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Transcriptional Profiling Reveals Key Regulatory Roles of the WUSCHEL-Related Homeobox Gene Family in Yellowhorn (*Xanthoceras sorbifolia* Bunge)

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Abstract: The WUSCHEL-related homeobox (WOX) gene family plays a crucial role in regulating embryonic development, organ formation, and stress resistance. Yellowhorn (*Xanthoceras sorbifolia* Bunge), a drought-resistant tree known for its oil production, lacks sufficient information regarding the WOX gene family. To understand the evolutionary mechanisms and potential functions of this gene family in yellowhorn, we conducted a comprehensive investigation on its expression patterns and evolutionary characteristics. Our analysis revealed the presence of nine XsWOX genes in the yellowhorn genome, which could be categorized into three distinct clades through a phylogenetic analysis. A chromosomal localization analysis indicated that these nine XsWOX genes were situated on six out of the fifteen chromosomes. An intra-species collinear analysis revealed only one pair of tandem duplicated genes within the XsWOX family. The promoter regions of the XsWOX family were found to contain responsive cis-acting elements associated with plant growth and development, stress responses, and hormone signaling. Moreover, an analysis of the gene expression profiles in different developmental stages of callus revealed significant expressions of *XsWOX1*, *XsWOX4*, and *XsWOX5* in embryogenic callus and somatic embryo formation, suggesting that they have special roles in regulating yellowhorn's somatic embryogenesis. Furthermore, the expression level of *XsWOX5* indicated its potential involvement not only in organ formation but also in responding to low temperature, salt, and saline-alkali stresses. Overall, our findings lay a solid foundation for future in-depth studies on the functionality and evolution of XsWOX genes in yellowhorn.

Keywords: yellowhorn; XsWOX; organ formation; abiotic stress

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Citation: Zhang, W.; Xie, X.; Le, L.; Cao, F. Transcriptional Profiling Reveals Key Regulatory Roles of the WUSCHEL-Related Homeobox Gene Family in Yellowhorn (*Xanthoceras sorbifolia* Bunge). *Forests* **2024**, *15*, 376. https://doi.org/10.3390/f15020376

Academic Editor: John E. Carlson

Received: 29 January 2024
Revised: 15 February 2024
Accepted: 16 February 2024
Published: 18 February 2024

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1. Introduction

Yellowhorn (*Xanthoceras sorbifolia* Bunge) is a perennial woody plant that belongs to the *Sapindaceae* family and is native to northern and northwestern China [1]. In semi-arid and dry environments, yellowhorn is considered a pioneer tree species for ecological restoration and landscaping because of its powerful root system, which is characterized by excellent adaptation to adverse conditions such as drought, cold, and saline-alkali stresses [2,3]. Yellowhorn has been widely used to produce bioactive oils because of its high percentage of seed kernel oil (55%–65%), which is rich in unsaturated fatty acids (85%–93%) [3,4]. The pharmacological properties of yellowhorn [5] and the various techniques utilized for extracting seed oil [6] have been the subjects of numerous studies in recent years. However, yellowhorn faces challenges in seed reproduction due to its dormant period and low seed survival rate. These limitations hinder the market development of yellowhorn. Tissue culture technology, as an essential approach for plant regeneration, offers a promising solution. It enables low-cost expansion and rapid production of plants. Tissue culture techniques, specifically somatic embryogenesis, have demonstrated the potential to improve the reproductive efficiency of clonal seedlings [7]. Nevertheless, the application of somatic

embryogenesis is limited by its low frequency, which restricts its use in biotechnology-based propagation and enhancement [8]. Therefore, understanding the developmental mechanisms of somatic embryogenesis in yellowhorn becomes crucial.

Somatic embryogenesis is a fundamental process in plants, showcasing the remarkable ability of plant cells to regenerate into whole new plant organisms without the need for fertilization. This development process resembles the formation of zygote embryos through the fusion of somatic cells [9]. The process of somatic embryogenesis involves multiple stages, including dedifferentiation, cell division, and redifferentiation, ultimately leading to the formation of fully developed plants [10]. Two types of somatic embryogenesis exist—direct and indirect. Indirect somatic embryogenesis arises from embryogenic calluses (ECs) and non-embryogenic calluses (NECs). These two types of calluses can be easily distinguished based on their morphological and color characteristics. EC typically appears white or light yellow, with smooth surfaces, large nuclei, dense cytoplasm, and abundant organelles. In contrast, NEC exhibits a yellowish-brown color, rough tissue surfaces, large vacuoles, and minimal inclusions [11]. The progression of somatic embryogenesis is intricately regulated by transcription factors (TFs) that bind specific gene promoter regions in response to various physiological processes [12]. Notably, TFs like WUSCHEL-related homeobox (WOX) [13], BABY BOOM (BBM) [14], and LEAFY COTYLEDON (LEC) [15] have been found to play crucial roles in the regulation of embryogenesis in callus. For instance, in cotton, the *GhWOX11* and *GhWOX12* genes have simultaneous effects on callus initiation and adventitious root production [16]. Furthermore, the LEC1-ABI3-FUS3-LEC2 network, regulated by BBM, is involved in mediating somatic embryogenesis [14]. Additionally, exogenous phytohormones, such as high levels of auxin [17] and low concentrations of cytokinin [18], have been reported to induce somatic embryogenesis in *Arabidopsis thaliana*.

WOX, a plant-specific homeobox transcription factor, plays a crucial role in regulating the expression of target genes through its highly conserved structure called the homeodomain (HD) domain, which consists of approximately 60–66 amino acid residues [19]. Based on protein sequence alignments and phylogenetic analysis, WOX proteins can be classified into three distinct groups: ancient, intermediate, and modern/WUS clade [20]. In addition to the homeodomain, the modern clade of WOX genes also includes two conserved domains, namely the WUS motif and the EAR domain [21,22]. WOX family genes are widely present in various plant species, with increasing identification through transcriptome studies. For instance, *Arabidopsis thaliana* [22] has 16 WOX genes, *Oryza sativa* has 13 [23], *Juglans regia* L. has 12 [24], and *Citrus sinensis* has 11 [25]. These genes have been found to play crucial roles in embryo and organ development, as well as in the response to abiotic stress [26–28]. In *Arabidopsis*, *AtWUS* and *AtWOX5* are key regulators of shoot, inflorescence, and root apical meristem development [29]. *AtWOX5* is activated during early lateral and adventitious root development and enhances the frequency of lateral root formation [30]. *AtWOX11* and *AtWOX12* are involved in root development and callus formation [31]. *AtWOX6* is responsible for ovule development and responds to cold stress [32]. In poplar, *PeWOX11a* and *PeWOX11b* promote various developmental processes, including adventitious root formation [33]. In oil palm, the *EgWOX2*, *EgWOX7-9*, and *EgWOX12* genes are involved in regulating somatic embryogenesis [34]. Recent studies have also shown that WOX genes respond to abiotic stress and hormonal treatments. In physic nut, five *JcWOX* genes are highly sensitive to salt and drought stresses [35]. *PbWUS* expression in *Phoebe bournei* embryogenic callus is induced by IAA, ABA, and MeJA [36]. With the continuous accumulation of evidence, the importance of WOX genes in plant development, as well as their responses to abiotic stress and hormonal treatments, is becoming increasingly evident.

The potential role of WOX genes in yellowhorn during somatic embryogenesis and organogenesis is not well understood and requires further investigation. In this study, we utilized the recently sequenced high-quality yellowhorn genome [37] to identify nine WOX genes (referred to as XsWOX genes) and examined their compositions, phylogenies, gene structures, conserved motifs, and gene replication events. Moreover, we induced

embryogenic calluses, somatic embryos, and adventitious roots from yellowhorn leaves and analyzed the expression profiles of XsWOX genes at these three stages using transcriptomic data. These findings were further validated through a qRT-PCR analysis. Additionally, we investigated the expression patterns of XsWOX genes in different organs and under various abiotic stress conditions. The objective of this study was to gain insights into the enhancement of yellowhorn's rapid propagation technology and the regulatory mechanism of XsWOX genes in growth, development, and stress resistance.

2. Materials and Methods

2.1. Identification of WOX Genes in Yellowhorn

The genome and protein sequences of yellowhorn were obtained from the yellowhorn genome project [37]. WOX family of proteins from *A. thaliana* and *O. sativa* were downloaded from PlantTFDB v5.0 (http://planttfdb.gao-lab.org/, accessed on 22 June 2022). The candidate WOX proteins in yellowhorn were identified through a BLASTp search with an e-value of $\leq 1 \times e^{-10}$. These candidate WOX genes were subsequently validated in the NCBI Conserved Domain Database (CDD) [38] and the simple modular architecture research tool (SMART) [39]. Further analysis was conducted only on the WOX proteins that contain the homeodomain [40].

2.2. Structural Analysis of WOX Proteins

The protein length, molecular weight (MW), theoretical isoelectric point (pI), and grand average of hydropathicity (GRAVY) of the WOX gene family in yellowhorn were analyzed with ExPASy (https://web.expasy.org/protparam/, accessed on 9 September 2022). The subcellular localization of yellowhorn WOX proteins was predicted utilizing the online CELLO software (v2.5, http://cello.life.nctu.edu.tw/, accessed on 9 September 2022). AlphaFold (https://alphafold.com/, accessed on 9 September 2022) was utilized to predict the tertiary structure of WOX proteins in yellowhorn and generate a per-residue confidence score known as pLDDT ranging from 0 to 100.

2.3. Phylogenetic Analyses of the WOX Proteins

Multiple sequence alignments of WOX proteins from *A. thaliana*, *O. sativa*, and yellowhorn were performed using ClustalW implemented in MEGA v7.0 [41] with default parameters. The phylogenetic tree was constructed with MEGA 7.0 software using the neighbor joining (NJ) method with 1000 bootstrap replicates [41]. Finally, the phylogenetic tree was visualized using the online tool EvolView v2.0 (http://www.evolgenius.info/evolview, accessed on 29 September 2022).

2.4. Chromosome Localization and Synteny Analysis

The yellowhorn chromosomes were mapped with the XsWOX genes based on the genomic information provided in the gff3 file for genome annotation. To visualize the gene distribution on chromosomes, a chromosome localization map was created using TBtools [42]. The names of the XsWOX members were assigned based on the order of gene distribution on the chromosomes. For a syntenic analysis of the XsWOX genes, we utilized the MCScanX module within the TBtools software [43]. By running the program with default parameters, collinearity blocks were generated, covering the entire genome. Within these collinearity blocks, the gene pairs of XsWOX genes exhibiting collinearity were identified and displayed.

2.5. Multiple Sequence Alignment, Gene Structure, and Motif Feature Analysis

We performed multiple alignments of the conserved domain sequences of WOX family proteins using Clustalx [44] and GeneDoc [45] software to identify the conserved regions. Next, we used the MEME [46] function module of the TBtools software to analyze the conserved motifs of the WOX family proteins in yellowhorn. We set the number of motifs to 6, and the width of the motifs ranged from 6 to 100 [46]. Furthermore, we extracted

the exon–intron sequences from the XsWOX genes using the gff3 file. To analyze the gene structure, we utilized the TBtools software [42] to generate diagrams.

2.6. Cis-Elements Analysis

The 2000 bp upstream promoter regions of each WOX gene were submitted, and their cis-regulatory elements were predicted using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 10 October 2022). TBtools software [42] was used to display all cis-elements.

2.7. Plant Materials and Culture Conditions

Yellowhorn leaves at the age of four weeks were used as explants for this study. The leaves were carefully placed on a sterile work surface and then subjected to a 30 s immersion in 75% alcohol. Subsequently, the leaves were rinsed three times with distilled water in order to remove any remaining alcohol residue. After the surface disinfection, the explants were cultured in an MS agar medium supplemented with 30 g/L of sucrose, 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L of α-Naphthalene acetic acid (NAA), and 0.5 mg/L of 6-Benzylaminopurine (6-BA) for a period of one month. The aim of using this cultivation period was to obtain callus tissue. Embryogenic callus was carefully selected from the culture and then transplanted into a solid MS medium supplemented with 0.2 mg/L of NAA, 1 mg/L of 6-BA, and 30 g/L of sucrose. The callus was subjected to another month of cultivation to induce the formation of somatic embryos after one month of subculture. The somatic embryos were subsequently transferred to a 1/2 MS rooting medium containing 30 g/L of sucrose, 0.2 mg/L of NAA, and 1 mg/L of 6-BA. This culture medium facilitated the growth of adventitious roots, and the embryos were cultured for another month. Before the sterilization process, the pH value of the medium was adjusted to 5.8 ± 0.02. The cultures were maintained at a constant temperature of 25 ± 2 °C and exposed to a light period of 16 h followed by an 8 h period of darkness.

Three representative growth stage samples, namely embryogenic callus (S1), somatic embryos (S2), and adventitious roots (S3), were selected to investigate the expression of XsWOX genes. Immediately after sampling, the samples from all three stages were frozen in liquid nitrogen and stored at −80 °C. This ensured their preservation for subsequent RNA extraction and transcriptomic sequencing. The experiment was conducted in triplicate to ensure accuracy and reliability.

2.8. Expression Profile Analysis of XsWOX Genes

The total RNA was extracted from the tissue samples mentioned above using TRIzol® Reagent, following the manufacturer's instructions. To ensure high-quality RNA samples were obtained for sequencing library construction, RNA quality was assessed using the 5300 Bioanalyser from Agilent and quantified using the ND-2000 from NanoDrop Technologies. Majorbio Bio-pharm Biotechnology Co. Ltd. (Shanghai, China) was responsible for performing RNA purification, reverse transcription, library construction, pair-end 150 bp sequencing utilizing the NovaSeq 6000 sequencer, as well as raw data analyses. To ensure the quality of the raw paired-end reads, fast [47] was used for trimming and quality control, with default parameters applied. The resulting clean reads were then individually aligned to the reference genome using HISAT2 software [48] with the orientation mode. StringTie [49] was used to assemble the mapped reads of each sample in a reference-based approach. The expression level of each gene was calculated based on transcripts per million reads (TPM). A heatmap was constructed using TBtools based on the expression matrix of XsWOX genes.

The expression profiles of XsWOX genes in different organ types and under various abiotic stresses were studied using RNA-Seq data from yellowhorn. The dataset included five distinct organs: flower, young fruit, young leaf, young stem, and tender root. Additionally, three abiotic stresses were considered: low temperature, salt stress, and saline-alkali stress. All transcriptome data were obtained from the NCBI database with the follow-

ing BioProject codes: PRJNA665609, PRJNA608707, and PRJNA608707. Gene expression quantification and the construction of a heat map were performed as described previously.

2.9. Quantitative RT-PCR Analysis

Quantitative real-time PCR (qRT-PCR) was employed to investigate the expressions of XsWOX genes at different stages (S1, S2, and S3) and to confirm the accuracy of the transcriptome data. Total RNA was isolated following previously established protocols, and cDNA synthesis was performed using the MonScript^{TM} RT reagent kit (Monad, China) according to the manufacturer's instructions. Specific primers for the XsWOX genes were designed using DNAMAN [50], and the primer sequences can be found in Supplementary Table S1. The quality of the primers was confirmed by PCR amplification and agarose gel electrophoresis. All qRT-PCR experiments were carried out using SYBR Green Master Mix (Monad) on a LightCycler 96 (Roche, Basel, Switzerland). The reaction conditions were set as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with the XsACTIN gene serving as the internal reference gene for each reaction [51]. The experiment was repeated three times. Statistical analysis was performed using one-way ANOVA in SPSS software ($p < 0.05$) [52].

3. Results

3.1. Characterization of XsWOX Genes in Yellowhorn

A study identified nine XsWOX genes in the yellowhorn genome, specifically named *XsWOX1* to *XsWOX9* according to their chromosome positions (Table 1). The sequence analysis revealed that these XsWOX proteins varied in length, with XsWOX9 consisting of 193 amino acids and *XsWOX6* consisting of 319 amino acids. Consequently, their predicted molecular weights ranged from 22,498.07 Da (*XsWOX9*) to 35,920.22 Da (*XsWOX6*). Furthermore, their predicted PI values differed, with *XsWOX2* having a pI of 10.11 and *XsWOX6* exhibiting a pI of 5.33. Interestingly, all XsWOX proteins were determined to be hydrophilic based on their negative GRAVY values (−0.992 to −0.32). The subcellular localization predictions found that all of these proteins were present in the nucleus. Additionally, the three-dimensional structure of the XsWOX proteins in yellowhorn was investigated and exhibited remarkable similarity amongst all nine WOXs (Figure 1).

Table 1. Characteristics of XsWOX proteins.

Genome ID	Gene Name	Protein Length (aa)	MW (Da)	pI	GRAVY	Subcellular Localization
XS04G0106200.1	XsWOX1	289	31,923.78	5.77	−0.32	Nuclear
XS05G0232400.1	XsWOX2	264	30,132.33	10.11	−0.814	Nuclear
XS07G0006100.1	XsWOX3	253	28,800.05	5.79	−0.426	Nuclear
XS07G0075600.1	XsWOX4	221	25,333.47	9.11	−0.992	Nuclear
XS08G0109100.1	XsWOX5	257	28,795.94	5.41	−0.814	Nuclear
XS08G0130200.1	XsWOX6	319	35,920.22	5.33	−0.983	Nuclear
XS13G0025800.1	XsWOX7	232	26,842.18	9.5	−0.931	Nuclear
XS13G0128800.1	XsWOX8	254	28,201.88	7.79	−0.827	Nuclear
XS14G0083400.1	XsWOX9	193	22,498.07	7.00	−0.975	Nuclear

Notes: Protein composition and physiochemical characteristics of WOX proteins in yellowhorn, including protein length, molecular weight (MW), theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), and subcellular localization analysis.

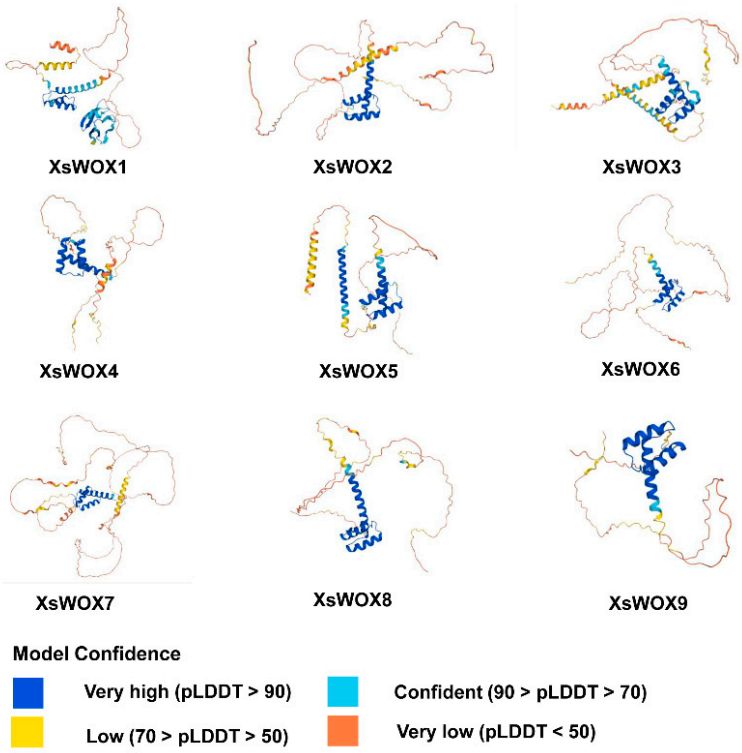


Figure 1. The tertiary structures of XsWOX proteins. The confidence level was represented by the blue to orange hues, which are organized from high to low, respectively.

3.2. Phylogenetic Analysis of XsWOX Genes in Yellowhorn

To investigate the evolutionary patterns of XsWOX genes in yellowhorn, we constructed a phylogenetic tree of WOX genes. This tree included 9 identified WOX protein sequences from yellowhorn, 15 from *Arabidopsis*, and 13 from rice (Figure 2). Based on the evolutionary classification of WOXs in *Arabidopsis*, we found that the WOX members in yellowhorn could also be divided into three clades: ancient, intermediate, and modern/WUS. Specifically, *XsWOX3* and *XsWOX5* were assigned to the ancient clade, while the intermediate clade contained only one member, *XsWOX1*. The modern clade contained the largest number of XsWOX genes, including *XsWOX2*, *XsWOX4*, and *XsWOX6-9*. It is important to note that protein structures clustered within the same clade exhibit high homology and may have similar functions. Therefore, the close relationship observed between the XsWOX proteins in yellowhorn and the WOX proteins in *Arabidopsis*, rather than rice, is highly significant.

3.3. Chromosomal Localization and Syntenic Analysis of XsWOX Genes in Yellowhorn

The chromosome localization analysis revealed that nine XsWOX genes were distributed unevenly across 6 out of the 15 chromosomes in the yellowhorn plant (Figure 3A). Specifically, *XsWOX1*, *XsWOX2*, and *XsWOX9* were found on chromosomes 4, 5, and 14, respectively. Moreover, there were two XsWOX genes, each on chromosomes 7, 8, and 13. An intra-species collinear analysis indicated that *XsWOX3* and *XsWOX5* were present as tandem repeat genes, suggesting the occurrence of gene duplication during the evolution of these two genes (Figure 3B).

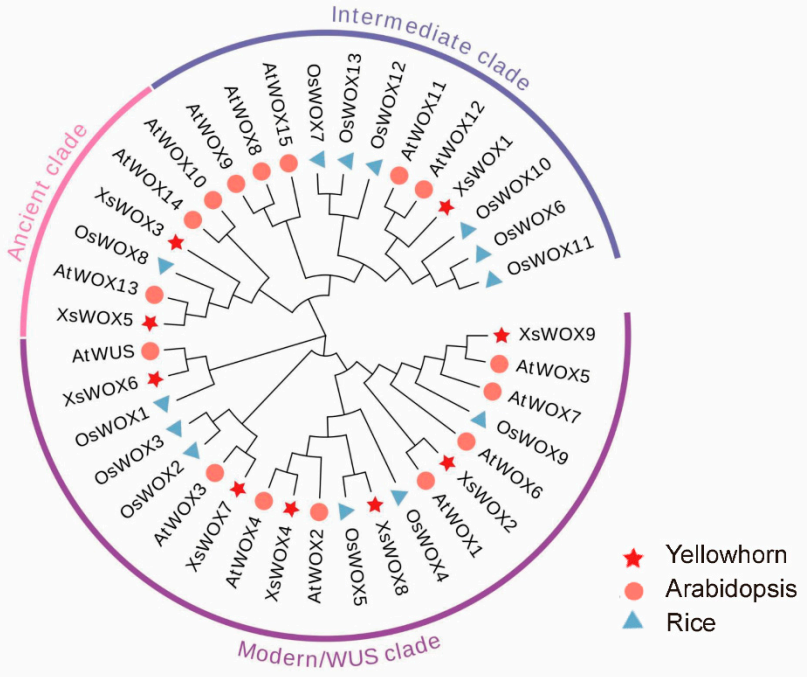


Figure 2. Phylogenetic tree of the XsWOX gene family in yellowhorn, *Arabidopsis*, and rice. Notes: The ancient clade, intermediate clade, and modern/WUS clade are highlighted using pink, blue, and purple bars, respectively. The XsWOX proteins are marked with a red star.

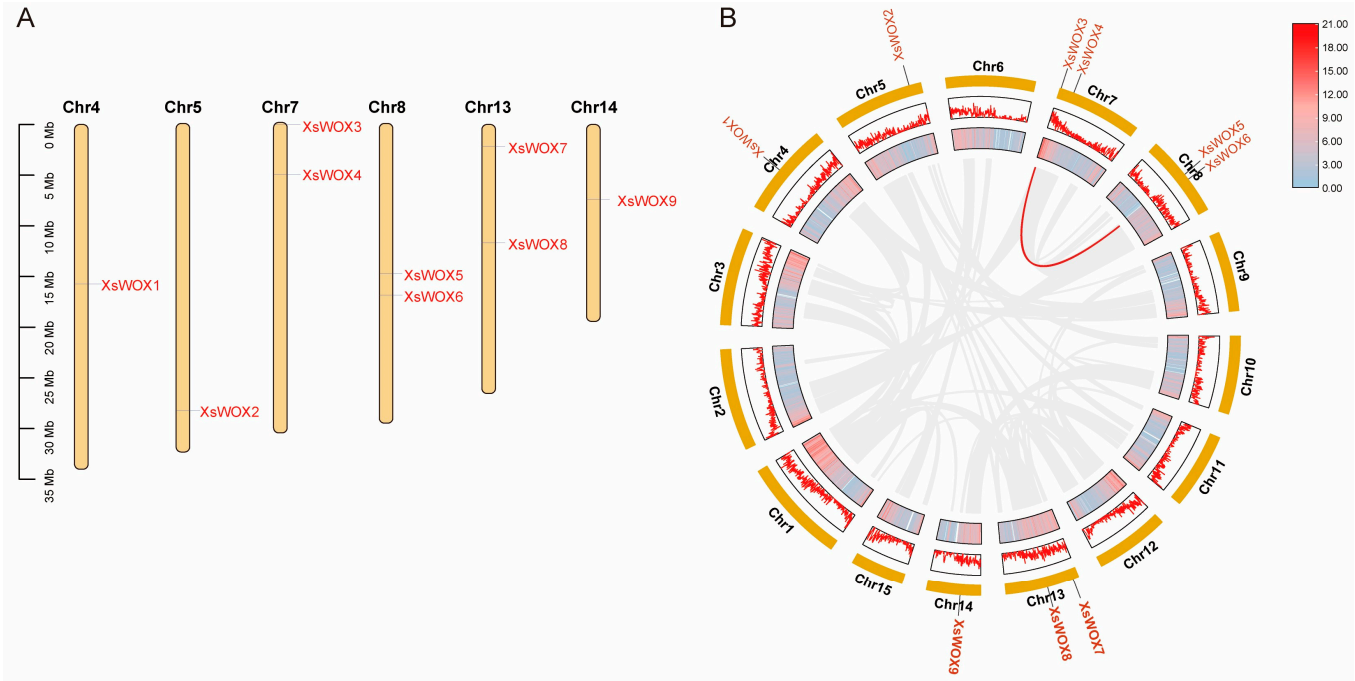


Figure 3. Physical chromosomal location and collinearity analysis of XsWOX genes in yellowhorn. (**A**) Distribution diagram of XsWOX genes on 6 chromosomes. (**B**) Duplicated relationship of XsWOX genes.

3.4. Domain, Motif Identification, and Gene Structure Analysis

The multiple sequence alignment revealed that XsWOX proteins possess a highly conserved domain, HLHTH, which consists of 60 amino acid residues. This conserved folding sequence is crucial for maintaining the functional integrity of plant WOX proteins (Figure 4).



Figure 4. Multiple sequence alignments of XsWOX proteins in yellowhorn. The HD domain is marked with red outlined boxes. "*" indicated the position of multiple sequence alignments.

The numbers and locations of motifs, exons, and introns in XsWOX proteins closely corresponded to their phylogenetic relationships (Figure 5A). In yellowhorn WOXs, six conserved motifs, labeled as motifs 1–6, were identified (Figure 5B). It was observed that all predicted XsWOX proteins contained motifs 1 and 2, which were exclusively present in the intermediate clade (*XsWOX1*). Only the ancient clade exhibited the presence of motif 3 and motif 6, while motif 4 and motif 5 were unique to the WUS clade. The number of exons varied from two to nine, with the WUS clade showing a distinct pattern of exon differentiation. Notably, the lengths of the introns of *XsWOX2* and *XsWOX7* exceeded the total lengths of their exons (Figure 5C).

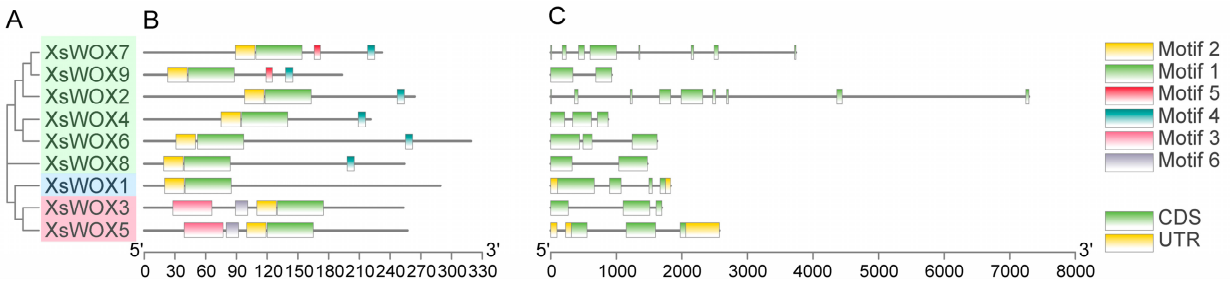


Figure 5. Phylogenetic tree, compositions of conserved protein motifs, and gene structures of the XsWOX genes in yellowhorn. (**A**) Analysis of phylogenetic tree of XsWOX family. The backgrounds of the various clades are displayed in various colors. (**B**) Conserved motif patterns of XsWOX proteins. Different conserved motifs are displayed in differently colored boxes. (**C**) Gene structures of XsWOXs. Exons and introns were visually distinguished by green boxes and black lines, respectively.

3.5. Analysis of Cis-Acting Elements in the XsWOX Promoters

Cis-elements play a crucial role in the regulation of transcription and have a profound impact on gene function. To gain a better understanding of the regulatory mechanisms and functions of XsWOXs, the 2000 bp upstream regions of each yellowhorn WOX gene were analyzed to predict the presence of cis-elements. The analysis revealed significant differences in the numbers and distributions of these cis-acting elements among the nine XsWOX gene promoters (Figure 6B). In total, 15 cis-acting elements were identified within the XsWOXs, falling into three distinct categories: plant growth and development, biotic and abiotic stress responses, and hormone-induced responses (Figure 6C). The largest proportion, consisting of 122 elements, was associated with plant growth and development. Interestingly, a considerable number of cis-acting elements related to plant hormone responses, such as MeJA, abscisic acid, gibberellin, salicylic acid, and auxin, were found in the promoter regions of XsWOXs. These findings suggest that the regulation of XsWOX genes is complex and involves various cis-acting elements associated with essential processes, like plant growth, responses to stress, and hormone signaling. This analysis provides valuable insights into the potential regulatory mechanisms governing the expression of XsWOXs in yellowhorn plants.

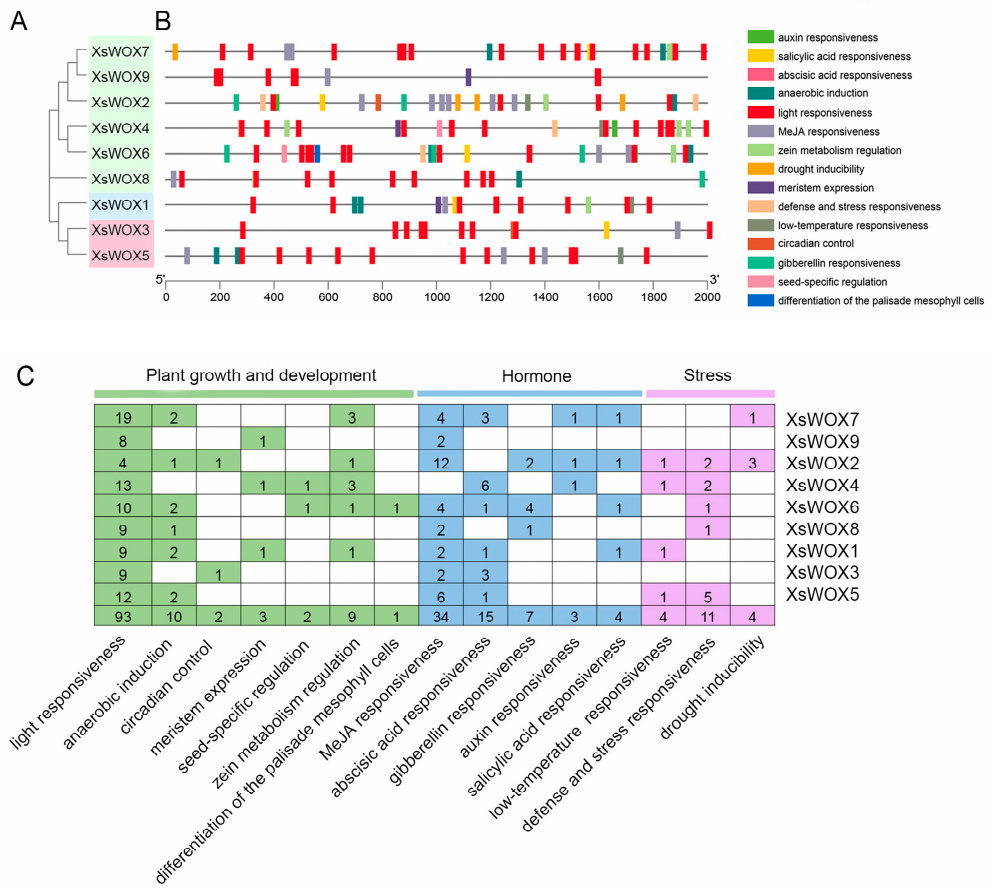


Figure 6. Prediction of cis-acting elements on the promoter of XsWOX genes in yellowhorn. (**A**) The phylogenetic tree of XsWOX proteins. (**B**) Distribution of cis-regulatory elements in the promoter region of the XsWOXs. (**C**) Numbers of cis-elements in XsWOXs. The corresponding colors indicate the presence of different categories of cis-acting elements.

3.6. Phenotypes of Callus at Different Developmental Stages of Yellowhorn and Transcriptome Analysis

The yellowhorn leaves induced a predominantly white, dense, and vigorous embryonic callus at the S1 stage (Figure 7A,D). Filamentous protrusions were observed around somatic embryos during the S2 stage (Figure 7B,E). As the rooting process progressed to the S3 stage, the tissue gradually underwent browning, and after approximately 10 days, root induction occurred. After 30 days, an abundance of adventitious roots was observed (Figure 7C,F).

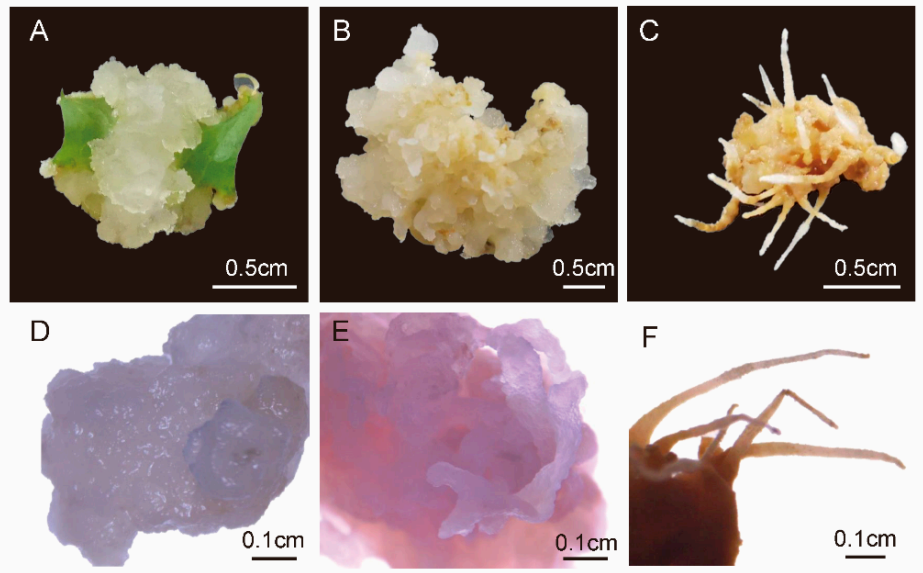


Figure 7. Phenotypes of calluses in different growth stages of yellowhorn. (**A**,**D**) Embryogenic callus (S1); (**B**,**E**) somatic embryos (S2); (**C**,**F**) adventitious root (S3).

Based on the expression profiles of XsWOX genes in different stages (Figure 8A, Table S2), we identified four XsWOX genes (*XsWOX1*, *XsWOX3*, *XsWOX4*, and *XsWOX5*) with relatively high expression levels throughout the three stages. Specifically, *XsWOX4* exhibited the highest expression level during the S1 and S2 stages, while *XsWOX5* was highly expressed during the S3 stage. The *XsWOX2*, *XsWOX6*, *XsWOX7*, and *XsWOX8* genes were minimally expressed at different time points. To further validate the expression patterns of the XsWOX genes, a qRT-PCR analysis was conducted during the S1, S2, and S3 stages. The qRT-PCR results showed similar trends to the transcriptome data (Figure 9). The expression levels of the XsWOX1, XsWOX3, XsWOX4, and XsWOX5 genes were high in the early stage of the callus and gradually decreased with the maturity of the callus.

3.7. Transcriptome Analysis of Yellowhorn in Different Organs and under Different Stress Treatments

To examine the expression of the XsWOX gene across different organs, our study explored the transcript abundance in flower, fruit, leaf, stem, and root samples (Figure 8B and Table S3). Our findings revealed distinct organ-specific expression patterns for the XsWOX genes. Specifically, *XsWOX5* exhibited the highest expression level in the stems, followed by the roots, and also demonstrated a moderate expression level in other organs. *XsWOX2* expression was predominantly observed in the fruits, and *XsWOX4* was slightly expressed in the stems, while the remaining XsWOX genes were barely expressed in these five organs.

Finally, we assessed the responses of the XsWOX genes to abiotic stresses by analyzing transcriptome data from the yellowhorn seedlings subjected to salt, salt-alkali, and low-temperature treatments (Figure 8C,D and Tables S4 and S5). Our findings confirmed that the *XsWOX5* gene expression was significantly affected by the stress treatment. *XsWOX1* expression gradually decreased with prolonged exposure to salt and salt-alkali stresses. However, no significant response to any of the three abiotic stress treatments was observed in the remaining genes.

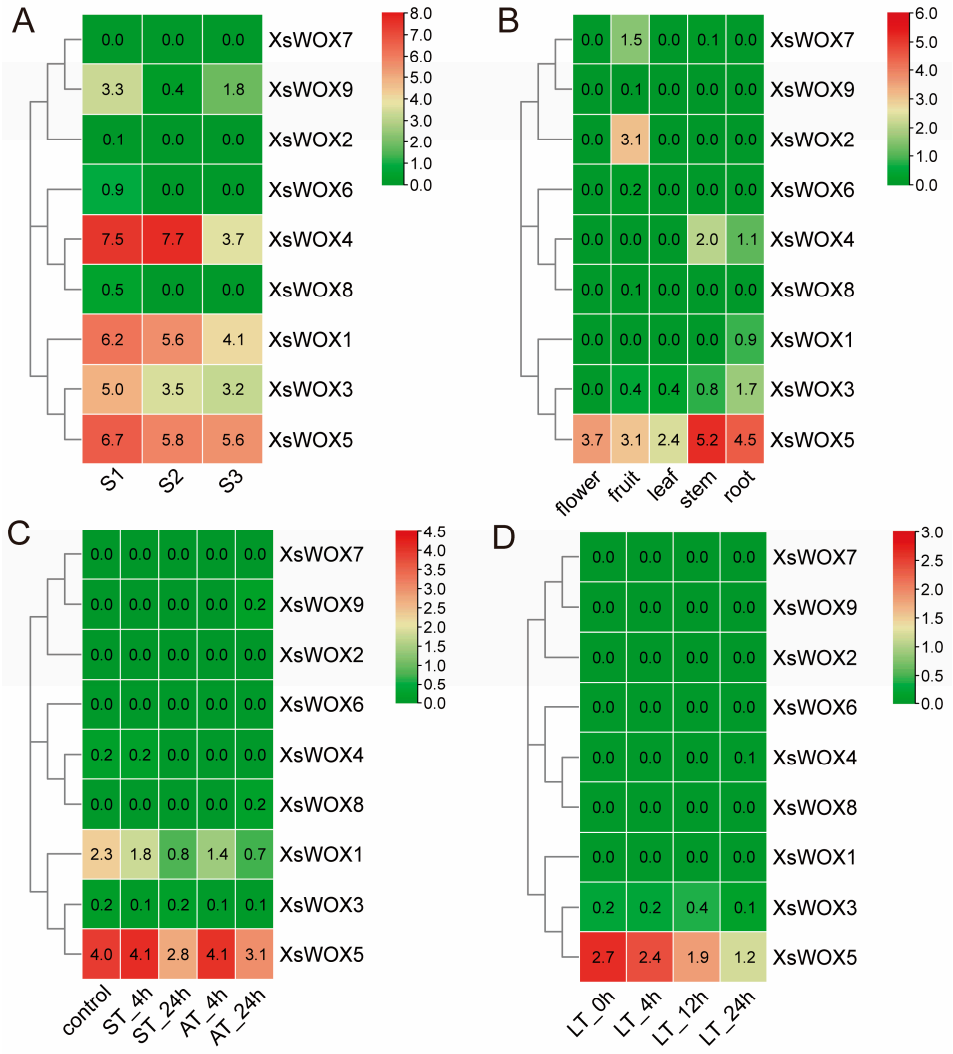


Figure 8. Expression profiles of XsWOX genes in different developmental stages, organs, and under different treatments. (**A**) Expression profiles of XsWOXs in three developmental stages. (**B**) Expression profiles of XsWOXs in five organs. (**C**) Expression profiles of XsWOXs in yellowhorn seedlings treated with salt and saline-alkali stresses. ST, salt treatment; AT, saline-alkali treatment. (**D**) Expression profiles of XsWOXs in yellowhorn seedlings treated with cold stress. LT, low-temperature treatment. Gene expression levels are indicated by numbers and color scale. The numbers on the heatmap represent the log scale, and the visual representation displays high expression levels in red and low expression levels in green.

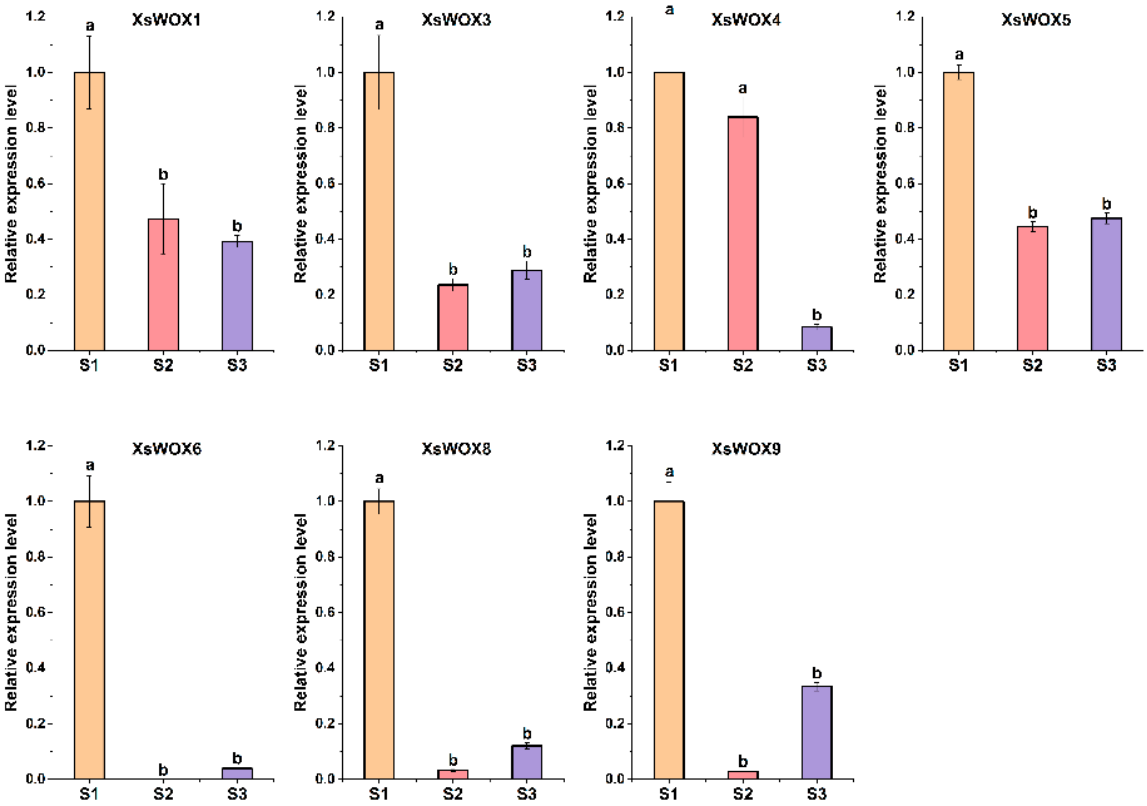


Figure 9. Expression profiles of seven selected XsWOX genes at the S1, S2, and S3 stages. Notes: The error bar represents the standard error of three repetitions, and different letters indicate differences between groups.

4. Discussion

The WOX gene family plays a crucial role in various developmental processes in plants, such as embryonic development and organ formation [53]. In recent years, multiple plant genome sequences have been published, leading to investigations on the WOX gene family in different woody plants [24,25]. However, there is a lack of comprehensive studies on the analysis and function of the XsWOX genes in yellowhorn on a genome-wide scale. Therefore, it is crucial to explore and understand the role of the XsWOX genes in the development of yellowhorn.

In our recent research, we conducted a comprehensive analysis of XsWOX genes in the yellowhorn genome using bioinformatics techniques. We found a total of nine candidate XsWOX genes and investigated their roles in organ development and the response to abiotic stress in yellowhorn. Based on the classification of WOX genes in *Arabidopsis* and rice, we categorized the XsWOX genes into three clades: ancient, intermediate, and WUS clades (Figure 2). The WUS clade, which consists of 20 members, including 6 from yellowhorn, 8 from *Arabidopsis*, and 6 from rice, exhibited the largest representation among the clades. This finding suggests that the WUS clade is highly conserved across different plant species, including yellowhorn, which supports previous research [20]. Nevertheless, the homologs of *Arabidopsis AtWOX6*, *AtWOX8*, *AtWOX9*, and *AtWOX15* could not be found in yellowhorn. Furthermore, while the GmWOX genes in soybean are evenly distributed among all chromosomes [54], the XsWOX genes in yellowhorn are irregularly distributed on only 6 out of 15 chromosomes (Figure 3A). Within our study, we identified *XsWOX3* and *XsWOX5* as members of the ancient clade, showing collinearity and suggesting that these genes might enhance yellowhorn's adaptability to environmental changes through gene replication [34]. In addition, we identified six conserved motifs in yellowhorn WOXs (Figure 5B). The distributions of these motifs and the exon–intron structures were consistent

with the evolutionary relationship, thereby maintaining the functional integrity of WOX genes. Overall, our study provides valuable insights into the diversity and conservation of XsWOX genes in yellowhorn, shedding light on their role in organ development and the response to abiotic stress. These findings contribute to our understanding of the genetic basis of yellowhorn and provide a foundation for future research in this area.

Although the regulatory role of transcription factors and genes in the growth and development of various plants has been extensively studied, there is limited knowledge of their role in yellowhorn. Thus, in this study, we aimed to address this gap by conducting transcriptome sequencing on samples collected from yellowhorn embryonic callus, somatic embryos, and adventitious roots. Our focus was on investigating the expression abundance of XsWOX genes. The transcriptome analysis revealed that most XsWOX genes exhibited high expression levels in the early stage of callus formation in yellowhorn. This finding suggests that XsWOX genes may play a crucial role in initiating callus formation (Figure 8A). Notably, previous research has demonstrated that, in older plants, the enhanced transcription of *EIN3* (the ethylene-sensitive TF ETHYLENEINSENSITIVE3) and the repression of *WOX5* and *WOX11* expression resulted in difficulties in regenerating roots [55]. Based on this knowledge, we speculated that the expression levels of WOX genes might be closely associated with the ages of the explants. Consequently, selecting younger tissues could potentially facilitate organ regeneration.

The key TFs, *XsWOX1*, *XsWOX3*, *XsWOX4*, and *XsWOX5*, were found to induce somatic embryos and adventitious roots (Figures 8A and 9). Among these, *WOX4* and its homologs not only regulated the development of vascular tissue and stimulated procambium and cambium cell proliferation, but they also played a role in regeneration in various plant species [53]. For instance, *WOX4* orthologs were expressed in the developing embryogenic callus of *Coffea canefora* [56], as well as in the somatic embryogenesis of *V. vinifera* [57] and other species. In our study, *XsWOX4* exhibited significantly higher expression than other genes during the S2 stage, highlighting its role in promoting embryogenic callus proliferation and transition to adventitious roots. This observation is supported by the presence of an auxin response element in the *XsWOX4* promoter (Figure 6C), and previous studies have shown that auxin and cytokinin can stimulate WOX expression and somatic embryogenesis in plants [58–60]. Therefore, it is plausible that XsWOX genes regulate somatic embryogenesis through their interaction with phytohormone signaling in yellowhorn. Furthermore, *XsWOX1* showed high expression levels during the S2 stage, and its Arabidopsis homologs, *AtWOX11* and *AtWOX12*, have been found to regulate callus formation and adventitious root development [28]. These homologs are known to activate *AtWOX5* and *AtWOX7*, which are crucial upstream genes in root primordia initiation and organogenesis [28]. Similarly, in rice, *OsWOX11* promotes adventitious root growth by activating target genes involved in auxin transport, metabolism, and cell wall construction via histone acetylation [61]. In cotton, both *GhWOX11* and *GhWOX12* are important genes that promote somatic embryogenesis and organogenesis, with conserved functions in callus induction and root formation [16]. Collectively, these findings suggest that *XsWOX1* and *XsWOX4* may be involved in somatic embryogenesis and play significant roles in root organ formation.

XsWOX5 not only regulated organ development, but also responded to abiotic stress (Figure 8). High expression levels were seen in all organs, but especially in the stems and roots, indicating that XsWOX5 is essential for the development of these structures. To our knowledge, *AtWOX13*, a homolog of *XsWOX5*, was dynamically expressed during embryo development, root formation, and growth in *Arabidopsis* [21]. This suggests that *XsWOX5*, like *AtWOX13*, may also have a significant impact on yellowhorn development and responses to environmental stresses. Similar WOX genes in the ancient clade have been identified in other tree species as well. For example, in Camellia sinensis, *CsWOX13*, *CsWOX15*, and *CsWOX16* exhibit significant upregulation under phytohormone and abiotic stress treatments [62]. In *Populus* × *xiaohei*, the expression levels of *PsnWOX13b* and *PsnWOX13c* in roots are regulated in response to short-term drought stress [63]. However,

the specific role of *XsWOX5* in stress resistance through root development remains unclear. Future studies should focus on further investigating the precise functionality of *XsWOX5*.

5. Conclusions

In this study, we investigated the WOX gene family in yellowhorn at a genome-wide level. A total of nine XsWOX genes were successfully identified and classified into three clades. The analysis of XsWOX gene promoters revealed the presence of elements related to plant growth and development, as well as responses to biological and abiotic stresses and hormone-induced responses. Through a transcriptome analysis, we observed that the *XsWOX1*, *XsWOX4*, and *XsWOX5* genes played crucial roles in somatic embryogenesis, while *XsWOX5* was specifically associated with organ formation and stress resistance. These findings provide valuable insights into the function, evolution, and regulation of the XsWOX gene family in the context of regenerative systems and abiotic stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15020376/s1, Table S1: The primer sequences of XsWOX genes for qRT-PCR. Table S2: Expression profiles of XsWOX genes in different developmental stages. Table S3: Expression profiles of XsWOX genes in different organs. Table S4: Expression profiles of XsWOX genes under salt and saline-alkali stress. Table S5: Expression profiles of XsWOX genes under cold stress.

Author Contributions: W.Z.: Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation, and Software. X.X.: Investigation and Visualization. L.L.: Data Curation and Project Administration. F.C.: Supervision, Funding Acquisition, and Writing—Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Natural Science Foundation of Jiangsu Provincial Science and Technology Department (BK20210611).

Data Availability Statement: All data from this study are presented in the article.

Conflicts of Interest: The authors declare no conflict of interest.

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