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Genome-Wide Identification of *WOX* Gene Family and Expression Analysis during Rejuvenational Rhizogenesis in Walnut (*Juglans regia* L.)

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Abstract: Rejuvenation is an efficient approach used in the cuttings of trees and horticultural crops, to improve their rooting ability, especially in difficult-to-root trees. *WOX* gene family members are involved in cell-fate transformation through balancing the maintenance and proliferation of the stem cells. However, there are no reports about the *WOX* gene family in Walnut (*Juglans regia* L.) and its relationship between rejuvenation and adventitious roots formation (ARF). Here, a genome-wide identification of *JrWOX* genes and their physical and chemical properties, phylogeny, and expression profiles in different organs and during rejuvenation-induced ARF is reported. The phenotype and histology characteristics of mature and rejuvenated cuttings (Mc and Rc) are also observed. In this study, 12 genes were identified and clustered into three groups based on phylogenetics, special domains, and conserved motifs. The gene structures and conserved motifs were relatively conserved, while the 12 sequences of the JrWOXs domain were diversified. Gene expression in root, stem, leaf, female flower, immature fruit, and zygotic embryo revealed that the expression levels of *JrWOX4a*, *JrWOX4b*, *JrWOX5*, *JrWOX11*, and *JrWOX13* in the root were significantly higher than those of other *JrWOXs*, while only the expression of *JrWOX11* was exclusive to the root organ. Additionally, rejuvenation treatment significantly induced almost all *JrWOX* genes, except *JrWOX4a*, *JrWOX4b*, and *JrWOX13* (Rc 0 vs. Mc 0). During the ARF process, the transcripts of *JrWOX11* and *JrWOX5* were consecutively increased on a significance level; in contrast, the transcription levels of the other *JrWOXs* decreased or changed insignificantly. The phenotype and histology observation indicate that rejuvenation treatment made the base of the stem expand and reduced the thickness and density of sclerenchyma between the cortex and phloem. This might provide the conditions for the formation of new meristem niches. The results provided insight into the *JrWOX* genes' general characteristics and their roles in rejuvenation-induced ARF.

Keywords: WOX family; *Juglans regia*; rejuvenation; ARF

1. Introduction

Adventitious root formation (ARF) is a common and widespread adaptive trait that is vital for vegetative propagation in woody plants. In some economically important trees, the poor rhizogenesis of stem cuttings has been a main bottleneck for the development of elite cultivars. To overcome the

problems associated with the low rooting abilities of stem cuttings in difficult-to-root trees, as well as to better understand the regulation mechanism underlying rhizogenesis, extensive studies have been conducted around rejuvenation and rhizogenesis [1,2]. Rejuvenation, including serial grafting, etiolation, repeated in vitro subculturing, heavy pruning, and mound-layering [3,4], could not only regress the stem cuttings to a more juvenile state, but also significantly improve the competence of ARF. The previous research of ARF improved by rejuvenation has been carried out in terms of phenotype, microanatomy, and physiology [5–7]. However, the molecular mechanisms that regulate ARF in difficult-to-root trees are poorly understood.

WUSCHEL-related homeobox (*WOX*) genes, coding WOX transcription factors, are plant-specific homeobox subfamily genes and are widely found in green plants [8]. *WOX* genes play crucial roles in key developmental processes, by organizing stem cell maintenance and proliferation in the shoot apical meristem (SAM), root apical meristem (RAM), and cambium (CAM) [8–12]. *AtWUS*, a member of the modern WOX clade (MC), is involved in the feedback loop with CLV3 for SAM maintenance and also regulate the floral patterning [13,14]. *AtWOX5*, mainly expressed in the quiescent center and regulates the stem cell identity of RAM via a negative feedback signal provided by CLE40 [15,16]. *AtWOX4*, is also a *MC-WOX* gene, primarily regulates stem cell maintenance of CAM through the CLE41/CLE44-PXY-WOX4/WOX14 pathway [17]. *AtWOX1* act redundantly with *PRESSED FLOWER1* (*PRS1/AtWOX3*) during the formation of the lateral domains of vegetative and floral organs [18]. *AtWOX2* is expressed specifically in zygotes, and is required for apical patterning [19]. *AtWOX6/PRETTY FEW SEEDS2* (*PFS2*) is expressed abundantly in developing ovules, and influence the ovule formation [20]. *AtWOX8* and *AtWOX9* regulate embryonic development [19,21]. *AtWOX11* and *12* are involved in the first-step cell fate transition during ARF of detached leaves [22,23]. *AtWOX13* and *AtWOX14* are also have effects on the regulation of primary and lateral root development in *Arabidopsis* [24]. The roles of some *WOXs* in adventitious root formation and development have also been well studied in annual plant rice (*Oryza sativa* L.) [25–27], and the easy-to-root tree poplars (*Populus tomentosa* Carr) [9,28]. By contrast, there is less information about their mechanism in difficult-to-root trees.

Walnuts are generally recognized as being difficult-to-root species and even more difficult to propagate from cuttings. With the completion of the walnut genome sequence and multiple analysis of the rejuvenation in walnut [5,29–31], the walnut could be adopted as an important material for difficult-to-root trees to investigate the regulation mechanism in ARF. The patent created from walnut also significantly improve the competence of ARF in numerous other difficult-to-root trees, such as cork oak (*Quercus suber* L.), teak (*Tectona grandis* L.), and California redwood (*Sequoia sempervirens* (Lamb.) Endl.) [32]. In recent years, the transcriptome landscape of walnut has also been reported, and that gene transcriptional regulatory network is undergoing building with the RNA-seq data and bioinformatics tools during ARF by rejuvenation in walnut [33,34]. While two *WOX* genes are expressed dynamically in the process of walnut rooting [35], whether *WOX* genes are induced by rejuvenation or whether other *WOX* genes are involved in ARF of walnut trees has not been investigated.

In this study, we systematically characterized 12 *WOX* genes in the common walnut. The basic local alignment search tool (BLAST) was used to identify *JrWOX* genes from the available genome in walnut [36], thereby detailed information about the physiological and biochemical characters, structural features, and conserve motifs of the 12 genes were revealed. There might exist differences between easy-to-root trees and difficult-to-root trees. We also constructed a phylogenetic tree of *WOX* genes and the evolution of the root. To understand the function of rejuvenation on *JrWOXs*, we studied the transcriptional levels of *JrWOXs* in mature and rejuvenated cuttings. Subsequently, the expression profiles of *JrWOXs* at critical time points in ARF of mature and rejuvenated cuttings were also detected. Our results provide useful theoretical support for the functional characterization of these *JrWOXs* genes that are involved in the ARF, improved by rejuvenation in common walnut, and provide insight regarding the molecular mechanisms of rejuvenation in difficult-to-root trees.

2. Materials and Methods

2.1. Identification and Bioinformatics Analysis of WUSCHEL-Related Homeobox Genes in J. regia Genomes

The genomic sequences of common walnut *J. regia* were first downloaded from NCBI (http://planttfdb.cbi.pku.edu.cn) [37], and the predicted WOX genes in *J. regia* were found in PlantTFDB (http://ftp.ccb.jhu.edu/pub/dpuiu.Walnut/English_Walnut/v.2.0d). The genomic sequences were conducted to search homologous *WOX* genes, using known WOX proteins from Arabidopsis thaliana as queries, with a cutoff E-value of $<10^{-10}$. Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was performed to confirm the integrity of homeobox domain (PF00046) in these candidate proteins, and the proteins lacking HD domain were removed. Subsequently, Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/), ProtParam (http://web.expasy.org/protparam/), and Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) were used to analyze *JrWOX* genes' structure, basic physical and chemical properties, and subcellular location, respectively. Alignment of canonical conserved residues in JrWOX homeodomain sequences were analyzed by WebLogo (web-based sequence logo generating application; Weblogo.berkeley.edu). Finally, MEME program was applied for the elucidation of Motifs in the candidate proteins. Each *JrWOX* gene was given a unique name based on the BAST results with *AtWOX1–15* (Table 1).

Table 1. Basic characteristics of the proteins encoded by *JrWOXs*.

Gene	Gene ID	Protein Length/aa	Molecular Weight (Mw)/Da	Isoelectric Point (pI)	Protein Hydrophobi-city	Aliphatic Amino Acid Index	Subcellular Localization
JrWUSa	XM_018977489.1	285	31,531.34	5.70	−0.966	43.79	Nucleus
JrWUSb	XM_018974029.1	283	31,356.19	6.15	−0.925	44.77	Nucleus
JrWOX1	XM_018987154.1	360	41,092.06	9.43	−0.891	58.00	Nucleus
JrWOX2	XM_018980445.1	251	27,979.82	6.40	−0.726	53.63	Nucleus
JrWOX3a	XM_018969027.1	204	23,370.43	8.77	−0.590	61.72	Nucleus
JrWOX3b	XM_019004669.1	206	23,981.82	6.15	−0.879	62.96	Nucleus
JrWOX4a	XM_018964283.1	226	25,844.20	8.92	−1.000	54.87	Nucleus
JrWOX4b	XM_019000128.1	226	25,541.73	9.16	−0.908	54.82	Nucleus
JrWOX5	XM_018966208.1	184	20,891.56	8.73	−0.708	65.16	Nucleus
JrWOX9	XM_018977986.1	387	42,711.37	6.62	−0.597	58.71	Nucleus
JrWOX11	XM_018977839.1	258	28,171.61	5.86	−0.434	71.36	Nucleus
JrWOX13	XM_019002154.1	275	31,410.80	5.61	−0.923	65.56	Nucleus

2.2. Phylogenetic Analysis

The JrWOX protein sequences from green alga (*Ostreococcus tauri, Ot*), moss (*Physcomitrella patens, Ph*), lycophyte (*Selaginella moellendorffii, Sm*), fern (*Ceratopteris richardii*, *Cr*; and *Cyathea australis*, *Ca*), gymnosperm (*Ginkgo biloba*, *Gb*; *Picea abies*, *Pa*), Monocotyledon angiosperm (*Oryza sativa*, *Os*; and *Zea mays*, *Zm*), Dicotyledon angiosperm (*Arabidopsis thaliana*, *At*; *Populus trichocarpa*, *Ptr*; *Spirodela polyrhiza*, *Sp*; *Quercus suber*, *Qs*; *Juglans regia*, *Jr*), and representative species were used for phylogenetic analysis. The gene ID were listed in Table A1. Clustal X 2.0 software [38] was used for the multiple alignment, and unrooted phylogenetic trees were constructed using MEGA 7.0 software [39] with the neighbor-joining (NJ) method by 1000 bootstrap replicates.

2.3. Analysis of Regulatory Elements in the Promoter Regions of the JrWOX Gene Family

The upstream regions (2.0 kb) of the translation initiation sites (ATG) of *WOX* genes were used as promoter fragments. In addition, the elements were located in the promoter sequences, using the program PlantCARE online (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.4. Plant Materials, Growth Conditions, and Treatments

The walnut stock cultivar "Zhongningsheng" [ZNS, (*Juglans hindsii* × *Juglans regia*)] [30], which is selected with a steady and high yield, a good adaptation, and high graft compatibility, is grown in the

Luoning county, Henan Province. To analyze expression profiles of *JrWOX* genes in different organs, three 23-year-old ZNF trees were chosen for the collection of root (R), stem (S), leaf (L), female flower (FF), immature fruit (IF), and zygotic embryo (ZE) samples. The cuttings of 1-year-old ZNS shoots were rejuvenatedand rooting. Then the root (R) was collected, washed and dried. Stem (S) and leaf (L) were also obtained from the middle part of 1-year-old saplings. Female flower (FF), immature fruit (IF), and zygotic embryo (ZE) were harvested, respectively, 15, 45, and 60 days after flower, when the trees were at the stage of pollination, after pollination, and starting to swell, respectively. Six samples of each organ from the same tree were mixed as one biological replicate. All the above materials were rapidly frozen in liquid nitrogen and stored at −80 °C.

Rejuvenated soft cuttings (Rc) of ZNS were obtained as described as follows: (1) serial grafting, the buds of new ZNS shoots were used as scions and were grafted to 1-year-old seedlings (rootstock) for sprouting; (2) etiolation, 1-year-old grafted seedlings were placed horizontally on the greenhouse seedbeds and covered with 4–5 cm of clean wet sand. The sand temperature and humidity were respectively held at 15 ± 5 °C and 50%, which ensured maximum side bud germination rate. After about two months, the new shoots of 14–18 cm height were harvested as rejuvenated soft cuttings [5]. Mature soft cuttings (Mc) of the same height were directly collected from new shoots of the 23-year-old ZNF trees. The 8–10 cm base of both cutting materials was treated with 9 mM of IBA (dissolve in 30% (*v*/*v*) ethanol) for about 30 s and then submerged in seedbeds. Under the conditions of field, 80% relative humidity and 23 ± 5 °C temperature were controlled by automatic intermittent spraying. After rooting induction 0, 1, 2, 3, and 5 days, cambium and phloem tissues surrounding the xylem of the 1 cm base of the both cuttings were scraped by a sterile scalpel blade. The harvested materials were, in turn, named Rc0, 1, 2, 3, 5 days and Mc0, 1, 2, 3, 5 days, then rapidly frozen in liquid nitrogen and stored at −80 °C to detect the role of *JrWOX* genes in rooting. Three biological replicates were performed in different pots for each treatment.

2.5. Expression Pattern Analysis of the JrWOX Genes

RNA isolation, quantitative reverse transcription qRT-CR, and RT-PCR were performed according to Ma et al. [33]. *GAPHD* was used as an endogenous control gene. Then, qRT-PCR was performed by using the SYBR Premix Ex Taq™ II Kit (TaKaRa, Dalian, China) on a LightCycler 480 system (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The primers used for qRT-PCR were designed by using Primer 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and listed in Table A2. All experiments were carried out at least three times, and data analysis was performed by using the $2^{-\Delta\Delta Ct}$ method [40].

3. Results

3.1. Identification and Sequence Characteristics of JrWOX Genes

To identify *WOX* genes in *J. regia*, the protein sequences of 15 *A. thaliana* WOX (AtWOX) were used as a query to perform basic local alignment search tool algorithms (BLASTP) and Hidden Markov models (HMMs) search against the *J. regia* genome database. A total of 12 nonredundant proteins were identified, and protein length, molecular weight (Mw), isoelectric point (pI), protein hydrophobicity, aliphatic amino acid index, and subcellular localization prediction are shown in Table 1. The length of proteins varied from 184 aa (JrWOX5) to 387 aa (JrWOX9), the predicted pI ranged from 5.61 to 9.43, and the Mw ranged from 23.37 to 42.71 kDa (Table 1). The protein hydrophobicity is <0, indicating that the 12 JrWOX proteins are hydrophilic. Aliphatic amino acid index is >40, indicating that JrWOX proteins are thermally stable. All the 12 WOX proteins are predicted to localize in the nucleus based on the signal peptides. JrWOXs act as a transcription factor in the nucleus, regulating the expression of the downstream target genes.

3.2. Gene Structure, Conservative Domain, and Motif Analysis of JrWOXs

To gain further insight into the structural diversity of different groups of JrWOX genes, the simplified neighbor-joining (NJ) phylogenetic tree of their homeobox domains was constructed, as well as the analysis of gene structures and functional motifs (Figure 1). As shown in Figure 1A, the 12 JrWOX members are divided into three subfamilies. The members close on the phylogenetic tree shared similar gene structures and intron phases (Figure 1B). Extron number ranged from 2 to 4. All members have the homeobox domain, while only the members clustered in the first subfamily (WC subfamily) have the transcription activation domain WUS-box. Three members of the nine WC-WOX also contained an ethylene-response-factor-like domain (ERF-like)—JrWUSa, JrWUSb, and JrWOX5. The same subfamily also shared a similar motif-structure, as shown in gene structure (Figure 1C). There were ten conserved motifs identified in JrWOXs; among them were motif1 and 2, and motif3 and 7 might correspond to conservative functional elements distributing in homeobox, WUS-box and ERF-like domains, respectively. JrWOX13 only contained the common conserved motif1 and 2; however, IC-subfamily members JrWOX9 and JrWOX11 contained an additional Motif5 at the C-terminus. In regard to WC-WOXs, JrWUSa/b specifically contained motif4, while JrWOX4a/b exclusively contained motif5, 9, and 10. These results suggested that gene structures and formation of motifs are associated with the sub-functionalization and neofunctionalization of WOX members.

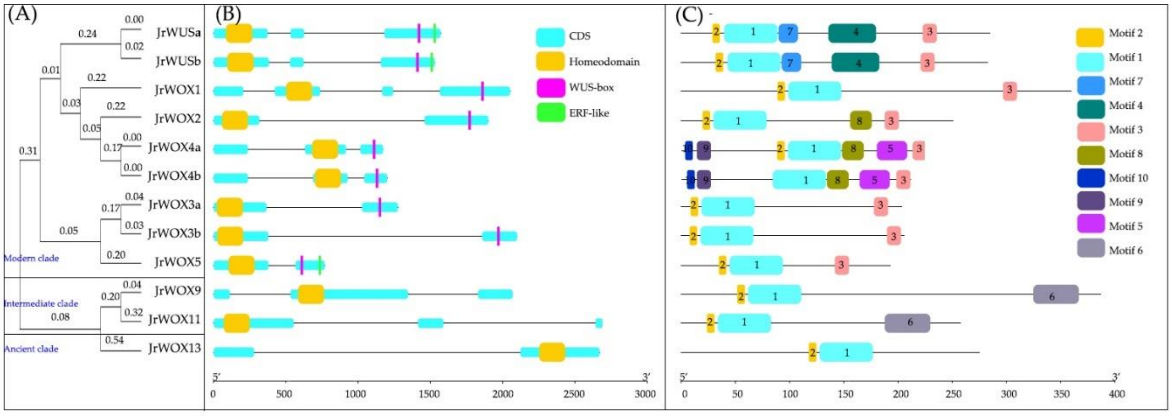


Figure 1. Phylogenetic relationships, gene structure, and motif phases of the *WOX* genes in *J. regia*. (**A**) Phylogenetic tree based on JrWOXs homeobox domains. (**B**) The gene structures and intron phases of *JrWOXs* gene. (**C**) The distribution profiles of conserved motifs on JrWOXs proteins.

3.3. Multiple Alignment the WOX Protein Sequence in J. regia

Members of the WOX family contain a conserved homeodomain, which consists of the "helix-loop-helix-loop-helix" (Figure 2A). Alignment of the homeodomains of 12 WOX genes in *J. regia* revealed the residues were highly conserved. There were 16 highest conserved residues in the homeo-domain; among them, Leucine (L), Valine (V), and Tryptophan (W), which are marked with asterisks, are also the most conserved of the other 50 species. These three residues are located inside the structure of the homeodomain, and they might influence the factor function by changing the domain structure. The residues within the two black boxed motifs have positional correlation, which may also influence the protein function [9]. The red boxed residues within helix 3 can distinguish the three clades of WOX proteins (Figure 2A): The 'YN' to 'FY' change in the sequence occurred during the evolution from the ancient to the intermediate clade, and the 'R' to 'H' change may have occurred during the evolution from the intermediate clade to the MC. Only the members of MC harbor the WUS-box and the EAR-like domain appeared exclusively in JrWUSa/b and WOX5 (Figure 2B,C). Interestingly, C-terminal region of IC-WOXs contained a specific motif, while there were none found in MC and AC. There are other conservative structural domains in the JrWOX family, while the functions need to be further studied.

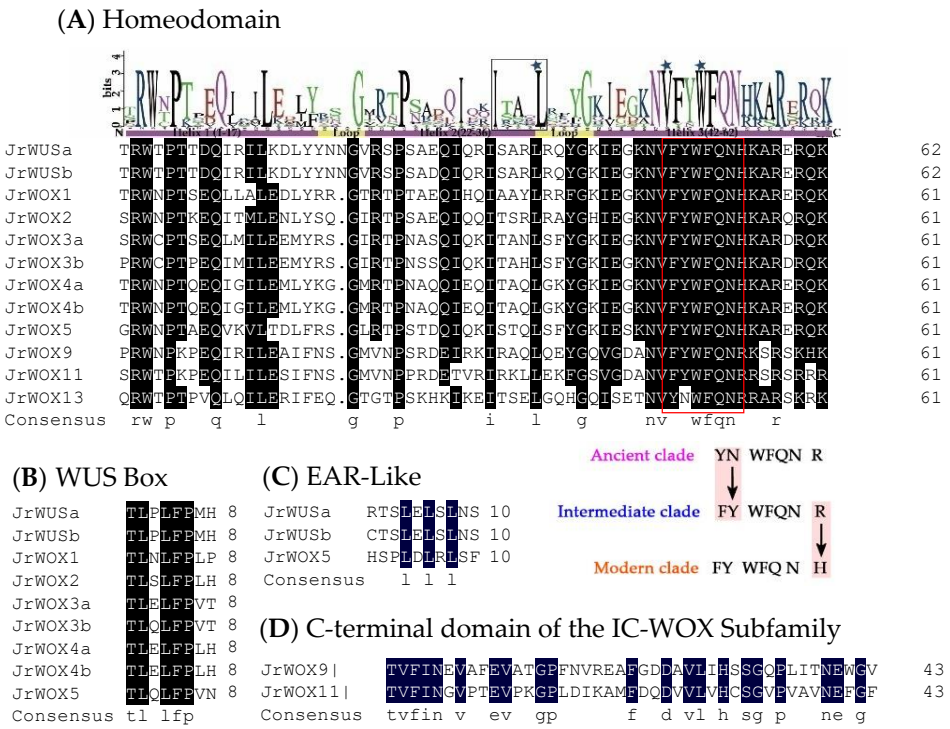


Figure 2. Comparison of the WOX protein family in *J. regia*. (**A**) Alignment of the homeodomain sequences. Asterisks indicate residues that are highly conserved in homeodomains (Gehring et al., 1990). (**B**) Alignment of the WUS-box that is located downstream of the homeodomain. (**C**) Alignment of the EAR-like domain of the MC-WOXs. The identical residues are in black boxes. (**D**) Alignment of the C–terminal region in a subset of IC-WOX subfamily.

3.4. Characterization of Cis-Acting Elements in the Promoter Regions of JrWOXs

To further investigate the possible regulation mechanism of WOX genes in *J. regia*, the cis-elements in the promoter regions of *JrWOX* genes were analyzed, using the PlantCARE database. A series of hormone-related (e.g., Auxin, ABA, ZT, GA, MeJA, and Ethylene), and development-related (e.g., meristem expression, seed-specific regulation, endosperm expression, circadian control, and palisade mesophyll cells), stress-related (e.g., heat, drought, low temperature, and anaerobic induction), and light response cis-acting elements were identified (Figures 3 and A1). In hormone-related cis-acting elements, 58.33% of JrWOXs (seven genes) had the auxin response elements, 91.67% of JrWOXs (11 genes) had the ABA response elements, which was the most abundant cis-acting element, with its number reaching 54. Moreover, as-1 (Plant hormone responsiveness), O2-site (zein metabolism regulation), ERE (ethylene responsiveness), CGTCA-motif (MeJA-responsiveness), GARE-motif (MeJ-responsiveness), TCA-element (salicylic acid responsiveness), P-box (gibberellin responsiveness), TATC-box (gibberellin responsiveness), GARE-motif (gibberellin responsiveness), TGA-element (auxin responsiveness), and AuxRR-core (auxin responsiveness) were identified in the promoters of 8, 8, 5, 8, 8, 10, 5, 3, 3, 6, and 1 JrWOXs, respectively. In development-related cis-acting elements, there were 10 root-specific elements (W-box), seven seed-specific regulation elements (RY-element), eight endosperm-specific expression elements (GCN4_motif and AACA_motif), eight meristem-specific regulatory (CAT-box and CCGTCC motif), and two circadian cis-acting elements, that were located in 7, 4, 6, 6, and 2 JrWOXs promoters, respectively. Furthermore, large numbers of cis-acting elements related to drought resistant (MRE, MBS, and DRE), abiotic resistant (ARE, STRE, WRE, and GC-motif), low temperature resistant (LTR), etc., were detected. This result implied that JrWOXs might not only be involved in the developmental processes, but might also be recruited in biotic and abiotic stress.

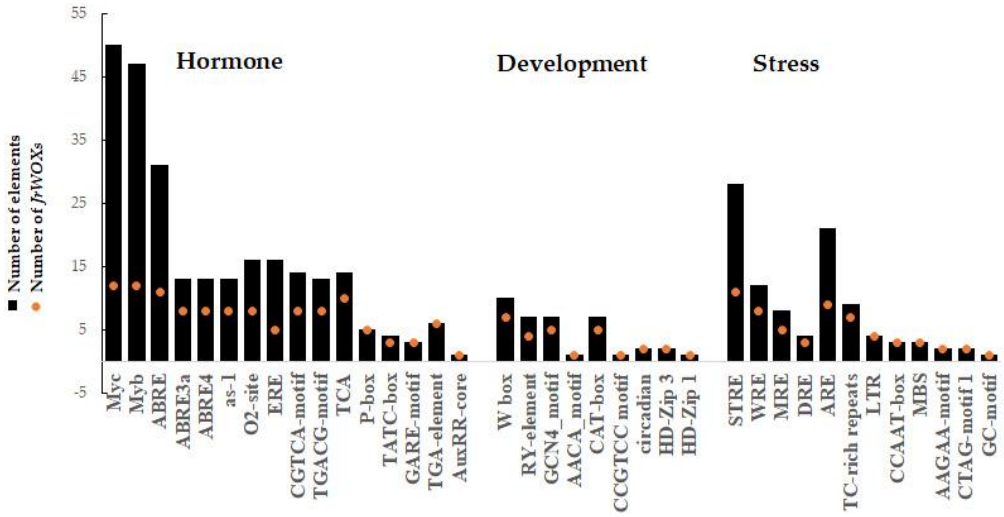
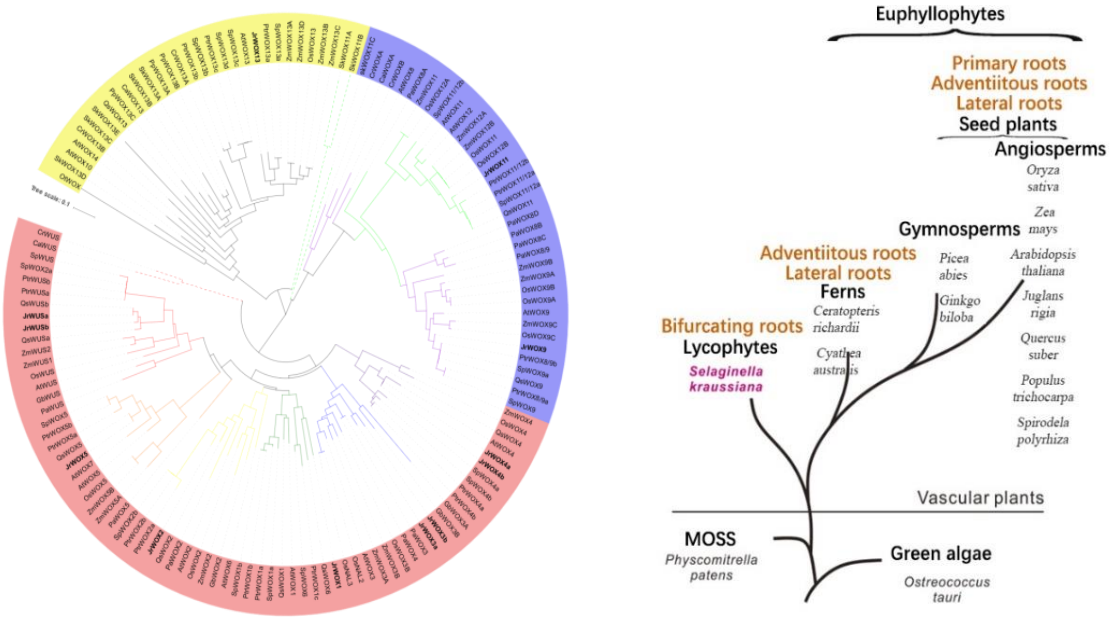


Figure 3. Various cis-acting elements in the promoter of *JrWOX* genes.

3.5. Phylogenetic Analysis of J. regia WOXs and Root Evolution in Green Plants

To further investigate the possible function of JrWOXs during evolutionary change, a phylogenetic tree of 138 WOX proteins from 14 species was conducted, using the neighbor-joining (NJ) method. The phylogenetic results show that all the WOX proteins were allocated into three clades: the modern clade (MC), the intermediate clade (IC), and the ancient clade (AC) (Figure 4A). Green algae and mosses, having no root structures, are only distributed with AC-WOX genes. The *WOX* gene may originate from green algae, as *WOX* genes can be found in the genomes of euphyllophytes, lycophytes, moss, and green algae, but do not exist in the red algae genome [13,41]. *S. kraussiana* encodes four *WOX* proteins in AC and one WOX protein in IC; it is worth noting was that there were two lycophyte-specific SkWOX proteins, SkWOX11A and SkWOX11B, marked with a purple dotted line in Figure 4A. SkWOX11A and SkWOX11B appear to be transitional between the AC and IC, in line with the fact that the first root-evolution event was in the lycophytes [42]. The second root-evolution event was in the ancestor of the ferns. Although CrWUL were included in the WC, they might represent a transitional evolutionary stage from IC [43]. This suggests that IC WOX proteins are recruited in adventitious and lateral root formation. The WC genes have further evolved in gymnosperms and angiosperms. During the seed plant stage, primary roots appeared in addition to adventitious and lateral roots (Figure 4B). In *J. regia*, 12 WOXs were also classified into three clades: as for AC, *JrWOX13* are homologous to *Arabidopsis WOX10*, *WOX13*, and *WOX14*. *JrWOX9* and *JrWOX11* are homologous to *Arabidopsis WOX8*, *WOX9*, *WOX11*, and *WOX12* belonging to IC. The WUS Clade contained nine *JrWOX* genes, including *JrWUSa*, *JrWUSb*, *JrWOX1*, *JrWOX2*, *JrWOX3a*, *JrWOX3b*, *JrWOX4a*, *JrWOX4b*, and *JrWOX5*. This indicated that the differentiation of transcription factor family members may be earlier than that of seed species. However, the possible function of *WOX* family members during root morphological variation in seed plants, especially woody plants, is not enough.



(**A**) (**B**)

Figure 4. Dendrogram based on the full-length protein sequences of the WOXs and root evolution in green plants. (**A**) Phylogenetic analysis of WOXs. Bootstrap values are given (1000 rounds). The tree contains sequences from green alga (*Ostreococcus tauri*, *Ot*), moss (*Physcomitrella patens*, *Ph*), lycophyte (*Selaginella kraussiana*, *Sk*), fern (*Ceratopteris richardii*, *Cr*; and *Cyathea australis*, *Ca*), gymnosperm (*Ginkgo biloba*, *Gb*; *Picea abies*, *Pa*), and Monocotyledon angiosperm (*Oryza sativa*, *Os*; and *Zea mays*, *Zm*) and Dicotyledon angiosperm (*Arabidopsis thaliana*, *At*; *Populus trichocarpa*, *Ptr*; *Spirodela polyrhiza*, *Sp*; *Quercus suber*, *Qs*; *Juglans regia*, *Jr*) representative species. The unrooted amino acid sequence similarity trees were generated by using the MEGA7.0 software by the neighbor-joining method. Modern clade (red; M); intermediate clade (blue; I); ancient clade (black; A). (**B**) Simplified evolutionary route of green plants, showing two existing branches of vascular plants; lycophytes and euphyllophytes.

3.6. Rejuvenation-Changed Phenotype and Histological Features of Cuttings

The leafy twigs were used as cuttings for detecting morphological characteristic changes. The bark of mature cuttings (Mc) is a uniform green (Figure 5A). The base of the rejuvenated cuttings (Rc) became swollen, and the bark was yellowish-white, while the upper bark was also yellowish-white (Figure 5B).

Histological observation of mature and rejuvenated cuttings showed that a large number of histologic sections indicated that there was no latent rooting primordial group in Mc and Rc, implying walnut rooting in a type of induction model. The sections of Mc and Rc both consisted of periderm, cortex, cortical vascular tissue, and pith. Interestingly, the sclerenchyma distributing between the cortex and phloem was arranged more closely in Mc and contained more layers of cells than that in Rc (Figure 5C,D), which might be one of the histological reasons for rejuvenation improving the rooting ability of walnut cuttings.

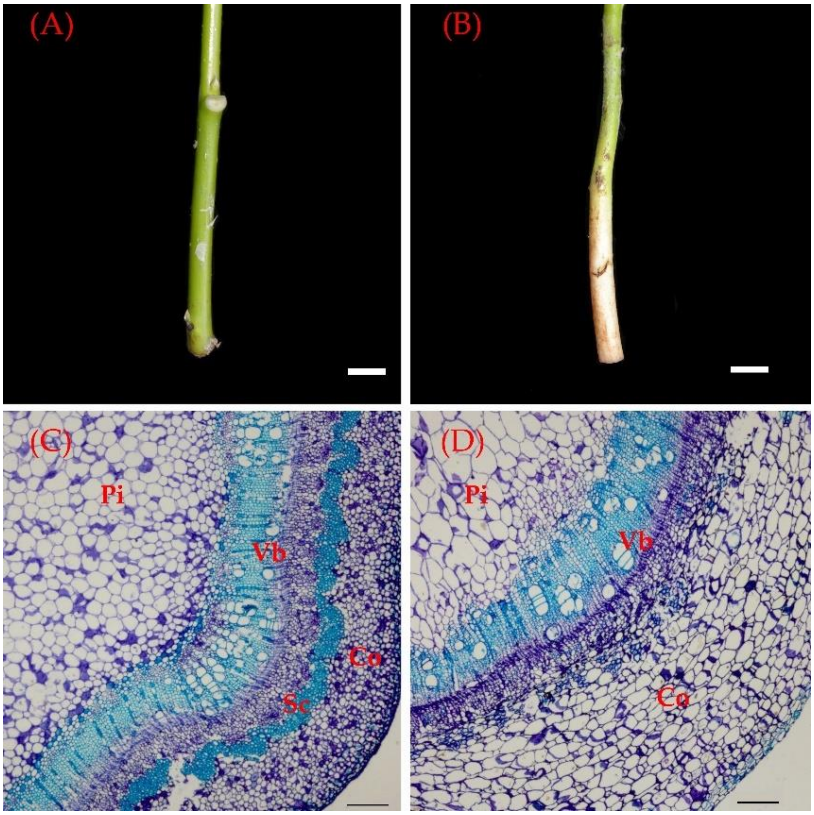


Figure 5. Phenotype and histological comparison between mature cuttings and rejuvenated cuttings. (**A**,**C**) Phenotype and histological investigation of Mc. (**B**,**D**) Phenotype and histological investigation of Rc. Co, cortex. Vb, cortical vascular tissue. Pi: pith. Sc: sclerenchyma. Scale bars: 10 mm (**A**,**B**), 100 μm (**C**,**D**).

3.7. Diversified Expression Patterns of JrWOX Genes

3.7.1. Expression Patterns of JrWOX Genes in Diversified Organs

To gain insights into possible developmental and physiological functions of JrWOX proteins, the expression pattern of *WOX* genes in ZNS were analyzed by semi-quantitative RT-PCR and relative quantitative RT-PCR (Figure 6). The results showed that the performances of 12 family members were different in different organs. In WC, *JrWUSa/b*, *JrWOX1* and *JrWOX2* were expressed specifically in the zygotic embryo (ZE). There were slightly different expression profiles between two subgenes. The highest expressions of *JrWOX3a* and *JrWOX3b* were in female flowers (FF) and zygotic embryos (ZEs), respectively, although they both had lower expressions in the root (R), stem (S), leaf (L), and immature fruit (IF). The expression level of *JrWOX4a* and *JrWOX4b* were both higher in vegetative organs than reproductive organs; however, the highest expressions of *JrWOX4a* and *JrWOX4b* were in the root (R) and stem (S), respectively. Similar to *PtoWOX5b*, *JrWOX5* was ubiquitously expressed, but *AtWOX5* was restricted to the RAM [15,44]. In IC, *JrWOX9* was expressed highest in female flowers (FF), while the highest expression of *JrWOX11* was in the root (R) system. The only subclass of the ancient WOX clade in *J. regia*, *JrWOX13,* also exhibited a ubiquitous expression pattern.

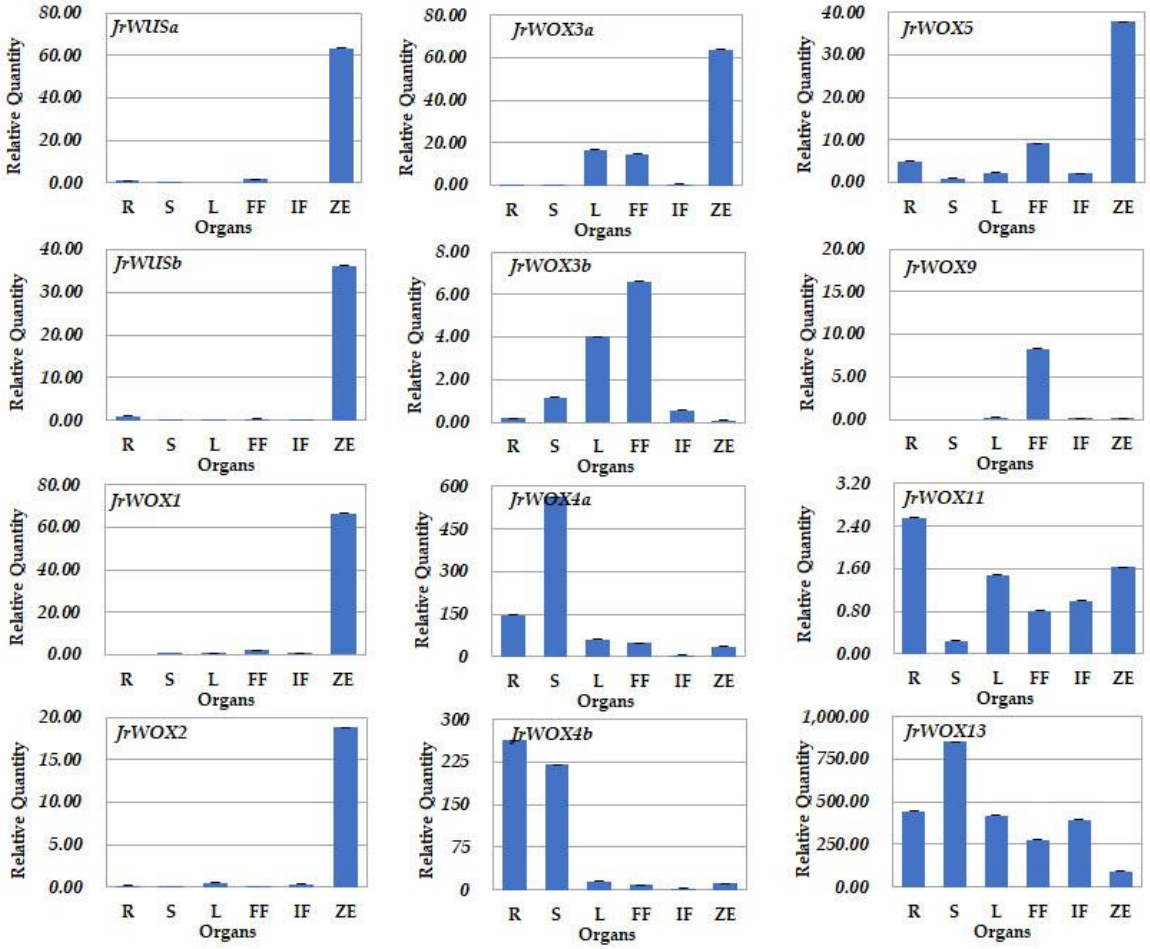


Figure 6. Expression analysis of walnut *WOX* family's genes in different tissues, using qRT-PCR.

3.7.2. Expression Patterns of *JrWOX* Genes in ARF of Mature and Rejuvenated Cuttings

Rejuvenation is an essential factor to improve the soft-shoot rooting ability of mature woody species. In this study, the rejuvenated and mature cuttings of walnut were used as materials. The results of quantitative analysis indicated that rejuvenation increased the expression level of *JrWOX* genes, other than *JrWOX4a*, *JrWOX4b*, and *JrWOX13*. In the mature cuttings, *JrWUSa*, *JrWUSb*, *JrWOX1*, *JrWOX2*, *JrWOX3a*, *JrWOX3b*, and *JrWOX5* nearly did not express during the process of ARF. In the rejuvenated cuttings, *JrWUSa/b*, *JrWOX1*, *JrWOX2*, *JrWOX3a/b*, and *JrWOX4a/b* showed a slender declining trend, while *JrWOX5* exhibited a significant increase trend. The expression level of *JrWOX11* sharply increased before 2 days, and then it descended in the rejuvenated cuttings; however, in mature cuttings, *JrWOX11* displayed high expression until the last rooting stage (Figure 7). The results of histology show that the cambium cells started to divide strongly three days after rejuvenated cuttings and induced rooting [5]. Then, cambium thickened, and the root meristem formed on the fourth day [5]. It can be presumed that *JrWOX11* was essential for adventitious root primordia formation, and *JrWOX5* was crucial for root development.

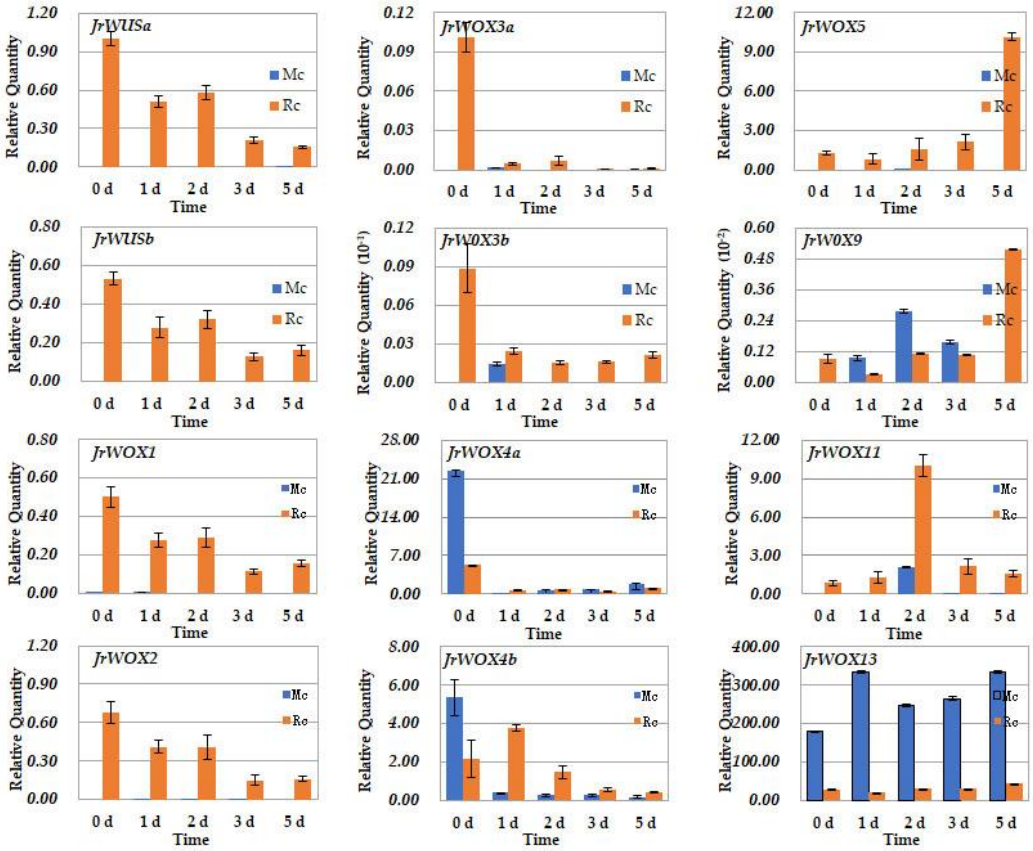


Figure 7. Expression analysis of walnut *WOX* family genes during adventitious roots formation in rejuvenated and mature soft shoots.

4. Discussion

4.1. Diversification of WOX Genes in J. regia

With the rapid development of sequencing technology and the increase in the efficiency and accuracy of sequencing, more and more species' genome and transcriptome data have been reported. This provides good data resources for analyzing the gene structure and function prediction on the scope of whole-genome identification and comparison. The genomes of plants are different; for example, *O. tauri*, *P. patens*, *S. kraussiana*, *P. trichocarpa*, *Z. mays*, *A. thaliana*, and *O. sativa* genome are 14, 472, 115, 423, 2300, 125, and 389 Mb, respectively. These plants contain *WOX* genes 1, 3, 8, 18, 18, 15, and 14, respectively. It could be speculated that the genome size was not the main determinant of the number of gene families [45]. Along with this, the common walnut genome is 667 Mb, while 12 WOX encoding genes were identified in *J. regia* (Table 1), which could be categorized into modern, intermediate, and ancient clades. While there were no *JrWOX* genes classified together with *AtWOX6*, *AtWOX7*, *AtWOX10*, or *AtWOX14*, other *WOX* genes gathered in the same clade may perform similar functions. For example, WUS-box in *WOX6* and *WOX7* is involved in leaf-blade outgrowth and floral organ development in *Nicotiana Sylvestris* [46]. In *A. thaliana*, while the role of *AtWOX14* is unknown, it may be restricted to the early stages of lateral root formation and the floral transition, as the *AtWOX13* gene is involved in replum formation during fruit development [24,47]. This study also identified two copies of *JrWOXs* which could be classified as *WUS* (*JrWUSa* and *b*), *WOX3* (*JrWOX3a* and *b*), and *WOX4* (*JrWOX4a* and *b*), and this might result from chromosomal duplication events.

4.2. The Characteristics of the WOX Gene Family of Walnut

Similar physical and chemical properties indicate that members may have some similar functions. In *A. thaliana* and *P. trichocarpa*, WOX proteins' length range from 122 aa (AtWOX7) to 378 aa (AtWOX9) and from 181 aa (PtrWOX5) to 390 aa (PtrWOX8/9a). Similarly, the length of JrWOX proteins varied from 184 aa (JrWOX5) to 387 aa (JrWOX9). Because *JrWOX5* and *PtrWOX5* are homologous to *AtWOX7*, *JrWOX5* may perform the same functions as *PtrWOX5* and *AtWOX7* in adventitious root formation and development [44,48,49]. Although the physicochemical properties of different JrWOX proteins were different, the sister copies of the same JrWOX protein were similar (Table 1). Proteins normally perform their biological functions depending on their own functional domains. Through protein structure analysis, it was found that all the 12 WOX transcription factor family members in walnut contained a conserved homologous domain composed of 60 amino acid residues (Figures 1B and 2A). Comparing these with the 350 WOX proteins from 50 species, homeodomain has the structure of "helix-loop-helix-turn-helix", which plays an important role in DNA recognition and binding [10]. Homeodomain contains many highly conserved amino acid residues, among which the last amino acid residue L (leucine) of the second helix structure, the last amino acid residue I/V (isoleucine/valine) of the turn structure, and the amino acid residue V (valine) of the middle position of the second helix structure are the most conserved (Figure 2A). Three-dimensional structure imaging shows that these three amino acid residues are located in the interior of homeodomain, indicating that they play a key role in the function of the WOX transcription factor [46]. Moreover, the angle of formation of "helix-turn-helix" structure in ancient branches is smaller than that of intermediate clades and modern clades, which may lead to functional changes in the evolution process of WOX transcription factor. In this study, the three residues were also highly conserved in the homeodomain of the JrWOX protein family (Figure 2A). The residues marked by two black boxes (Figure 2A) may highly correlate with each other and play a role in the evolutionary process. In the homeodomain structure, there were other residues marked by two red boxes (Figure 2A); the "YN" to "FY" change in the sequence occurred during the evolution from the ancient to the intermediate clade, and the "R" to "H" change may have occurred during the evolution from the intermediate clade to the modern clade [41,50]. In addition, the WUX-box domain containing "LXL" might have a transcriptional inhibitory activity and plays an important role in leaf development and flower formation [46,50] (Figure 2B). The EAR-like domain may supplement the transcriptional inhibition function of WUS-box (Figure 2C). There was a specific motif in the C-terminal region of IC-WOXs (Figures 1C and A1), and multiple sequence alignment showed that this fragment was conserved (Figure 2D). Other studies, accordingly, have found that IC-WOX genes play a role in root evolution [12,42,51].

4.3. The Promoter Regions of JrWOXs

As the binding sites of transcription factors, cis-acting elements in the promoter of the gene determine its expression patterns. In our study, a series of hormone-, development-, and stress- related cis-acting elements were detected in the promoter of *JrWOXs* (Figure 3). The maximum number of cis-acting elements were Myb elements, which play a role in controlling plants' secondary metabolism and regulating cell morphological formation and signal transduction pathways [52]. Previous reports have testified that auxin directly induces *AtWOX11* expression, owing to the AuxRE (TGTCTC) in the promoter of *AtWOX11* [13]. *OsWOX11* interacting with ERF3 can promote crown root through the cytokinin signaling pathway [25]. In poplar, *PtoWOX11* improves drought resistance by interacting with AP2 [53]. In difficult-to-root trees, exogenous hormones, such as NAA and IBA, are often used for rooting induction [54]. Sometimes, however, even if exogenous auxin is applied to the cuttings, they cannot take root, such as walnut, Quercus species, and teak. It can be speculated that only the supplement exogenous auxin is not enough for difficult-to-root trees. The relationship of supplement exogenous auxin, rejuvenation and protein–protein interactions in rooting is a question deserving of further research. There were also a number of organ-specific cis-elements in JrWOXs promoters, such as ACGT-core, CANNTG-motifs, GATA-motifs, and W-box ((T)TGAC(C)). Among them, ACGT,

CANNTG, and GATA act as transcription-factor binding sites for other transcription factors that are specifically expressed in roots [55]. This suggests that *JrWOXs* might be involved in biotic and abiotic stress responses and plant development under regulation of these cis-acting elements.

4.4. The Evolution of JrWOX Genes and Root-Evolution Events

Based on phylogenetic analysis, green alga and moss only harbor ancient clade WOX genes, subsequently, intermediate clade WOX genes present in the vascular species lycophyte (Figure 4A.). In gymnosperms, the phylogenetic classification and the developmental role of WOX genes have not been clearly characterized [56]. As *WOX* genes from angiosperm species, such as *O. sativa*, *Z. mays*, *A. thaliana*, *P. trichocarpa*, *S. polyrhiza*, and *Q. suber*, were separated into three distinct clades—ancient clade, intermediate clade, and modern clade—12 *JrWOXs* were also separated into the three clades (Figure 4A.). Both *Quercus* and *Juglans* are difficult-to-root trees for low adventitious root ratio from stem cuttings [5,57]. The evolutionary distance of *JrWOXs* and *QsWOXs* were near to each other, implying that they are closely related. Motif analysis shows that a Motif 7 and Motif 3 tandem repeats in *QsWOX11* and *JrWOX4a/b*, respectively (Figure A1). This provides clues for mechanism research on the AR formation of difficult-to-root trees.

For the evolution of the root, there is no differentiation of root in green alga and moss (Figure 4B). Fossil evidence and the root anatomy indicate that at least three root-evolution events happened in lycophytes, ferns, and seed plants, sequentially (Figure 4B) [42]. Accordingly, the morphology of roots evolved from bifurcating roots to adventitious roots and lateral roots, and then to primary roots. The evolution of roots in vascular plants is beneficial to the successful adaptation to different environments. There are more different root types in seed species, e.g., tap root system, fibrous root system, mycorrhiza, etc. The investigation of *WOX* genes and their functions in the polymorphic root organogenesis will help to improve the understanding of the molecular bases of root evolution. WOX genes not only influence root formation; for example, *AtWOX11* and *12* are involved in the first-step cell-fate transition during de novo root organogenesis, but also influence the AR number, relative strength, and histomorphology [21,58]. In walnut, endogenous hormone distribution could be changed by rejuvenation [5]; however, whether WOX genes are involved in these changes is unclear. Furthermore, morphological observation of the difficult-to-root trees and easy-to-root trees may provide guidance for improving agricultural production. It has been considered that the distribution of thick-walled tissues between phloem and cortex determines the degree of rooting difficulty to some extent, and that thick-walled tissues do not exist or are discontinuously distributed in the easy-to-root trees, while they are arranged in a circular pattern in the difficult-to-root trees with large thickness [59]. Current studies suggest that sclerenchyma is not a physical barrier to adventitious root formation, but rather a barrier to the initiation of adventitious root primordia [60]. In walnut, breaking through the barrier around the cross-sectional areas of the cambium and phloem is an important reason for the improvement of rooting rate.

4.5. Expression Patterns of JrWOX Genes

The tissue expression patterns of genes in different plants can be used to identify the functions of genes. In *A. thaliana* and *P. tomentosa*, the expression patterns of *WOX* genes are different, implying that the functions of *WOX* genes in annual plants and woody plants might be different [8]. In *A. thaliana*, only *WOX4* is involved in the differentiation of vascular meristem; however, poplars also need the *WUS* gene, as microtubule tissues are differentiated from the cambium every year in poplar [16,61]. The *AtWOX5* gene is specifically expressed in QC cells, while the *PtoWOX5* gene also can be found in the other tissues [44]. In this study, the expression patterns of *WOX* genes in walnut were more similar to those in poplar. The difference was that the *PtoWOX3* gene was not identified in poplar, while *JrWOX3a* and *JrWOX3b* were highly expressed in the leaves of walnut. *PtoWOX11/12s* and *PtoWOX5s* are uniquely expressed in roots; however, there also existed weak expression of *JrWOX11* gene in other tissues besides the high expression in roots (Figure 4). It can be speculated that the functions of

WOX genes in walnut might be more similar to poplar than annual plants. The differences between walnut and poplars might provide evidences that the rooting mechanisms of easy-to-root trees and difficult-to-root trees are different.

4.6. Expression Profiling Revealed JrWOX Genes' Responses to Rejuvenation and Involvement in ARF

Stem cuttings are the most extensively used method in woody plants. However, their capacity for forming adventitious roots becomes weaker as trees age. Some studies have shown that rejuvenation of mature trees or the induction of plants to regress from maturity to the juvenile state may significantly improve ARF. It might be owing to the changes of distribution of endogenous plant hormones after rejuvenation [5]. In this study, we further revealed the histological differences between rejuvenated and mature cuttings in walnut (Figure 5). Moreover, almost all the *JrWOX* genes were induced by being rejuvenated. This might be helpful to better understand the regulation mechanism of rejuvenation at the level of molecular.

The roles of WOX proteins in root formation and development have been well-documented in *Arabidopsis*, rice, tobacco, and poplar; for example, the leaf of *Arabidopsis* is used to study de novo root organogenesis, and the results show that *AtWOX5* regulate stem cells in root apical meristem (RAM) and *AtWOX11* mediate root primordium formation. In rice, *OsWOX11* is required to activate lateral root initiation, root hair formation, and responses to abiotic stresses [24]. In poplar, over-expression of *PtoWOX4*, *PtoWOX5a*, *PtoWOX11,* and *PtoWOX13* led to an increased AR number, decreased AR length, or increased AR roughness [21]. In tobacco, plant hormones, their intricate signaling networks and WOX play a crucial function in the process of ARF [62]. In this study, *JrWOX5*, *JrWOX9*, and *JrWOX11* were strongly induced in adventitious root regeneration of rejuvenation cuttings, and *JrWOX11* was also highly promoted in mature cuttings, implying *JrWOX5*, *JrWOX9*, and *JrWOX11* play a pivotal part in ARF. *JrWOX4* and *JrWOX13* may play an important role in later root development, although the relative expression of them showed significant changes during ARF. It is unclear if the other *WOX* genes are involved in AFR, in view of the fact that there was no significant change in their expression level.

5. Conclusions

J. regia contains 12 WOX proteins and can be divided into three clades: the ancient, intermediate, and modern clades. The sequences of domains are relatively conservative, and the gene structure and motifs contribute to phylogenetic analysis of *WOX* genes. *WOX* genes may be recruited in root evolution. *JrWOX4a/b*, *JrWOX11*, and *JrWOX13* were highly expressed in root, and *JrWOX5* and *JrWOX11* were strongly induced during adventitious root regeneration. Rejuvenation treatment reduced thickness and density of sclerenchyma between the cortex and phloem, and almost all *JrWOX* genes were expressed. This study also provides an approach for identifying and characterizing *WOX* genes in other species and reveals the possible functions of *WOX* genes in a difficult-to-root tree ARF. In future research, the rejuvenation function in ARF should be verified in more-difficult rooting species. The question of whether the hormone treatment for root induction is a standard treatment, and whether hormone treatment triggers the expression of *JrWOX* genes, should be focused on. Moreover, the genetic engineering means could be used to analyze the above genes' functions.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish.

Appendix A

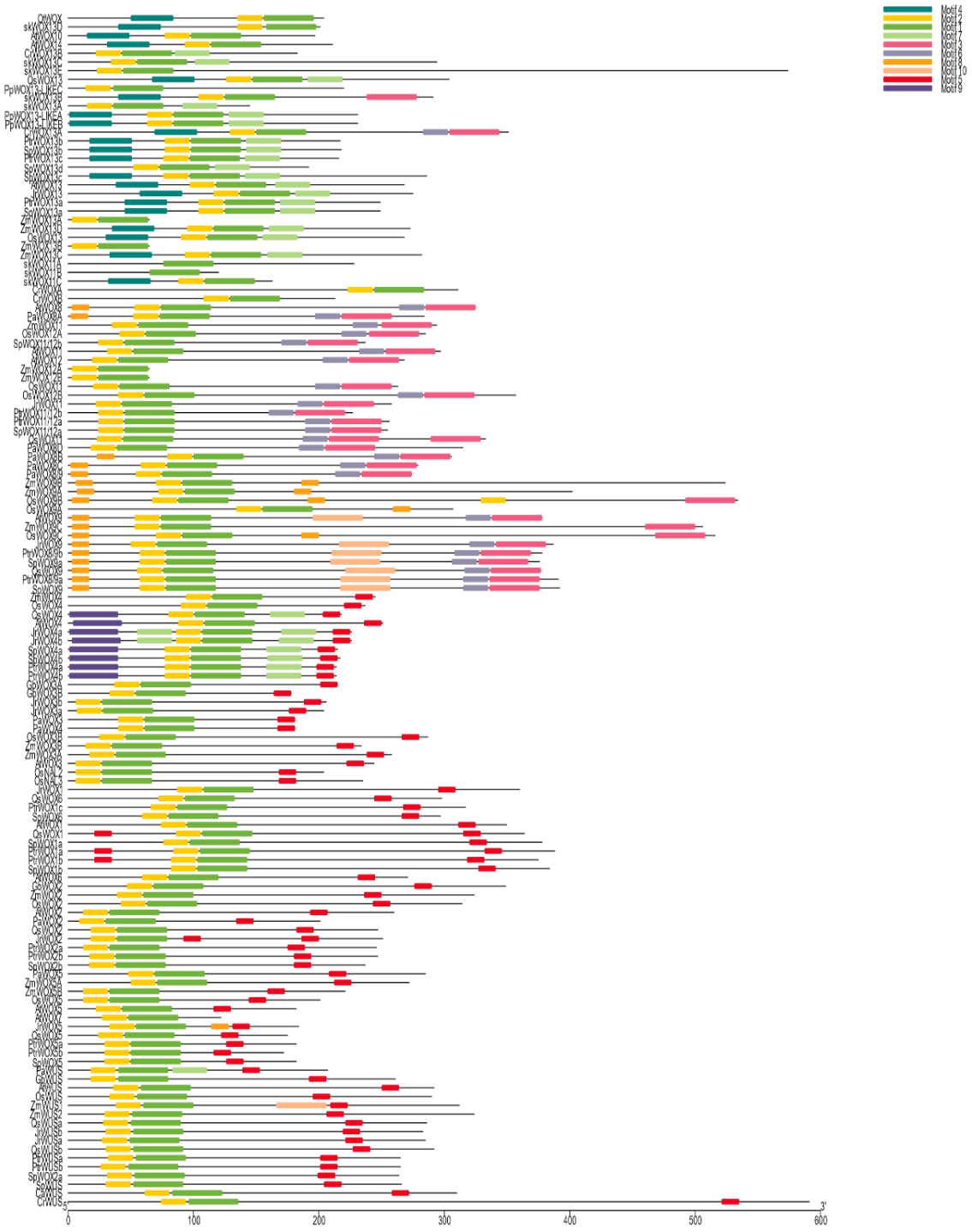

Figure A1. Motifs distribution of the whole protein sequences of OtWOXs, PpWOXs, CrWOXs, CaWOXs, GbWOXs, PaWOXs, AtWOXs, PtWOXs, ZmWOXs, OsWOXs, and SpWUS. QsWOXs and JrWOXs.

Appendix B

Table A1. Accession numbers and genome loci of the WOX sequences used for phylogenetic analysis. *Ot*: *Ostreococcus tauri*, *Pp*: *Physcomitrella patens*, *Sk*: *Selaginella kraussiana*, *Cr*: *Ceratopteris richardii*, *Ca*: *Cyathea australis*, *Gb*: *Ginkgo biloba*, *Pa*: *Picea abies*, *Os*: *Oryza sativa*, *Zm*: *Zea mays*, *At*: *Arabidopsis thaliana*, *Ptr*: *Populus trichocarpa*, *Sp*: *Spirodela polyrhiza*, *Qs*: *Quercus suber and Jr*: *Juglans regia* [63–66].

Gene Name	Accession No./Locus	Gene Name	Accession No.
Pa_WUS	JX512364	Zm_WUS1	AM234744
Pa_WOX2	AM286747	Zm_WUS2	AM234745
Pa_WOX3	JX411947	Zm_WOX2	AM490235
Pa_WOX4	JX411948	Zm_WOX3B	AM491777
Pa_WOX5	JX411949	Zm_WOX3A	AM490236
Pa_WOX8/9	GU944670	Zm_WOX4	AM490237
Pa_WOX8A	JX411950	Zm_WOX5A	AM490238
Pa_WOX8B	JX411951	Zm_WOX5B	AM490239
Pa_WOX8C	JX411952	Zm_WOX9A	AM490240
Pa_WOX8D	JX411953	Zm_WOX9B	AM490241
At_WOX1	AT3G18010	Zm_WOX9C	AM490242
At_WOX2	AT5G59340	Zm_WOX11	EU954172
At_WOX3	AT2G28610	Zm_WOX12A	AM234774
At_WOX4	AT1G46480	Zm_WOX12B	AM234775
At_WOX7	AT5G05770	Zm_WOX13A	AM234776
At_WOX5	AT3G11260	Zm_WOX13B	AM234777
At_WOX6	AT2G01500	Zm_WOX13C (ZmWOX14B)	EU952747
At_WUS	AT2G17950	Zm_WOX13D (ZmWOX14A)	EU961475
At_WOX9	AT2G33880	Vv_WUS	AM447418
At_WOX8	AT5G45980	Vv_WOX1	AM439847
At_WOX11	AT3G03660	Vv_WOX2	AM488389
At_WOX12	AT5G17810	Vv_WOX3	AM429035
At_WOX13	AT4G35550	Vv_WOX4	AM447494
At_WOX10	AT1G20710	Vv_WOX5	AM454567
At_WOX14	AT1G20700	Vv_WOX6 (VvWOX1B)	AM463144
Gb_WUS	FM882128	Vv_WOX9	AM488026
Gb_WOX2	FM882124	Vv_WOX11	AM435207
Gb_WOX3A	FM882125	Vv_WOX13A	AM486367
Gb_WOX3B	FM882126	Vv_WOX13B	AM473516
Pp_WOX13-LIKE A	AB699867	Vv_WOX13C	AM463736
Pp_WOX13-LIKE B	AB699868	Sp_WOX4A	KF982703
Pp_WOX13-LIKE C	XM_024511279	Sp_WOX4B	KC691250
Sm_WOX13	XM_002962367	Sp_WOX1B	KF982700
Sm_WOX8	XM_024668721	Sp_WOX1A	KF982699
Sm_WOX10	XM_002981839	Sp_WOX9A	KF982708
Sm_WOX11	XM_024684472	Sp_WOX13B	KF982713
Sm_WOX6	XM_024673314	Sp_WOX13C	AJ010810
Sm_WOX5	EFJ20992	Sp_WOX13D(8)	AY479970
Sm_WOX9	EFJ33362	Sp_WOX13A	KF982712
Sm_WOX1	EFJ33357	Sp_WOX6	KF982707
Sm_WOX3	EFJ33027	Sp_WOX11/12B	KF982709
Sm_WOX4	EFJ21095	Sp_WOX11/12A	KF982710
Sm_WOX2	EFJ35854	Sp_WUSA	KF982698
Ca_WUS	FR716459	Sp_WOX2B	KF982702
Ca_WOXA	FR716461	Sp_WOX2A	FJ232064
Ca_WOX13	FR716460	Sp_WOX5B	KF982706
Cr_WUS	FR716458	Sp_WOX9B	KF982709
Cr_WOXA	FR716456	PtrWUSa	Potri.005G114700

Table A1. *Cont.*

Gene Name	Accession No./Locus	Gene Name	Accession No.
Cr_WOXB	FR716457	PtrWUSb	Potri.007G012100
Cr_WOX13A	FR716454	PtrWOX1a	Potri.012G047700
Cr_WOX13B	FR716455	PtrWOX1b	Potri.015G039100
Os_WOX3a	LOC_Os11g01130.1	PtrWOX1c	Potri.010G111400
Os_WOX3b	LOC_Os12g01120.1	PtrWOX2a	Potri.001G237900
Os_WOX3c	LOC_Os11g01130.2	PtrWOX2b	Potri.009G029200
Os_WOX2	LOC_Os05g02730.1	PtrWOX4a	Potri.002G124100
Os_WOX4	LOC_Os04g55590.1	PtrWOX4b	Potri.014G025300
Os_WOX5	LOC_Os01g62310.1	PtrWOX5a	Potri.008G065400
Os_WOX1	LOC_Os04g56780.1	PtrWOX5b	Potri.010G192100
OsWOX9	LOC_Os01g63510.1	PtrWOX8/9a	Potri.004G051600
OsWOX7	LOC_Os01g47710.1	PtrWOX8/9b	Potri.011G061400
OsWOX12	LOC_Os05g48990.1	PtrWOX11/12a	Potri.013G066900
OsWOX11	LOC_Os07g48560.1	PtrWOX11/12b	Potri.019G040800
OsWOX6	LOC_Os03g20910.1	PtrWOX13a	Potri.005G101800.
OsWOX13	LOC_Os07g34880.1	PtrWOX13b	Potri.005G252800
OsWOX10	LOC_Os08g14400.1	PtrWOX13c	Potri.002G008800
OsWOX8	LOC_Os01g60270.1		

Table A2. The primer sequences of qRT-PCR.

Genes	Primer Sequence (F: 5′→3′)	Primer Sequence (F: 5′→3′)	Production/bp
GAPDH	ATGATGTCAAGGAAGGACTC	CACAATGATCTCAGCTCCG	102
JrWUSa	ACTCCTTCATCCAACGGCTT	TGAGCTCCAGGGAAGTACGA	88
JrWUSb	TCAATGGCTTCTGCAATGTTAG	AGAACCGCTCTTGCCACTGT	104
JrWOX1	ACGAACACCAAAGTGCCCAT	ATCGGCTGGCTGTTTTCCT	105
JrWOX2	CTCACGCTGGAATCCGACA	CAATGTGACCGTAAGCCCG	128
JrWOX3a	ACGGCTATGATGGGAATGCTA	AACAGGGAAGAGTTCAAGGGTC	92
JrWOX3b	CTGCTCTTCAACAACTTTCCCA	TTTGAGTTGTCTATCTCGCCCA	157
JrWOX4a	CAAGCAGGCTAACTCCGACA	GGGGAATCTCGTTTGTCGTC	126
JrWOX4b	AGCACGAACCCTCCCTCAC	AAAAGCCGCAACACCAGC	106
JrWOX5	GACCCTCCAACTTTTCCCATT	AGTGCTCCATTTCTGTCCCAA	122
JrWOX9	ACACAACTGCGGTCTCTCCCT	ACCCGATTCCCCAACACCT	95
JrWOX11	GACGTAGTGTTGGTCCATTGCT	GCCCTCTTTCTTCCCCTTTAA	135
JrWOX13	CAGGGCTGAAGATTTGTGCT	TACTGCCATCTGACGGGAAC	107

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