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RESEARCH

# Genome-Wide Identification of WUSCHEL-Related Homeobox (WOX) Gene Family in Hazelnut (*Corylus avellana* L.) and Their Expression Profiles During Adventitious Root Development

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**Abstract**

The propagation of high-yield and high-quality hazelnut varieties through vegetative methods, such as micropropagation, is crucial for large-scale production. However, low adventitious root (AR) formation and regeneration remain major limitations in hazelnut micropropagation. AR formation is regulated by various environmental and internal factors, with the WUSCHEL-related homeobox (WOX) gene family playing a central role in its molecular control. Although WOX transcription factors (TFs) are known regulators of AR development in several plant species, this gene family has not yet been characterized in hazelnut. In this study, a genome-wide analysis identified WOX gene family members in hazelnut and examined their roles in AR development. Ten CaWOX genes were unevenly distributed across six chromosomes. Phylogenetic analysis grouped these proteins into three clades (WUS, intermediate, and ancient), consistent with other plant species. Conserved motif analysis revealed structural similarity among CaWOX proteins. The proteins ranged from 167 to 351 amino acids with molecular weights of 19.10–40.20 kDa, and three had isoelectric points below 7. All CaWOX proteins were predicted to localize in the nucleus. Promoter analysis revealed cis-acting regulatory elements associated with hormone signaling, development, and stress responses, indicating complex transcriptional regulation. *CaWOX4* transcripts showed a statistically significant 1.82-fold increase on day 7 compared to day 1, whereas *CaWOX13* transcripts showed a 1.36-fold increase that was not statistically significant. Overall, these results suggest that CaWOX4 and CaWOX13 may be associated with AR formation in hazelnut and represent candidate genes for future functional studies aimed at improving rooting efficiency in woody plants with recalcitrant regeneration systems.

**Keywords** Adventitious root · *Corylus avellana* L. · Hazelnut · Micropropagation · WOX

## Introduction

Transcription factors are key regulatory proteins involved in various cellular and biological functions, as well as in controlling multiple signaling pathways during plant growth and development, by binding to specific regions of the genome. The mechanisms of action and biotechnological applications of transcription factors have attracted considerable interest due to their potential roles in improving plant development, stress tolerance, and crop productivity (He et al. 2023). TFs function by binding to specific regions of the genome, particularly within the promoter regions of target genes, through specialized DNA-binding domains, thereby regulating transcription. Depending on their mode of action, TFs either enhance or repress the expression of their target genes, thereby acting as transcriptional activators or repressors (Kummerfeld and Teichmann 2006). Homeobox proteins are

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members of one of the largest superfamilies of TFs and are characterized by a highly conserved DNA-binding domain known as the homeodomain (HD). The WOX gene family, a plant-specific subgroup of homeobox TFs, contains a conserved homeobox domain that is slightly longer than the typical 60-amino-acid homeodomain, often extending to approximately 66 amino acids. This extended domain plays an essential role in DNA binding and transcriptional regulation (Viola and Gonzalez 2016). Among homeobox TFs, the plant-specific WOX gene family has emerged as a key regulator of various developmental processes, including embryonic development, meristem maintenance, organogenesis, and stress responses (Tvorogova et al. 2021). Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana* (Haecker et al. 2004). The WOX gene family is evolutionarily conserved across a wide range of plant species, reflecting its fundamental and indispensable role in plant development. To date, the WOX gene family members have been characterized in model plants as well as in several agriculturally and economically important species, providing insights into their conserved and species-specific functions (Liu et al. 2014a; Tvorogova et al. 2021; Xu et al. 2022).

Roots play a vital role in plant functions, including water and nutrient uptake. Plants develop three types of root systems: primary, lateral, and adventitious roots (Gogna et al. 2022). ARs form post-embryonically from nonroot tissues and are essential for many plant species that propagate asexually. Their development is a complex process influenced by environmental factors such as nutrient deficiencies, heavy metal exposure, and wounding during plant growth (Da Costa et al. 2013; Steffens and Rasmussen 2016; Naz et al. 2022). In addition, AR formation is regulated by multiple internal factors. While genetic regulation of AR formation has been studied extensively in herbaceous plants, the molecular mechanisms remain largely unknown in woody plants, where root formation is more challenging (Niemi 2009; Pizarro and Díaz-Sala 2019; Vielba et al. 2020). Recent studies have highlighted that the WOX TFs play a significant role in root development, particularly in the formation of ARs, which are essential for biotechnological processes such as tissue culture.

In *A. thaliana*, *AtWOX11* and *AtWOX12* play a role in the reprogramming of leaf procambium into root founder cells during AR formation (Liu et al. 2014a). In rice, auxin activates the transcription of *OsWOX11*, which forms the YUC-auxin-WOX11 module for crown root development, subsequently directing the initiation and development of crown roots (Zhang et al. 2018). It has been found that *PtWOX11* increases AR formation in *Populus tomentosa* (Liu et al. 2014b). In *J. regia*, a woody plant that is difficult to root, *JrWOX11* is hypothesized to be a critical factor in AR formation (Chang et al. 2020, 2022). However, the role of WOX gene family members in hazelnuts, a species known for its difficult rooting process, remains unknown.

Hazelnuts (*Corylus avellana* L.) are essential agricultural products worldwide and are widely used in the confectionery, chocolate, and bakery industries. In addition to their economic significance, hazelnuts provide considerable health benefits due to their rich nutrients containing protein, fatty acids, vitamins, and amino acids (Alasalvar et al. 2003; Köksal et al 2006; Ferrao et al. 2021). Hazelnut consumption has been shown to positively influence cardiometabolic health by enhancing lipid metabolism and regulating blood glucose levels, particularly in individuals with obesity and type 2 diabetes (Damavandi et al. 2013; Brown et al. 2022). Moreover, various parts of the hazelnut plant—including the kernels, skins, shells, husks and leaves—are abundant in health-promoting bioactive constituents such as phenolic acids, flavonoids, tannins, and dietary fibers, which exhibit notable antioxidant, anti-inflammatory, and vascular protective effects, making them valuable candidates for further biomedical and nutritional research (Alasalvar et al. 2006; Oliveira et al. 2007; Cikrikci et al. 2016; Krol et al. 2020; Rivas et al. 2020; Balik 2021).

The propagation of high-yield, high-quality, and elite hazelnut varieties through vegetative methods is essential for large-scale production. However, low rooting and regeneration capacity of hazelnut cuttings poses a major challenge for rapid clonal propagation techniques. Consequently, elucidating the molecular basis of AR formation is crucial for improving clonal propagation. In this study, we first performed a genome-wide analysis of the *WOX* gene family to identify and characterize the *WOX* genes in hazelnuts. Then, we examined their expression patterns at different stages of AR development. This study provides valuable insights into the transcriptional regulation of AR formation in hazelnut, contributing to more efficient tissue culture applications and future genetic improvement strategies.

## Materials and Methods

### Identification of WOX Gene Family Members in Hazelnut

The predicted WOX protein sequences of *C. avellana* L. were first downloaded from PlantTFDB (https://planttfdb.gao-lab.org/). Besides, BLAST programs (BLASTP and BLASTN) were used to identify *C. avellana* WOX genes (CaWOX) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Fifty-one WOX protein sequences of *Arabidopsis thaliana*, *Oryza. sativa*, and *Juglans. regia* were downloaded from PlantTFDB v5.0 (//planttfdb.gao-lab.org/). Local BLASTp-based homology search was performed against the genome database of *C. avellana* using the protein sequences *A. thaliana*,

*O. sativa*, and *J. regia* as the query (search score of ≥ 100 and an *e* value of $\leq 1 \times 10^{-10}$).

### Analysis of CaWOX Protein Structure

The conserved domains of WOX proteins were validated using the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and the Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/). The sequences containing the homeodomain were considered for further analysis.

### Analysis of Physicochemical Properties of the CaWOX Proteins

The physical and chemical properties of CaWOXs such as molecular weights (MWs), isoelectric points (pIs), grand average of hydropathicity (GRAVYs), and lengths of the WOX proteins were analyzed using ProtParam on the ExPASy server (//web.expasy.org/protparam/). The subcellular localization of WOX proteins was predicted using the CELLO web-based software (v2.5, http://cello.life.nctu.edu.tw/).

### Gene Structure, Motif Characteristics, and Cis-Element Analysis

The genomic and coding sequences of WOX genes in *C. avellana* were extracted. The gene structures of WOXs were analyzed using the Gene Structure Display Server (GSDS 2.0, http://gsds.cbi.pku.edu.cn/). Conserved motifs in CaWOX proteins were identified using MEME (//memesuite.org/tools/meme) with a maximum of 10 motifs (Bailey et al. 2009).

To analyze cis-elements in the promoter regions of *CaWOX* genes, 2 kb upstream sequences were predicted using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002) and visualized using TBtools II.

### Phylogenetic Analysis of WOX Proteins

Multiple sequence alignments of WOX proteins from *A. thaliana*, *O. sativa*, *J. regia*, and *C. avellana* were performed using Clustal T-coffee tool with default parameters (Notredame et al. 2000). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates in MEGA-X software (Kumar et al. 2018). The phylogenetic tree was uploaded into the iTOL website (https://itol.embl.de/) for a better display, annotation, and management of phylogenetic trees.

### Plant Materials and Treatments

The hazelnut (*Corylus avellana* L.) cultivar "Palaz," propagated by subculturing in tissue culture for approximately 6 months, was used as plant material. Plantlets were cultured in a modified Driver and Kuniyuki (DKW) medium (Hand and Reed 2014) containing 30 g/L sucrose, 200 mg/L Sequestrene 138 Fe EDDHA, 2 mg/L thiamine, 2 mg/L nicotinic acid, 2 mg/L glycine, 1 g/L myo-inositol, 5 mg/L N6-benzyladenine (BA), and 0.6% (*w*/*v*) agar, with the pH adjusted to 5.7 prior to autoclaving. Cultured plants were maintained in a growth chamber under controlled conditions: 24 ± 1 °C temperature, a 16/8 h light/dark photoperiod, and a light intensity of 80 µmol•$m^{-2}$•$s^{-1}$. Microshoots measuring 3–4 cm in length were excised and transferred into test tubes containing 2 mL of DKW medium supplemented with 30 g/L sucrose and 1 mg/L indole-3-butyric acid (IBA) to induce adventitious rooting. The emergence of the first roots (measured in millimeters) was recorded as day 0, which served as the reference for subsequent time points. Samples were collected at four time points (days 1, 2, 4, and 7), with five plants per time point. Approximately 5-mm tissue segments were excised from the basal region of each plantlet. The collected root tissues were rapidly frozen in liquid nitrogen and stored at − 80 °C until RNA isolation.

### Total RNA Isolation and cDNA Synthesis

Root tissues (approximately 5 mm from the basal region) were collected from five independent plantlets at each sampling time point. Total RNAs were extracted from the root samples using the Quick-RNA Miniprep Plus Kit (Zymo Research, USA), following the manufacturer's instructions. RNA concentrations (A260) and purities (A260/A280 and A260/A230 ratios) were measured using a UV-Vis spectrophotometer. RNA integrity was evaluated by running 1% agarose gel electrophoresis at 80 V for 50 min. For cDNA synthesis, 400 ng of total RNA from each sample was reverse-transcribed using the EcoScript 5 × First Strand cDNA Synthesis Kit (Ecotech, Turkey). The resulting cDNA samples were diluted to 10 ng/µL and stored at − 20 °C for further use in quantitative real-time PCR (qRT-PCR) analyses.

### Quantitative Real-Time PCR (qRT-PCR) Analysis

Gene-specific primer pairs for *CaWOX* genes (*CaWOX1*, *CaWOX2*, *CaWOX3*, *CaWOX4*, *CaWOX5*, *CaWOX6*, *CaWOX9*, *CaWOX11*, *CaWOX13*, and *CaWUS*) were designed using the Primer Quest program (Table 1). A total reaction volume of 20 µL was prepared, containing 40 ng cDNA template, 0.25 µM of each forward and reverse primer, 1X SYBR Green master mix (Abmgood, BlasTaq™, G891),

**Table 1** List of primer sequences used for gene expression analysis

| Gene | NCBI accession | Primer sequences (5′–3′) | Amplicon size (bp) |
|---|---|---|---|
| *CaWOX1* | XM_059598459 | F: TGGCTGAGCAGAACAAGAAG<br>R: GAGTCCCACGTCGATACAATTC | 114 |
| *CaWOX2* | XM_059573263 | F: AGGAGAAATCAAGAGCCTTCG<br>R: CTGTTGGGTGCAATGGAAATAG | 93 |
| *CaWOX3* | XM_059574913 | F: AACCACAAGGCAAGGGATAG<br>R: GGTGGGTGATTGAGGTGATAAT | 104 |
| *CaWOX4* | XM_059598797 | F: GGCAAGATCGAAGGGAAGAA<br>R: GGACAATGGCTAAGACCAAGA | 101 |
| *CaWOX5* | XM_059602525 | F: GGACGAGGGAATGTCAATGT<br>R: CTTTGTTCCAGTGCTTCCATTAC | 85 |
| *CaWOX6* | XM_059592672 | F: GCTTCGGTAGGATTGAAGAGAA<br>R: GCTGATCTGGGAAGTGTAGTG | 114 |
| *CaWOX9* | XM_059584047 | F: AACCCTCCTAGAGACGAGATAAG<br>R: TGAAACCAGTAGAAGACGTTGG | 89 |
| *CaWOX11* | XM_059572412 | F: CTTCAACAGCGGAATGGTAAATC<br>R: GGTTCTGGAACCAGTAGAAGAC | 113 |
| *CaWOX13* | XM_059583029 | F: CCTACAGATGGCGGCTTAAA<br>R: CAGTCAGGTGGTCGTTTCTT | 94 |
| *CaWUS* | XM_059584660 | F: ACTACTCCTTGCCCAACATATC<br>R: CAGAGATCCATATCCACAGTTCC | 101 |
| *18S rRNA* | XR_009441211.1 | F: GAAAGACGAACAACTGCGAAAG<br>R: GGTTGAGACTAGGACGGTATCT | 102 |

and nuclease-free water. The 18S rRNA gene was used as the internal reference to normalize the threshold cycle (Ct) values of the *CaWOX* genes (Kavas et al. 2019). The qRT-PCR was performed on a Bio-Rad CFX-connect, and conditions were as follows: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. For the melt curve analysis, samples were heated from 65 to 95 °C with a 0.5 °C increase every 2–5 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All qRT-PCR experiments were conducted with two biological replicates and two technical replicates.

### Statistical Analysis

The qRT-PCR data were analyzed using one-way ANOVA with the least significant difference (LSD) test via the R programming language (RStudio, Version 1.3.1093) and the agricolae package.

## Results and Discussion

### Genome-Wide Identification of the CaWOX Gene Family Members in *Corylus avellana* L.

A total of 51 WOX proteins were downloaded in FASTA format, including 18 WOX proteins from *A. thaliana*, 20 WOX proteins from *O. sativa*, and 13 WOX proteins from *J. regia* from PlantTFDB v5.0. Protein sequences of all these WOX family members were used as query sequences against the hazelnut genome in the BLASTp tool, and 10 candidate *CaWOX* genes were identified. The HD (PF00046) was confirmed in all putative CaWOX sequences using CDD and SMRT.

The physicochemical properties of the CaWOX proteins are presented in Table 2. The amino acid lengths ranged from 167 to 351 aa, and molecular weights varied between 19.10 kDa (CaWOX5) and 40.20 kDa (CaWOX1). The theoretical isoelectric point (pI) ranged from 5.30 (CaWOX) to 9.43 (CaWOX5), with seven proteins having pI values above 7. The negative grand average of hydropathy (GRAVY) values, ranging from − 1.043 (CaWOX13) to − 0.430 (CaWOX11), indicated that all of the proteins possess hydrophilic properties. The aliphatic index (AI) ranged from 50.99 (CaWOX) to 70.36 (CaWOX11), suggesting that these proteins may be unstable in vitro. The instability index (II) ranged from 53.63 to 69.50, with lower values indicating greater stability. While most proteins have high solubility, CaWOX3, CaWOX9, and CaWOX11 show low solubility. This analysis reveals that the WOX proteins in hazelnuts exhibit diversity in terms of hydrophobicity, stability, and solubility. Subcellular localization predictions indicated that all these proteins were localized in the nucleus.

The chromosomal locations of the *WOX* genes revealed that these genes were unevenly distributed across six of the

**Table 2** Physicochemical properties of the predicted CaWOX proteins

| Protein | Length (aa) | MW (kDa) | pI | GRAVY | AI | II | Charge | KSP | Protein solubility |
|---|---|---|---|---|---|---|---|---|---|
| CaWOX1 | 351 | 40.20 | 8.61 | −0.787 | 60.91 | 56.5 | 3.6 | 40 | High |
| CaWOX2 | 232 | 25.90 | 6.76 | −0.761 | 54.74 | 62.11 | −0.2 | 22 | High |
| CaWOX3 | 210 | 24.21 | 9.03 | −0.632 | 59.52 | 67.56 | 5.5 | 25 | Low |
| CaWOX4 | 222 | 25.32 | 9.17 | −1.014 | 55.41 | 63.35 | 6.4 | 25 | High |
| CaWOX5 | 167 | 19.10 | 9.43 | −0.787 | 62.46 | 53.63 | 6.2 | 20 | High |
| CaWOX6 | 299 | 34.69 | 9.40 | −0.852 | 62.68 | 68.76 | 11.3 | 38 | High |
| CaWOX9 | 339 | 36.90 | 7.77 | −0.532 | 62.39 | 57.38 | 1.9 | 44 | Low |
| CaWOX11 | 249 | 27.33 | 6.71 | −0.430 | 70.36 | 57.75 | −0.3 | 25 | Low |
| CaWOX13 | 299 | 34.12 | 5.85 | −1.043 | 58.03 | 54.01 | −9.5 | 36 | High |
| CaWUS | 283 | 31.78 | 5.30 | −0.976 | 50.99 | 69.50 | −6.8 | 37 | High |

Molecular weight (MW), kinase-specific phosphorylation (KSP) sites, isoelectric point (pI), grand average of hydropathy (GRAVY), aliphatic index (AI), instability index (II), and charge of proteins were predicted based on amino acid composition

11 chromosomes of the *C. avellana* L. genome (Table 3). Three WOX genes were located on Chr2, two WOX genes on Chr6, two WOX genes on Chr10, and one WOX gene each on Chr4, Chr7, and Chr9. No WOX genes were detected in chromosomes 1, 3, 5, 8, and 11. The length of coding sequences ranged from 504 (CaWOX5) bp to 1059 bp (CaWOX1). The number of exons in WOX genes ranged from 2 to 4. The exon lengths of the WOX genes varied between 504 and 1059 bp (Table 3). Fine mapping of CaWOX genes on the respective chromosomes is presented in Fig. 1.

## Phylogenetic Analysis of CaWOX Proteins

The phylogenetic tree was constructed to evaluate the evolutionary relationships of WOX proteins from *C. avellana* (10 members) together with WOX proteins from *A. thaliana* (14), *O. sativa* (15), and *J. regia* (12). A total of 51 WOX proteins from the four species were classified into three well-defined clades: the WUS clade, the intermediate clade, and the ancient clade (Fig. 2). According to the phylogenetic analysis, the WUS clade contained the smallest number of CaWOX members and was represented solely by CaWUS. In contrast, the intermediate clade comprised the largest proportion of CaWOX genes, including CaWOX1–CaWOX6, which clustered with intermediate WOX proteins from other species. The ancient clade included CaWOX9, CaWOX11, and CaWOX13, which grouped with ancient WOX homologs (Fig. 2).

## Gene Structure and Conserved Motif Analysis of CaWOX Genes

The phylogenetic relationships and gene structures were constructed to clarify the evolution of the WOX family in hazelnuts (Fig. 3). Analysis of *WOX* genes showed that they contain between one intron (CaWOX2, 3, and 5) and four introns (CaWOX6 and CaWOX9). The *CaWOX13* gene contains two very long intronic regions that span the entire gene. This suggests that the transcriptional regulation of this gene might be complex. CaWOX5, on the other hand, is the shortest gene, with a single, short intron. This could

**Table 3** Summary of the *CaWOX* genes identified in the hazelnut genome

| Gene name | Accession no. | Chromosome | Gene location (5′−3′) | CDS (bp) | Exon no. |
|---|---|---|---|---|---|
| CaWOX1 | XP_059454442.1 | Chr6 | NC_081546.1 (22898799..22901547, complement) | 1059 | 4 |
| CaWOX2 | XP_059429246.1 | Chr10 | NC_081550.1 (18053158..18054619, complement) | 699 | 2 |
| CaWOX3 | XP_059430896.1 | Chr10 | NC_081550.1 (17022026..17024097) | 633 | 2 |
| CaWOX4 | XP_059454780.1 | Chr6 | NC_081546.1 (6701845..6703003, complement) | 669 | 3 |
| CaWOX5 | XP_059458508.1 | Chr7 | NC_081547.1 (28,686,431..28687036, complement) | 504 | 2 |
| CaWOX6 | XP_059448655.1 | Chr4 | NC_081544.1 (20119506..20121581) | 900 | 4 |
| CaWOX9 | XP_059440030.1 | Chr2 | NC_081542.1 (40609171..40611501) | 1020 | 5 |
| CaWOX11 | XP_059428395.1 | Chr9 | NC_081549.1 (12765168..12766635) | 750 | 3 |
| CaWOX13 | XP_059439012.1 | Chr2 | NC_081542.1 (866212..870291, complement) | 900 | 3 |
| CaWUS | XP_059440643.1 | Chr2 | NC_081542.1 (1850693..1851976, complement) | 852 | 3 |

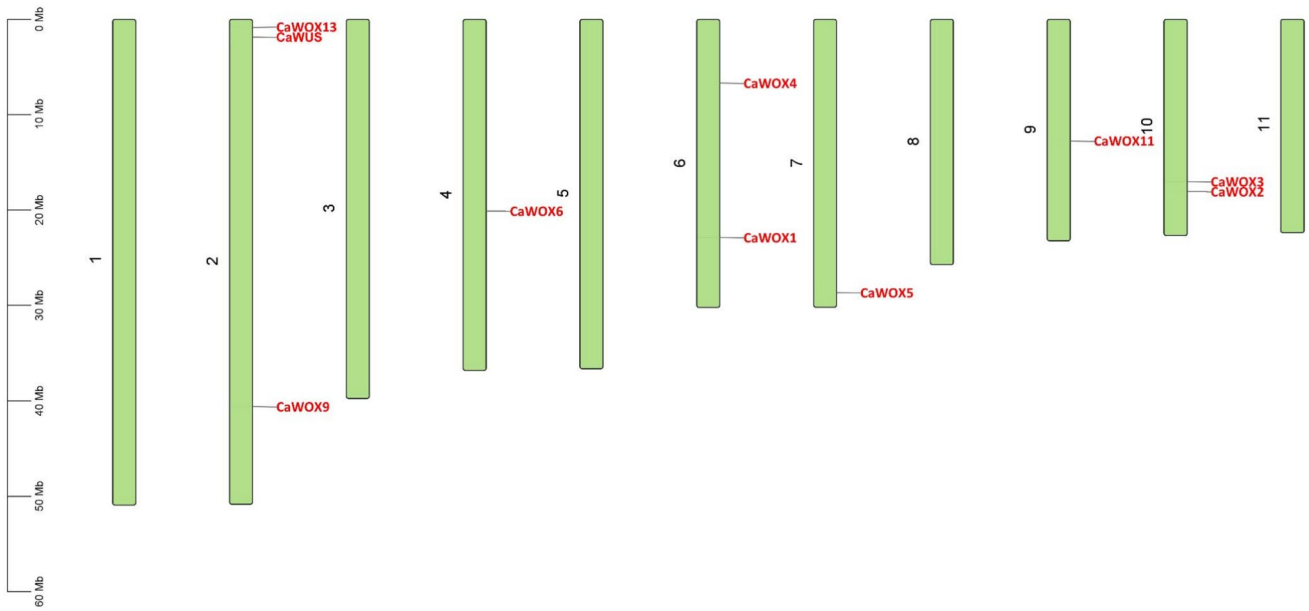



**Fig. 1** Chromosomal positions of *CaWOX* genes in the hazelnut genome. Chromosome numbers are indicated as 1 to 11. The left axis represents the length of each chromosome, measured in megabases (Mb)

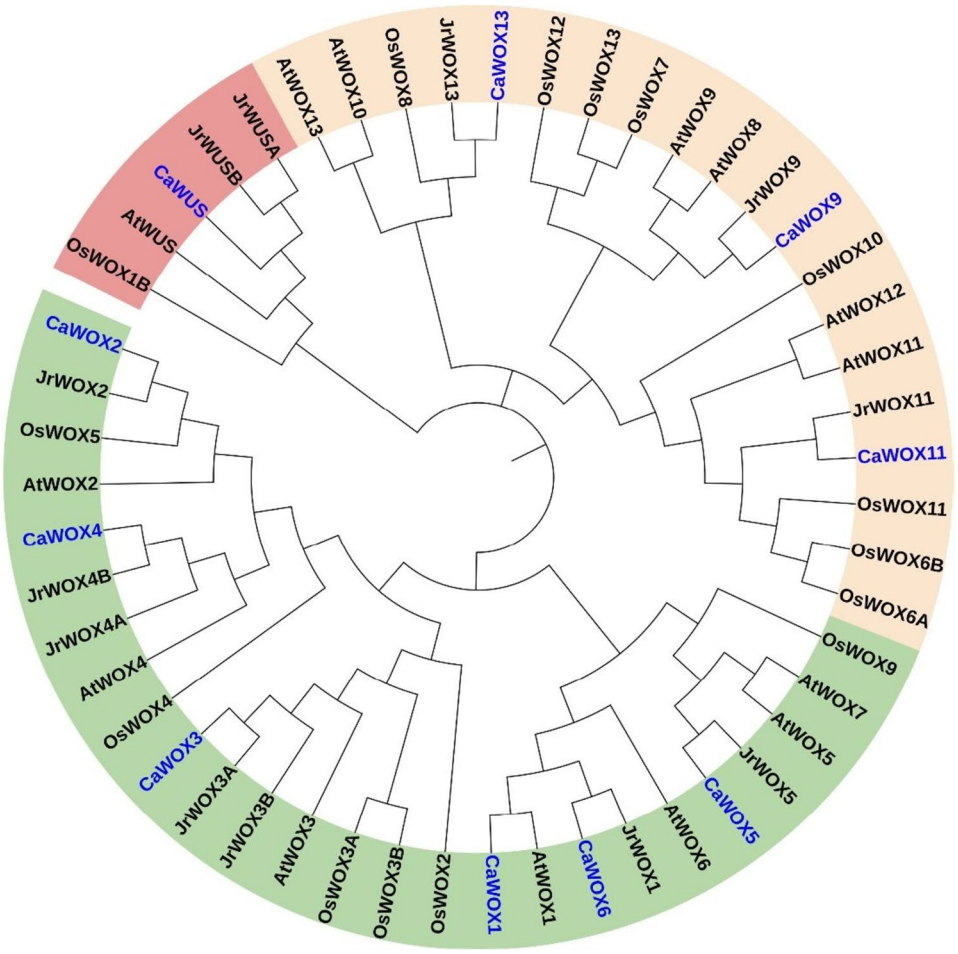



**Fig. 2** Phylogenetic analysis of WOX proteins in *C. avellana*, *A. thaliana*, *O. sativa*, and *J. regia*. The phylogenetic tree was constructed using a total of 51 WOX proteins using MEGA-X

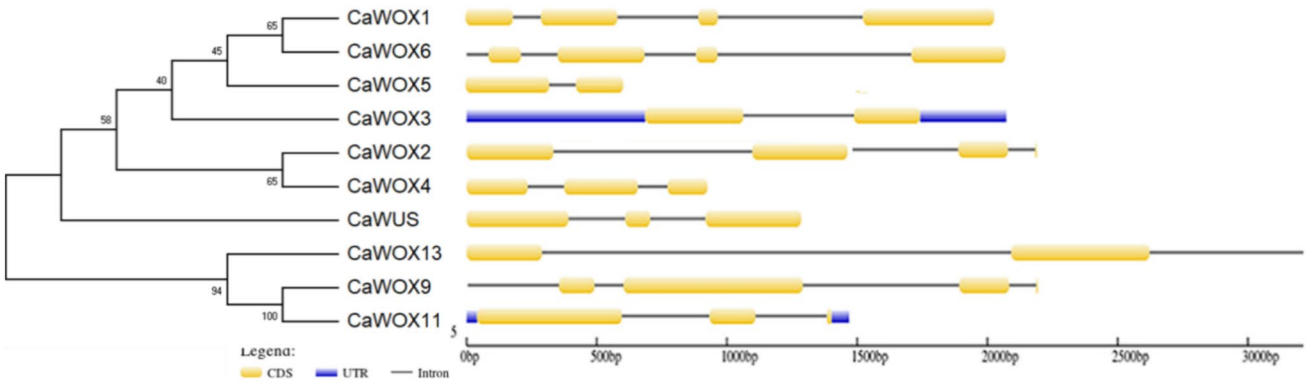



**Fig. 3** Phylogenetic relationships and gene structures in CaWOX genes. The unrooted tree was constructed, using the MEGA-X

imply that it may reflect structural differences that could influence gene regulation. The remaining *CaWOX* genes contained 2 introns, except for CaWOX1, which included 3 introns. In terms of UTR regions, the analysis revealed two long UTR regions in CaWOX3 and shorter UTR regions in CaWOX11. This suggests that these genes may play a crucial role in translational and post-transcriptional regulation processes.

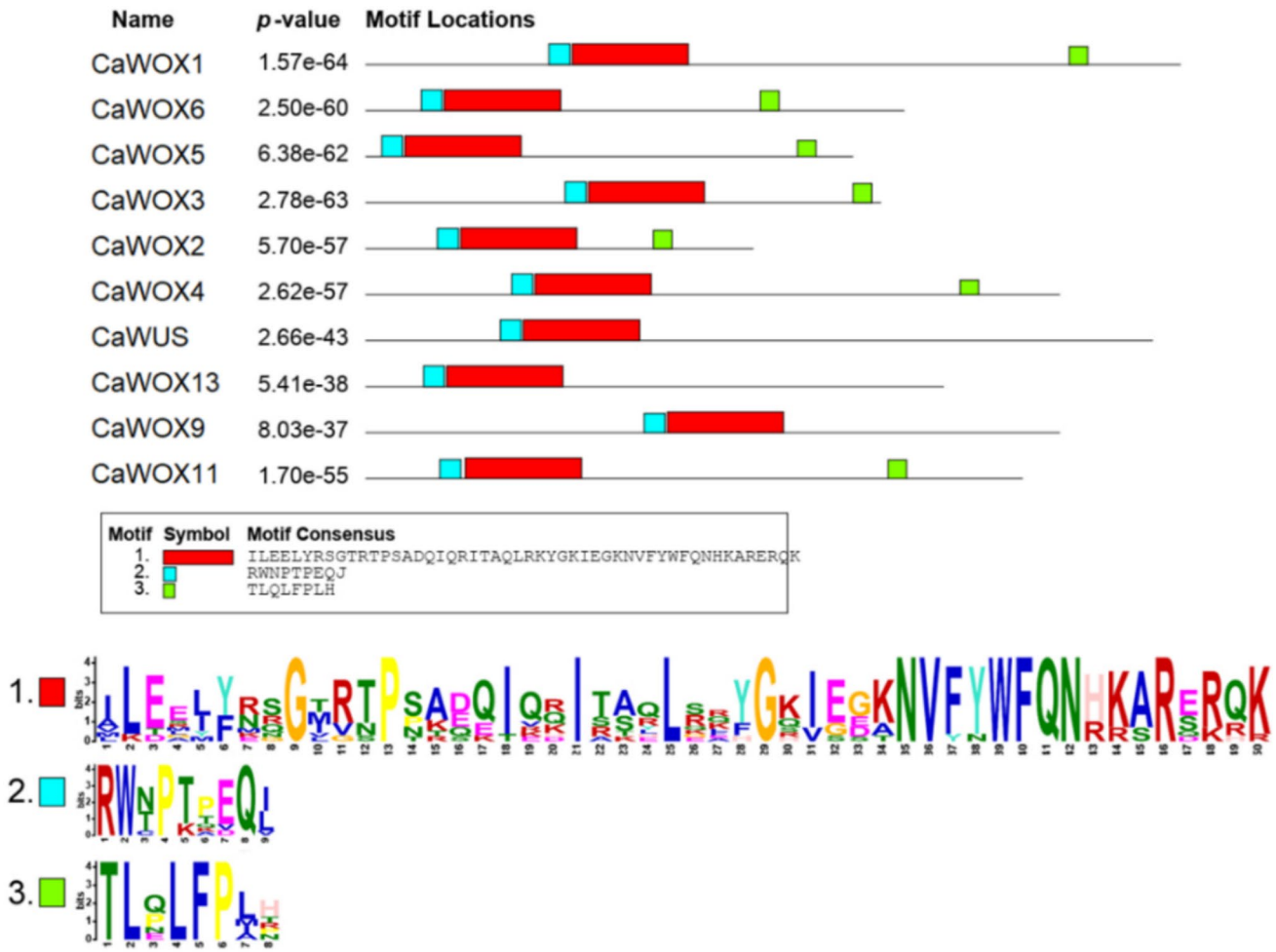
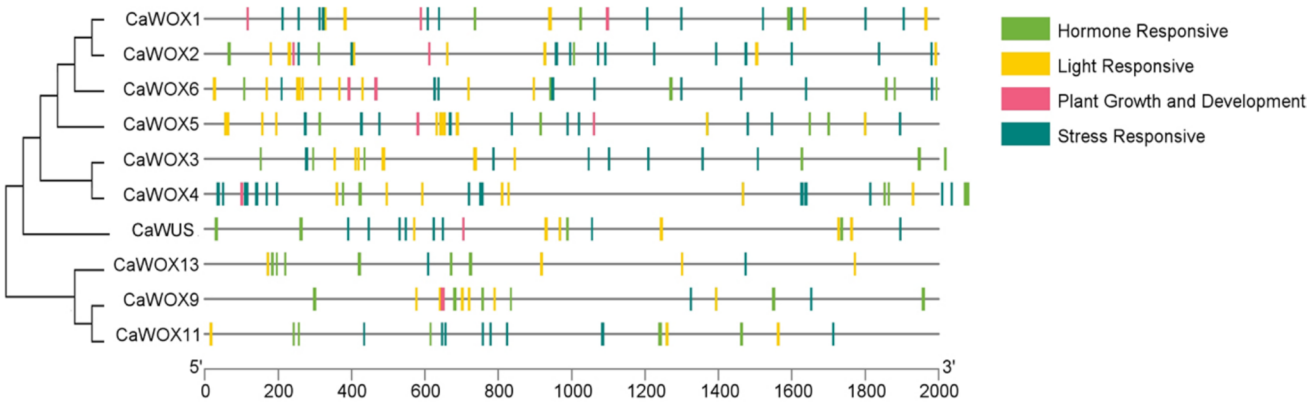



**Fig. 4** The conserved motifs and sequence logos of HD domains in CaWOX protein sequences




| | Plant Growth and Development | | | | Hormone Responsive | | | | | | | Stress responsive | | | | | | | | | | Light Responsive | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAT-box | CCGTCC motif | Circadian | RY-element | AAGAA-motif | ABRE | as-1 | CGTCA-motif | GARE-motif | TCA-element | TGA -element | TGACG-motif | ARE | Box S | MBS | MYB | MYC | LTR | STRE | TC-rich repeats | W box | WRE3 | WUN- motif | Box 4 | G-box | GATA-motif | GT1-motif | TCCC-motif |
| CaWUS | 1 | | | | 2 | 1 | | | | 1 | | | | | | 5 | 2 | | 1 | | | | | 1 | 2 | | 1 | |
| CaWOX1 | 2 | 1 | | | 3 | | | 1 | | | | | 4 | | | 2 | | 2 | 3 | | | | | | 2 | 1 | 1 | |
| CaWOX2 | 2 | | | | 1 | 1 | 1 | | | | 1 | 1 | 1 | | 1 | 2 | 1 | 1 | | | 3 | 3 | 1 | | 3 | | 2 | 1 |
| CaWOX3 | | | | | 1 | 1 | 2 | | 1 | 1 | | 2 | | | 2 | 4 | | | 2 | 1 | | | | 5 | 1 | | | |
| CaWOX4 | | | | 1 | | 2 | 1 | | | 3 | 1 | 1 | 1 | 1 | | 6 | 1 | 1 | | 2 | | 1 | 2 | 2 | 2 | | | 2 |
| CaWOX5 | | 1 | | 1 | 1 | 1 | | 1 | | | 1 | | 2 | 1 | | 4 | 1 | | | | | 1 | 1 | 2 | 3 | | | |
| CaWOX6 | | | 1 | 1 | 2 | 1 | 2 | | | | | 2 | | | | 2 | 4 | | 1 | | | | 1 | 7 | | | | |
| CaWOX9 | | | | | | 1 | | 1 | 1 | 2 | | | | | | | 1 | | | | | 1 | | 4 | 1 | 1 | | |
| CaWOX11 | | | | | | | 2 | 1 | | | 1 | 2 | | | | 1 | 1 | 1 | 1 | | 2 | 1 | 1 | | | | | |
| CaWOX13 | | | | | | 3 | | 1 | | | | 2 | | | | | 2 | | | | | | | | 2 | | | |

**Fig. 5** Distribution and comparative abundance of cis-acting regulatory elements identified within the 2000 bp upstream promoter regions of *CaWOX* genes in hazelnut, categorized according to hormone responsiveness, plant growth and development, light responsiveness, and stress responsiveness

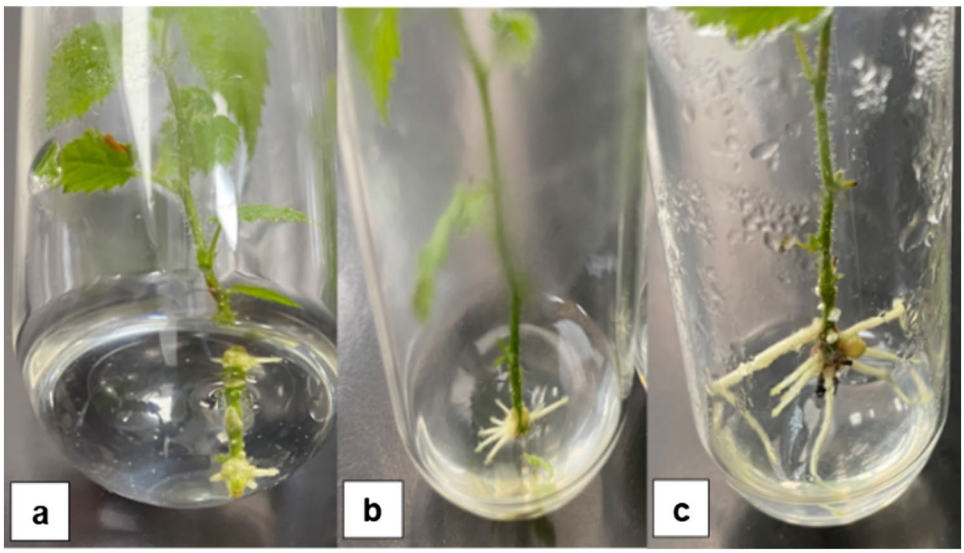



**Fig. 6** Representative images of AR development in hazelnut microshoots cultured in liquid rooting medium. Images represent different plantlets sampled at (**a**) day 1, (**b**) day 2, and (**c**) day 4 after rooting initiation

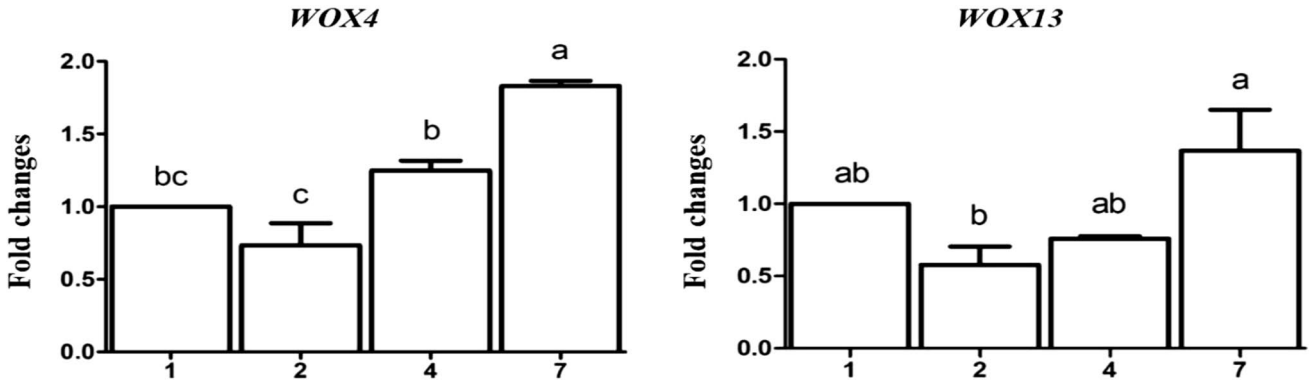



**Fig. 7** Time-dependent (1–7 days) fold changes of *CaWOX4* and *CaWOX13* transcripts in hazelnut root tissues

Three conserved motifs of CaWOX proteins were identified using the MEME program. Motif 1 (red) and motif 2 (blue) were present in all WOX proteins. Motif 3 (green), however, was a less common motif and was found in genes other than CaWOX9, CaWOX11, and CaWOX13 (Fig. 4).

## Cis-Acting Regulatory Elements (CAREs)

Identification of CAREs provides valuable insights into the regulation and functions of genes. Seventy-four CAREs were identified in the 2000-bp upstream regions of each *CaWOX* gene. The most abundant CAREs found in all WOX were the core elements TATA-box and CAAT-box. Among CAREs, we selected elements associated with hormone signaling, plant growth and development processes, and stress responses for further analysis. A comparative analysis of the abundance and distribution of these cis-acting regulatory elements across all *CaWOX* promoters is presented in Fig. 5. In terms of the hormone-responsive elements, we identified AAGAA-motif and ABRE, associated with abscisic acid; as-1, CGTCA-motif, TGACG-motif, and JERE, related to jasmonic acid; AuxRR-core and TGA-element linked to auxin; GARE-motif, TCA-element, TATC-box, and P-box involved in gibberellic acid; and ERE associated with ethylene response. Light-responsive elements were the most abundant among the plant growth and development elements, with 18 CAREs. CAT-box, CCGTCC motif, and RY-element were related to meristem development, meristem, and seed-specific expression, respectively. The circadian element was found to play a crucial role in regulating the circadian rhythm. Several stress-responsive elements were also identified, such as box S, CCAAT-box, GC-motif, LTR, MBS, MRE, MYB, MYC, STRE, TC-rich repeats, W box, WRE3, and WUN-motif. These findings suggest potential transcriptional regulation of *CaWOX* genes in response to hormone signaling, plant developmental cues, and stress-related factors in hazelnut.

## Adventitious Root Formation Stages in Hazelnut Microshoots

Hazelnut microshoots selected for rooting had an average length of 3–4 cm and contained 3–4 leaves at the apical region. After transfer to the liquid rooting medium, AR development was monitored over time. The appearance of the first millimeter-scale roots was defined as day 0, which served as the reference point for subsequent observations. AR formation progressed gradually, and samples were collected at 1, 2, 4, and 7 days after rooting initiation (Fig. 6). For each time point, samples from 5 plants were pooled. Basal tissue (approximately 5 mm) from the lower part of the shoots was used for downstream molecular analyses.

## Gene Expression Analysis

To investigate the expression patterns of *CaWOX* genes during AR formation in *C. avellana* Palaz variety, expression profiles of 10 *CaWOX* genes (*CaWOX1*, *CaWOX2*, *CaWOX3*, *CaWOX4*, *CaWOX5*, *CaWOX6*, *CaWOX9*, *CaWOX11*, *CaWOX13*, and *CaWUS*) were analyzed by qRT-PCR. During the early stages of AR formation, among the tested genes, only *CaWOX4* and *CaWOX13* exhibited detectable expression under the experimental conditions. An increase in transcript was detected in *CaWOX4* gene on days 4 and 7 compared to day 1. The increase on day 7 was statistically significant ($p < 0.05$) with a 1.82-fold change. A 1.36-fold transcript increase was detected in *CaWOX13* only on day 7 relative to day 1. However, this increase was not statistically significant (Fig. 7).

Adventitious root formation occurs in response to various stress conditions, such as nutrient deprivation, heavy metal stress, and injury, as well as in vegetative propagation of plants. The WOX gene family plays a significant role in plant root development and tissue reprogramming. Studies conducted on various plants have revealed the functions of *WOX* genes in AR formation. In *Paeonia ostii*, *PoWOX4*,

*PoWOX5*, *PoWOX11*, and *PoWOX13b* were high expression levels during the early stages of AR primordium formation (Lou et al. 2024). Similarly, in *Eucalyptus species*, *EupWOX1*, *EupWOX5*, and *EupWOX13* were shown to regulate AR development and lateral root growth (Chen et al. 2024). In studies conducted on *Larix kaempferi*, increased expression of *LkWOX4* was observed at the initiation of ARs, and its heterologous expression led to an increased number of adventitious roots (Wang et al. 2020). In tree species like *Populus nigra* and *Malus domestica*, the expression of *WOX4a*, *WOX4b*, *WOX5a*, *WOX5b*, *WOX11/12a*, and *WOX11/12b* was linked to AR formation (Baesso et al. 2020; Xu et al. 2022). Studies on *J. regia* revealed that *JrWOX4a*, *JrWOX4b, JrWOX5*, *JrWOX11*, and *JrWOX13* exhibit expression in roots and play roles in the AR formation process (Chang et al. 2020). These findings suggest that WOX genes are evolutionarily conserved in root morphogenesis and may have related roles across plant species. In our study, the expression patterns of *CaWOX4* and *CaWOX13* during AR formation in *C. avellana* were consistent with observations reported in other species. These results suggest that CaWOX4 and CaWOX13 may be associated with AR formation in hazelnut. Specifically, the observed increase in expression on day 7 suggests a temporal association between the expression of these genes and AR development under the experimental conditions used in this study.

This study has several limitations. First, the expression analysis was based on qRT-PCR data only, and no functional validation experiments, such as gene overexpression or silencing, were performed to confirm the biological roles of candidate genes in AR formation. Second, the analysis was conducted using a single hazelnut cultivar ("Palaz"), which may limit the generalizability of the findings across different genotypes. In addition, although a substantial number of plantlets were included across multiple sampling time points and pooled at each time point to reduce biological variation, the qRT-PCR analysis was conducted using two biological replicates.

## Conclusion

In this study, a comprehensive genome-wide analysis of the WUSCHEL-related homeobox (WOX) gene family in hazelnut (*Corylus avellana* L.) was conducted for the first time. A total of ten *CaWOX* genes were identified and characterized based on their chromosomal distributions, gene structures, conserved motifs, and physicochemical properties. Phylogenetic analysis classified these genes into three conserved clades—WUS, intermediate, and ancient—consistent with observations in both model and woody plant species. Promoter analysis revealed the presence of multiple cis-regulatory elements associated with hormone signaling and stress responses, suggesting complex regulatory mechanisms governing *CaWOX* gene expression. Expression profiling during AR development revealed detectable expression of *CaWOX4* and *CaWOX13*, with only *CaWOX4* showing a statistically significant increase by day 7. These findings suggest that *CaWOX4* and *CaWOX13* may be associated with AR formation in hazelnut. Overall, this study enhances our understanding of the molecular basis of AR formation in hazelnut and provides a foundation for future functional studies aimed at improving rooting efficiency in woody plant species.

**Author Contribution** F.T. supervised and completed the project, performed bioinformatic and molecular analyses, and wrote the manuscript draft. M.Ş. performed the bioinformatic analyses, conducted sample collection and experiments, and contributed to manuscript writing. C.U. performed the bioinformatic analyses and contributed to the manuscript editing. E.Y. performed the molecular analyses and contributed to the manuscript editing.

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**Data Availability** This study used publicly available genomic and protein sequence data from established databases cited in the manuscript for genome-wide identification and in silico analyses of the WOX gene family. Gene expression analysis was performed using quantitative real-time PCR (qPCR) with gene-specific primers designed in this study. No high-throughput sequencing or large-scale expression datasets were generated, and therefore no data were deposited in public repositories such as GenBank, the Gene Expression Omnibus (GEO), or the Sequence Read Archive (SRA). All data supporting the findings of this study are included within the manuscript and its supplementary materials. Additional information related to the analyses is available from the corresponding author upon reasonable request.

## Declarations

**Competing interest** The authors declare no competing interests.

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