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Comprehensive analysis of WUSCHEL-related Homeobox family in Chosenia arbutifolia (Pall.) A. Skv.: Characterization, Structure, evolutionary insights, and Expression Profiles

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Comprehensive analysis of WUSCHEL-related Homeobox family in *Chosenia arbutifolia* (Pall.) A. Skv.: Characterization, Structure, evolutionary insights, and Expression Profiles

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Abstract

WUSCHEL-related Homeobox (WOX) is a key transcription factor regulating plant meristems, and plays an important role in plant growth, development and stress resistance regulation. To analyze the molecular characteristics and functional potential of the *CaWOX* gene family in *Chosenia arbutifolia* (Pall.) A. Skv, genome-wide identification and bioinformatics analysis were performed in poplar in this study, and 14 *CaWOX* genes were identified. The 14 *CaWOX* genes were distributed on 11 chromosomes with 8 tandem duplications. Phylogenetic analysis divided the 14 CaWOX proteins into 4 conserved subgroups, and members in the same subgroup shared highly conserved conserved motifs and gene structures. The *CaWOX* family was highly evolutionarily conserved among closely related species in Salicaceae, but showed weak collinearity with *Arabidopsis thaliana*; cis-elements related to meristem development, hormone response and abiotic stress were enriched in the promoter regions. Tissue development analysis revealed that *CaWOX3/4/5/9/11* participated in leaf development and maintenance of physiological functions in mature leaves;

CaWOX1/6/10/14 regulated root development; and *CaWOX12* mediated secondary growth of stems in *C. arbutifolia*. This study clarified the evolutionary pattern and functional differentiation of the*CaWOX* family in *C. arbutifolia*, providing a key theoretical basis and gene resources for subsequent functional verification of this gene family, as well as growth regulation, wood property improvement and stress-resistant molecular breeding of Salicaceae trees.

Keywords:

WOX，*Chosenia arbutifolia*，Expression Profiles，evolutionary

Introduction

The WOX (WUSCHEL-related Homeobox) gene family encodes plant-specific transcription factors belonging to the Homeobox (HB) superfamily[1]. As a unique class of transcription factors in plants, WOX proteins contain a conserved Homeodomain (HD) consisting of 60-66 amino acid residues[2, 3]. This domain functions in binding specific DNA sequences and folds spatially into a three-dimensional structure comprising three α-helices, known as the helix-loop-helix-turn-helix (HLHTH) motif. Beyond the HD, members of the WUS clade possess a unique WUS-box motif in the carboxy-terminal region, with the consensus sequence T-L-X-L-F-P-X-X (specifically TLELFPLH)[4]. This WUS-box is crucial for WUS function, regulating the stem cell population in the Shoot Apical Meristem (SAM) and controlling floral development. Notably, members of the WUS clade have a dipeptide motif at the start of the WUS-box, whereas WOX proteins from other clades show variations at these positions[5, 6]. Some WOX family members also contain an EAR-like (ERF-associated amphiphilic repression) domain and the repression-associated WUS-box domain (with conserved sequences LxLxLx and TLxLFP, respectively) at their C-termini. Recent research highlights that various of the *WOX* family play significant roles at different stages of plant development[7, 8].

In *Arabidopsis thaliana*, the *WOX* family comprises 15 members, while in rice, there are 12 members. Based on the conservation of protein sequences, the *WOX* family is phylogenetically divided into three clades: the Modern/WUS clade (including WUS

and *AtWOX1-7*), the Intermediate clade (including *AtWOX8, AtWOX9, AtWOX11*, and *AtWOX12*), and the Ancient clade (including *AtWOX10, AtWOX13*, and *AtWOX14*). For instance, *AtWUS* and *AtWOX5* are expressed in the stem and root apical meristems, respectively, maintaining meristem cell activity and exhibiting functional redundancy in root stem cell regulation[9]. *AtWOX4* and *AtWOX14* co-regulate vascular cambium differentiation, ensuring vascular bundle and stem cell maintenance, while *AtWOX6* inhibits primordium differentiation. *AtWOX11* and *AtWOX12* are induced in the root cambium and promote adventitious root formation[10].

The *WOX* gene family is pivotal throughout plant development. *WOX1* has a conserved function in promoting lateral organ growth in species like pea, *A. thaliana*, cucumber, and rice[11]. In *A. thaliana*, *WOX2* and *WOX8* exhibit functional redundancy in regulating apical embryo patterning[12]. *WOX3* is key for cell proliferation during lateral organ growth in *Medicago truncatula* and *A. thaliana* [13, 14]. Studies show that *WOX4* role in promoting vascular stem cell proliferation/maintenance is conserved across *A. thaliana*, rice, and poplar[15]. *WOX5* is expressed in the Quiescent Center and involved in maintaining the root stem cell niche[16-18]. Silencing of *WOX6* delays primordium differentiation and maturation, and regulates ovule patterning in *A. thaliana*. In rice, *WOX6* is auxin-responsive and important for linking shoot gravitropism with tiller angle control. In *A. thaliana*, *WOX7* suppresses lateral root formation in a sugar-dependent manner[19], *WOX9* is essential for meristem growth and development and positively regulates WUS expression, and *WOX11* plays a key role in shoot development[20-22]. Furthermore, *WOX11* and its homolog *WOX12* are involved in adventitious root formation in rice and Liriodendron[23-25]. Research also indicates that *A. thaliana WOX13* promotes fruit development and coordinates patterning, while *WOX14* regulates vascular tissue proliferation[26].

Given the critical roles of the *WOX* gene family in plant growth, development, and developmental plasticity in response to the environment, research on this family has been conducted in multiple species. However, it has not yet been reported in *Chosenia arbutifolia* (Pall.) A. Skv. *C. arbutifolia*, a member of the Salicaceae family, possesses pendulous male catkins, and both male and female flowers lack nectaries, resembling

features of the genus polar. This makes it scientifically valuable for studying Salicaceae phylogeny[27]. Characterized by fast growth, an elegant form, and strong ecological benefits, it is often found along riverbanks and streams, serving as an excellent species for bank protection.

Considering the impact of plant morphogenesis on productivity and the influence of *WOX* family members on embryogenesis, organ formation, and environmentally responsive plasticity, identifying key *WOX* genes affecting various meristem activities in *C. arbutifolia* could provide candidate target genes for molecular breeding aimed at trait improvement or for the molecular screening of high-yield variants. As the *WOX* gene family plays vital roles in processes like plant cell development and tissue regeneration, this study will employ bioinformatics methods to identify and systematically analyze the *WOX* gene family in *C. arbutifolia*. The aim is to provide theoretical support for subsequent genetic transformation and regeneration studies in this species.

Material and methods

Genome-wide Identification of *CaWOX* Genes

The genomic files, GFF3 annotation file, and CDS sequence file for *C. arbutifolia* were downloaded from the NCBI database[27]. Protein sequences of the *A. thaliana* *WOX* gene family were obtained from the TAIR database. A blastp search was performed against all protein sequences from the *C. arbutifolia* genome, and genes with an e-value < 0.001 were selected for preliminary identification. Candidate genes were further manually analyzed and confirmed to contain conserved domains using the CDD database. The chromosomal locations of the identified *WOX* genes were retrieved from the GFF3 file using their gene IDs. The physicochemical properties of the proteins, including relative molecular weight, theoretical isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY), were analyzed using the ExPASy online tool. Subcellular localization was predicted using Cell-PLoc 2.0.

Characterization, Chromosomal Distribution, and Phylogenetic Analysis of *CaWOX* Genes

The protein sequences encoded by *WOX* genes from *C. arbutifolia*, *Salix suchowensis*, *A. thaliana*, and *Populus trichocarpa* were downloaded from the Phytozome database. Multiple sequence alignment of these WOX protein sequences was performed using ClustalW [28] within the MEGA11 software[29]. A phylogenetic tree was subsequently constructed using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates based on the alignment results. The tree was visualized and edited using Evolview.

Gene Structure and Conserved Motif Analysis of *CaWOX* Genes

Gene structure information (exon-intron organization) for the *CaWOX* genes was extracted from the *C. arbutifolia* genome GFF3 file based on gene IDs and visualized. Protein sequences of *CaWOX* members were retrieved using their protein IDs. Conserved motifs were analyzed using the MEME software[30] suite with parameters set to identify 10 motifs, a maximum motif width of 50, and a minimum motif width of 6. The identified motifs were annotated using meme-v4.12.0.

Analysis of Cis-acting Elements and Collinearity Relationships of *CaWOX* Genes

The promoter regions (2,000 bp upstream of the transcription start site) of *CaWOX* genes were extracted. Cis-acting regulatory elements within these promoter sequences were predicted and analyzed using the PlantCARE database and visualized with the GSDS online tool. Interspecific collinearity analysis was conducted among four species (*A. thaliana*, *P. trichocarpa*, *C. arbutifolia*, and *S. suchowensis*) using McscanX. Tandemly duplicated gene pairs within the *CaWOX* family were identified based on screening criteria. The Ka/Ks ratio for each identified tandem duplicate gene pair was calculated using the Ka/Ks Calculator function in TBtools software[31, 32]. A circos plot was generated to illustrate the tandem duplication events[33].

Plant Growth Conditions

Branches were collected from superior individuals of *Chosenia arbutifolia* (Peter Simon Pallas) A. Skvortsov growing in Manjiang Town, Fusong County, Jilin Province, China (41°47′10.55″ N, 127°55′56.13″ E). The specimen materials of Chosenia arbutifolia were stored in the Chinese virtual herbarium （https://www.cvh.ac.cn/index.php）. The specimen number is BJFC00076636 and the identifier is Zhixiang Zhang. The branches were stored and hydroponically cultured in the laboratory of Jiangsu Academy of Forestry until leaves emerged. *C. arbutifolia* were grown in a greenhouse at the Jiangsu Academy of Forestry. The growth conditions were maintained at 23°C, 74% relative humidity, with a photoperiod of 16 hours light / 8 hours dark. After approximately one month of growth, different tissues were harvested. All collected samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from samples using an RNA extraction kit (TIANGEN, Beijing). cDNA was synthesized from the RNA using the Evo M-MLV Reverse Transcription Kit (Accurate Biology, China). Specific primers for the *CaWOX* gene family were designed using Primer5, validated, and the optimal primer pairs were selected for subsequent use. qRT-PCR was performed using SYBR Green Premix Pro Taq HS (Accurate Biology) on a qPCR instrument with the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The reference gene used was PtActin (Accession number: XM-006370951.1). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Three independent biological replicates were performed for each treatment, with three technical replicates for each biological sample.

Results

Identification and Analysis of the *CaWOX* Gene Family

Based on BLASTP search results and CDD domain analysis, a total of 14 *CaWOX* genes were identified in the *C. arbutifolia* genome (Table 1). Following the *WOX* gene nomenclature rules established in Arabidopsis thaliana, these 14 genes were designated *CaWOX1* to *CaWOX14*. The 14 *CaWOX* genes are distributed across 11 chromosomes: Chromosomes 4, 9, 10, 11, 12, 13, 14, 15, and 19 each contain 1 gene; Chromosome 2 contains 2 genes; Chromosome 5 contains the highest number with 3 genes. The CDS lengths of the 14 *CaWOX* genes range from 642 bp (*CaWOX2*) to 1221 bp (*CaWOX9*), encoding proteins with amino acid lengths between 213 and 406 aa. The relative molecular weights of the proteins range from 24,481.4 Da to 45,121.3 Da. Among the 14 CaWOX proteins, 8 have theoretical isoelectric points (pI) between 4.87 and 6.91, classifying them as acidic proteins; 6 have theoretical pI values between 7.66 and 9.99, classifying them as basic proteins. Subcellular localization prediction indicates that all 14 CaWOX proteins are localized to the Nucleus.

Table1 CaWOX Gene Family

GN	GID	CID	C	PL	CDS	MW	PI	NE	SL
CaWOX1	Salix001816	Salix001816.1	Chr02	215	648	24600.6	4.87	3	Nucleus
CaWOX2	Salix002763	Salix002763.1	Chr02	213	642	24481.4	9.7	3	Nucleus
CaWOX3	Salix006034	Salix006034.1	Chr04	391	1176	43212.9	7.86	3	Nucleus
CaWOX4	Salix008073	Salix008073.1	Chr05	250	753	28278.2	5.59	3	Nucleus
CaWOX5	Salix008176	Salix008176.1	Chr05	266	801	30264.7	6.29	3	Nucleus
CaWOX6	Salix009306	Salix009306.1	Chr05	217	654	24731.7	5.66	3	Nucleus
CaWOX7	Salix014938	Salix014938.1	Chr09	245	738	27878	7.66	2	Nucleus
CaWOX8	Salix017014	Salix017014.1	Chr10	309	930	34793.8	9.99	4	Nucleus
CaWOX9	Salix018617	Salix018617.1	Chr11	406	1221	45121.3	7.86	4	Nucleus
CaWOX10	Salix019842	Salix019842.1	Chr12	365	1098	41117.1	6.52	4	Nucleus
CaWOX11	Salix021133	Salix021133.1	Chr13	255	768	28051.9	5.95	2	Nucleus
CaWOX12	Salix021874	Salix021874.1	Chr14	217	654	24976.8	8.48	3	Nucleus
CaWOX13	Salix023427	Salix023427.1	Chr15	383	1152	42941.1	6.91	4	Nucleus
CaWOX14	Salix029936	Salix029936.1	Chr19	235	708	25850.5	5.74	2	Nucleus

NOTE: GN:Gene Name. GID: GeneID. CID: Mrnaid. Chr.PL: length of Pro in AA. MW: Molecular Weights (MV). PI: Protein Isoelectric Points. NE: Number of Exons. SL: subcellular localization.

Phylogenetic Analysis of the *CaWOX* Gene Family

A phylogenetic tree was constructed using 15 *A. thaliana WOX* genes, 18 *P. trichocarpa WOX* genes, 16 *S. suchowensis WOX* genes, and the 14 *CaWOX* genes, as shown in the figure 1. According to the clustering pattern of the *A. thaliana WOX* gene family, the 63 *WOX* genes were divided into three major clades. Clade 1 contains 36 genes, Clade 2 contains 12 genes, and Clade 3 contains 15 genes. *WOX* genes from all four species are distributed across each clade, indicating the relatively conserved evolution of the *WOX* gene family in plants. The 14 *CaWOX* genes are clustered into these clades as follows: Clade 1 contains the largest number (7 members): *CaWOX2, CaWOX5, CaWOX7, CaWOX8, CaWOX10, CaWOX12,* and *CaWOX13*. Clade 2 contains 4 members: *CaWOX3, CaWOX9, CaWOX11*, and *CaWOX14*. Clade 3 contains 3 members: *CaWOX1, CaWOX4,* and *CaWOX6*.

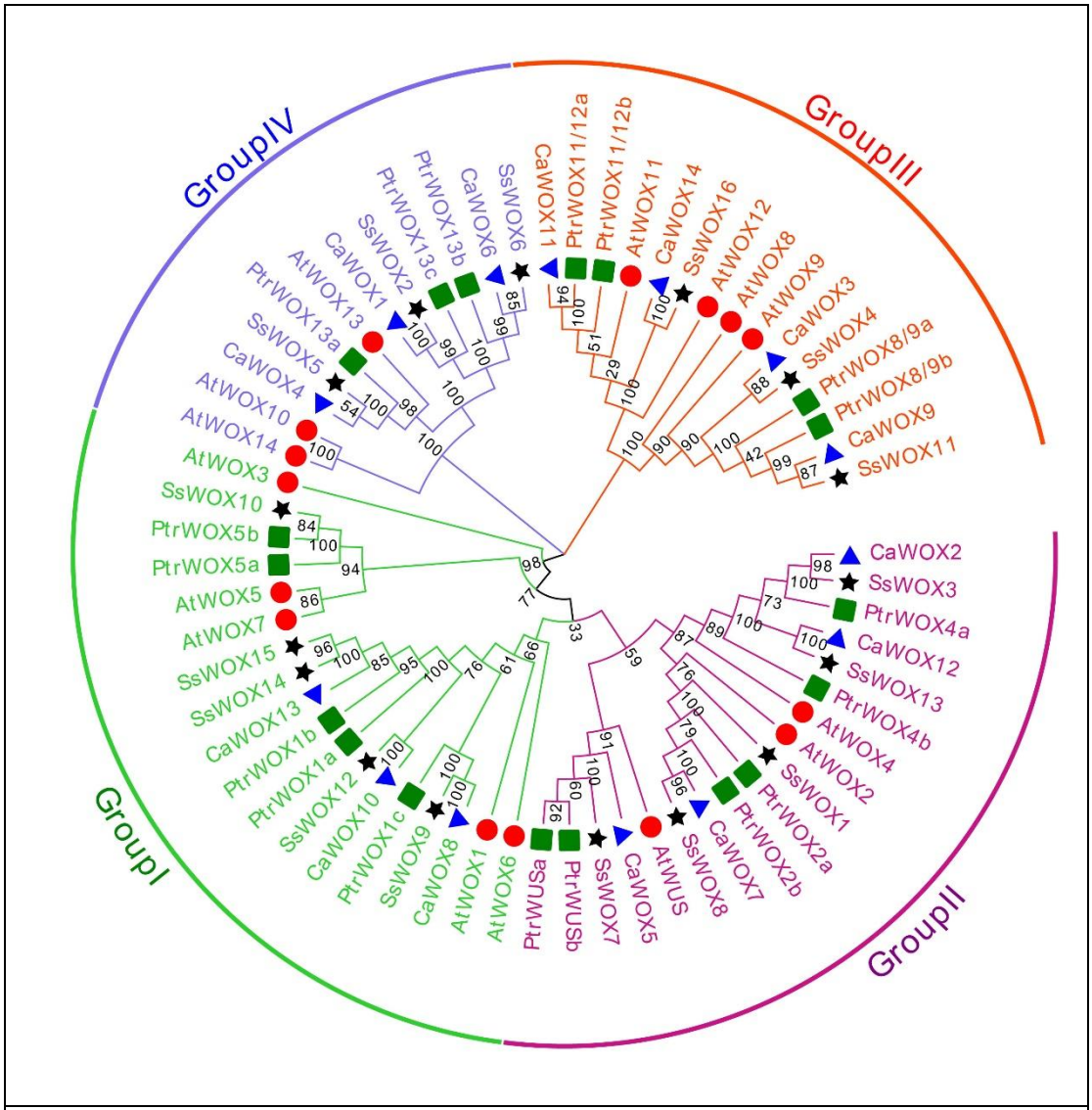


Figure 1. Phylogenetic tree of the *WOX* gene family across different species. The evolutionary relationships among WOX genes from *A. thaliana*, *P. trichocarpa*, *C. arbutifolia*, and *S. suchowensis* are shown.

Conserved Motif Analysis and Gene Structure Analysis of the Gene Family

The results from the MEME software analysis indicate that the number and arrangement of conserved protein motifs within the 14 CaWOX protein sequences exhibit a degree of similarity and conservation (Figure 2). All 14 CaWOX protein sequences contain motif 1 and motif 2. Within Clade 3, the three CaWOX protein sequences share identical types and numbers of conserved motifs: motif 1, motif 2, motif 4, and motif 6. In Clade 1, the seven CaWOX protein sequences show some variation in their conserved motif composition. CaWOX2 and CaWOX12 share

identical motif composition and order: motif 1, motif 2, motif 5, motif 8. CaWOX5 and CaWOX7 share identical motif composition and order: motif 2, motif 3, motif 8. CaWOX10 and CaWOX13 share identical motif composition and order: motif 1, motif 2, motif 8, motif 10. In Clade 2, the conserved motifs also show specific patterns. CaWOX3 and CaWOX9 share identical motif composition and order: motif 1, motif 2, motif 3, motif 7, motif 9. CaWOX11 and CaWOX14 share identical motif composition and order: motif 1, motif 2, motif 3. Analysis of the gene structures (exon-intron organization) of the 14 *CaWOX* genes reveals a relatively high degree of similarity among genes within the same clade. In contrast, notable differences in both motif composition and gene structure are observed between different clades. This suggests that the *CaWOX* genes may have undergone functional differentiation in *C. arbutifolia*, potentially participating in distinct physiological processes within the plant.

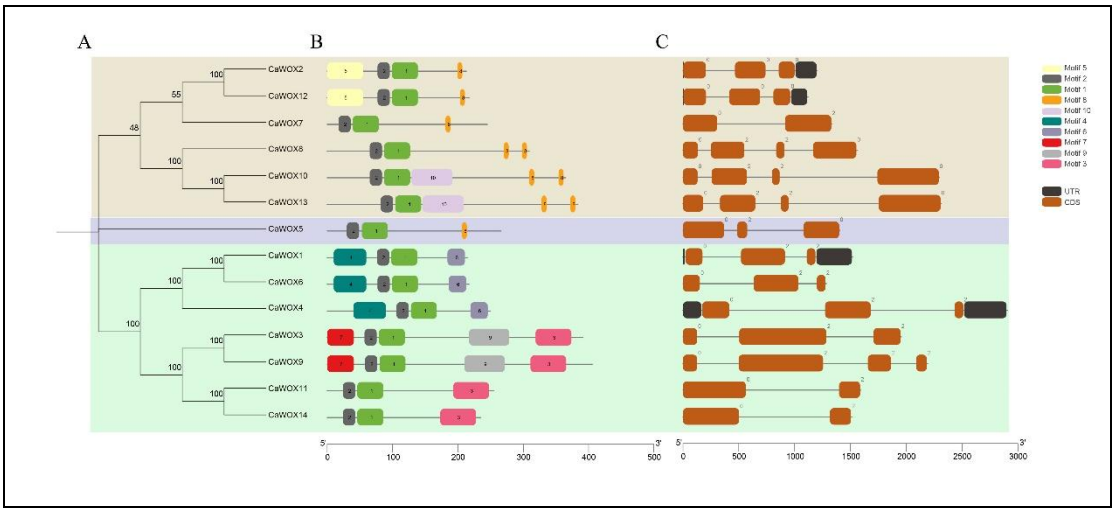


Figure 2. Gene structure analysis of the *CaWOX* gene family. (A) Phylogenetic tree of CaWOX proteins. (B) Gene structure and distribution of conserved motifs. Different colored blocks represent different types of conserved motifs. (C) Schematic of gene structure, where different colored blocks correspond to structural elements such as UTR and CDS.

Analysis of Cis-acting Elements in Gene Promoter Regions

Cis-acting elements located in promoter regions can regulate gene expression by interacting with trans-acting factors. Analysis of these elements can reveal a gene's potential responsiveness to various biotic and abiotic stresses. Examination of the 2,000 bp upstream sequences indicates that the *CaWOX* gene family can be clearly clustered into two distinct evolutionary clades, with significant genetic differentiation

characteristics between them (Figure 3). The first clade includes *CaWOX2, CaWOX12, CaWOX7, CaWOX8, CaWOX10*, and *CaWOX13*. High bootstrap support values, often reaching 100%, among genes within this clade suggest a reliable and close evolutionary relationship. The second clade comprises *CaWOX1, CaWOX4, CaWOX6, CaWOX3, CaWOX9, CaWOX11*, and *CaWOX14*, whose evolutionary relationships appear more stable and conserved. The promoter regions of the *CaWOX* genes contain a rich variety of cis-acting elements with specific distribution patterns, primarily falling into three functional categories. Abiotic Stress Response Elements: These include the low-temperature-responsive LTR, drought-responsive MBS, and dehydration/salt stress-responsive DRE elements. For instance, the *CaWOX1* promoter contains both LTR and DRE elements, while *CaWOX4* and *CaWOX5* promoters are enriched with MBS elements, suggesting these genes may be involved in adaptive responses to multiple abiotic stresses. Hormone Signaling Response Elements: These involve ABA-responsive ABRE, jasmonate-responsive CGTCA/TGACG-motif, and gibberellin-responsive P-box elements. For example, the *CaWOX2* promoter contains both ABRE and CGTCA-motif, and *CaWOX6* contains the gibberellin-responsive P-box, indicating this gene family may participate in physiological process regulation through hormone signaling pathways. Growth and Development-Related Elements: These include meristem expression-related CAT-box and endosperm development-related GCN4_motif. Specifically, the *CaWOX2* promoter contains a CAT-box, and *CaWOX13* contains a GCN4_motif, reflecting the potential roles of some genes in plant growth and differentiation. Further correlation between phylogeny and element distribution reveals that genes within the same evolutionary clade share more similar compositions of cis-acting elements, suggesting that closely related genes tend to have more consistent functional regulation patterns. In contrast, genes from different clades show significant differences in element types, corresponding to distinct functional differentiations. In summary, the evolutionary relationships within the *CaWOX* gene family are well-defined. Through diverse promoter cis-acting elements, these genes are likely broadly involved in abiotic stress responses, hormone signal transduction, and growth regulation. This analysis provides crucial clues for the subsequent targeted

validation of gene functions.

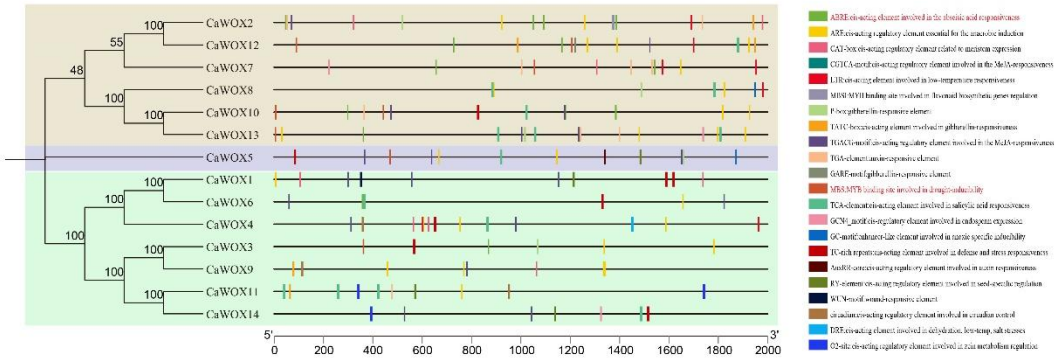


Figure 3. Evolutionary association analysis of cis-acting elements in the promoters of the *CaWOX* gene family. The left panel shows the phylogenetic tree of *CaWOX* genes. The right panel uses colored bars (with the legend indicating the functional categories of the elements, covering hormone response, stress response, growth and development regulation, etc.) to represent the distribution of different cis-acting elements.

Ka/Ks Analysis of the *CaWOX* Gene Family

The results of the Ka/Ks analysis for the 14 *CaWOX* genes showed that all homologous gene pairs have Ka/Ks ratios less than 1, ranging from 0.145 to 0.391 (Supplementary data). The pair *CaWOX1*-*CaWOX6* has the smallest Ka/Ks value (0.145), while *CaWOX11*-*CaWOX14* has the largest (0.391). This indicates that homologous genes within the *CaWOX* family have undergone purifying selection during evolution, with varying intensities of selective pressure across different gene pairs. Regarding sequence characteristics, the effective lengths of homologous gene pairs range from 630 to 1143 bp, with *CaWOX3*-*CaWOX9* having the longest effective length (1143 bp). The average number of synonymous sites (AverageS-sites) ranges from 133.92 to 265.67, and the average number of non-synonymous sites (AverageN-sites) ranges from 493.42 to 877.33. For all gene pairs, the frequency of synonymous substitutions (pS) is higher than that of non-synonymous substitutions (pN), further supporting the role of purifying selection in maintaining the functional stability of *CaWOX* genes.

Collinearity Analysis

Analysis of tandem duplication among the 14 *CaWOX* genes revealed a dispersed distribution pattern (Figure 4A), with genes not clustered on a few chromosomes but rather widely distributed across multiple chromosomes including Chr02, Chr04, Chr11, Chr12, and Chr15. Chr04 appears to be an enriched region, harboring multiple family members such as *CaWOX3, CaWOX6*, and *CaWOX5*. A total of 8 gene pairs were identified as tandem duplications. Significant collinearity relationships were observed across several chromosomes: genes on Chr04 (*CaWOX3, CaWOX6*) show collinearity with genes on Chr15 (*CaWOX13*) and Chr12 (*CaWOX10*). Similarly, *CaWOX9* on Chr11 forms collinear pairs with *CaWOX* genes on chromosomes such as Chr04 and Chr12. These findings indicate that the CaWOX gene family in *C. arbutifolia* has experienced gene duplication events during genome evolution, which is a core mechanism driving the expansion of this gene family. The collinear regions are often located on dispersed, non-homologous chromosomes, suggesting that genes were relocated following duplication events during chromosomal evolution. This dispersed genomic distribution provides a structural basis for the functional differentiation of *CaWOX* genes. In summary, the *CaWOX* gene family in *C. arbutifolia* expanded through duplication events, and its dispersed chromosomal distribution and extensive collinearity relationships provide crucial chromosomal-level evidence for future investigations into the functional specialization and evolutionary origins of this gene family. Interspecific collinearity analysis was performed among the genomes of four species: *P. trichocarpa*, *A. thaliana*, *S. suchowensis*, and *C. arbutifolia* (Figure 4B). The collinearity results for the *CaWOX* gene family were plotted to obtain insights into its evolutionary history across these lineages. The results show a significant positive correlation between phylogenetic relatedness and the degree of collinearity conservation. The densest network of collinear connections is observed between *C. arbutifolia* and *P. trichocarpa*, with multiple chromosomes of *C. arbutifolia* forming numerous collinear pairs with most chromosomes of *P. trichocarpa*. This reflects the highest degree of evolutionary conservation within the *WOX* gene family between these

two species. The number of collinear connections between *C. arbutifolia* and *A. thaliana* is intermediate, reflecting their family-level phylogenetic relationship. In contrast, collinear connections between *C. arbutifolia* and *S. suchowensis* are relatively sparse, which may be attributed to their greater phylogenetic distance and consequently lower gene conservation. Furthermore, *CaWOX* genes can form collinear associations with homologous genes from multiple species, indicating that the core sequences and functions of this gene family have retained a degree of evolutionary conservation throughout plant evolution. In conclusion, this cross-species collinearity analysis not only confirms the evolutionary conservation of the *CaWOX* gene family but also clarifies the influence of phylogenetic relationships on gene collinearity. It provides crucial evolutionary evidence for inferring the potential biological functions of *CaWOX* genes in *C. arbutifolia* by leveraging known functions from model species.

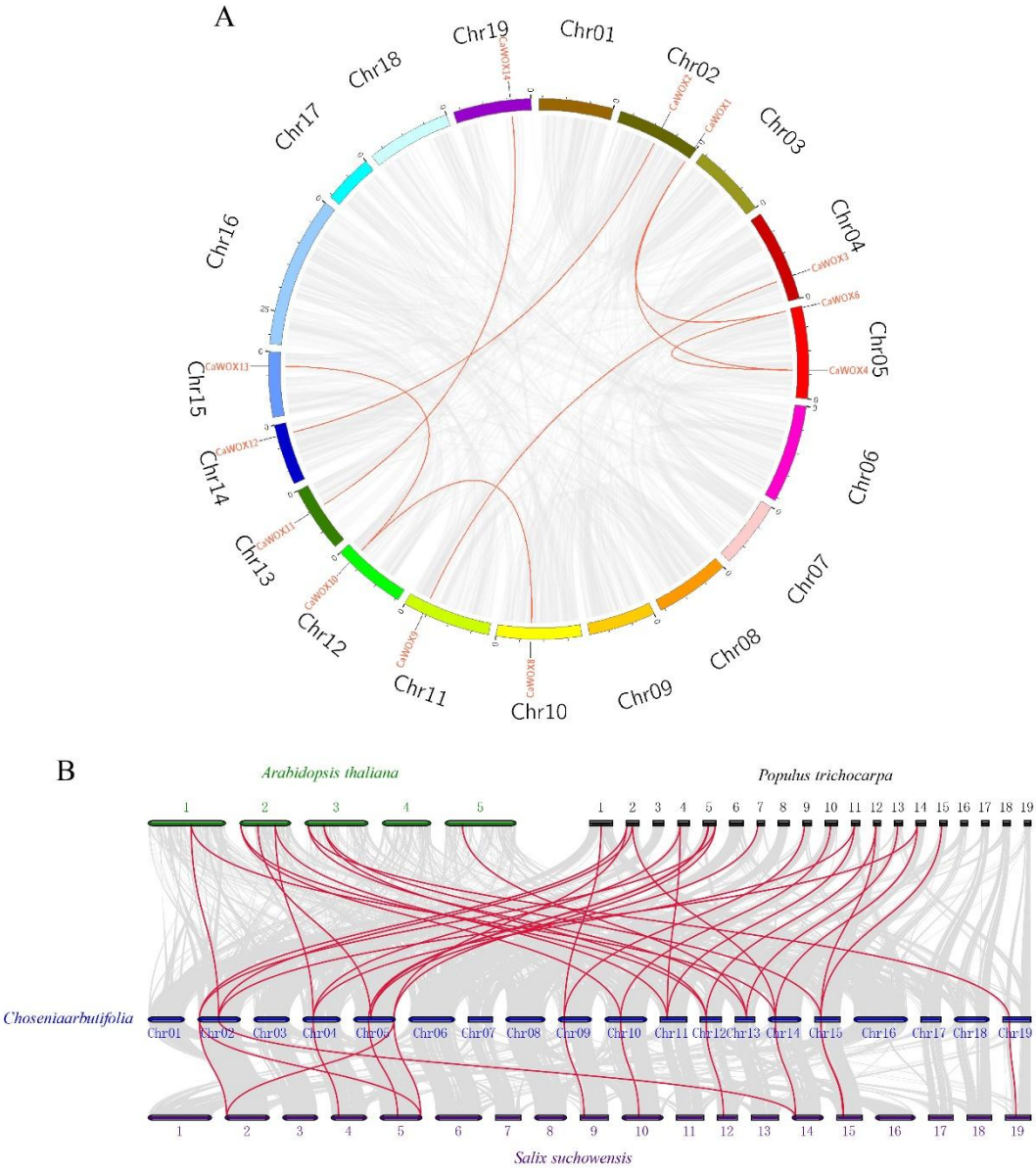


Figure 4. Chromosomal collinearity analysis of the *WOX* gene family. (A) Intra-species collinearity plot among chromosomes of *C. arbutifolia*. (B) Inter-species collinearity plot showing chromosomes of four species: *A. thaliana*, *P. trichocarpa*, *C. arbutifolia*, and *S. suchowensis*. Red connecting lines represent collinear gene pairs between different species, illustrating conserved evolutionary relationships of *WOX* genes across species.

Expression Profiles of *CaWOX* Genes

To investigate the roles of *CaWOX* genes in the growth and development of *C. arbutifolia*, we analyzed their differential expression across various tissues. Quantitative real-time PCR (qRT-PCR) was performed to systematically analyze the

317 expression patterns of 10 *CaWOX* family genes across five different tissues, namely
318 upper stem, lower stem, root, young leaf, and mature leaf (Figure 5). The results
319 revealed that *CaWOX* family genes exhibited significant tissue-specific expression
320 characteristics, with distinct expression profiles of different members during the
321 development of various plant organs. In leaf tissues, *CaWOX3, CaWOX4, CaWOX5,*
322 *CaWOX9*, and *CaWOX11* all showed dominant expression in mature leaves, with their
323 relative expression levels in mature leaves significantly higher than those in other
324 tissues including stems and roots ($P < 0.05$). Among them, *CaWOX3, CaWOX5*, and
325 *CaWOX11* also maintained high expression levels in young leaves, indicating that these
326 genes may be widely involved in the whole process of leaf initiation, development, and
327 maturation in *C. arbutifolia*. In root tissues, *CaWOX1, CaWOX6, CaWOX10*, and
328 *CaWOX14* displayed a prominent root-specific high expression pattern. Their relative
329 expression levels in roots were the highest among all tested tissues, and were
330 significantly higher than those in stem and leaf tissues ($P < 0.05$), suggesting that these
331 four genes play a core regulatory role in root growth, development, and morphogenesis.
332 In stem tissues, only *CaWOX12* exhibited stem-specific dominant expression. The
333 expression level of this gene was the highest in the lower stem, followed by the upper
334 stem, and was significantly downregulated in root and leaf tissues ($P < 0.05$). In contrast,
335 the expression levels of other *CaWOX* family members were generally low in stem
336 tissues, indicating that *CaWOX12* may be a key member of the *WOX* family that
337 mediates stem growth and development. In summary, the expression patterns of
338 different members of the *CaWOX* family are clearly differentiated in a tissue-specific
339 manner, with each member showing unique tissue-specific expression characteristics
340 during the development of roots, stems, and leaves. These findings provide a key
341 expression-level basis for the subsequent functional characterization of *CaWOX* family
342 genes in plant organ morphogenesis.
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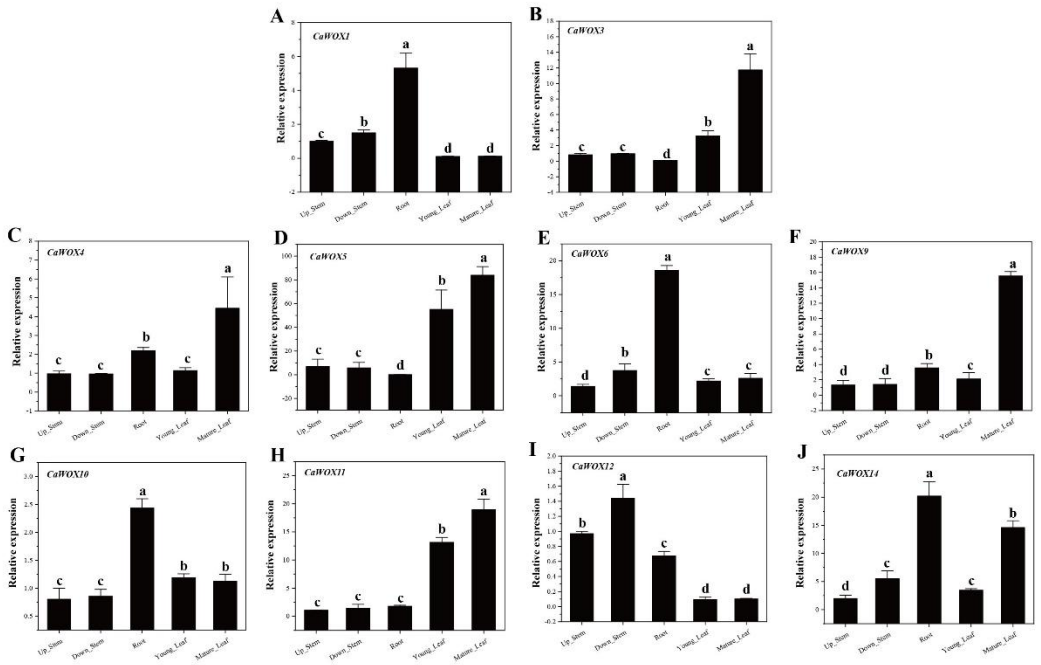


Figure 5. Relative expression levels of *CaWOX* family genes in different tissues of *C. arbutifolia*. The relative expression levels of 10 *CaWOX* family genes were determined in five tissues of *C. arbutifolia*, namely upper stem, lower stem, root, young leaf, and mature leaf. Panels A-J correspond to the tissue expression profiles of *CaWOX1, CaWOX3, CaWOX4, CaWOX5, CaWOX6, CaWOX9, CaWOX10, CaWOX11, CaWOX12*, and *CaWOX14*, respectively. Different lowercase letters above the bars indicate significant differences in the expression level of the same gene across different tissues.

Discussion

The *WOX* gene family represents a class of plant-specific transcription factors characterized by a conserved homeodomain (HD), making it a focal point in plant developmental biology and molecular genetics. As a key regulator in plant growth, development, and stress responses, deciphering its evolutionary patterns, structural features, and expression profiles is fundamental to understanding its function. However, this family had not been previously identified or analyzed in the woody species *C. arbutifolia*. In this study, a BLASTP search against the *C. arbutifolia* database led to the identification of 14 *CaWOX* genes. Comparative analysis shows that while the number of *WOX* genes varies across species. 15 in *A. thaliana*, 13 in rice, 18 in flax, and 12 in grape. the overall count remains relatively stable[11]. This suggests a high degree of evolutionary conservation within the *WOX* family across the plant kingdom.

Variations in protein physicochemical properties hint at potential functional diversification[34, 35]. The *CaWOX* members exhibit significant differences in amino acid length (213–406 AA) and molecular weight (24,481.4–45,121.3 Da). For instance, *CaWOX9* (406 AA) has a substantially higher molecular weight than *CaWOX2* (213 AA), a disparity that may correspond to the gain or loss of functional domains. The range in isoelectric points (pI 5.59–9.99) could influence protein stability within the nucleus and its affinity for DNA or other interacting proteins. For example, the relatively basic *CaWOX2* (pI=9.7) and the acidic *CaWOX4* (pI=5.59) may function in different pH microenvironments or distinct regulatory pathways. Subcellular localization predictions indicate that all *CaWOX* proteins are localized to the nucleus, which is entirely consistent with their core function as transcription factors. This nuclear localization ensures their capacity to bind DNA regulatory regions, forming the structural basis for their transcriptional activity and further underscoring the evolutionary conservation of this family's function.

The core structural feature of the *WOX* family is the HD domain, composed of 60–66 amino acids responsible for binding DNA regulatory elements. Beyond this, members can be further classified based on additional domains such as the WUS-box, EAR repression motif, and VLWV motif[36-38]. Phylogenetically, the family is typically divided into three clades: the ancient clade, found in basal plants like mosses and ferns; the Intermediate clade, exclusive to angiosperms; and the WUS clade, also angiosperm-specific and containing distinctive domains like the WUS-box. This structural differentiation among clades lays the groundwork for their functional diversity. Our phylogenetic analysis, which included *WOX* genes from *P. trichocarpa* and Arabidopsis thaliana, investigated the origin and evolution of *CaWOX* genes. The cross-species phylogenetic tree grouped *CaWOX* genes with those from *A. thaliana*, *P. trichocarpa*, and *Eutrema salsugineum* into distinct evolutionary groups with high bootstrap support. This reflects both the evolutionary conservation of the *WOX* family in higher plants where conserved core domains like the HD maintain basic regulatory functions and the species-specific expansion of *CaWOX* in *C. arbutifolia*. Intra-species collinearity analysis in *C. arbutifolia* revealed collinear gene pairs across multiple

chromosomes, confirming that segmental or whole-genome duplication events are the primary drivers of *CaWOX* family expansion. The subsequent dispersed chromosomal distribution of these duplicated genes provides a genomic structural basis for their functional divergence.

Gene structure and conserved motif analyses further support the functional correlations implied by phylogenetic grouping[39, 40]. Genes within the same evolutionary clade share highly similar motif compositions and exon-intron structures. Motif1 corresponds to the conserved HD, the core element for DNA binding and transcriptional regulation, ensuring basic functional consistency within the clade. Unique motifs and differential exon distributions likely serve as structural markers of functional divergence following gene duplication. These unique motifs may participate in novel protein interactions or regulatory networks, enabling the *CaWOX* family to adapt to specific growth requirements of *C. arbutifolia*. Variations in exon number directly reflect structural diversification. Some members contain only 2 exons, while others like *CaWOX10* and *CaWOX9* possess 4 exons. This variation may be related to alternative splicing events or domain reorganization, where a greater number of exons could confer increased structural diversity, allowing participation in more complex regulatory networks.

Analysis of the promoter regions of the *CaWOX* gene family revealed enrichment for three core categories of cis-acting elements. Hormone-responsive elements, such as ABA-responsive ABRE, jasmonate-responsive CGTCA/TGACG-motif, and gibberellin-responsive P-box, indicating broad involvement in plant hormone signaling pathways. The presence of ABRE elements in the promoters of *CaWOX1* and *CaWOX4* suggests potential roles in responding to stresses like drought via ABA signaling. Abiotic stress-responsive elements, including low-temperature-responsive LTR, drought-responsive MBS, and dehydration/salt-responsive DRE, highlighting the potential function of *CaWOX* genes in stress adaptation in *C. arbutifolia*. The presence of MBS elements in *CaWOX5* and *CaWOX3* promoters aligns with recently reported drought-resistance functions of the *WOX* family. Growth and development-related elements, such as the meristem-specific CAT-box and the endosperm development-

related GCN4_motif, corroborating the classic growth-regulatory roles of the *WOX* family. For example, CAT-box elements in the promoters of *CaWOX2* and *CaWOX12* closely match their high expression in root meristems, suggesting a role in root meristem maintenance.

Correlating phylogeny with element distribution shows that *CaWOX* genes within the same evolutionary clade share more consistent compositions and distributions of promoter cis-elements[41, 42]. For instance, *CaWOX1, CaWOX4*, and *CaWOX6*, clustered in one clade, all contain both hormone-responsive and stress-responsive elements, indicating conserved regulatory pathways among evolutionarily related genes. In contrast, genes from different clades show significant divergence in element types, the *CaWOX2* clade is enriched for meristem-related CAT-box elements, while the *CaWOX3* clade contains more stress-responsive elements. This differentiation corresponds to functional specialization, an outcome of gene duplication adapting to diverse regulatory demands. The diversity of promoter cis-elements further explains the functional complexity of the *CaWOX* family. For example, the *CaWOX12* promoter contains both CAT-box and LTR elements, implying a dual role in root meristem development and cold stress response, showcasing its potential for "growth-stress response" coordination. The specific distribution of these elements within the promoter region may influence signal sensitivity and regulatory efficiency. The characteristics of *CaWOX* promoter cis-elements not only reflect the relationship between evolutionary conservation and functional divergence but also reveal the family potential as a regulatory hub connecting growth and development with stress response and integrating hormonal, stress, and growth signals to build a multi-dimensional regulatory network, providing a molecular foundation for *C. arbutifolia* to adapt to complex environments and coordinate growth.

Gene duplication events, including tandem and segmental duplications, are closely associated with gene family expansion and evolution[43-45]. The evolution of the *WOX* family follows a pattern of species differentiation and gene duplication". Lower plants possess only 1–2 *WOX* members, while higher angiosperms show significant expansion, driven primarily by whole-genome and segmental duplications. The expansion of the

Arabidopsis *WOX* family is linked to ancient hexaploidization events, and the collinear characteristics of poplar *WOX* genes also confirm the role of duplication. Concurrently, highly conserved homologous relationships exist between *WOX* families of different species, reflecting their evolutionary conservation. In this study, the 14 *CaWOX* genes are unevenly distributed across 11 chromosomes. The clustering of *CaWOX1/2* on Chr02 and *CaWOX4/5/6* on Chr05 aligns with the conclusion that "gene duplication drives family expansion": members on the same chromosome likely originated from segmental duplication events, subsequently diverging in physicochemical properties and exon structures to form specialized functions, adapting to the regulatory needs of different developmental stages or environmental conditions in *C. arbutifolia*. Regarding tandem duplication, we identified 8 pairs of duplicated genes within the *CaWOX* family in the poplar genome, indicating that gene duplication has provided impetus for *CaWOX* evolution, promoting the evolutionary process of these genes in *C. arbutifolia*. Interspecific collinearity analysis with Arabidopsis, rice, and *S. suchowensis* genomes revealed significant differences in the number of tandemly duplicated genes between the CaWOX family and WOX families of other species. Notably, the collinear connections between *C. arbutifolia* and *P. trichocarpa* were the most dense, while connections with Arabidopsis were relatively sparse. This pattern is highly consistent with phylogenetic relatedness, indicating that the conservation of WOX genes decreases with increasing phylogenetic distance. Based on this, known functions of WOX genes in closely related species like *P. trichocarpa* can be used to infer the core regulatory roles of *CaWOX* genes, providing targeted references for subsequent functional validation.

The WUSCHEL-related homeobox family is a plant-specific family of homeobox transcription factors that play critical roles in core biological processes, including meristem homeostasis maintenance, organ morphogenesis, and temporal regulation of growth and development in plants[2]. In this study, we characterized the spatial expression profiles of 10 *CaWOX* family genes across root, stem, and leaf tissues of *C. arbutifolia*, which exhibited highly tissue-specific expression patterns. *CaWOX3, CaWOX4, CaWOX5, CaWOX9*, and *CaWOX11* showed high expression levels in leaf

tissues, with expression patterns highly consistent with the reported functions of their homologous genes. *WOX* family members are well-recognized key regulators of plant leaf development[46]. In Arabidopsis thaliana, *AtWOX3* determines leaf margin morphogenesis by regulating the activity of marginal meristems, while *AtWOX9* is involved in leaf primordium initiation and the regulation of cell proliferation in young leaves[13]. In the present study, the expression levels of the above five genes in mature leaves were significantly higher than those in other tissues, and *CaWOX3*, *CaWOX5*, and *CaWOX11* also maintained high expression levels in young leaves. This result suggests that these *CaWOX* genes are not only involved in the early developmental processes of *C. arbutifolia*, including leaf primordium initiation and cell proliferation and differentiation in young leaves, but may also function in maintaining the physiological functions of mature leaves. Given the biological characteristics of *C. arbutifolia* as a deciduous tree species, mature leaves serve as the core organs for photosynthetic carbon fixation and stress response in the plant. Therefore, in addition to regulating leaf morphogenesis, these leaf-specific *CaWOX* genes may also participate in maintaining photosynthetic homeostasis and mediating abiotic stress responses in mature leaves by regulating the expression of downstream target genes. This finding provides a new direction for subsequent studies on their application value in improving photosynthetic efficiency and stress resistance breeding of forest trees. *CaWOX1, CaWOX6, CaWOX10,* and *CaWOX14* exhibited root-specific high expression, which is highly consistent with the conserved regulatory function of the WOX family in root development. The root system is the core organ of forest trees for water and nutrient uptake, as well as the perception of soil environmental signals, and WOX transcription factors are key regulators for maintaining root apical meristem homeostasis and the initiation of lateral and adventitious roots[47, 48]. Studies in model plants have confirmed that *AtWOX11* and *AtWOX12* synergistically regulate adventitious root initiation, and *AtWOX5* sustains continuous root growth by maintaining cell viability in the root apical quiescent center[49-51]. In this study, the expression levels of these four *CaWOX* genes in roots were significantly higher than those in aboveground tissues, indicating that these genes are core regulators of root

development in *C. arbutifolia* and may determine root architecture by modulating root apical meristem activity and lateral root formation. Notably, the root developmental status of *C. arbutifolia* directly determines the drought and barren tolerance of the plant. These root-specific *CaWOX* genes are not only regulators of root morphogenesis, but may also serve as key targets mediating stress adaptation in forest trees. This is consistent with our previous finding that *WOX* family genes are involved in drought response from transcriptome analysis of drought-stressed *C. arbutifolia*, and provides core candidate genes for subsequent dissection of the synergistic regulatory mechanism between root development and stress resistance in forest trees.

In stem tissues, only *CaWOX12* exhibited stem-specific dominant expression, with a significantly higher expression level in the lower stem than in the upper stem. This expression pattern is highly matched with the secondary growth characteristics of woody plants. Unlike herbaceous plants, the stems of woody plants undergo continuous secondary growth, a process in which vascular cambium activity drives xylem and phloem differentiation and ultimately wood formation[52, 53]. Furthermore, the lower stem of the plant has a more vigorous secondary growth process and a higher degree of lignification. Previous studies have shown that *WOX* family members play important regulatory roles in vascular development and secondary cell wall biosynthesis in woody plants. For example, *PtoWOX4* in Populus regulates secondary growth by modulating vascular cambium activity[1, 54]. In this study, *CaWOX12* was specifically highly expressed in the lower stem but had extremely low expression levels in roots and leaves, suggesting that this gene may be a key transcription factor regulating stem secondary growth, vascular tissue differentiation, and lignification in *C. arbutifolia*. This result provides an important research target for subsequent dissection of the molecular mechanism of wood formation and molecular breeding for wood property improvement in Salicaceae tree species.

The spatial expression patterns of different *CaWOX* family members in *C. arbutifolia* showed significant functional differentiation, and such tissue-specific expression characteristics are a core manifestation of subfunctionalization during the evolution of the gene family[55, 56]. During long-term evolution, different copies of

the *CaWOX* family acquired tissue-specific cis-regulatory elements, thus differentiating into specific functions that separately regulate the development of root, stem, and leaf organs, and forming a transcriptional regulatory network with clear functional division and synergistic regulation of plant growth and development[57, 58]. The tissue expression characteristics of the *CaWOX* family identified in this study lay a solid foundation for subsequent functional validation of these genes via technologies such as gene editing and genetic transformation, and also provide an important theoretical basis for the regulation of growth and development, as well as molecular breeding for stress resistance and wood property improvement of *C. arbutifolia* and even other Salicaceae trees.

Conclusion

In this study, 14 members of the *CaWOX* gene family were identified in *C. arbutifolia*, and the evolutionary characteristics and functional differentiation patterns of this family were systematically elucidated. Phylogenetic analysis classified the CaWOX proteins into four conserved subgroups, and members within the same subgroup exhibited highly similar gene structures and conserved motifs, confirming subfunctionalization during the family's evolution. Collinearity analysis verified that whole-genome duplication constituted the core driving force for gene expansion in this family; furthermore, the *CaWOX* family displayed extremely high conservation among closely related species in Salicaceae, but weak collinearity with *Arabidopsis thaliana*, reflecting species-specific evolution after the divergence of monocotyledons and dicotyledons. Cis-element analysis revealed that the promoter regions of these genes were rich in elements associated with hormone response, stress response, and meristem regulation, indicating their dual functions in regulating organ development and stress adaptation. Tissue expression analysis further uncovered distinct functional differentiation: *CaWOX3/4/5/9/11* were predominantly expressed in leaves and participated in leaf development and physiological homeostasis maintenance; *CaWOX1/6/10/14* exhibited root-specific high expression and regulated root growth and development; *CaWOX12* was specifically highly expressed in stems and mediated

stem secondary growth and lignification. This study lays a solid foundation for subsequent functional verification of *CaWOX* family genes in *C. arbutifolia*, and also provides vital theoretical support and high-quality gene resources for growth regulation, wood property improvement, and stress-resistant molecular breeding of Salicaceae trees.

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Author contributions

P.W. designed the experiments and drafted and prepared the manuscript. J.Z., R.H., W.W., and H.W. reviewed and edited the manuscript. D.S. and X.H. conceived and funded the study. All authors read and approved the final version of the manuscript.

Competing Interests

The authors declare no known competing financial interests or personal relationships that could influence the work reported in this paper.

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Not applicable.

Abbreviations

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data will be made available on request.

Data availability

The genomic data supporting this study are publicly available to ensure accessibility and reproducibility. Genome sequences of *C. arbutifolia* and *S suchowensis* can be accessed in the NCBI database under accession number ASM2516995v1 and ASM2903076v1. Reference genome sequences and annotations for *A. thaliana*, *P. trichocarpa*, were obtained from Phytozome V14(https://phytozome-next.jgi.doe.gov/info).

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