

Transcriptome-wide identification and characterization of WUSCHEL-related homeobox (WOX) gene family in *Pinus yunnanensis*

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Abstract

WUSCHEL-related homeobox (WOX), a specific gene family in plants, plays a critical role during stem cell regulation, plant regeneration and upgrowth. However, our understanding of WOX functions in conifers is limited compared to angiosperms. To address this gap, we investigated the presence, expression profiles and protein characteristics of WOX gene in *P. yunnanensis*. Our findings revealed that 10 *PyWOX* genes were dispersed across three existing clades, and their expression profiles were presented in specific developmental stages and tissues. The ancient-clade members (*PyWOX13*, *PyWOXG*, *PyWOXA*) exhibited constitutive expressions in the majority of tissues and developmental stages, indicating that they are the oldest and conserved WOX genes. Members of the intermediate-clade (*PyWOXB*, *PyWOXE*) were primarily expressed during callus formation and seed germination, suggesting a role in promoting embryogenesis and plant regeneration. Most members of WUS-clade (*PyWUS*, *PyWOX3*, *PyWOX4*, *PyWOX5*, *PyWOXX*) showed high transcripts level in cluster buds, which may be related to stem cell regulation and the formation of axillary meristems. The self-activation assay demonstrated that *PyWOX4* has transcriptional activation activity. Our study also suggested that there were highly conserved and clear orthologs of WOX genes present in *Pinus*. Together, these findings provide a foundation for further clarifying the function and regulatory mechanism of WOX genes in *P. yunnanensis* growth and development.

1 Introduction

WUSCHEL-related homeobox (WOX) is a critical transcription factor (TF) family responsible for regulating stem cell niche in plants, playing a crucial role in plant growth and development, including embryogenesis and development, cell division and differentiation, morphogenesis, and stress response [1–5]. The members of the WOX family feature a classic and conserved homeodomain (HD), which includes a “helix-loop-helix-turn-helix” structure consisting of 60–61 amino acids [6]. Additionally, the “helix-turn-helix” motif binds to specific downstream region of target genes to regulate their transcriptional expression [7]. The highly conserved HD across species is essential for maintaining the integrity of WOX genes and plant growth [8]. Fifteen genes of WOX family in *Arabidopsis thaliana* have been identified, divided into three clades according to their evolutionary relationship in plants [2, 3]. Among them, ancient-clade members (*AtWOX10*, *AtWOX13*, *AtWOX14* and their orthologous) are found in the earliest differentiated green plants, such as green algae and moss. Intermediate-clade members (*AtWOX8*, *AtWOX9*, *AtWOX11*, *AtWOX12* and their orthologous) exist in vascular plants. The modern clade, also known as WUS-clade (*AtWUS*, *AtWOX1-7* and their orthologous), is found exclusively in ferns and seed plants. Studies have shown that the members of WUS-clade contain a WUS-box (T-L-x-L-F-P) in the downstream of HD, and the expression profiles of WOX genes suggesting that genes with a WUS-box have overlapping and redundant functions in stem cell regulation and meristem differentiation [2]. Moreover, WUS, WOX5 and WOX7 have an EAR-motif (L-x-L-x-L) near the C-terminal of sequence, which may perform transcriptional inhibition function and play a critical role in certain periods of plant upgrowth [4].

At present, functions of the WOX family on plant growth and development have been extensively studied in angiosperms such as *Arabidopsis*, *petunia hybrida*, *Zea mays*, *Oryza sativa* and *Populus* [2, 3, 5, 9, 10]. All the detected WOXs have shown specific expression profiles spatially and temporally, and most of them interact with phytohormones to regulate plant growth and development [1, 2, 5, 7, 9]. In *Arabidopsis*, *AtWUS* is expressed in the organizing center (OC) of the shoot apical meristem (SAM) and regulates the balance between proliferation and differentiation of stem cells in SAM by establishing a negative regulatory loop with *CLAVATA*, which has been demonstrated to be functionally conserved in other species [11–14]. Meanwhile, *AtWOX5* maintains the homeostasis of stem cells in root apical meristem (RAM) under the CLE40-WOX5 regulatory loop, indicating that the regulatory mechanism of meristematic stem cells homeostasis is considered evolutionally conserved for some WOX genes [15, 16]. *AtWOX1* is preferentially expressed in the primary vascular bundle of cotyledon and juvenile leaf, and is involved in leaf development [17]. *AtWOX3/PRS* is expressed around SAM, which regulates the development of lateral organs such as leaf primordia and floral organs [18, 19]. *AtWOX4* is involved in the regulation of cambium cell division and differentiation in developing root and stem through the TDIF-TDF signaling pathway [20, 21]. *AtWOX2* promotes embryogenesis by auxin transporter PIN1, while *AtWOX8* and *AtWOX9* have overlapping functions that improve embryonic development [2, 22]. *AtWOX11* and its homolog *AtWOX12* are induced in root cambium, contributing to the transformation of the procambium or its adjacent parenchyma cells into root initiation cells, and ultimately promoting the formation of callus and adventitious root [23, 24]. *AtWOX13* and *AtWOX14* may prevent their premature organs differentiation [2].

However, most studies have been widely focused on model plants, especially in angiosperms. It remains unclear whether the expression profiles of the WOX family during growth and development in model plants may apply to conifers. There may be many differences and unknowns between gymnosperms and angiosperms. Recently, some studies in conifers have reported that *PaWOX2* and *PaWOX8/9* are intensively expressed in zygotic and somatic embryos of *Picea abies* during embryonic development [25–27]. This finding suggests that PAT-PIN-WOX plays an ancient and conserved role in the embryonic development of spermatophytes [25]. Moreover, somatic embryogenesis and organogenesis of *Pinus pinaster* and *Arabidopsis* were enhanced by overexpression of *PpWOX2*, possibly by affecting

the expression of LEC1 and WUS [28]. *PaWOX3* is expressed specifically in the cotyledon and its adjacent area, *PaWOX3-RNAi* lines lead to abnormal growth at the lateral margin of the cotyledon and young needle [29]. Previous reports have found that WOX3 has a similar function in ensuring lateral organ growth in *Gnetum gnemon*, *Pinus sylvestris*, and *P. pinaster* as well, which seems to be conserved in spermatophytes [30].

Pinus yunnanensis, one of the primarily silvicultural conifers with high economic and ecological value in southwestern China, has strong adaptability and vigorous growth, providing forest products and sustaining the stability of the regional ecosystem. In consideration of the important role of WOX family in plant growth and development, the identification and characterization of the WOX gene family members in *P. yunnanensis* are presented. Ten *PyWOX* genes were screened and cloned, and three clades were divided by phylogenetic analysis. Their physicochemical characteristics and protein structures were analyzed. Then, the expression profiles for each of the *PyWOXs* were detected and analyzed in different tissues and developmental stages. Furthermore, the transcriptional self-activation assay of *PyWUS*, *PyWOX3* and *PyWOX4* were performed. These results supplement and provide more information on the WOX family in conifers, elucidating the similarities and differences in underlying mechanisms of plant growth. Our findings also provide a basis for further functional studies of WOX genes in conifers

2 Materials and Methods

2.1 Plant materials and growth

The seeds of *P. yunnanensis* were treated with 0.5% KMnO_4 solution for 30 minutes and then soaked in water for 48 hours to improve germination. They were transferred to a wet napkin in tissue culture flasks and maintained at 26°C with a 16 h-light/8 h-dark cycle. Materials from different developmental stages and tissues were collected according to Alvarez et al. [1]. Germinated seeds with radicle between 1 and 2 cm were selected. Stem apex, cotyledons, stems and roots of three-week-old seedlings were isolated. Callus induced from cotyledons and cluster buds induced from stem apex were conserved by our laboratory. Each treatment contained three biological replicates, and every replicate had 10–15 samples. All isolated materials were frozen in liquid nitrogen and stored at -80°C until the following experiment (Fig. 1).

2.2 Identification and cloning of the *PyWOX* genes family

Firstly, the WOX protein sequences of *Arabidopsis* as query sequences were downloaded from the TAIR (<https://www.arabidopsis.org/>), and the raw HMM model file of homeodomain (WOX) (PF00046) was obtained from Pfam (<http://pfam.xfam.org/>). Then, BLASTp and HMM searches in TBtools were performed to identify potential WOX protein sequences in *P. yunnanensis* transcriptomes derived from previously determined three quality grades (PRJNA1065384), nitrogen and phosphorus application (PRJNA1086820), different shading environment (PRJNA1097002) and cytokinin application (PRJNA108856). Combining these two methods, candidate WOX sequences were then identified and screened by HMMscan (<https://www.ebi.ac.uk/Tools/hmmer/>) and NCBI-CDD (<https://www.ncbi.nlm.nih.gov/cdd/>) to ensure they contained a full homeobox domain or homeodomain, after excluding redundant and domain-lacking sequences.

The cloned candidate *PyWOX* sequences were searched using NCBI-ORFfinder (<https://www.ncbi.nlm.nih.gov/cdd/>) to confirm their coding sequence (CDS). Specific amplification primers for CDS were designed by SnapGene 3.2 with the restriction endonuclease sites of *BamH*I and *Xho*I (Table S1). The full-length CDS of *PyWOX* were obtained using 2 × Phanta Max Master Mix (Vazyme, China) and purified using FastPure Gel DNA Extraction Kit (Vazyme). The PCR products were ligated into pET30a + vector (Solarbio, China) by ClonExpress II One Step Cloning Kit (Vazyme) and then transferred to *E. coli* DH5α cell (Vazyme). The recombinant bacterial solution was coated on LB solid medium (containing antibiotic Kanamycin) and cultured inverted at 37°C for 16 h. The positive monoclonal colony was selected, and its band was detected using bacterial solution PCR (primers are shown in Table S1), followed by sequencing by Sangon Biotech (Shanghai, China). The results were assembled by Lasergene 7.10 software (DNASTar, USA) and then compared with transcriptome data and homologous sequences of other species using BioEdit software (Borland, USA).

2.3 phylogenetic analysis of the *PyWOX* genes family

Multiple sequence alignment for representative WOX protein sequences from algae (*Ostreococcus tauri*, *O. lucimarinus*), moss (*Physcomitrella patens*), ferns (*Selaginella moellendorffii*, *Ceratopteris richardii*, *Cyathea australis*), gymnosperms (*Gnetum gnemon*, *P. abies*, *P. pinaster*, *P. taeda*), angiosperms (*A. thaliana*, *Z. mays*, *P. trichocarpa*, *Vitis vinifera*) was carried out using MAFFT 7.5 (all protein sequences are referred to Alvarez et al.) (Fig. S1). To obtain the unrooted phylogenetic tree and reduce noise, only the parts of conserved domain (Fig. S2) were constructed using MEGA X by Maximum Likelihood (ML) method with 500 bootstraps and Jones-Taylor-Thornton (JTT) model. The tree was beautified using iTOL online tool (<https://itol.embl.de/>). The full protein sequences in the WUS-clade of

P. abies, *P. pinaster*, *P. taeda*, *P. tabuliformis*, *Arabidopsis*, *P. trichocarpa* and *PyWOXs* were used to construct a Maximum Likelihood tree using all site and JTT model with 500 bootstraps (Fig. S3).

2.4 Characterization of *PyWOX* genes and proteins

The ProtParam tool in ExPASy (<https://www.expasy.org/>) was used for the physicochemical characteristics analysis. Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>) and WoLF PSORT Prediction (<https://psort.hgc.jp/>) were used to predict the subcellular localization of *PyWOX* genes. To characterize the conserved domain and motifs of *PyWOX* proteins, NCBI-CDD and MEME (<https://meme-suite.org/meme/>) were performed. The structure of *PyWOX* sequences were visualized and displayed by TBtools and Jalview. PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred>) and SWISS-MODEL (<https://swissmodel.expasy.org/>) were executed for the prediction of protein secondary and tertiary structure, respectively.

2.4 Expression analysis of the *PyWOX* genes (RT-qPCR)

The RNAprep pure plant kit (TianGen, China) was used to extract total RNA from samples, following the manufacturer's instructions. The concentration and purity of RNA were qualified by NanoDrop 2000 (Thermo Fisher Scientific, USA), and its integrity was examined by 1% agarose gels. 1 ug of each RNA was reverse transcribed using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China) as described by the protocol. The expression level of the target genes was measured with Taq Pro Universal SYBR qPCR Master Mix (Vazyme) and performed using Rotor-Gene Q 5plex Platform (QIAGEN, Germany). All primers were designed in Oligo 7.0 and *Tubulin* was used as a reference gene (Table S1). The reaction mixtures were prepared according to the kit's description and protocol as follows: 95°C 2 min, 40 cycles of 95°C for 5 sec, 60°C for 5 sec, and 72°C for 25 sec, with each sample containing three biological replicates. The relative abundance of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method [31]. The genes expression level in different tissues were expressed as mean normalized expression values $\pm$ standard error (SE) and determined by ANOVA analysis using GraphPad prism 9.5 (GraphPad, USA).

2.5 Transcriptional activation activity assay

To examine the transcriptional self-activation activity of *PyWUS*, *PyWOX3*, and *PyWOX4*, the CDS of *PyWUS*, *PyWOX3*, and *PyWOX4* were cloned into pGBKT7 (BD) to produce BD bait vectors. *BamH I* and *EcoR I* were selected as the restriction endonuclease sites and specific primers used for vector construction are shown in Table S1. The empty pGBKT7 vector was used as a negative control. The constructed BD vectors and empty pGADT7 (AD) were co-transformed into AH109 yeast strain (Weidi Biotec., China), which were then screened on DDO medium (SD/-Trp-Leu) at 28°C with 48–96 h. The positive single yeast colony with 2–3 mm was suspended in ddH₂O until OD₆₀₀ reached 0.2. The yeast solution was diluted to OD₆₀₀ (10^{-1} , 10^{-2} , 10^{-3}), and then added to DDO medium and QDO medium (SD/-Trp-Leu-Ade-His), respectively. Finally, the plates were inverted placed in a dark incubator at 28°C for 72 h.

3 Results

3.1 Identification and phylogenetic analysis of the *PyWOX* genes family

Ten candidate *PyWOX* genes with intact CDS and conserved homeodomain were identified after searching and screening by HMM and BLASTp in transcriptomes. To further confirmed that they're not pseudogenes, the CDS of candidate *PyWOX* genes were cloned by RT-PCR and sequenced using Sanger sequencing (Fig. S4). We found clear orthologues of *P. pinaster* and *P. taeda* that were designated as *PyWUS*, *PyWOX3*, *PyWOX4*, *PyWOX5*, *PyWOXX*, *PyWOXB*, *PyWOXE*, *PyWOX13*, *PyWOXG* and *PyWOXA*, respectively. All sequences and accession number for all *PyWOXs* identified in this study can be found in Table S2. To understand and analyze the evolutionary relationship between *PyWOXs* and *WOXs* in other species, a multiple sequence alignment and phylogenetic tree of *PyWOXs* were performed with the 14 representative plants from algae, moss, fern, gymnosperm, angiosperm (Fig. 2). The results showed that the 10 *PyWOX* genes are divided into three evolutionary clades. The WUS-clade, which consisted of 5 members (*PyWUS*, *PyWOX3*, *PyWOX4*, *PyWOX5*, *PyWOXX*). The intermediate-clade, which consisted of 2 members (*PyWOXB*, *PyWOXE*). The ancient-clade, which consisted of 3 members (*PyWOX13*, *PyWOXG*, *PyWOXA*). All *PyWOX* genes had a closely genetic relationship with their orthologues of conifers.

Sequence alignment was performed by comparing the WUS-clade members of *P. pinaster*, *P. taeda*, and *P. tabuliformis*. The analysis showed that *PyWOXs* and their orthologues in *Pinus* had high similarity in nucleotide sequences (Fig. S5) and polypeptide sequences (Fig. S6), comprising the highly conserved HD characteristic of *WOX* family members. *PyWOXX* and its orthologues constituted a monophyletic group that was only found in conifers. In addition, we also found that *PyWOXs* were closely related to the *WOXs* of *Pinus* in the phylogenetic tree, they had high homology, which implicates that the evolution of *WOX* genes has a closely genetic relationship between conifers and may play similar functions (Fig. 3). However, we had not identified and cloned the orthologues of *WOX2* in *P. yunnanensis* nor searched in the genome of *P. tabuliformis*.

3.2 Characteristics of *PyWOX* genes and proteins

The result of physicochemical characteristics showed in Table 1 indicate that the sequence lengths of 10 *PyWOX*s ranged from 169 amino acids (aa) (*PyWOXX*) to 482aa (*PyWOX4*), and the protein molecular masses ranged from 19.06KDa to 53.12KDa. The isoelectric points (pI) of the *PyWOX*s were predicted to range from 5.44 to 9.38, with 3 alkaline proteins having a pI > 7 and 7 acidic proteins with a pI < 7. The negatively charged residues of each protein were nearly equivalent to the positively charged residues, and the aliphatic index was in range of 54.88 to 67.69. The hydropathicity of *PyWOX* proteins were negative, in the range of -0.586 to -0.911, indicating they are hydrophilic proteins. The instability coefficient had the smallest value for *PyWOXA* (43.82) and the largest value for *PyWOXB* (67.05). However, all *PyWOX*s exceeded the value of 40, indicating that *PyWOX* family members might be unstable hydrophilic proteins. The results of subcellular localization prediction showed that 10 genes were uniformly located in the nucleus (excluding *PyWOX3*) using the two methods, while *PyWOX3* was located in the mitochondrial matrix according to PSORT (Table S3). Therefore, the predicted *PyWOX* genes are considered to be characteristic of transcription factors.

Table 1
Physicochemical characterization of the WOX family proteins from *P. yunnanensis*.

Gene name	Clade	Molecular weight (Da)	Protein length (aa)	pI	Negatively charged residues	Positively charged residues	Aliphatic index	GRAVY	Instability index	Stability /Instability
<i>PyWUS</i>	Modern/WUS	24813.88	215	8.04	28	30	54.88	-0.911	61.18	unstable
<i>PyWOX3</i>	Modern/WUS	20818.7	181	9.18	22	28	67.69	-0.745	63.71	unstable
<i>PyWOX4</i>	Modern/WUS	53125.35	482	6.81	48	47	63.01	-0.65	56.66	unstable
<i>PyWOX5</i>	Modern/WUS	27840.54	245	5.44	32	26	59.31	-0.953	59.93	unstable
<i>PyWOXX</i>	Modern/WUS	19060.98	169	5.75	21	17	62.37	-0.72	65.97	unstable
<i>PyWOXB</i>	Intermediate	29189.46	260	9.38	25	31	59.96	-0.801	67.05	unstable
<i>PyWOXE</i>	Intermediate	28093.22	253	6.38	22	20	66.64	-0.586	60.45	unstable
<i>PyWOX13</i>	Ancient	40220.67	356	6.21	49	43	65.17	-0.856	46.38	unstable
<i>PyWOXG</i>	Ancient	33190.48	294	6.61	32	31	67.01	-0.649	54.01	unstable
<i>PyWOXA</i>	Ancient	40675.11	366	5.92	42	33	59.62	-0.783	43.82	unstable
Note: theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY).										

To further understand the structural characteristic of WOX genes, we analyzed the conserved domains and motifs of these genes. The results showed that motif 1 and motif 3, containing a “helix-loop-helix-turn-helix” structure consisting of 60–61 amino acids, were detected in all *PyWOX* proteins, which constitute conserved HD of WOX (Fig. 4B, C and Fig. 5). Moreover, *PyWUS* has an extra amino acid residue Y (tyrosine), between helix 1 and loop, which is missing in other *PyWUS* proteins (Fig. 5). Motif 5 was specific to the members of the WUS-clade, comprising a WUS-box(T-L-x-L-F-P)after the HD. In addition, *PyWUS* and *PyWOX5* had an EAR-motif (L-x-L-x-L) near the C-terminal (Fig. 5). Motif 8 and 10 were presented in *PyWOXB* and *PyWOXE* in the intermediate-clade with a bootstrap value of 100, suggesting a strong homology and that they may perform the same functions (Fig. 4A). Motif 4, composed of 32 aa, was specifically only present in the members of the ancient-clade after HD (Fig. 4C and Table S4). These results suggested that 10 candidate *PyWOX*s are members of the WOX family, and that genes in different clades have distinct and conserved structure and motifs.

The results of secondary structure prediction of the 10 *PyWOX* proteins showed that they contained the structure of α-helix, β-corner, extended strand and random coil, with α-helix and β-corner accounting for more than 30% of the total protein structure (Table S3). The α-helix and β-corners constituted the construction of “helix-turn-helix” (HTH), which might be involved in binding to specific DNA sequences. Additionally, the tertiary structure of the *PyWOX* proteins were constructed and predicted using the homologous modeling method that identified templates with high similarity to the target protein. Most of models matched protein models of species in *Pinus* and *Picea*, with identities higher than 84%, which could accurately represent the true structure of the proteins (Table S3, Fig. 6). In summary, the sequence analysis indicates that the 10 *PyWOX* genes we identified possess the typical structural characteristics of the WOX gene family, and they exhibit a high degree of homology with WOX genes from *Pinus* and *Picea* species.

3.3 The expression profiles of *PyWOX*s during developmental stages and tissues

To understand the specific expression patterns of each *PyWOX* gene in plant growth, their expression profiles during seeds germination, seedlings growth, cluster buds proliferation and callus induction were detected and analyzed by RT-qPCR. Our result showed that all identified *PyWOX* genes were detected at least in some of the tissues and developmental stages in this study, and showed a certain degree of difference in their expression levels (Fig. 7). The members of the ancient-clade were expressed in all detected stages and tissues, suggesting that they are probably constitutively expressed. The expression levels of *PyWOX13* and *PyWOXA* in the cluster buds were the highest among all the tested samples. The members of intermediate-clade were mainly expressed in the callus and germination stage. *PyWOXB* was also expressed in cluster buds, while the expression of tissues during seedlings growth were not detected. The expression levels of *PyWUS* and *PyWOX3* were quite constant during callus, seeds germination and seedlings growth, and increased dramatically during cluster buds, although transcripts from these two genes were not found in the roots. The transcripts of *PyWOX4* was expressed in all tissues, preferentially located in the cluster buds, with the lowest expression during seeds germination. *PyWOX5* was not expressed in callus and cluster buds, and there was no significant difference in expression during germination and seedlings growth. *PyWOXX* transcripts were high expressed in the cluster buds and stem apex of seedlings, and only a little expression was detected during seeds germination. Moreover, we found that *PyWUS*, *PyWOX3*, *PyWOX4* and *PyWOXX* showed a similar expression profile, as transcripts of these genes reached their maximum in the cluster buds. These findings suggested that *PyWOX* members characterized the expression profiles of tissue specificity, and members in the same clade exhibited similar expression patterns.

3.4. The transcriptional activation activity of *PyWUS*, *PyWOX3*, and *PyWOX4*

To further examine whether *PyWUS*, *PyWOX3* and *PyWOX4* have transcriptional activation activity, the CDS of these three genes were cloned into pGBKT7 vector and transformed into AH109 yeast strains (Fig. 8). The yeast cultures of three genes were able to grow on DDO medium and exhibited similar growth patterns. While only the pGBKT7-*PyWOX4* yeast cultures grew on QDO medium after being diluted by 10-, 100-, and 1000-fold. These results indicate that *PyWOX4* has transcriptional activation activity and can function as a transcription factor in *P. yunnanensis*.

4 Discussion

The public availability of genome sequencing in Chinese pine and the increasing availability of genomic data of conifers have facilitated the study of the WOX gene family in conifers [32]. This study reported the screening and analysis of ten WOX members in *P. yunnanensis* transcriptomes, showing that the characteristics of *PyWOX* genes were conserved and that the self-activation of *PyWUS*, *PyWOX3* and *PyWOX4* were performed. Recently, the screening and characterization of WOX genes in *P. abies* and *P. pinaster* have shown that they comprised 11 and 14 WOX genes, respectively [1, 29]. Our phylogenetic analysis suggested that all *PyWOX* genes were dispersed in the three clades established in WOX phylogeny based on previous results, which supports the hypothesis that a great diversification in the WOX family had occurred before the split among seed plants [3].

The ancient-clade take up the initial position of phylogenetic tree and is regarded as the oldest and highly conserved clade in the WOX gene family [33]. In *P. yunnanensis*, three genes belonging to the ancient-clade were screened, along with those non-vascular plants from green algae and *P. patens*, which may represent the earliest differentiated WOX genes. Two genes of the intermediate-clade showed a very close relationship from phylogenetic analysis. Two phylogenetic trees revealed that five genes were found in the WUS-clade, which exclusively comprised ferns and spermatophytes. The number of their members was greater than in other clades, such as most species of *Pinus* and other angiosperms, indicating that they are probably the result of expansion and evolution that occurred before the splitting of spermatophytes [30, 34]. Furthermore, most members of the WUS-clade, excluding *PyWOXX*, were found to have clearly definite homologous sequences for *Arabidopsis*. *PaWOX2* and *PpWOX2* were exclusive expressed during somatic embryogenesis, and no transcripts were detected after the embryo had germinated [1]. The orthologue of WOX2 was not detected and isolated in *P. yunnanensis* owing to the somatic embryos have not been induced, therefore, the function and regulatory mechanism of WOX2 in *P. yunnanensis* remain obscure, and further researches will be need for elucidation. The additional residue Y at the position between the first helix and loop in *PyWUS*, showed the utmost consistency with WUS in many species, indicating that it may be involved in transcriptional activation, which implicates the conservation of this gene during evolution [1, 30, 35]. Notably, *PyWOXX* and its orthologues WOXX emerged exclusively in conifers, which have not been found and described in *Arabidopsis* and any other angiosperm. The result is consistent with the previous report on *P. pinaster*, where *PyWOXX* and its homologues of conifers constituted a monophyletic cluster at the base of the WUS-clade, presumably representing a transition stage between the members of intermediate-clade and WUS-clade during evolution [1]. Therefore, based on these findings, it is reasonably supposed that the members of the WUS-clade in *P. yunnanensis* exist in the last common ancestor of vascular plants and probably underwent gene expansion and diversification before the split of seed plants.

Available information for gene function in conifers is limited and obscured, due to the huge and complex genomes that exceed the size of 20GB, about 90% of the genes are thought to have originated from duplication, and the difficulty in performing genetic transformation in

many conifers until the moment. To characterize each WOX gene in *P. yunnanensis*, therefore, the expression profiles of 10 *PyWOXs* were analyzed in different tissues of seedlings and developmental stages using RT-qPCR. The monophyletic WOX gene that regulates the differentiation and regeneration of stem cells is identified by previous studies in green algae and bryophytes [5]. Recently, it was reported in *Arabidopsis* and *P. pinaster* that the expression patterns of the ancient-clade members are similar. They are expressed extensively in the majority of tissues and developmental stages, and their function could be correlated with embryonic development, radicle initiation and floral organogenesis [35]. In this study, the transcripts of all ancient members were expressed in callus as well as during seeds germination and seedlings development, consequently, they could be considered as the oldest and most conserved members in the *PyWOX* family [4]. The expression of the intermediate-clade members in *P. abies* is restricted to the periods of embryonic initiation and development, and *PaWOX8/9-RNAi* lines exhibit embryonic deformity [2, 25, 26, 36]. The expression of the intermediate-clade members in *P. yunnanensis* was detected during seeds germination, which is highly homologous to that of Norway spruce, suggesting that their expression patterns and functions could be similar. Besides, the study shows that *AtWOX11* and its homolog *AtWOX12* contribute to the cell fate determination from procambium and adjacent parenchyma cells into root founder cells [23, 37, 38]. The expression of the intermediate-clade members in callus supported our view that their expression patterns might be approximate.

Previous studies have shown that WUS-clade members are related to stem cells maintenance, cell division and differentiation, and morphogenesis [7, 39, 40]. WUS and WOX5 in *Arabidopsis* and *P. pinaster* maintain the balance of stem apex and root apex by regulating cell division and differentiation, and are expressed in SAM and RAM respectively [34, 41]. In the present study, the homologs (*PyWUS* and *PyWOX5*) were screened, and their transcripts were detected, which is consistent with the results in conifers [42]. Based on the mechanism of the WUS-CLV regulatory loop, WUS activation is required to establish stem cell niche and regulate the homeostasis of meristematic cells when the buds are induced and initiated, thereby forming a complete and functional SAM [2, 12, 14]. We found that *PyWUS* was highly expressed in the cluster buds, which fits well with the function of homologs in *Arabidopsis* and apple, suggesting that the function of WUS in regulating bud growth may be conserved between species [38, 43]. The transcripts of *PyWOX5* were not only expressed in stems, stem apex and cotyledons but also in roots, which are also reported in the studies of spruce and *Pinus* [1, 34]. Besides, in the sub-tree of WUS-clade, we found that *AtWOX5* and *AtWOX7* were grouped tightly in a sub-clade, and *PyWOX5* and its conifer homologs were also presented together with them in a group. This finding could indicate some functional similarity of *PyWOX5* and its homologs between gymnosperms and angiosperms. Recent studies on herbs and woody plants demonstrate that WOX3 and its orthologues are expressed in the periphery of SAM near leaf primordia, promoting lateral expansion and leaves growth through the proliferation of leaf marginal cells [19, 44]. The mutant of *PaWOX3* and *ZmWOX3/NS1* exhibit deficient development of the cotyledon and leaf lateral margin [29, 45]. The transcripts of *PyWOX3* were preferentially expressed during the proliferation of cluster buds, slightly expressed in other organs and seeds germination, suggesting that it may perform a conserved function in leaf expansion and development possibly. *PyWOX4*, the ortholog of WOX4, was the unique gene whose transcripts were detected in all tested samples in the WUS-clade. As the initial cells of vascular tissue in many species, WOX4 genes are predominantly transcribed in the developing vascular cambium and play a crucial role in the CLE41-WOX4 pathway [46–48]. Moreover, the expression of *PyWOXX* was restricted to cluster buds and shoot apex, it is a WUS-clade member that has not been described in angiosperms. Sequence alignment and phylogenetic analysis revealed that *PyWOXX* was homologue to WOX4 in conifers, and the CDS was also cloned in this study, which verified that it was not a pseudogene. The result of fluorescence in situ hybridization in *P. pinaster* shows that the expression domain of *PpWOXX* is similar to that of WUS in the stem apex region of seedlings, implicating that *PyWOXX* may also be related to the development of SAM [1]. These results suggest that some WUS-clade members (such as *PyWUS*, *PyWOX3*, *PyWOX4*, *PyWOX5* and *PyWOXX*) are probable involved in regulation of meristematic cells and organ differentiation, and their specific functions will be studied and clarified.

Declarations

Supplementary Materials: The following supporting information can be downloaded at:

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Data Availability Statement: The WOX gene sequences in *P. yunnanensis* have been deposited in the GenBank: *PyWUS*, PP660559; *PyWOX3*, PP660560; *PyWOX4*, PP660561; *PyWOX5*, PP660562; *PyWOXX*, PP660563; *PyWOXB*, PP977271; *PyWOXE*, PP977272; *PyWOX13*, PP977273; *PyWOXG*, PP977274; *PyWOXA*, PP977275.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

References

1. Alvarez JM, Bueno N, Cañas RA, Avila C, Cánovas FM, Ordás RJ. Analysis of the *WUSCHEL-RELATED HOMEODOMAIN* gene family in *Pinus pinaster*. New insights into the gene family evolution. *Plant Physiol Biochem*. 2018;123:304–18.
2. Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, et al. Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning. *Arabidopsis thaliana Dev*. 2004;131:657–68.
3. van der Graaff E, Laux T, Rensing SA. The WUS homeobox-containing (WOX) protein family. *Genome Biol*. 2009;10(12):248.
4. Wu CC, Li FW, Krame ME. Large-scale phylogenomic analysis suggests three ancient superclades of the *WUSCHEL-RELATED HOMEODOMAIN* transcription factor family in plants. *PLoS ONE*. 2019;14(10):e0223521.
5. Jha P, Ochatt SJ, Kumar V. WUSCHEL: a master regulator in plant growth signaling. *Plant Cell Rep*. 2020;39(4):431–44.
6. Gehring WJ, Müller M, Affolter M, Percival-Smith A, Billeter M, Qian YQ, et al. The structure of the homeodomain and its functional implications. *Trends Genet*. 1990;6:323–9.
7. Zeng DQ, He CM, Yu ZM, Si C, Chen J, Huang L, et al. Roles of WOX Transcription Factors in Plant Development. *J Trop Subtropical Bot*. 2022;30(5):742–52.
8. Ariel FD, Manavella PA, Dezar CA, Chan RL. The true story of the HD-Zip family. *Trends Plant Sci*. 2007;12(9):419–26.
9. Liu B, Wang L, Zhang J, Li JB, Zheng HQ, Chen J, et al. *WUSCHEL-related Homeobox* genes in *Populus tomentosa*: diversified expression patterns and a functional similarity in adventitious root formation. *BMC Genom*. 2014a;15:296.
10. Zhang X, Zong J, Liu JH, Yin JY, Zhang DB. Genome-wide analysis of WOX gene family in rice, sorghum, maize, *Arabidopsis* and poplar. *J Integr Plant Biol*. 2010;52(11):1016–26.
11. Schoof H, Lenhard M, Haecker A, Mayer KF, Jürgens G, Laux T. The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell*. 2000;100(6):635–44.
12. Xin W, Wang ZC, Liang Y, Wang YH, Hu YX. Dynamic expression reveals a two-step patterning of WUS and CLV3 during axillary shoot meristem formation in *Arabidopsis*. *J Plant Physiol*. 2017;214:1–6.
13. Xue ZZ, Liu LY, Zhang C. Regulation of shoot apical meristem and axillary meristem development in plants. *Int J Mol Sci*. 2020;21(8):2917.
14. Yang T, Jiao Y, Wang Y. Stem Cell Basis of Shoot Branching. *Plant Cell Physiol*. 2023;64(3):291–6.
15. Gonzali S, Novi G, Loreti E, Paolicchi F, Poggi A, Alpi A, et al. A turanose-insensitive mutant suggests a role for WOX5 in auxin homeostasis in *Arabidopsis thaliana*. *Plant J*. 2005;44(4):633–45.
16. Stahl Y, Wink RH, Ingram GC, Simon R. A signaling module controlling the stem cell niche in *Arabidopsis* root meristems. *Curr Biol*. 2009;19(11):909–14.
17. Vandenbussche M, Horstman A, Zethof J, Koes R, Rijpkema AS, Gerats T. Differential recruitment of WOX transcription factors for lateral development and organ fusion in *Petunia* and *Arabidopsis*. *Plant Cell*. 2009;21(8):2269–83.
18. Matsumoto N, Okada K. A homeobox gene, *PRESSED FLOWER*, regulates lateral axis-dependent development of *Arabidopsis* flowers. *Genes Dev*. 2001;15(24):3355–64.

19. Shimizu R, Ji JB, Kelsey E, Ohtsu K, Schnable PS, Scanlon MJ. Tissue specificity and evolution of meristematic WOX3 function. *Plant Physiol.* 2009;149(2):841–50.
20. Eeda SK, Werr W. Transcription of the *WUSCHEL-RELATED HOMEBOX 4* gene in *Arabidopsis thaliana*. *Gene Expr Patterns.* 2020;38:119150.
21. Ji J, Strable J, Shimizu R, Koenig D, Sinha N, Scanlon MJ. WOX4 promotes procambial development. *Plant Physiol.* 2010;152(3):1346–56.
22. Ueda M, Zhang ZJ, Laux T. Transcriptional activation of *Arabidopsis* axis patterning genes WOX8/9 links zygote polarity to embryo development. *Dev Cell.* 2011;20(2):264–70.
23. Liu J, Sheng L, Xu Y, Li J, Yang Z, Huang H, Xu L. WOX11 and 12 are involved in the first-step cell fate transition during de novo root organogenesis in *Arabidopsis*. *Plant Cell.* 2014b;26(3):1081–93.
24. Wan Q, Zhai N, Xie D, Liu W, Xu L. WOX11: the founder of plant organ regeneration. *Cell Regen.* 2023;12(1):1.
25. Palovaara J, Hallbergm H, Stasolla C, Hakman I. Comparative expression pattern analysis of *WUSCHEL-related homeobox 2* (WOX2) and WOX8/9 in developing seeds and somatic embryos of the gymnosperm *Picea abies*. *New Phytol.* 2010;188(1):122–35.
26. Zhu T, Moschou PN, Alvarez JM, Sohlberg JJ, von Arnold S. *WUSCHEL-RELATED HOMEBOX 8/9* is important for proper embryo patterning in the gymnosperm Norway spruce. *J. Exp. Bot.* 2014, 65, 6543–6552.
27. Zhu T, Moschou PN, Alvarez JM, Sohlberg JJ, von Arnold S. *WUSCHEL-RELATED HOMEBOX 2* is important for protoderm and suspensor development in the gymnosperm Norway spruce. *BMC Plant Biol.* 2016;16:19.
28. Hassani SB, Trontin J-F, Raschke J, Zoglauer K, Rupps A. Constitutive overexpression of a conifer WOX2 homolog affects somatic embryo development in *Pinus pinaster* and promotes somatic embryogenesis and organogenesis in *Arabidopsis* seedlings. *Front Plant Sci.* 2022;13:838421.
29. Alvarez JM, Sohlberg J, Engstrom P, Zhu TQ, Englund M, Moschou PN, Arnoldet SV. The *WUSCHEL-RELATED HOMEBOX 3* gene *PaWOX3* regulates lateral organ formation in Norway spruce. *New Phytol.* 2015;208(4):1078–88.
30. Nardmann J, Werr W. Symplesiomorphies in the WUSCHEL clade suggest that the last common ancestor of seed plants contained at least four independent stem cell niches. *New Phytol.* 2013;199:1081–92.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2- $\Delta\Delta$ CT. *Methods.* 2001;25(4):402–8.
32. Titievsky A, Putintseva YA, Taranenko EA, Baskin S, Oreshkova NV, Brodsky E, et al. Comparative genomics analysis of repetitive elements in ten gymnosperm species: Dark Repeatome and its abundance in conifer and *Gnetum* species. *Life.* 2021;11(11):1234.
33. Deveaux Y, Toffano-Nioche C, Claisse G, Thareau V, Morin H, Laufs P, et al. Genes of the most conserved WOX clade in plants affect root and flower development in *Arabidopsis*. *BMC Evol Biol.* 2008;8:291.
34. Zhang Y, Jiao Y, Jiao H, Zhao H, Zhu YX. Two-step functional innovation of the stem-cell factors WUS/WOX5 during plant evolution. *Mol Biol Evol.* 2017;34(3):640–53.
35. Hedman H, Zhu T, von Arnold S, Sohlberg JJ. Analysis of the *WUSCHEL-RELATED HOMEBOX* gene family in the conifer *Picea abies* reveals extensive conservation as well as dynamic patterns. *BMC Plant Biol.* 2013;13:89.
36. Breuninger H, Rikirsch E, Hermann M, Ueda M, Laux T. Differential expression of WOX genes mediates apical-basal axis formation in the *Arabidopsis* embryo. *Dev Cell.* 2008;14:867–76.
37. Hu X, Xu L. Transcription factors WOX11/12 directly activate WOX5/7 to promote root primordia initiation and organogenesis. *Plant Physiol.* 2016;172(4):2363–73.
38. Wang J, Tian C, Zhang C, Shi B, Cao X, Zhang TQ, et al. Cytokinin signaling activates WUSCHEL expression during axillary meristem initiation. *Plant Cell.* 2017;29:1373–87.
39. Ikeda M, Mitsuda N, Ohme-Takagi M. *Arabidopsis* WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. *Plant Cell.* 2009;21(11):3493–505.
40. Lin H, Niu L, McHale NA, Ohme-Takagi M, Mysore KS, Tadege M. Evolutionarily conserved repressive activity of WOX proteins mediates leaf blade outgrowth and floral organ development in plants. *Proc. Natl. Acad. Sci. U. S. A.* 2013, 110(1):366–371.
41. Kong DY, Hao YL, Cui HC. The WUSCHEL related homeobox protein WOX7 regulates the sugar response of lateral root development in *Arabidopsis thaliana*. *Mol Plant.* 2016;9(2):261–70.
42. Bueno N, Cuesta C, Centeno ML, Ordás RJ, Alvarez JM. In vitro plant regeneration in conifers: the role of WOX and KNOX gene families. *Genes.* 2021;12:438.

43. Li GF, Tan M, Ma JJ, Cheng F, Li K, Liu XJ, et al. Molecular mechanism of *MdWUS2–MdTCP12* interaction in mediating cytokinin signaling to control axillary bud outgrowth. *J Exp Bot.* 2021;72(13):4822–38.
44. Honda E, Yew C-L, Yoshikawa T, Sato Y, Hibaram K-I, Itoh J-I. *LEAF LATERAL SYMMETRY1*, a member of the *WUSCHEL RELATED HOMEODOMAIN* gene family, regulates lateral organ development differentially from other paralogs, *NARROW LEAF2* and *NARROW LEAF3* in rice. *Plant Cell Physiol.* 2018;59(2):376–91.
45. Conklin PA, Johnston R, Conlon BR, Shimizu R, Scanlon MJ. Plant homeodomain proteins provide a mechanism for how leaves grow wide. *Development.* 2020;147(20):193623.
46. Hirakawa Y, Kondo Y, Fukuda H. TDIF Peptide signaling regulates vascular stem cell proliferation via the *WOX4* homeobox gene in *Arabidopsis*. *Plant Cell.* 2010;22(8):2618–29.
47. Kucukoglu M, Nilssonm J, Zheng B, Chaabouni S, Nilsson O. *WUSCHEL-RELATED HOMEODOMAIN* (*WOX4*)-like genes regulate cambial cell division activity and secondary growth in *Populus* trees. *New Phytol.* 2017;215(2):642–57.
48. Ohmori Y, Tanaka W, Kojima M, Sakakibara H, Hirano H-Y. *WUSCHEL-RELATED HOMEODOMAIN* is involved in meristem maintenance and is negatively regulated by the *CLE* gene *FCP1* in rice. *Plant Cell.* 2013;25(1):229–41.

Figures

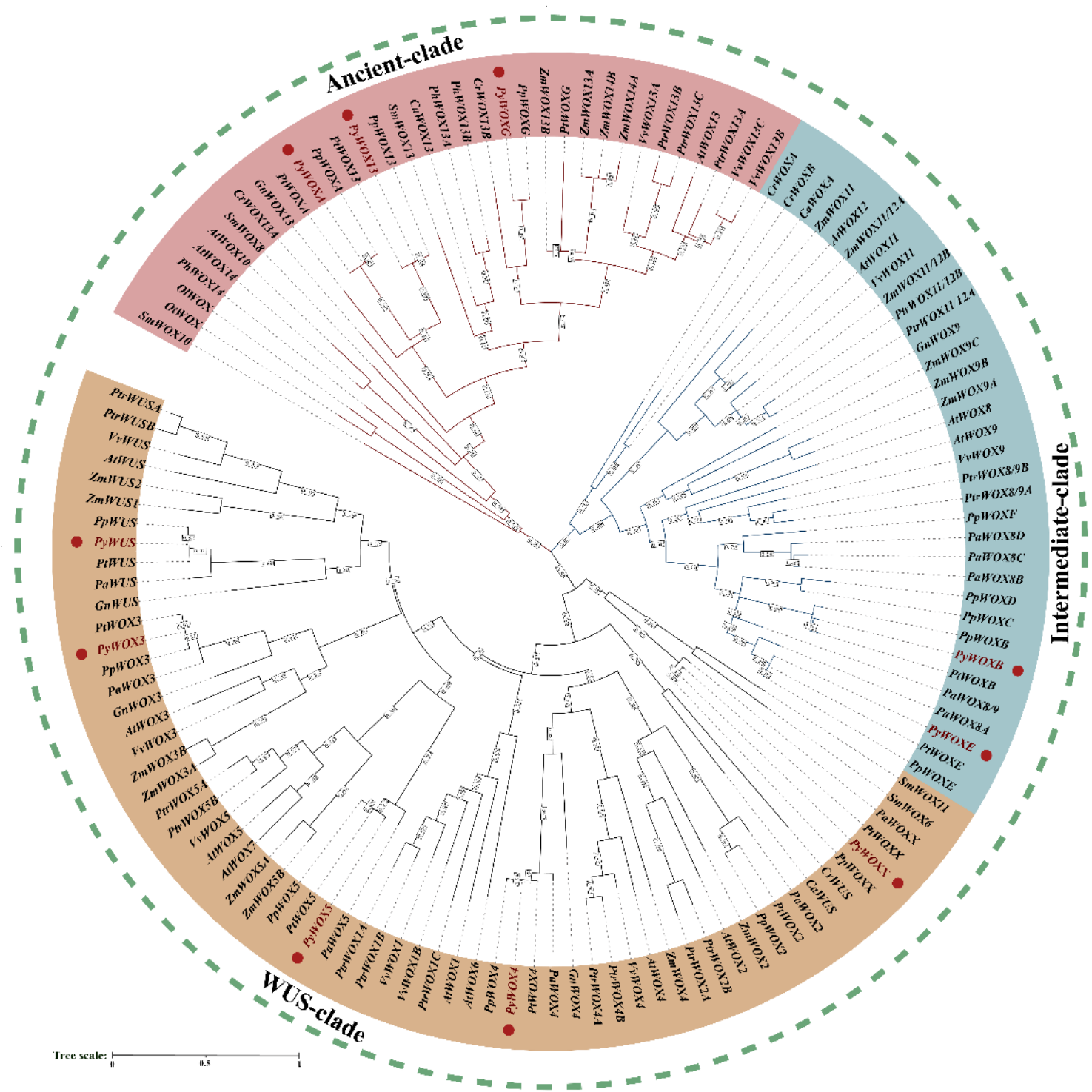


Figure 2

Phylogenetic tree of the WOX proteins. The tree comprises the representative WOX protein sequences from algae (*Ostreococcus tauri*, Ot; *O. lucimarinus*, Ol), moss (*Physcomitrella patens*, Ph), ferns (*Selaginella moellendorffii*, Sm; *Ceratopteris richardii*, Cr; *Cyathea australis*, Ca), gymnosperms (*G. gnemon*, Gn; *P. abies*, Pa; *P. pinaster*, Pp; *P. taeda*, Pt), angiosperms (*A. thaliana*, At; *Z. mays*, Zm; *P. trichocarpa*, Ptr; *Vitis vinifera*, Vv). WUS-clade (orange); intermediate-clade (blue); ancient-clade (pink); ten *PyWOX* genes are highlighted with red and dot; the box values in the branches represent bootstrap value.

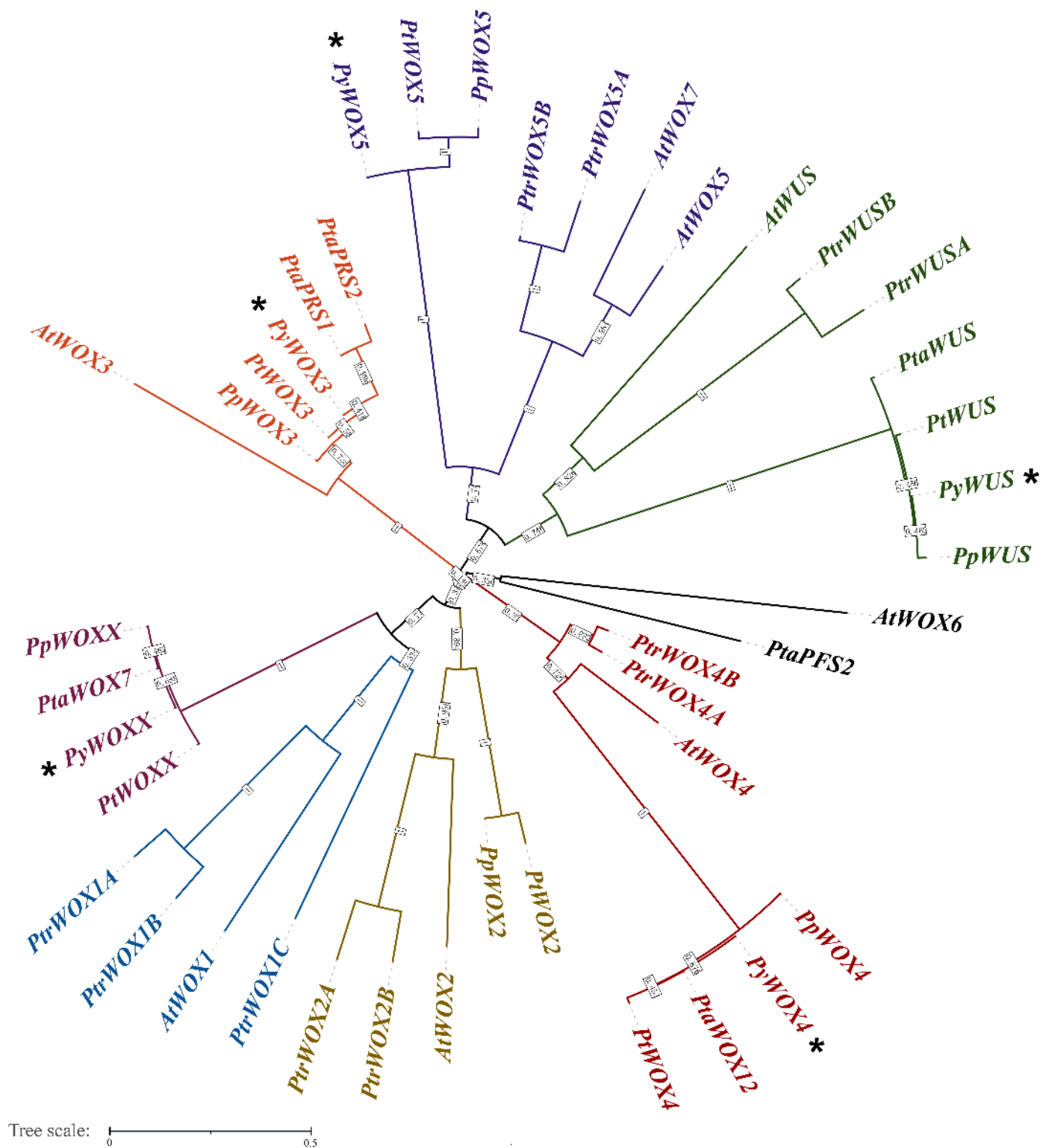


Figure 3

Phylogenetic tree of the WUS-clade members from full-length protein sequences of *P. pinaster*, *P. taeda*, *P. tabuliformis*, *Arabidopsis*, *P. trichocarpa* and *P. yunnanensis*. Five *PyWOX* genes from WUS-clade are highlighted with an asterisk; each group of orthologous genes is highlighted in a different colour; the box values in the branches represent bootstrap value.

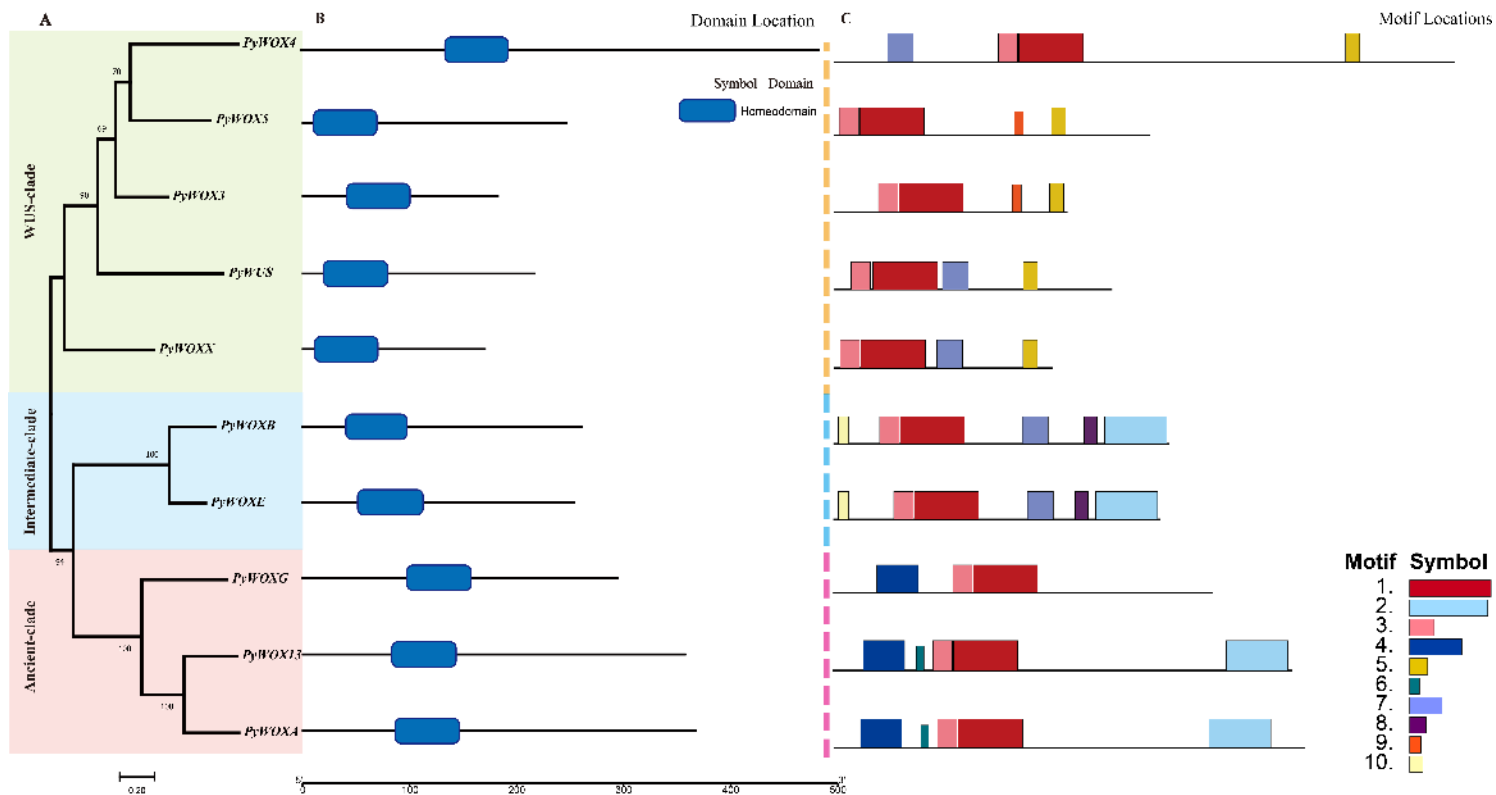


Figure 4

Analysis of phylogenetic tree, conserved domain and motif of WOX proteins from *P. yunnanensis*. **(A)** phylogenetic tree of 10 protein sequences from the WOX family of *P. yunnanensis*. **(B)** Conserved domain identified by NCBI-CDD are visualized. Homeodomain (blue). **(C)** Schematic representation of the conserved motifs in all *PyWOX* family proteins. The distribution of domains and motifs on the lines in the (B) and (C) represents their relative positions and lengths on the *PyWOX* protein sequences. The specific sequences of motifs are shown Table S3.

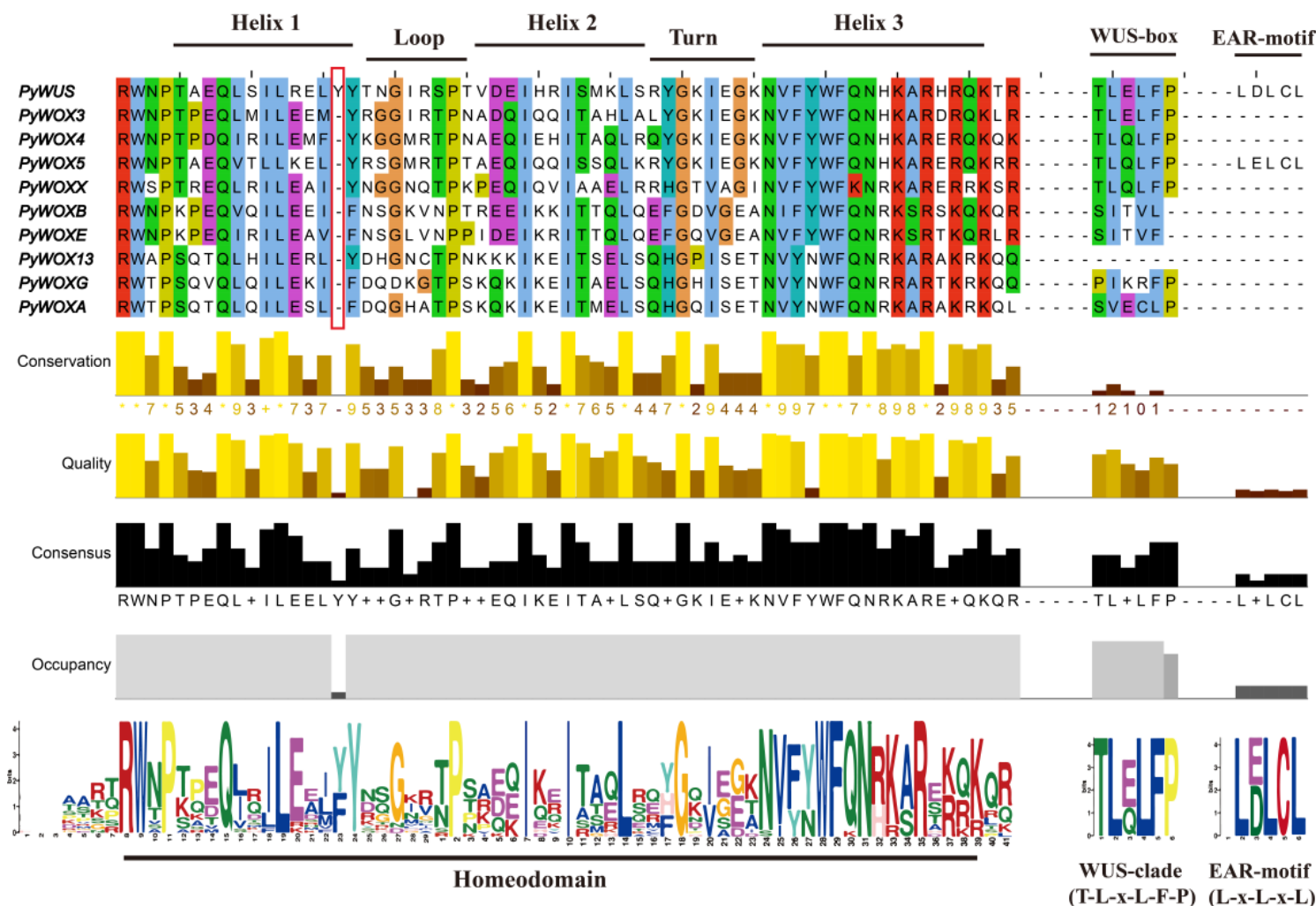


Figure 5

Alignment, schematic representation and sequence logo of conserved domain from ten *PyWOX* proteins. Note the extra Y residue (tyrosine) between helix 1 and loop in *PyWUS* sequence (red box).

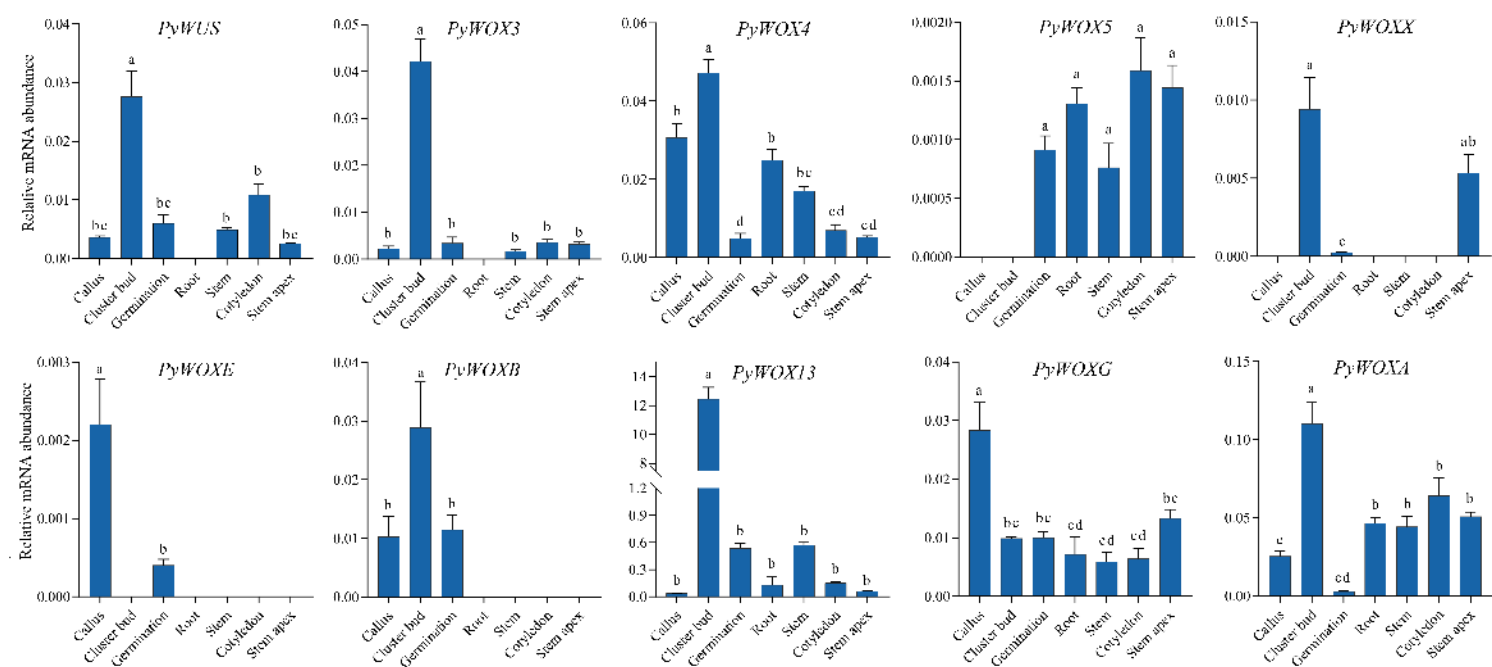


Figure 6

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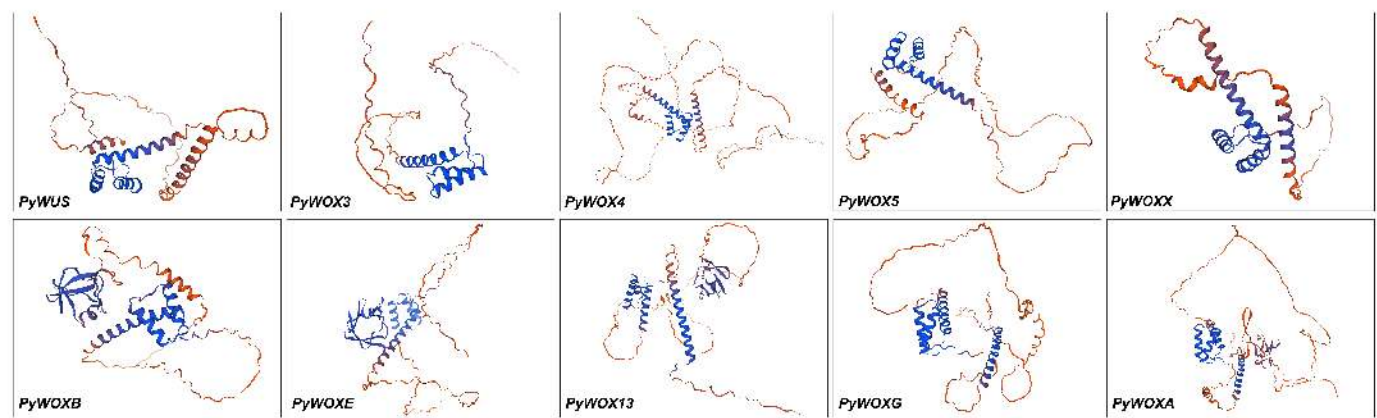


Figure 7

RT-qPCR analysis of the relative mRNA abundance of the WOX gene family in *P. yunnanensis* during the different developmental stages and tissues. Error bars stand for the standard error (SE) of three replicates, and different letters indicate statistically significant differences (determined by one-way ANOVA followed by Duncan's method for multiple comparisons).

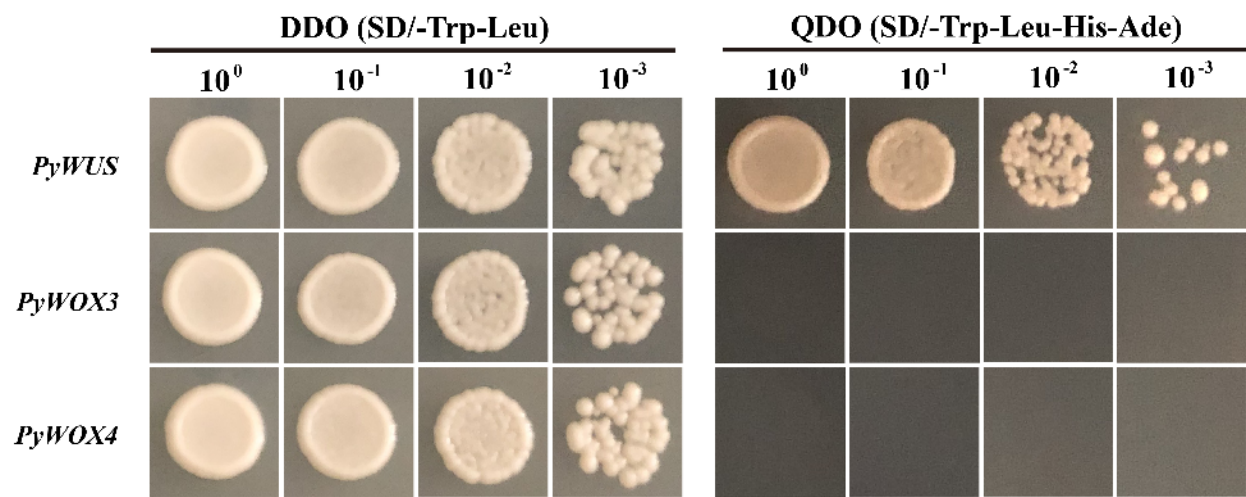


Figure 8

Transcriptional activation activity of three WOX genes in *P. yunnanensis*. The full length of *PyWUS*, *PyWOX3* and *PyWOX4* were fused to BD vector. Co-transformed yeast strains (diluted to 10⁰, 10⁻¹, 10⁻², and 10⁻³) were grown on DDO medium and selected on QDO medium.

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