 8B), *EuKNOX4* exhibited a high expression level of 713.81 at the early stage, which then progressively declined to 99.10 by September. Similarly, *EuKNOX1* was strongly expressed at the early stage but decreased to below 1 FPKM at later stages. *EuKNOX3* also showed significantly higher expression in the early stage compared to later stages. In contrast, *EuKNOX5* remained consistently highly expressed throughout fruit development, peaking in June (FPKM = 281). *EuKNOX2* displayed a unimodal expression pattern, increasing to a peak in June (FPKM = 220) and then gradually decreasing. By contrast, *EuKNOX6*, *EuKNOX7*, and *EuKNOX8* exhibited relatively low and stable expression levels throughout fruit development, suggesting that these three genes are not core regulators in the fruit development process.

3.8 Expression dynamics of *EuKNOX* genes during floral development

E. ulmoides is a dioecious perennial woody plant, with male and female in florescences that greatly vary, and thus, the quantities of important compounds in male and female plant are different[60].

Studies have shown that male *E. ulmoides* flowers are rich in flavonoids and amino acid derivatives[61], which hold medicinal value for humans and are effective in treating hypertension[62]. In flowering plants, the sex difference is mainly manifested as different flower organs, and therefore, genes related to flower development may participate in the process of sex differentiation[63-64]. KNOX proteins are essential in regulating plant organ differentiation. In order to explore the function of *EuKNOXs* gene in the flower organ development of *E. ulmoides*, the expression profiles were shown by including transcriptomes from NCBI, including female floral organ morphological differentiation initial stage (F-1), female floral organ induction stage (F-2), female flower organ maturity stage (F-3), male floral organ morphological differentiation initial stage (M-1), male floral organ induction stage (M-2), and male flower organ maturity stage (M-3). The result showed that 8 *EuKNOX* genes displayed distinct expression profiles at different flowering developmental stages (Fig. 9; Table S6 and S7). No significant differences were observed in the expression level of *EuKNOX* genes between male and female flowers of *E. ulmoides*. Overall, the *EuKNOXs* genes exhibited slightly higher expression in female flowers compared to male flowers, with the exception of *EuKNOX6* and *EuKNOX8*, which showed prominent and specific expression in male flowers. Furthermore, distinct expression peaks of individual *EuKNOX* genes were detected across different floral developmental stages: *EuKNOX2* and *EuKNOX4* displayed the highest expression levels during the initial stage of female floral organ morphological differentiation, while *EuKNOX1*, *EuKNOX3*, *EuKNOX5* and *EuKNOX7* peaked at female floral organ induction stage. In contrast, *EuKNOX6* and *EuKNOX8* reached their highest expression levels during the male floral organ induction stage. Notably, all *EuKNOX* genes exhibited the lowest expression levels in the mature stages of both male and female flowers. Collectively, these results indicate that the *EuKNOX* gene family is involved in the floral development of *E. ulmoides*.

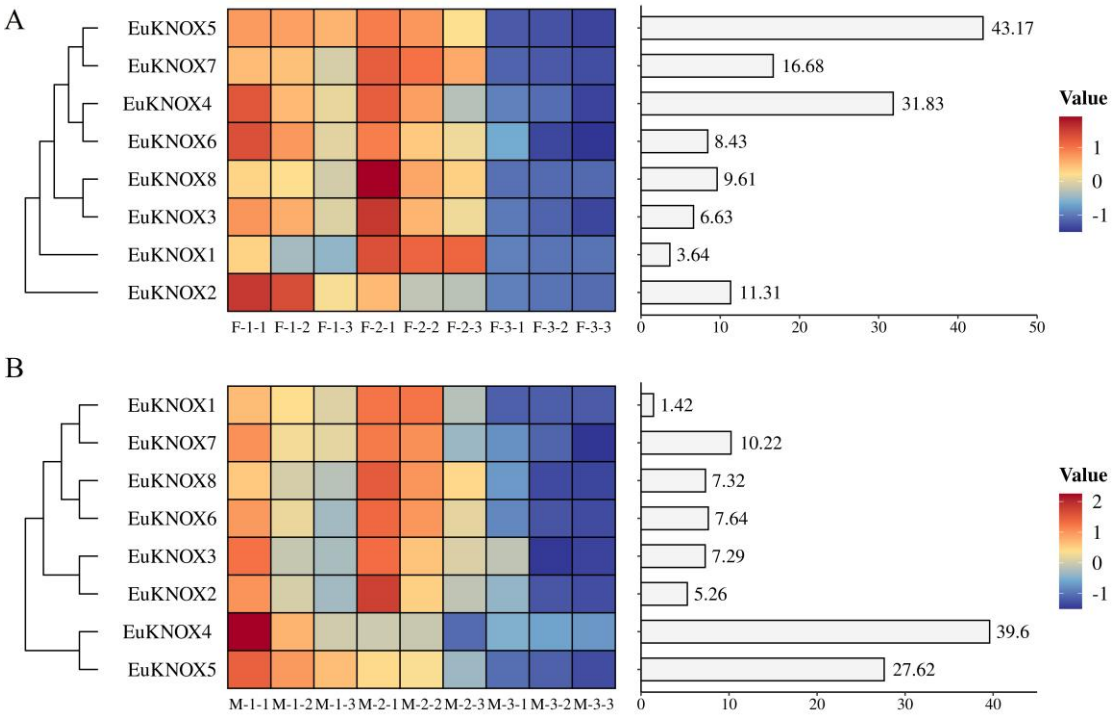


Fig. 9 Expression profiles of *EuKNOX* genes in flower at different growth stages. Note: F-1: initial stage of female floral organ morphological differentiation, F-2: female floral organ induction stage, F-3: female flower organ maturity stage, M-1: the initial stage of male floral organ morphological differentiation, M-2: male floral organ induction stage, M-3: male flower organ maturity stage. The FPKM value of *EuKNOX* genes were normalized by

the min-max method, according to the rows, and presented on the right side. Green represents a low level and red indicates a high level of transcript abundances.

3.9 The expression pattern of the *Eu*KNOX gene in the formation of Eu-Rubber and leaf color, as well as its correlation

It has been reported that at least 52 genes in *E. ulmoides* are involved in the biosynthetic pathway of Eu-rubber[65]. The molecular structure of Eu-rubber is trans-1,4-polyisoprene (TPI), which is synthesized from the precursor ispentenyl diphosphate (IPP) via the MVA and MEP pathways. To understand the possible relationship between *KNOX* genes in *E. ulmoides* and Eu-rubber biosynthesis-related-genes, we collected leaves from the high Eu-rubber-yielding cultivar 'Qinzhong 2' and low-yielding cultivar 'Xiaoye'. The result showed that the expression patterns of *EuKNOX* genes were highly similar between 'Qinzhong 2' and 'Xiaoye' (Fig. 10A; Table S8), with most *EuKNOX* genes exhibiting low expression levels. Specifically, among the eight *EuKNOXs* genes, five *EuKNOXs* had a low level of expression in all detected samples (FPKM <1). In contrast, *EuKNOX5* exhibited preferential expression across all detected tissues, with an average FPKM value exceeding 400 in the six samples. Meanwhile, six *EuKNOX* genes were expressed in all six tested samples, among which *EuKNOX4*, *EuKNOX5* and *EuKNOX8* showed constitutive expression patterns in all samples. In the 'Qinzhong 2' cultivar, the expression levels of *EuKNOX4*, *EuKNOX5* and *EuKNOX8* were significantly high, indicating that the three genes potentially play a positive regulatory role in the formation of *E. ulmoides* rubber, and the specific functions need to be further verified.

To clarify the potential association between *EuKNOX* genes and genes involved in the *E. ulmoides* rubber biosynthesis pathway in *E. ulmoides*, we constructed a co-expression network encompassing 8 *EuKNOX* genes and two key *E. ulmoides* rubber biosynthesis genes—*EuHMGR* (3-hydroxy-3-methylglutaryl-CoA reductase) and *EuFPS* (farnesyl pyrophosphate synthase) (Fig. 10E). The screening thresholds were $|r| \geq 0.50$ and $p < 0.5$. The analysis results showed that there was a highly significant positive correlation between *EuKNOXs* and these core *E. ulmoides* rubber biosynthesis genes. In the positive regulatory co-expression network, we identified 4 pairs correlated between 3 *E. ulmoides* rubber structural genes and 4 *EuKNOX* genes (*EuKNOX1*, *EuKNOX3*, *EuKNOX7* and *EuKNOX8*), suggesting that these four *EuKNOX* genes may play crucial positive regulatory roles in *E. ulmoides* rubber biosynthesis, though their specific functions require further experimental validation.

To investigate the role of *EuKNOX* genes in leaf color formation in *E. ulmoides*, we analyzed their expression patterns using transcriptome data from green and red leaves. As shown in Fig. 10C and Table S9, among the 8 *EuKNOX* genes, 4 *EuKNOX* were highly expressed in the red-leaf *E. ulmoides*, while only *EuKNOX4* and *EuKNOX5* showed a slightly higher expression level in the green-leaf *E. ulmoides* than in the red-leaf, suggesting most of the *EuKNOX* genes may regulate anthocyanin biosynthesis. Furthermore, only *EuKNOX4*, *EuKNOX5* and *EuKNOX7* exhibited high expression levels in both leaf color variants, whereas the remaining *EuKNOX* genes showed low expression abundance with FPKM values below 2. Notably, *EuKNOX2* and *EuKNOX6* displayed no transcriptional activity (FPKM = 0) across all tested samples.

To clarify the potential association between *EuKNOX* genes and those involved in the flavonoid synthesis pathway in *E. ulmoides*, we constructed a co-expression network encompassing 8 *EuKNOX* genes and three key flavonoid synthesis genes-*EuCHI* (Chalcone isomerase), *EuCHS* (Chalcone

synthase), *EuDFR* (Dihydroflavone 4-reductase), and *EuFLS* (Flavonol synthase) (Fig. 10F). The screening thresholds were set at $|r| \geq 0.60$ and $p < 0.01$. Analysis results revealed a highly significant positive correlation between *EuKNOX* and these core flavonoid synthesis genes. Within the positive regulatory co-expression network, 10 correlated pairs were identified between the 8 flavonoid synthesis structural genes and 4 *EuKNOX* genes. Statistical analysis showed that *EuKNOX7* had a correlation degree of 7, while *EuKNOX1*, *EuKNOX3*, and *EuKNOX8* each had a connection degree of 1. These findings suggest that these four *EuKNOX* genes may play crucial roles in positively regulating flavonoid biosynthesis, though their specific functions require further experimental validation.

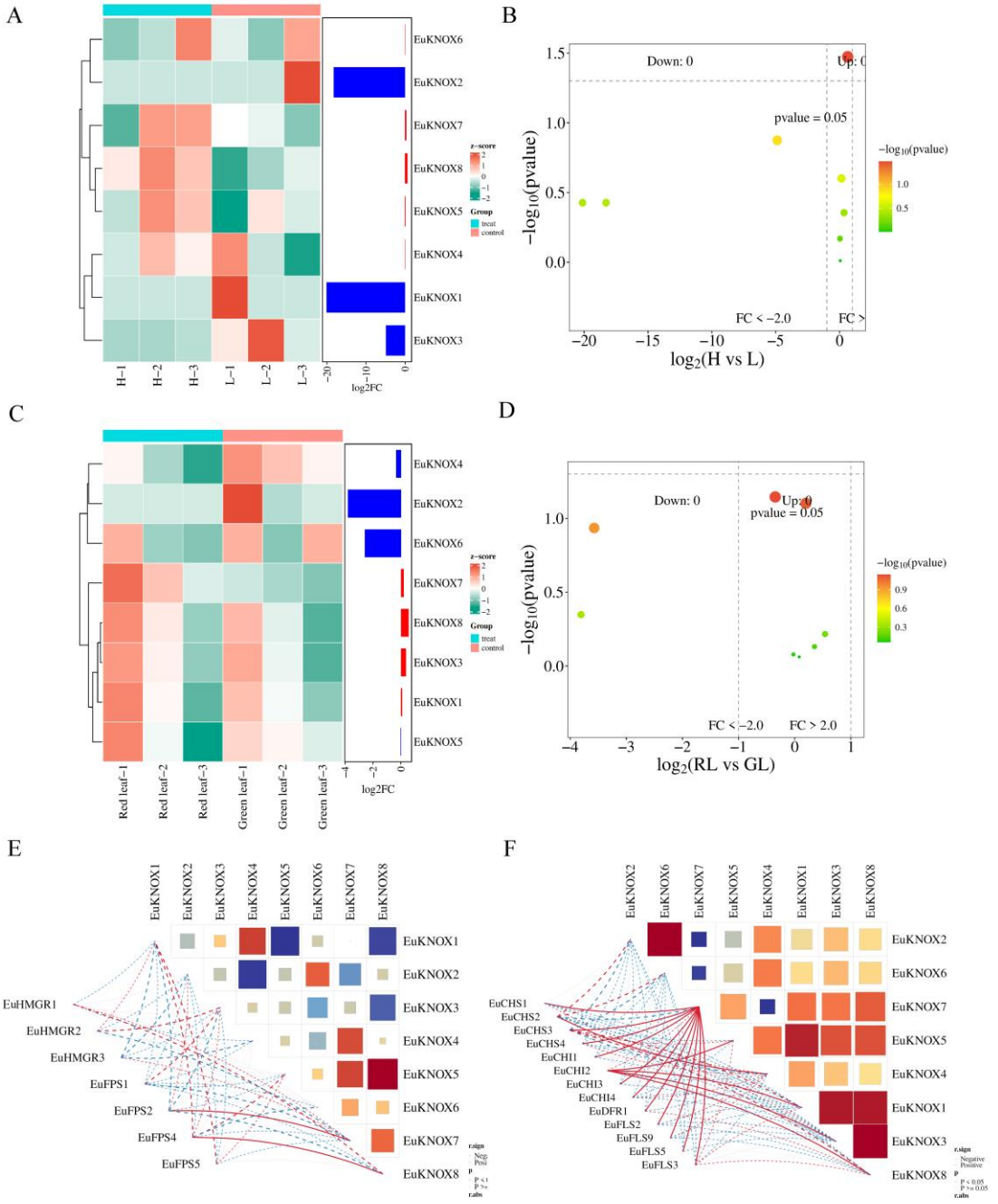


Fig. 10 Expression patterns of *EuKNOXs* in leaves with different *E. ulmoides* rubber content (A) and correlation analyses between *EuKNOXs* genes and *E. ulmoides* rubber biosynthesis genes (B). Expression patterns of *EuKNOXs* genes in different colored leaves of *E. ulmoides* (C). and correlation analyses between *EuKNOXs* genes and *EuCHS*, *EuCHI*, *EuDFR* and *EuFLS* genes (D). Note: Green represents a low level and red indicates a high level of transcript abundances. Three replicates per group, L-1-L-3: low Eu-rubber contents (1.39%, the average

rate of Eu-rubber); H-1-H-3: high Eu-rubber contents (4.80%); GL-1--GL-3: Green leaf of *E.ulmoides*; RL-1--RL-3: Red leaf of *E.ulmoides*. The color key on the right represents the relative expression level in FPKM values, the red and blue bars (FPKM) indicate high and low expression, respectively. Blue represents negative correlation, while red represents positive correlation. The solid line indicates that the correlation results reached a statistically significant level; the dashed line indicates that the correlation results did not reach a significant level.

3.10 Quantitative expression analysis of *EuKNOX* genes in various tissues by qRT-PCR

To primarily investigate the functions of *EuKNOX* genes, their expression patterns were analyzed across three different tissues (root, stem and leaf) using qRT-PCR. As shown in Fig. 11, eight *EuKNOX* genes exhibited different expression patterns. Specifically, four *EuKNOXs* (*EuKNOX1*, *EuKNOX2*, *EuKNOX3* and *EuKNOX5*) showed the highest expression in root, with *EuKNOX2* additionally displaying the lowest expression in leaves, and the remaining three had the lowest expression levels in the stems. In contrast, *EuKNOX6* was most highly expressed in stems, while *EuKNOX4* and *EuKNOX8* peaked in leaves and showed the lowest expression in roots. Furthermore, *EuKNOX7* exhibited the highest expression in leaves and the lowest in stems. These results indicate that the eight *EuKNOXs* genes posses tissue-specific expression patterns, suggesting functional divergence among family members.

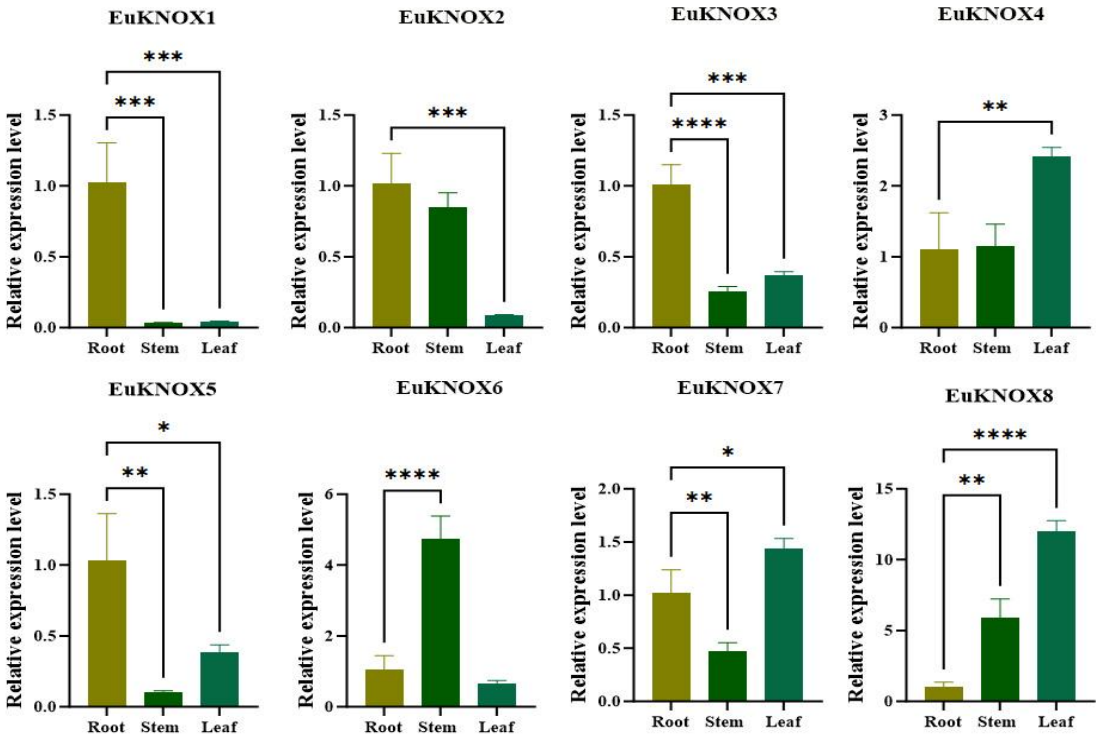


Fig. 11 Expression patterns of *EuKNOX* genes in roots, stems and leaves of *E. ulmoides*. qRT-PCR data were normalized using *UBC* as the reference gene. Results were expressed as mean±standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates. The * indicated significant differences compared to the roots, as determined by t-test (*p<0.05, **p<0.01,***p<0.001,****p<0.0001).

3.11 Expression analysis of *EuKNOXs* under under salt and drought stress

Various abiotic stresses can affect the growth and development of plants, and ultimately regulate the expression of a series of stress-responsive genes[66]. During the growth of *E. ulmoides*, some environmental factors, such as drought and low-temperature stress, can prevent its full genetic potential, resulting in reduced yield and even plant death[67]. Consequently, it becomes imperative to clarify the regulatory pathway and identify pivotal regulatory factors implicated in abiotic stress responses of *E. ulmoides*. To determine whether *EuKNOXs* gene expression is responsive to different abiotic stresses,

we analyzed their expression patterns following exposure to NaCl and drought stress. Specifically, qRT-PCR was used to detect the expression of the eight *EuKNOX* genes in leaves sampled at 0, 3, 6, 12, 24, 48, and 72 h after treatment. The results showed that the eight *EuKNOX* genes exhibited divergent expression patterns under both NaCl and drought stress treatments.

Under 200 mM NaCl treatment, *EuKNOX* genes expression exhibited distinct trends (Fig. 12). Specifically, *EuKNOX1* and *EuKNOX8* genes were induced by NaCl, with their expression peaking at 72 h post-treatment, reaching 17.8- and 6.8-fold of the control, respectively. In contrast, six *EuKNOXs* (*EuKNOX2*, *EuKNOX3*, *EuKNOX4*, *EuKNOX5*, *EuKNOX6* and *EuKNOX7*) showed a trend of first increasing and then decreasing, Among these, *EuKNOX6* and *EuKNOX7* peaked at 6 h and 48 h post-treatment, with expression levels 47- and 11-fold higher than the control, respectively. *EuKNOX3* reached its maximum expression at 3 h (29-fold of the control), while *EuKNOX2* and *EuKNOX5* peaked at 24 h, showing 19- and 343-fold increases relative to the control, respectively. Additionally, *EuKNOX4* expression peaked at 48 h, with a 15-fold increase compared to the control. These divergent expression patterns of *EuKNOX* genes under NaCl stress suggest functional differentiation within the gene family.

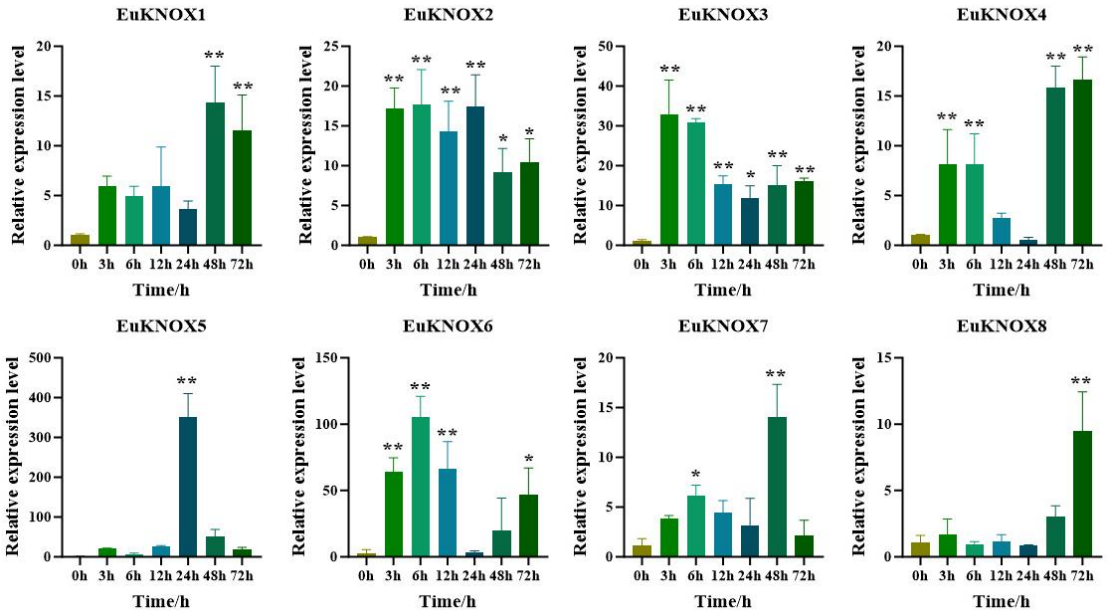


Fig. 12 The expression patterns of *EuKNOX* genes under NaCl treatments were examined by qRT-PCR. Two-month-old seedling leaves of *E. ulmoides* were sprayed with 200 mg/L NaCl). Spray water was used as the control treatment. qRT-PCR data were normalized using *UBC* as the reference gene and were displayed relative to 0 h. Results were expressed as mean±standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates, the * indicated significant differences by t-test (*p<0.05, **p<0.01).

Under drought treatment, *EuKNOX* genes exhibited a triphasic expression trend (first increasing, then decreasing, and subsequently increasing) (Fig. 13). Specifically, *EuKNOX1* and *EuKNOX4* reached their expression peaks at 24 h post-treatment, with levels 369- and 403-fold higher than the control, respectively. In contrast, *EuKNOX2* and *EuKNOX6* reached a peak at 72 h, which was 44 and 63-fold increases relative to the control. Meanwhile, the transcriptional levels of *EuKNOX3*, *EuKNOX7* and *EuKNOX8* achieved maximum transcriptional levels at 1 h, which was 74, 41 and 11 times that of the control, respectively, while the expression abundance of *EuKNOX5* peaked at 3 h, which was 12 times that of the control. Moreover, the expression levels of three genes (*EuKNOX2*, *EuKNOX5* and

EuKNOX8) decreased to their lowest levels at 48 h post-treatment, reaching 0.4, 0.5 and 0.03-fold of the control, respectively. In contrast, five *EuKNOX* genes (*EuKNOX1*, *EuKNOX3*, *EuKNOX4*, *EuKNOX6* and *EuKNOX7*) were the lowest expression at 6 h, with levels 0.2, 0.2, 0.6, 0.6 and 0.2-fold of the control, respectively. On the whole, *EuKNOXs* displayed divergent expression patterns under drought stress, suggesting they may exert distinct functional roles in the plant's drought response. Their specific biological functions, however, require further validation.

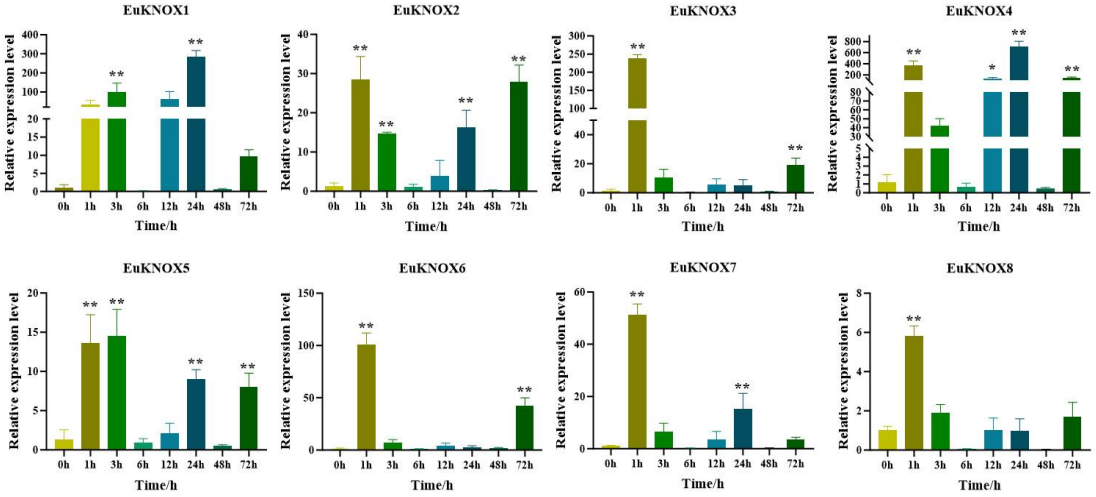


Fig. 13 The expression patterns of *EuKNOX* genes under drought treatments were examined by qRT-PCR. The horizontal axis represented PEG6000 treatments, and the vertical axis represented relative gene expression levels. qRT-PCR data were normalized using *UBC* as the reference gene and were displayed relative to 0 h. Results were expressed as mean±standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates, the * indicated significant differences by t-test (*p<0.05, **p<0.01).

3.12 Transcriptional responses of *EuKNOX* genes to hormonal treatments

To further investigate the roles of *EuKNOX* genes in the hormonal response mechanism of *E. ulmoides*, seedlings were treated with ABA, GA_3 and MeJA. Samples were collected at 0, 6, 12, 24, 36, 48 and 72 h post-treatment, with the 0 h sample as the control, the expression patterns of the eight *EuKNOX* genes were subsequently analyzed using qRT-PCR. Under ABA treatment, the relative expression levels of different *EuKNOX* genes showed significant differences (Fig. 14), indicating that all these genes are responsive to ABA. Although the expression patterns of each gene were not identical, several similarities were observed. Most genes exhibited a trend of initial increase, then decrease, and then rapid upregulation. Specifically, *EuKNOX6* showed a 63.82-fold increase at 6 h, while *EuKNOX2* and *EuKNOX1* increased by 39.30-fold and 13.41-fold at 1 h, respectively, followed by *EuKNOX5*, *EuKNOX2* and *EuKNOX3* by 18.93, 8.78 and 6.49 folds, respectively. In addition, the remaining *EuKNOX* genes also showed increases to varying degrees. However, the expression of *EuKNOX8* exhibited a distinct pattern of initial decrease followed by increase: it decreased to 0.14-fold of the control at 12 h, then increased to 4.97-fold at 72 h. This finding implied that *EuKNOX* genes might play multiple regulatory roles at different stages of ABA signal transduction.

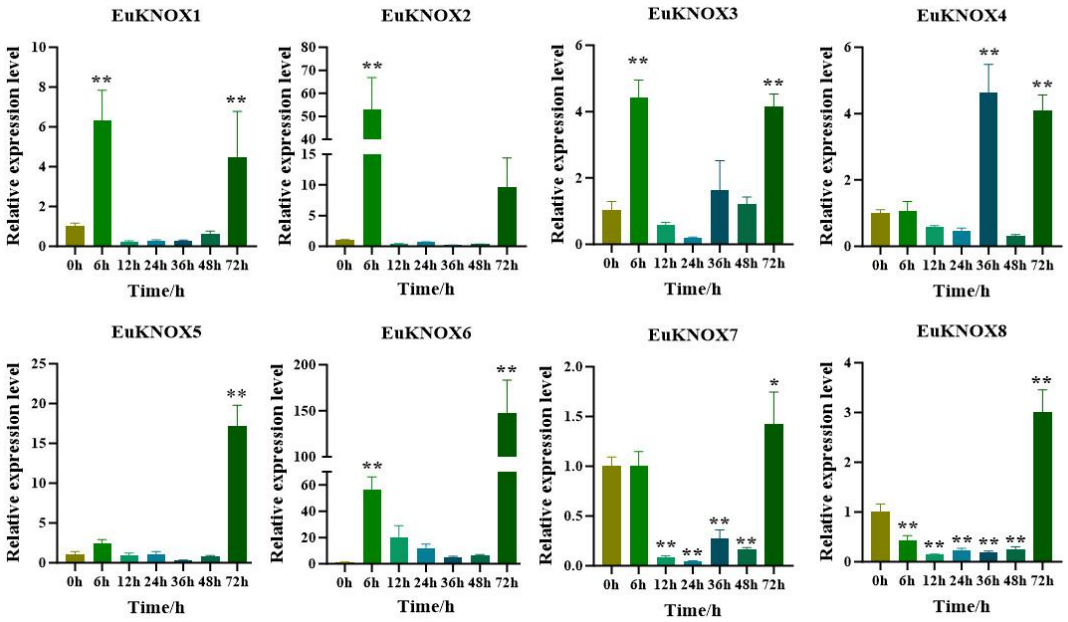


Fig. 14 Expression patterns of *EuKNOXs* under ABA treatments. The horizontal axis represented ABA treatments, and the vertical axis represented relative gene expression levels. 0 h was selected as the control group. qRT-PCR data were normalized using *UBC* as the reference gene and were displayed relative to 0 h. Results were expressed as mean±standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates, the * indicated significant differences by t-test (*p<0.05, **p<0.01).

Under GA_3 treatment, *EuKNOX* genes exhibited divergent expression patterns (Fig. 15). Specifically, Seven *EuKNOX* genes (*EuKNOX2*, *EuKNOX3*, *EuKNOX4*, *EuKNOX5*, *EuKNOX6*, *EuKNOX7* and *EuKNOX8*) showed a trend of initial increase followed by decrease. Among these, *EuKNOX4* increased 310.71-folds at 24 h, then quickly decreased to 14.32-folds at 36 h, *EuKNOX3* peaked at 90.23-fold at 24 h, subsequently decreasing to 0.71-fold at 72 h, *EuKNOX5* and *EuKNOX6* reached their maxima 36 h with 100.34-fold and 92.03-fold increases, respectively, followed by *EuKNOX7*, *EuKNOX8* and *EuKNOX2* by 19.12, 47.41 and 24.26 folds at 36 h, respectively. However, *EuKNOX1* genes exhibited the opposite patterns, that is, first decreased and then induced in GA_3 treatments, *EuKNOX1* decreased to 0.11 folds at 12 h, and then increased to be 1.28 folds at 36 h. These data supported the hypothesis that *EuKNOX* genes participated in ABA and GA_3 response pathways, consistent with findings reported in other plant species.

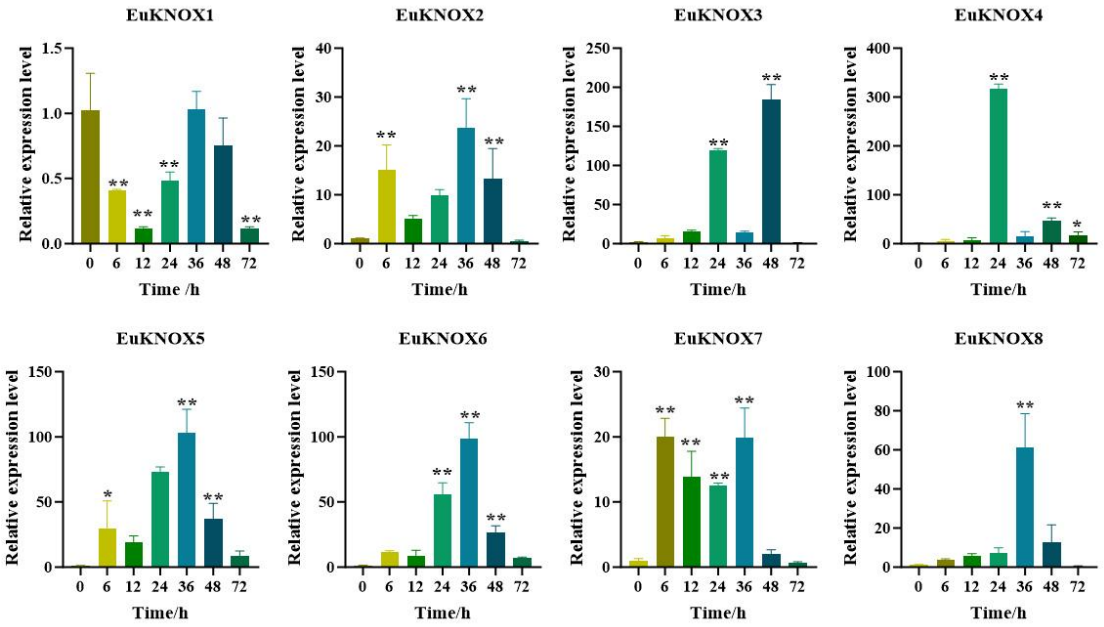


Fig. 15 Expression patterns of *EuKNOXs* under GA_3 treatments. The horizontal axis represented GA_3 treatments, and the vertical axis represented relative gene expression levels. qRT-PCR data were normalized using *UBC* as the reference gene and were displayed relative to 0 h. Results were expressed as mean ± standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates, the * indicated significant differences by t-test (*p<0.05, **p<0.01).

Under the treatment of MeJA, *EuKNOXs* genes exhibited diverse dynamic expression characteristics (Fig. 16). Specifically, the expression levels of *EuKNOX1*, *EuKNOX4*, *EuKNOX5*, *EuKNOX7* and *EuKNOX8* showed fluctuating expressions by first decreasing, then increasing, reaching their lowest expression levels at 6 h, which were 1/2, 1/10, 1/10, 1/50 and 1/5 of those of the control group. On the contrary, the expression level of *EuKNOX6* gene showed an initial increase followed by a decrease, peaking at 6 h of treatment, reaching 1.58 times that of the control group. Furthermore, MeJA treatment inhibits the expression of *EuKNOX2*, and *EuKNOX3* genes. After treatment with MeJA, the expression pattern shows a downward trend, with expression reaching its lowest point at 24 h post-treatment. At this time, their levels had decreased to 0.46-, and 0.20-fold of those in the control group, respectively. These results suggest that *EuKNOX* genes are differentially regulated under hormone treatment and abiotic stress, with most members contributing to the plant's adaptive response through temporally distinct expression profiles. The dynamic regulation observed highlights the functional specialization within the *EuKNOX* gene family and their potential involvement in hormone treatment and abiotic stress tolerance mechanisms in *E. ulmoides*.

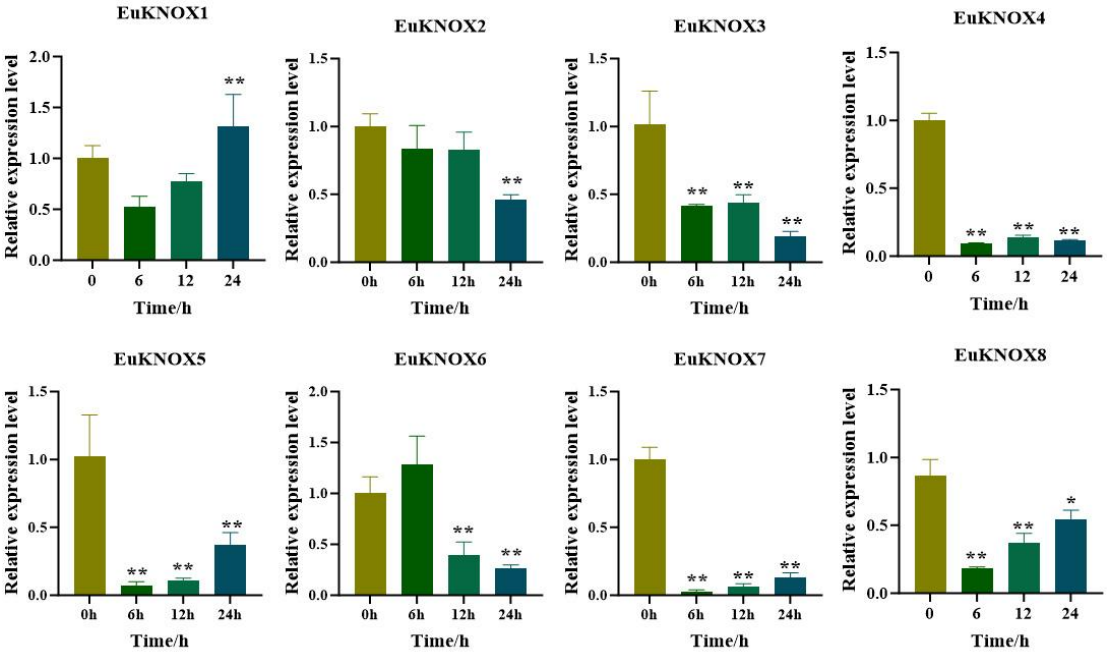


Fig. 16 Expression patterns of *EuKNOXs* under MeJA treatments. The horizontal axis represented MeJA treatments, and the vertical axis represented relative gene expression levels. qRT-PCR data were normalized using *UBC* as the reference gene and were displayed relative to 0 h. Results were expressed as mean ± standard deviation (SD). Error bars represented the standard error of the mean of three independent replicates, the * indicated significant differences by t-test (*p<0.05, **p<0.01).

3.13 protein interaction network and GO analysis of *EuKNOX* genes

To elucidate the protein interaction patterns and biological functions of the KNOX gene family in *E. ulmoides*, this study constructed a protein interaction network based on the STRING database and conducted a systematic study in combination with GO functional annotations. The analysis of the protein interaction network (Fig. 17A) revealed that six proteins were predicted to interact with each other, and a total of 15 pairs of interactions were identified. The remaining two proteins did not show significant interactions, and they might function independently or have species-specific interaction mechanisms. GO enrichment analyses were performed to clarify the biological functions of *EuKNOX* genes. The GO results showed that these genes were significantly enriched in tissue and xylem development, which are highly consistent with the typical functional characteristics of KNOX transporters (Fig. 17B).

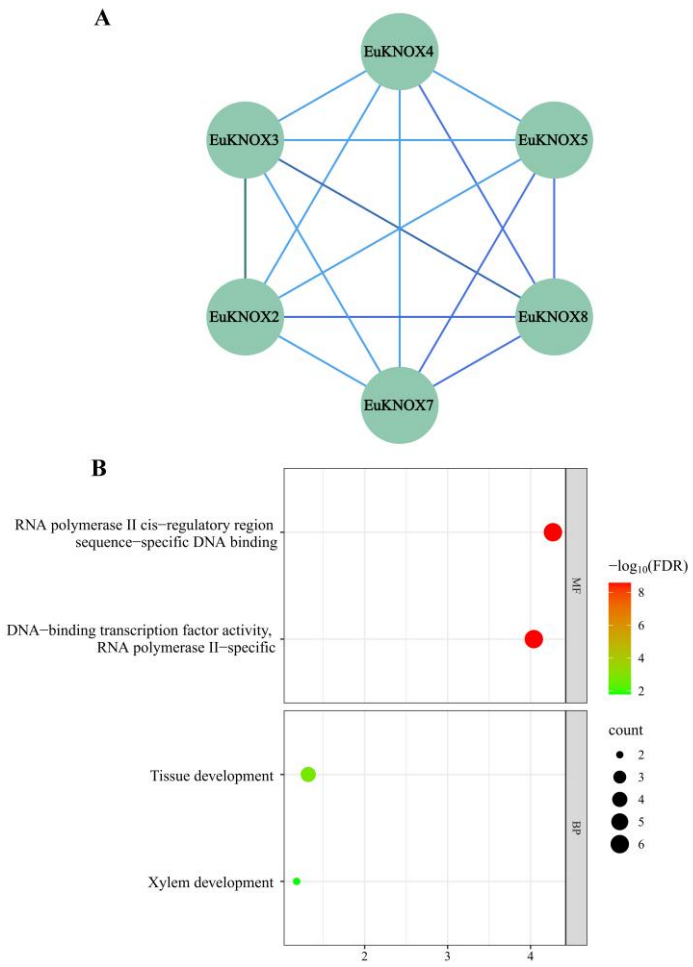


Fig. 17 Interacting protein and GO enrichment analysis of the *EuKNOX* genes. The ordinate is the main GO classification categories, and the abscissa is the enrichment factor.

4 Discussion

KNOX genes play a critical role in modulating activities of cells at meristems, and their expression is tightly controlled by a complex network of transcription factors[68-69]. To date, *KNOXs* have been identified and extensively studied in various plant species, however, *KNOXs* in *E. ulmoides* remain largely uncharacterized. In this study, eight *KNOX* genes family members were identified in the genome of the *E. ulmoides*. This number is nearly identical to that of tomato (8 members)[21], and higher than that of *Pinus pinaster* (6 members)[24], this might stem from gene duplication in plants, which is considered a fundamental driver of the evolution of genomes. However, the number of *KNOX* genes in *E. ulmoides* is fewer than that reported in *Arabidopsis* (9 members)[22], *Salvia miltiorrhiza* (10 members)[70], Garlic (10 members)[71], *Medicago truncatula* (12 members)[72], apple (22 members)[73], pear (19 members)[25], moso bamboo (24 members)[74], soybean (34 members)[75], and other species. This suggests that most *EuKNOX* genes have not been selectively eliminated by the environment, and no obvious gene family amplification events have occurred.

The analysis of physicochemical properties reveals a high variation among the different members. The variation range of the amino acid sequence of EuKNOXs was 212 aa to 426 aa, with molecular weights ranging from 24.70 to 47.35 kDa, and the expected the oretical isoelectric point ranging from 5.03 to 6.19, instability indices ranged between 39.90 and 62.53, aliphatic indices fell within 60.27–

82.26, and GRAVY values varied from -0.916 to -0.598. Chromosomal distribution analysis showed that these eight genes were located on six different chromosomes, while subcellular localization analysis revealed that among the eight *EuKNOXs* were predicted to localize to the nucleus.

KNOX proteins are known to possess four conserved structural domains, each serving a distinct biological function. At the N-terminus, the MEINOX domain comprises two subdomains, KNOX1 and KNOX2, involved in inter-protein interactions, homodimerization, and the modulation of target gene interactions[76]. The differentiation in the functionality of KNOX genes can likely be attributed to variations in the the number of conserved structural domains across subclasses[72]. To explore the evolutionary pattern of *KNOX* genes, a phylogenetic tree was constructed incorporating sequences from *E. ulmoides*, *Arabidopsis*, pear, *Triticum aestivum*, *Malus domestica*, and rice. The analysis revealed that the *E. ulmoides* KNOX family was divided into two calsses (Class I, and Class II), which were subsequently clustered into five subfamilies (BP like, STM like, KNOX2/6 like, KNOX3-5 like and KNOX7 like). Class I and Class II each contain four members, and no *EuKNOX* genes were assigned to the KNATM subfamily. Class I KNOX genes are specific in temporal and spatial expression patterns, displaying both functional redundancy and unique specific roles, they eventually affect the morphogenesis of lateral organs by regulating cell differentiation[18,77]. Notably, *EuKNOX2*/*3*/*6*/*8* are closely related to *STM*, *KNAT1*, *KNAT2*, and *KNAT6* in *Arabidopsis*, indicating that these are class I genes. Given that Arabidopsis *KNAT1*, *KNAT2* and *KNAT6* are mainly expressed in flower meristem and affect the process of flower development[29,78], suggesting that *EuKNOX2*/*3*/*6*/*8* may have similar functions. Class II KNOX proteins operate as repressors in the transcriptional control of their target genes, in contrast to Class I KNOX proteins, which appear to be activators[79]. In this study, *EuKNOX1*/*4*/*5*/*7* showed close phylogenetic relationships with Arabidopsis *KNAT3*, *KNAT4*, *KNAT5*, and *KNAT7*, which were expressed ubiquitously across plant tissues and associated with seed germination and secondary wall formation[80]. Class M *KNOX* genes are mainly expressed at the boundary between plant organ primordium and mature organs, where they may be involved in sensing or generating flowering signals[22]. Conservative domain analysis of the protein revealed that these proteins lack the homeobox domain. Previous studies have shown that KNOX proteins belonging to the KNATM family occur exclusively in eudicots, and some species contain more than one homolog of the KNM protein[81]. However, no EuKNM paralogs were identified in *E. ulmoides*.

Considering the functional significance of conserved domains within the KNOX protein, we focused on investigating the KNOX protein domains in *E. ulmoides*. The regulatory capacity of gene transcription is often affected by the presence of introns, with different numbers of introns influencing sensitivity. In the current study, the analysis of *EuKNOX* genes structure revealed that both the class I and II families have four to five exons in each gene (Fig. 4A). In addition, the differences in the exon and intron structure of genes are the main reasons for the diversification of gene functions, 8 *EuKNOX* genes contain different numbers of exons or introns, which indicates that the KNOX gene family of *E. ulmoides* has great diversity. We discovered that most members of the same subgroup have at least one KNOX protein with the same motifs, such as *EuKNOX4*/*EuKNOX7* and *EuKNOX2*/*EuKNOX6*. This may explain the functional differences and diversity of closely related *EuKNOX* genes, such as *EuKNOX*2 and *EuKNOX*3, as well as *EuKNOX*6 and *EuKNOX*8.

Cis-acting elements play a crucial role in regulating gene expression: by binding to transcription factors, they enable precise control over the initiation of gene transcription and transcription efficiency. Notably, those located upstream of transcriptional initiation sites are primarily responsible for mediating gene expression regulation, and are therefore essential for plant development, growth, and signaling processes[82]. In the current study, the predicted cis-acting elements of *EuKNOXs* genes showed that the promoter regions of these 8 *EuKNOXs* gene family members were rich in a large number of growth and development-related elements, hormone response-related cis-acting elements and stress related elements. Therefore, the *EuKNOXs* gene may affect plant growth, development and morphogenesis by influencing hormonal and metabolic pathways. Plant growth and development-associated cis-elements, such as MYC, can be recognized by basic helix loop helix (bHLH) TFs to activate gene expression, thereby regulating plant growth and development in response to Jasmonic Acid (JA) signaling[83]. The cis-acting elements of each KNOX member exhibited significant diversity, all of KNOX members within the *E. ulmoides* contained cis-acting elements related to adversity stress. Additionally, KNOX members containing these elements in *Salvia miltiorrhiza*, *Vitis amurensis*, *Gossypium hirsutum* and soybean were involved in modulating the plant's response to abiotic stresses like high temperatures, salt, cold and drought[14, 70, 84, 85]. Therefore, it is postulated that *E. ulmoides* KNOX members are involved in regulating plant abiotic stress response, ultimately enhancing its tolerance against adversity stresses.

KNOX proteins play important roles in regulating plant organ differentiation [13]. GO enrichment analyses confirmed that EuKNOXs play a crucial role in tissue development. By examining the KNOX family gene expression in various tissues of *E. ulmoides*, as well as in different periods of *E. ulmoides* (Fig. 7 and Fig. 11), the expression patterns of many *KNOXs* showed tissue specificity, indicating that *KNOXs* might participate in regulating organ differentiation of *E. ulmoides.* At the same time, we noticed that *EuKNOXs* was highly expressed in the xylem and edge of the peel of *E. ulmoides*, indicating that *EuKNOXs* played an important role in maintaining the meristem potential, which is consistent with previous research findings. *EuKNOX6* and *EuKNOX8* are homologous genes of *KNAT2* and *KNAT6* in *Arabidopsis*. *KNAT6* and *KNAT2* exhibit high homology and play important roles in the development of inflorescences in *Arabidopsis*[78]. In *Populus* and *Arabidopsis*, *KNAT2/6b* homologs are highly expressed in vascular tissues. Overexpression of PagKNAT2/6b in poplar reduces secondary xylem with thinner cell walls and lower cellulose, xylose, and lignin[35]. In citrus, *CiKN6*, expressed mainly in stems, promotes branching but inhibits shoot elongation[86]. In *Populus*, *PagKNAT5a* overexpression increased plant height and stem diameter, correlating with elongated xylem vessels/fiber cells and elevated auxin levels. These results suggest that *PagKNAT5a* promotes vascular cell elongation via auxin modulation and xylem radial growth through MYB46-mediated lignin biosynthesis[87]. Expression analysis revealed that *EuKNOX6* is highly expressed in the xylem, speculating that *EuKNOX6* and *EuKNOX8* may play significant roles in the lateral root development, and xylem formation of *E. ulmoides*, however, their specific functions still need to be further verified. In *Brassica napus*, Class I BnKNOXs are moderately expressed in buds and inflorescence tips, Class III has low expression in seeds and stamens, and Class II is expressed in most tissues[88]. Additionally, leaf development is closely related to the expression pattern of KNOX I; overexpressing of *KNAT1* in

Arabidopsis results in deeper leaf serrations on the leaf margins[89]. Ectopic expression of oil palm *EgKNOX1* in *Arabidopsis* caused greener, lobed leaves and shorter, wider petioles, with severity increasing during development; in rice, it induced white knot-like structures and disrupted parallel vein organization[90]. In *Camellia sinensis*, overexpression of *CsKNOX6* reduced the leaf area in transgenic *Arabidopsis*, which initially determined that it is a key gene negatively regulating bud size[91]. In alfalfa, Class I *MsKNAT1*/*STM2*/*KNATM1*/*2*/*NATM* expressed higher in trifoliate leaves; Class II (except *MsKNAT8*) increased in seven-leaflets and with leaf age, indicating *MsKNOX* roles in leaf development[72]. In the study, we noticed that the majority of *EuKNOX* genes exhibit the highest expression levels in leaf buds. As *E. ulmoides* leaves develop, their expression levels gradually decrease, suggesting that *EuKNOX* genes mainly functions during the early stage of leaf development. In contrast, only *EuKNOX4* and *EuKNOX5* showed the highest transcriptional levels in old leaves.

Moreover, plants have complex systems to respond to both biotic and abiotic stresses. The *KNOX* gene family has been found to enable cotton plants to respond to different stresses while also participating in plant growth and development[92,74]: *GhKNOX2* silencing enhances salt tolerance; *GhKNOX10/14* reduces it[92]. *GhKNOX4-A/22-D* silencing induces oxidative stress (lower SOD, cell death) and promotes stomatal opening/water loss, contributing to drought response[85]. In poplar, the expression of *PagKNAT2/6b* was significantly upregulated under drought conditions; its overexpression inhibits cell elongation, causing shorter internodes, smaller leaves, and reduced/absent petioles[93]. Under the stress of *salvia miltiorrhiza* salt, *SmKNOX4* expression significantly decreased within 24 h, while *SmKNOX1*, *SmKNOX2*, *SmKNOX3*, *SmKNOX8*, and *SmKNOX9* showed significant increases[70]. In pear, the expression of *PbKNOX7/13* increased under drought stress and decreased after plants were covered with water, whereas the expression of *PbKNOX5/16* was inhibited under drought stress and increased after plants were covered with water[58]. To deeply understand the stress response mechanism of the *EuKNOX* genes, qRT-PCR experiments under different abiotic treatments, such as NaCl and drought in leaves. The *EuKNOX* genes then showed significant differential expression patterns under treatment with these two treatments. These treatments strongly upregulated some *EuKNOX* genes, such as *EuKNOX3* expression peaked at 3 h of salt exposure, reaching a level 29-fold higher than the control, additionally, *EuKNOX2* and *EuKNOX5* reached their peak expression at 24 h, with increases of 19- and 343-fold relative to the control, respectively. Following drought treatment, *EuKNOX1* and *EuKNOX4* reached their expression peaks at 24 h post drought treatment, with levels 369- and 403-fold higher than the control, respectively. Therefore, it can be speculated that *EuKNOXs* may play a significant role in drought and salt stress conditions. Based on this evidence, we hypothesized that *EuKNOX* genes could influence the stress response of plants.

In plants, genes that are commonly linked to hormone stress are responsible for generating stress responses. For example, the promoter regions of the *KNOX* gene in *Cymbidium* and Orchidaceae contain abundant MeJA and ABA-responsive cis-elements, as well as auxin and gibberellin components, respectively[94-95]. In our study, many common cis-acting elements, such as MBS, LTR, ARE, and ABRE, were identified in the promoter region of *EuKNOXs*. These *KNOX* genes contain at least hormone-acting elements, which indicates that the expression level of these genes will change under the action of hormones. Furthermore, it revealed that Class I and Class II *EuKNOX* genes have

distinct roles in regulating growth, development, and responses to stress. To deeply understand the hormone response mechanism of the *E. ulmoides* KNOX genes, qRT-PCR experiments under different hormone treatments, such as ABA, and GA_3 in leaves (Fig. 14-15). Consistent with these results, the present expression analysis of *EuKNOX* genes then showed significant differential expression patterns under treatment with these two hormones. In ABA treatment, *EuKNOX6* showed a 63.82-fold increase at 6 h, and *EuKNOX2* and *EuKNOX1* increased by 39.30-fold and 13.41-fold at 1 h, respectively. Following GA_3 treatment, seven *EuKNOX* genes showed a trend of initial increase followed by decrease, with *EuKNOX4* being the most responsive, showing a 310.71-fold increase at 24 h post-treatment compared to the control, then quickly decreased to 14.32-folds at 36 h. These results were similar to previously reported studies, for example, in apple, overexpression of *MdKNOX19* increased the ABA sensitivity of apple calli, resulting in a dramatic up-regulation in the transcription of the Arabidopsis ABI5-like *MdABI5* gene[73]. In tree peony, IBA or ABA treatment significantly enhanced transcriptional activity of *PoKNOX7* and *PoKNOX8*[96]. In wheat, IAA, and cytokinin (CTK) induced *TaKNOX2-A*, *11-A* and *14-D* genes expression, *TaKNOX14-D* overexpression also resulted in the ratio of CTK and IAA being changed in leaves[97]. The legume *KNOX3* gene regulates nodule formation through cytokinin biosynthesis and activation[98]. The tomato *KNOX* gene *Tkn4* participates in pollen and pollen tube development and the regulation of plant growth through the gibberellin and auxin pathways[99]. Above all, *EuKNOXs* genes were induced by multiple treatments such as ABA, GA_3, as well as by drought and salt stress. In our study, we postulate that *KNOX* genes in *E. ulmoides* respond to various stresses through different hormone signaling pathways, such as ABA-GA_3 stress crosstalk,or drought-salt stress crosstalk, regulating the growth and development of *E. ulmoides*.

5 Conclusion

In conclusion, the genome-wide analysis of the KNOX gene family identifed 8 *EuKNOX* genes in *E. ulmoides* and provided valuable insights into their roles in promoting growth development, abiotic stress and their regulatory networks. Subcellular localization analysis revealed that *EuKNOXs* proteins are primarily located in the nucleus, and the structural analysis unveiled highly conserved domains, such as KNOX1, KNOX2, ELK, and Homeobox_KN, emphasizing *EuKNOXs* role in transcriptional regulation and fundamental biological processes. Cis-acting element analysis in *EuKNOX* gene promoters revealed numerous elements associated with plant growth, development, hormone response, and stress tolerance. The phylogenetic analysis showed that *EuKNOXs* genes share a closer evolutionary relationship with *Arabidopsis KNOX* genes. Analysis revealed that several *EuKNOX* genes exhibited a strong positive correlation with the leaf development, and the majority of *EuKNOX* genes exhibited strongly preferential expression in buds and were significantly up-regulated during the flower induction period. Furthermore, the transcript levels of *EuKNOX* genes were responsive to treatments with flowering- and stress-related hormones, suggesting their involvement in regulating both plant growth and development, as well as abiotic stress responses. Collectively, this study provides valuable insights into the functional roles of the KNOX gene family in the growth, development, and abiotic stress adaptation of *E. ulmoides*.

Author Contributions: J.L. and Q.X.D. conceived and designed this research. J.L. wrote the manuscript. T.T.C., L.L.W., L.X.P., and C.L.L. performed the software analysis. J.C.L., W.M.F., and

J.F.Y performed the data analysis. Writing—review & editing, J. F. K. and S.Q.C. All authors modified the manuscript and approved the final MS. All authors have read and agreed to the published version of the manuscript.

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