forests

MDPI

Article

ChaWRKY40 Enhances Drought Tolerance of 'Dawei' Hazelnuts by Positively Regulating Proline Synthesis

Pengfei Zhang †, Ruiqiang Chao †, Liping Qiu, Wenjing Ge, Jinjun Liang and Pengfei Wen *

College of Horticulture, Shanxi Agricultural University, Jinzhong 030801, China; zhangpengfei166@163.com (P.Z.); chaoruiqiang1026@163.com (R.C.); qiuliping2468@163.com (L.Q.); jingwg1005@163.com (W.G.); liangjinjun1989@163.com (J.L.)

* Correspondence: sxauwpf@sxau.edu.cn

† These authors contributed equally to this work.

Abstract: Hazelnuts are among the most important nuts worldwide. Drought has severely restricted the development of the hazelnut industry in the wake of global warming and lack of water resources. Δ-1-pyrroline-5-carboxylic acid synthase (P5CS) is closely related to drought stress as the rate-limiting enzyme of proline synthesis. *WRKY40* had been proven to be an important transcription factor regulating drought tolerance in several plants. In this study, the hybrid hazelnut 'Dawei' exhibiting drought tolerance was used as the test material. Tests for simulated drought stress and *ChaWRKY40* overexpression, and the yeast one-hybrid assay were performed. The results showed that the relative water content of leaves gradually decreased, but the proline content, electrolyte leakage, and expression of *ChaWRKY40* and *ChaP5CS* increased with increasing PEG-6000 concentration in the leaves. A transient *ChaWRKY40* overexpression trial indicated that overexpression of *ChaWRKY40* improved the proline content and the transcription level of *ChaP5CS*. The Y1H experiment suggested that *ChaWRKY40* directly binds to the W-box-acting element (W-box) on the promoter of *ChaP5CS*. In conclusion, *ChaWRKY40* may increase the proline content by positively regulating the expression of the *ChaP5CS* gene, thereby improving the drought resistance of hazelnuts.

Keywords: hybrid hazelnut; drought; *ChaWRKY40*; *ChaP5CS*; proline

Citation: Zhang, P.; Chao, R.; Qiu, L.; Ge, W.; Liang, J.; Wen, P. *ChaWRKY40* Enhances Drought Tolerance of 'Dawei' Hazelnuts by Positively Regulating Proline Synthesis. *Forests* **2024**, *15*, 407. https://doi.org/10.3390/f15030407

Academic Editor: Stéphane Maury

Received: 10 January 2024
Revised: 11 February 2024
Accepted: 12 February 2024
Published: 21 February 2024

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

1. Introduction

The hazelnut tree, a *Corylus* plant of the Corylaceae family, bears one of the four largest nuts worldwide. It has rich nutritional and economic value [1,2]. As a main cultivar in China, 'Dawei' (*Corylus heterophylla* Fisch. × *Corylus avellana* L.) possesses the tolerance of *Corylus heterophylla* and bears large fruit similar to that of *Corylus avellana* [3]. However, shriveling restricts the development of the hazelnut industry in spring. Shriveling, also known as physiological drought, is directly associated with drought. This phenomenon is observed in cold and arid regions of the Northern Hemisphere, where temperatures rise more rapidly than the ground temperature during winter and spring. Consequently, the soil remains frozen and the roots enter a dormant state, facing difficulties in absorbing water and nutrients. The aboveground parts experience high temperatures, dryness, and strong winds, leading to excessive transpiration from branches, resulting in significant water loss. Consequently, the branch water content gradually decreases below 35%–40% until desiccation occurs [4]. One study found that 'Dawei' had a strong tolerance to shriveling, and its proline content was significantly higher than that of non-resistant varieties during the overwintering period [5]. At the same time, another study showed that the proline content of the '82-7' variety was much higher than that of the other varieties and lines with weak tolerance to shriveling in hybrid hazelnut during overwintering, and the proline content of 'Dawei' was second to that [6]. In summary, the drought tolerance of hybrid hazelnuts was closely related to their proline content [7]. Osmoregulation is a stress response that enhances the resistance of plants to osmotic stress. By regulating

the metabolic activity within plant cells, the concentration of osmoregulatory substances increases, leading to an increase in solute concentration and a reduction in cell permeability. This enables plants to continuously absorb water from environments with low external water potentials and maintain optimal cell turgor pressure, thus facilitating normal growth, development, and cellular metabolism [8]. The osmoregulatory substance content is closely associated with the ability of a plant to cope with osmotic stress [9]. As an osmotic adjustment substance for plants to resist external stress, proline accumulates rapidly when plants are subjected to abiotic stress, thereby reducing the harm caused by stress [10]. One study suggested that the proline content was the main index to reflect drought resistance of three poplar varieties [11]. After dehydration treatment, the proline content of leaves of 10 black poplar clones increased to varying degrees compared with that before stress [12]. Δ-1-pyrroline-5-carboxylic acid synthase (P5CS) is a key enzyme in the proline biosynthesis pathway. When plants are subjected to drought stress, proline accumulation is accompanied by the activation of P5CS coding genes. The *P5CS1* gene in poplar exhibited a highly responsive behavior towards drought stress, while *PagP5CS1* played a pivotal role in the synthesis and accumulation of free proline during drought stress conditions in poplar. Overexpression of *P5CS1* enhanced the drought tolerance of poplar by promoting increased proline accumulation [13]. Under dehydration conditions, the relative expression of P5CS1 increased with the extension of dehydration treatment time [14]. In *Petunia hybrida*, a large amount of proline accumulated after 14 days of drought, and plants transformed using *AtP5CS* or *OsP5CS* showed significantly improved drought tolerance compared to wild-type plants [15]. In a PEG-simulated drought experiment, the growth and yield of transgenic P5CS rice were higher than those of the control [16]. In summary, the expression of P5CS and synthesis of proline were promoted under drought stress, which improved the tolerance of plants to drought.

The WRKY transcription family, which is one of the largest transcription families in plants, derives its name from the conserved heptapeptide sequence WRKYGQK. WRKY transcription factors are generally composed of about 60 amino acids, while their C-terminal end contains a conserved C_2H_2 ($CX_{4\text{-}5}CX_{22\text{-}23}HXH$)-type or C_2HC ($CX_7CX_{23}HXC$)-type zinc-finger structure, and their N-terminal end is the highly conserved heptapeptide sequence, WRKYGQK, which recognizes W-boxes in the cis-acting element ((T)TGAC(C/T)), thereby regulating the expression of downstream genes in order to control various metabolic processes. WRKY family transcription factors are involved in plant growth and development. For example, *PdeWRKY75* directly regulates the expression of *PdeRBOHB* to catalyze the production of H_2O_2, thereby controlling the development of adventitious roots, lateral shoots, and healing tissues in poplar [17]. *PtrWRKY19* forms a *PtrMYB074-PtrWRKY19-PtrbHLH186* module with *PtrMYB074* and *PtrbHLH186* to synergistically regulate secondary xylem development in poplar [18]. In addition, the WRKY transcription family can also be involved in the synthesis of secondary metabolites. In tomato, *SlWRKY35* can directly activate *SlDXS1* expression, prompting metabolic recoding toward the MEP pathway and leading to enhanced carotenoid accumulation [19]. In pear, *PyWRKY26* and *PybHLH3* interacted and co-targeted the *PyMYB114* promoter, leading to anthocyanin accumulation in red-skinned pear [20]. WRKY transcription factors also play important roles in adversity stress. For example, *PalWRKY77* negatively regulates the expression of *PalRD26* and *PalNAC002* to reduce salt stress tolerance in poplar [21]. In apple, *MdWRKY115* improved tolerance to drought and salt stress by directly binding to the *MdRD22* promoter [22]. The findings of various studies have demonstrated that *JrWRKY21* and *JrPTI5L* interact synergistically to form a protein complex, which specifically binds to the GCCGAC motif on the *JrPR5L* promoter, thereby inducing its expression and ultimately enhancing the walnut's anti-anthrax capability [23].

WRKY40, a transcription factor of the WRKY family, is closely related to abiotic stress. In potato, the expression of *StWRKY40* in the leaves was the highest after 8 days of PEG-8000 stress [24]. Overexpression of *FcWRKY40* in kumquats enhanced the expression of *FcP5CS* and proline, ultimately increasing the salt tolerance of transgenic tobacco and

lemons. In silent lines, spraying with exogenous proline restored their tolerance to salt stress [25]. Similar results have been reported for *Zea mays* [26], *Fraxinus mandshurica* [27], and *Myrothamnus flabellifolia* [28].

Previous studies in the laboratory expressed that proline might be a key indicator of strong drought tolerance of the hybrid hazelnut 'Dawei,' and speculated that *ChaWRKY40* might be involved in the accumulation of proline in the hybrid, which leads to high drought tolerance in 'Dawei'. However, the mechanism of *ChaWRKY40* response to drought stress by affecting proline synthesis remains unclear. Therefore, we investigated the mechanism of *ChaWRKY40* regulating drought tolerance in the hybrid hazelnut 'Dawei' in this study.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Branches with similar leaf size and branch thickness from a 20-year-old hybrid hazelnut 'Dawei' tree were collected as test materials on 19 May 2023. The entire material was subsequently divided equally into four groups, each consisting of three branches, and then placed in a 100 mL conical bottle. Polyethylene glycol-6000 (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) was used to simulate drought stress. Four gradients were set: 0 g·L^{-1} (distilled water), 50 g·L^{-1} (50 g PEG-6000 was dissolved in 1 L of distilled water), 150 g·L^{-1} (150 g PEG-6000 was dissolved in 1 L of distilled water), and 250 g·L^{-1} (250 g PEG-6000 was dissolved in 1 L of distilled water). The four solutions were then added to conical bottles and placed in an LED incubator. The samples were hydroponically cultured in a light-emitting diode incubator. After treatment for 12 h, samples were taken and then frozen in liquid nitrogen and stored at –80 °C. The LED light incubator (GLD-450E-4) was purchased from Ningbo Ledian Instrument Manufacturing Co., Ltd., Ningbo, China. The environmental temperature was 25 °C, the light was 250 μmol m^{-2} s^{-1}, and the relative humidity was 70%. The wavelength types were rich and coincided with the spectral ranges of plant photosynthesis and light morphogenesis. Specific wavelengths of light can be concentrated to illuminate plants evenly. The experiments performed on the control and treatment groups were independently repeated three times.

2.2. RNA Extraction and qRT–PCR Analysis

Total RNA was extracted from the hazelnut leaves using an improved CTAB method and purified using DNase I [29]. Then, total RNA was reverse-transcribed with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The concentration of cDNA was diluted to 200 ng·μL^{-1}. The concentration of primers was 10 μM. Real-time qRT-PCR was performed using real-time PCR Super mix SYBR green with anti-Taq (Mei5 Biotechnology, Co., Ltd., Beijing, China). The reaction system was established as follows: 5 μL 2 × Realtime PCR Super mix, 0.5 μL cDNA, 0.5 μL primers, and 4 μL ddH_2O. The reaction program was set as follows: 95 °C for 60 s, 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 60 s. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The actin gene of *Corylus heterophylla* Fisch. × *Corylus avellana* L. was used as the reference gene [30]. Three biological replicates were analyzed for each sample. The primer sequences used in this study are listed in Table A1 (italics indicate the sites of enzyme digestion).

2.3. Gene Cloning and Vector Construction

By using *ChaWRKY40*-F/R (Table A1) at a concentration of 10 μM, the *ChaWRKY40* gene was amplified using the cDNA of 'Dawei' hybrid hazelnut as a template. The concentration of cDNA was 200 ng·μL^{-1}. The amplified fragments were confirmed using 1.2% agarose gel electrophoresis and recycled using a TIANgel Midi Purification Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Then, the gene was ligated into the pMD19-T vectors (TaKaRa) and used to transform competent *E. coli* Trans5α (TransGen Biotech Co., Ltd., Beijing, China). The positive colonies were sequenced by the Shanghai Bioengineering Company (Shanghai, China). Primers G-*ChaWRKY40*-F/R (Table A1) were designed according to gene specificity. The forward primer contains the *Xba*I (TaKaRa Bio)

digestion site sequences (TCTAGA) and the reverse primer contains the *Kpn*I (TaKaRa Bio) digestion site sequences (GGTACC). The concentration of primers was 10μM. For vector construction, the PCR amplification products were ligated to the overexpression vector pCAMBIA-35S-1300 with T4DNA ligase (TaKaRa) and used to transform competent *E. coli* Trans5α. Then, positive colonies were screened and sequenced. The *35S::ChaWRKY40* plasmid was produced using the TIANprep Mini Plasmid Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China).

2.4. Bioinformatics Analysis

A phylogenetic tree was constructed using MEGA7.0 software. BLAST protein comparisons were conducted using NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 9 August 2023) and sequences with high similarity to the ChaWRKY40 protein were downloaded. DNAMAN software was used for multiple sequence alignments of WRKY proteins from *Corylus heterophylla* Fisch. × *Corylus avellana* L., *Quercus_lobata*, *Quercus_robur*, *Quercus_suber*, *Juglans_regia*, *Morella_rubra*, *Carya_illinoinensis*, *Populus_alba*, and *Populus_trichocarpa*. The upstream 2000 bp sequence of *ChaWRKY40* was analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 9 August 2023). TBtools software was used to plot the data.

2.5. Transient ChaWRKY40 Overexpression in Hazelnut Leaves

The plasmid of *35S::ChaWRKY40* and an empty pCAMBIA1300 vector were transformed into *Agrobacterium tumefaciens* GV3101 (Coolaber, Beijing, China). A. tumefaciens solution with the target gene and empty vector was transferred to 150 mL LB liquid medium (containing 50 ng·mL^{-1} Kan and 25 ng·mL^{-1} Rif) and cultured to OD600 = 1.0. The bacteria were collected by centrifugation at 5000 rpm for 8 min. They were then resuspended in a suspension containing 200 μM AS, 10 mM $MgCl_2$, and 10 mM MES, the OD_{600} was adjusted to about 1.0, and they were cultured in dark conditions for 2–3 h for reserve use. Afterwards, the fungal solution was infiltrated into the hazelnut leaves by vacuum infiltration. The petiole was inserted into a conical flask containing 120 mL of distilled water and placed in an LED-type light incubator (light intensity: 250 μmol m^{-2} s^{-1}, relative humidity: 70%, temperature: 25 °C). The cells were incubated in the dark for 12 h and then conditions were alternated between light and dark for 16 h/8 h. Samples were collected at 36 and 60 h. Afterwards, leaf RNA was extracted for qRT-PCR analysis.

2.6. Analyses of Physiological Indices and Histochemical Staining

To further investigate the function of *ChaWRKY40*, plants were used as test materials after 36 h of overexpression. Part of the materials was placed in clean water, the other part was placed in 50 g·L^{-1} PEG, and they were all sampled after treatment for 2 days. Electrolytic leakage (EL), proline, and soluble sugars were examined as previously described [6]. Malondialdehyde (MDA) content was measured using thiobarbituric acid colorimetry. The soluble protein content was examined using Coomassie brilliant blue staining. The content of H_2O_2 was measured using a Hydrogen Peroxide (H_2O_2) Content Assay Kit (AKAO009C, Boxbio, Beijing, China). The content of $O_2^{\cdot-}$ was detected by the hydroxylamine hydrochloride oxidation method. The accumulation of H_2O_2 and $O_2^{\cdot-}$ was determined by histochemical staining with 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively. The relative water content was determined using the weighing method. SOD activity was measured using the nitro blue tetrazolium method. POD activity was determined by measuring the oxidation rate of guaiacol at 470 nm [31]. The water potential of PEG-6000 solution was determined with $\Psi_{PEG} = 1.29[PEG]^2T\text{-}140[PEG]^2\text{-}4[PEG]$. ($\Psi_{PEG}$: the water potential of PEG solution, the unit is bar; [PEG]: the concentration of PEG in g·g^{-1} (water); T: temperature (°C) [32,33]).

2.7. Yeast One-Hybrid Assay

To determine the interactions between *ChaWRKY40* and *ChaP5CS*, we performed Y1H experiments. A 2000 bp fragment of the promoter of *ChaP5CS* was cloned and a W-box was identified using PlantCARE for promoter analysis. By using Y1H-*ChaP5CS*-F/R (Table A1), the 180 bp promoter sequence (*ChaP5CS*) containing only the W-box was used to amplify target promoter sequences using the DNA of 'Dawei' hybrid hazelnut as a template. The concentration of DNA was 90 ng·mL^{-1}. The concentration of primers was 10 μM. The target fragment and the pAbAi vector were digested using *Kpn*I and *Sac*I, respectively. They were ligated by T4DNA ligase and used to transform competent *E. coli* Trans5α. Positive colonies were screened and sequenced. The pAbAi-*ChaP5CS* plasmid was then produced. The pAbAi-*Chap5cs* plasmid (TTGACC → TTAACC) was synthesized by Shanghai Bioengineering Company (Shanghai, China). Primers Y1H-*ChaWRKY40*-F/R (Table A1) at a concentration of 10 μM were designed according to gene specificity. The forward primer contains the *EcoRI* (TaKaRa Bio) digestion site sequences (GAATTC) and the reverse primer contains the *BamHI* (TaKaRa Bio) digestion site sequences (GGATCC). For vector construction, the PCR amplification products were ligated to the pGADT7 vector with T4DNA ligase (TaKaRa) and used to transform competent *E. coli* Trans5α. Then, positive colonies were screened and sequenced. The plasmid was produced using the TIANprep Mini Plasmid Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Linearized p*ChaP5CS* and p*Chap5cs* vectors (digested with BspT104I) were transformed into competent Y1HGold. Then, the aureobasidin A (AbA) concentrations for each bait vector were 0 ng·mL^{-1}, 50 ng·mL^{-1}, 150 ng·mL^{-1}, 200 ng·mL^{-1}, and 300 ng·mL^{-1}. p*ChaP5CS*-AbAi and p*chap5cs*-AbAi were inoculated onto SD/-Ura plates at the above concentrations, and their growth status was observed after 3 days of inversion at 30 °C. The optimal aureobasidin A (AbA) concentration for each bait vector was 300 ng·mL^{-1}. The prey and bait constructs were co-transformed into Y1H yeast strains, followed by culture on SD/−Leu-AbA 300 ng·mL^{-1} selective medium at 30 °C for 3 days.

2.8. Statistical Analysis

The stress treatments were independently repeated thrice. Data were analyzed using SPSS 25 software. Statistical significance was determined using a one-way ANOVA. Differences were considered statistically significant at $p < 0.05$. Graphs and tables were drawn and processed using GraphPad Prism 8 and Photoshop2023.

3. Results

3.1. Phylogenetic Tree Construction, Homologous Sequence Alignment, and Promoter Sequence Analysis of ChaWRKY40

The following 12 amino acid sequences, including QlWRKY40, QrWRKY40, QsWRKY40, MrWRKY40, CiWRKY40, JrWRKY40, JsWRKY59, PaWRKY40, PtWRKY40, CjWRKY40, AtWRKY40, and ChaWRKY40, were selected to construct a phylogenetic tree using MEGA7 software. The results suggested that ChaWRKY40, CiWRKY40, and AtWRKY40 were close in the phylogenetic tree, indicating that they were closely related and had high homology (Figure 1A). A comparison of homologous amino acid sequences indicated that ChaWRKY40 contains a WRKY superfamily conserved domain (103–159 aa) (Figure 1B). We found that it contained three action elements, an MBS (CAACTG) in response to drought stress, three MeJA response elements, a CGTCA-motif/TGACG-motif (CGTCA/TGACG), one SA response element, and a TCA-element (CCATCTTTTT) by analyzing the promoter of *ChaWRKY40* (Figure 1C). Therefore, it was speculated that *ChaWRKY40* may contribute to stress resistance in hazelnuts.

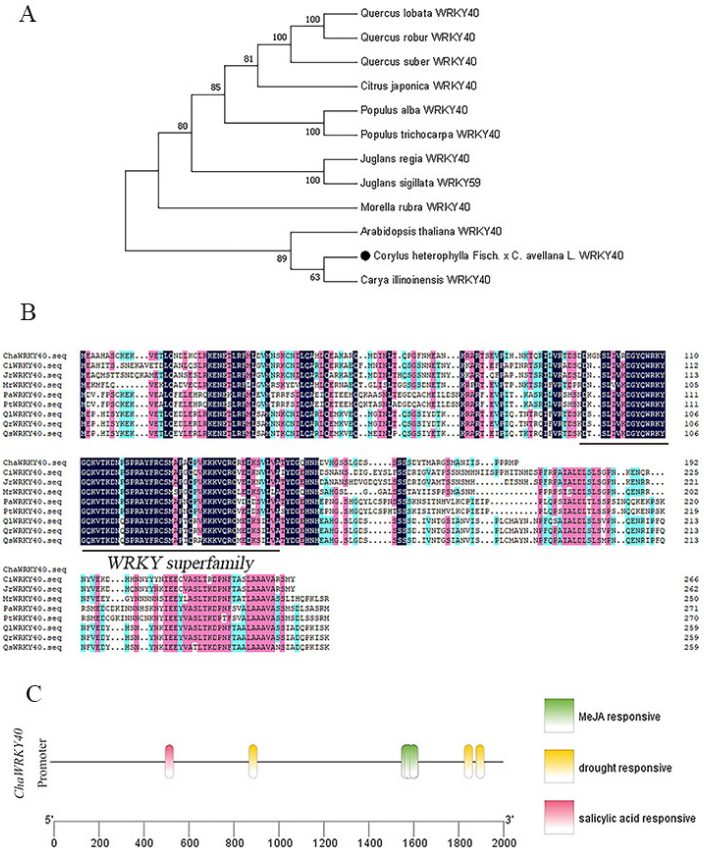

Figure 1. Phylogenetic tree, multiple sequence alignments, and promoter analysis. (**A**) Phylogenetic tree of WRKYs from several plants. (**B**) Amino acid sequence alignment of *WRKY40* from several plants. (**C**) Sequence analysis of *ChaWRKY40* promoter.

3.2. Phenotypic Observation and Determination of the Content of Proline, EL, RWC, and the Expression Levels of ChaWRKY40 and ChaP5CS under Drought Stress

To explore the response of *ChaWRKY40* to drought stress, we used PEG-6000 to stimulate drought stress. The water potentials of the four treatment solutions were 0 bar, −0.47 bar, −3.02 bar, and −7.73 bar in order (Table A2). Plants treated with clean water grew strongly and the leaves became dark green and stretched after 12 h. A large number of curls and water loss appeared in the leaves, and the growth tended to decline when the PEG-6000 concentration reached 250 g·L^{-1}. Phenotypic observations revealed that the leaves gradually lost water and curled, and the degree of curling increased with increasing drought stress (Figure 2A). qRT-PCR showed that the transcription levels of *ChaWRKY40* and *ChaP5CS* increased gradually with increasing PEG-6000 concentrations. The relative expression of *ChaP5CS* was approximately 9 times that of the control (Figure 2B), and that of *ChaWRKY40* was approximately 6 times that of the control when the PEG-6000 concentration was 250 g·L^{-1} (Figure 2C). We found that the content of proline in different treatments was significantly higher than in the control ($p < 0.001$), and the maximum value was 177.5 μg·g^{-1} FW when the PEG-6000 concentration was 250 g·L^{-1} (Figure 2D). We verified that the RWC decreased gradually with increasing PEG-6000 concentrations. The RWC of samples treated with PEG-6000 at different concentrations was significantly lower than that of the control ($p < 0.01$) (Figure 2E). On the contrary, electrolyte leakage gradually increased with an increase in drought stress. The EL of the various treatments increased significantly ($p < 0.0001$) compared to that of the water treatment (Figure 2F). The minimum RWC was 556.3 mg·g^{-1} and the maximum EL was 60.15% when the PEG-6000 concentration reached 250 g·L^{-1}. These results indicate that the proline content and transcript abundance of *ChaWRKY40* and *ChaP5CS* increased under drought conditions.

Therefore, we speculated that *ChaWRKY40* and *ChaP5CS* participate in regulating proline accumulation and improving hazelnut drought tolerance.

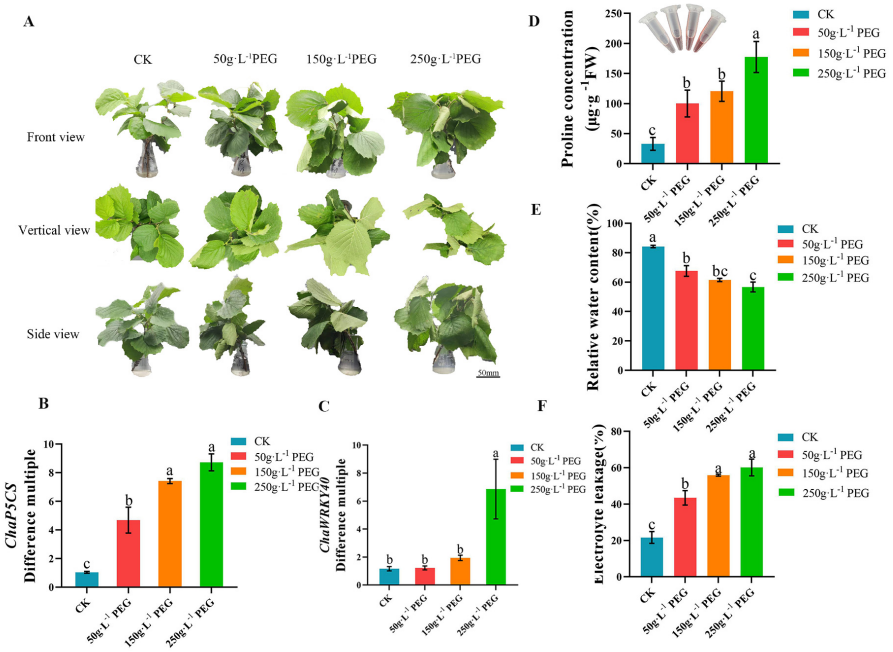

Figure 2. Measurement of related physiological indicators and genes in the hybrid hazelnut 'Dawei' under PEG-6000 treatment. (**A**) Phenotypes of 'Dawei' branches under different concentrations of PEG-6000 treatment conditions. (**B**,**C**) Relative expression levels of *ChaP5CS* (**B**) and *ChaWRKY40* (**C**) under drought stress. (**D**–**F**) Proline content, relative water content, and electrolyte leakage under different concentrations of PEG-6000 treatment conditions. Duncan's range test was used to test for significance (n = 3, $p < 0.05$).

3.3. The Effect of Transient ChaWRKY40 Overexpression on Proline and ChaP5CS

To determine the role of *ChaWRKY40* in drought tolerance, *ChaWRKY40* was introduced into the pCAMBIA1300 vector (Figure A1). The expression levels of *ChaWRKY40* in *OE-ChaWRKY40* plants were significantly higher than those in empty vector (EV) plants ($p < 0.0001$) after 36 and 60 h, as determined by qRT-PCR. These results implied that strains of *ChaWRKY40* overexpression were obtained. The transcript abundance of *ChaWRKY40* was ~38-fold and 9-fold higher after 36 h and 60 h, respectively (Figure 3B). We affirmed that the proline contents were dramatically higher ($p < 0.0001$) and approximately increased to 240.99 $\mu g \cdot g^{-1}$ FW in *OE-ChaWRKY40* plants after 36 h (Figure 3A,C). Similarly, the relative expression level of *ChaP5CS* was ~4 times higher ($p < 0.001$) (Figure 3D). These findings suggest that *ChaWRKY40* was successfully overexpressed. Transient overexpression of *ChaWRKY40* increased the proline content and expression levels of *ChaP5CS*.

3.4. Determination of Proline and ChaP5CS in OE-ChaWRKY40 and EV Plants under Drought Treatment

To further investigate the function of *ChaWRKY40*, the drought tolerance of plants after 36 h of overexpression was observed. The phenotypes of *OE-ChaWRKY40* and EV plants were similar after 2 days in clean water. Both plants wilted, but EV plants displayed more severe leaf curling and chlorosis than *OE-ChaWRKY40* plants under drought stress (Figure 4A,B). The proline content of *OE-ChaWRKY40* plants was significantly higher ($p < 0.05$) than that of EV plants under water and drought treatment. The content of proline of *OE-ChaWRKY40* plants reached 281.5 $\mu g \cdot g^{-1}$ FW, and that of EV plants was 180.55 $\mu g \cdot g^{-1}$ FW under drought stress (Figure 4C). The expression level of *ChaP5CS* in *OE-ChaWRKY40* plants was prominently higher ($p < 0.05$) than that in EV plants under drought treatment (Figure 4D).

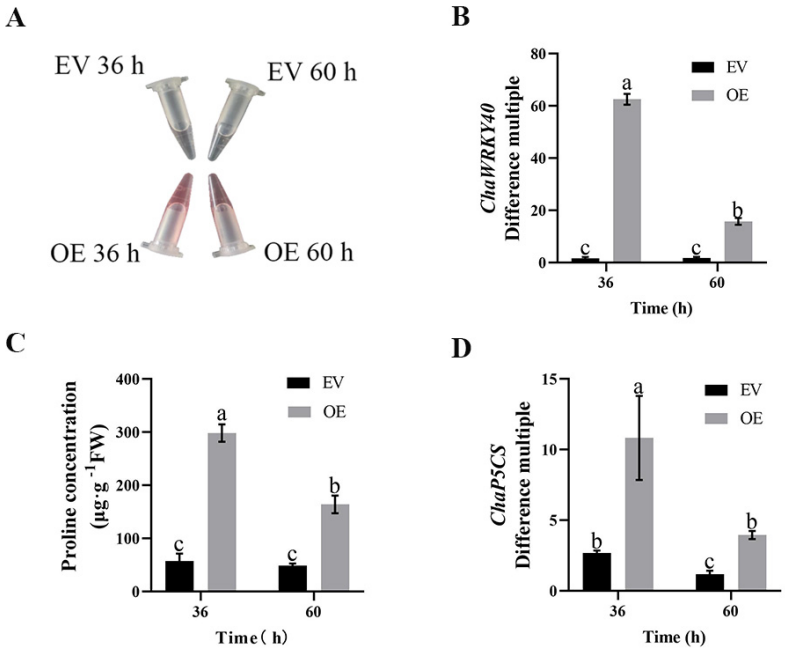

Figure 3. Transient overexpression of *ChaWRKY40* augmented the content of proline and the expression level of *ChaP5CS*. (**A**,**C**) The content of proline after overexpression. (**B**) The relative expression levels of *ChaWRKY40* after overexpression. (**D**) The relative expression levels of *ChaP5CS* after overexpression. Duncan's range test was used to test for significance (n = 3; $p < 0.05$). Note: EV: empty vector; OE: overexpression.

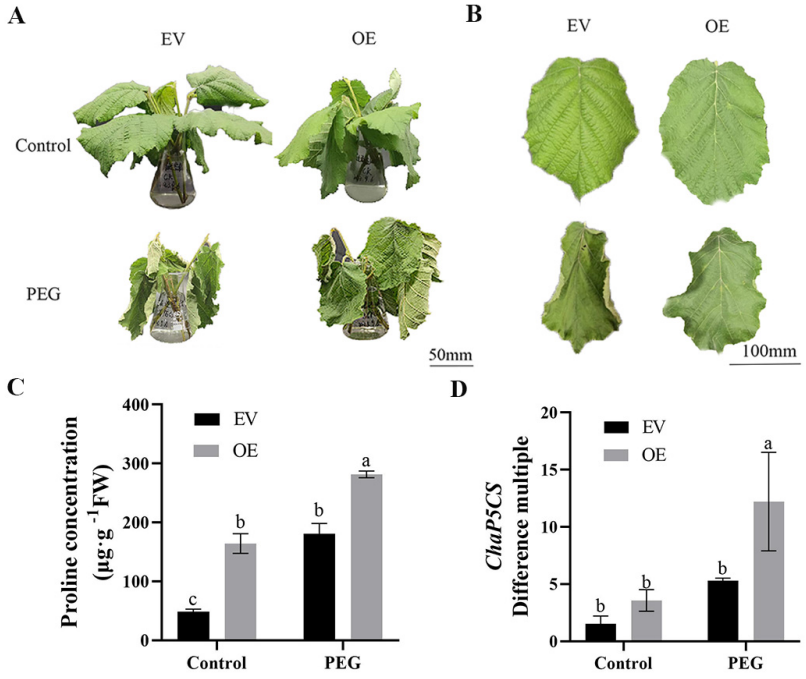

Figure 4. Determination of proline and *ChaP5CS* in *OE-ChaWRKY40* and EV plants under drought treatment. (**A**,**B**) Phenotypes of *OE-ChaWRKY40* and EV plants before and after stress. (**C**) Content of proline of *OE-ChaWRKY40* and EV plants before and after stress. (**D**) Expression levels of *ChaP5CS* in *OE-ChaWRKY40* and EV plants before and after stress. Duncan's range test was used to test for significance (n = 3; $p < 0.05$). Note: EV: empty vector; OE: overexpression.

3.5. Determination of the Content of RWC, EL, MDA, Soluble Sugar, and Soluble Protein in OE-ChaWRKY40 and EV Plants under Drought Treatment

The overexpression of *ChaWRKY40* enhanced the drought tolerance of 'Dawei' in a more comprehensive manner. There were no noticeable differences in the RWC, EL, and MDA contents between EV and *OE-ChaWRKY40* plants under normal conditions. The RWC of *OE-ChaWRKY40* plants was significantly higher than that of EV plants under drought stress. *OE*-ChaWRKY40 plants decreased by 280.8 $mg \cdot g^{-1}$ ($p < 0.05$) and EV plants decreased by 354.4 $mg \cdot g^{-1}$ ($p < 0.01$) compared with the normal condition (Figure 5A). However, *OE-ChaWRKY40* plants showed lower levels of EL and MDA under drought conditions. The content of EL markedly ($p < 0.05$) expanded by 40.02% (Figure 5B), and the content of MDA was dramatically ($p < 0.05$) augmented by 0.006 $\mu mol \cdot g^{-1}$ in *OE-ChaWRKY40* strains (Figure 5C). The content of EL substantially ($p < 0.01$) increased by 52.44%, and the content of MDA was significantly ($p < 0.01$) uplifted by 0.012 $\mu mol \cdot g^{-1}$ in EV plants. The results suggested that the contents of soluble sugar and soluble protein were improved by 0.12% ($p < 0.05$) (Figure 5D) and 0.08% ($p < 0.05$) in the EV plants under drought conditions (Figure 5E), respectively; and increased by 0.2% ($p < 0.01$) and 0.14% ($p < 0.01$) in *OE-ChaWRKY40* plants, respectively. Collectively, overexpression of *ChaWRKY40* reduced damage to 'Dawei' and improved the stress-resistant substances of 'Dawei' under drought stress.

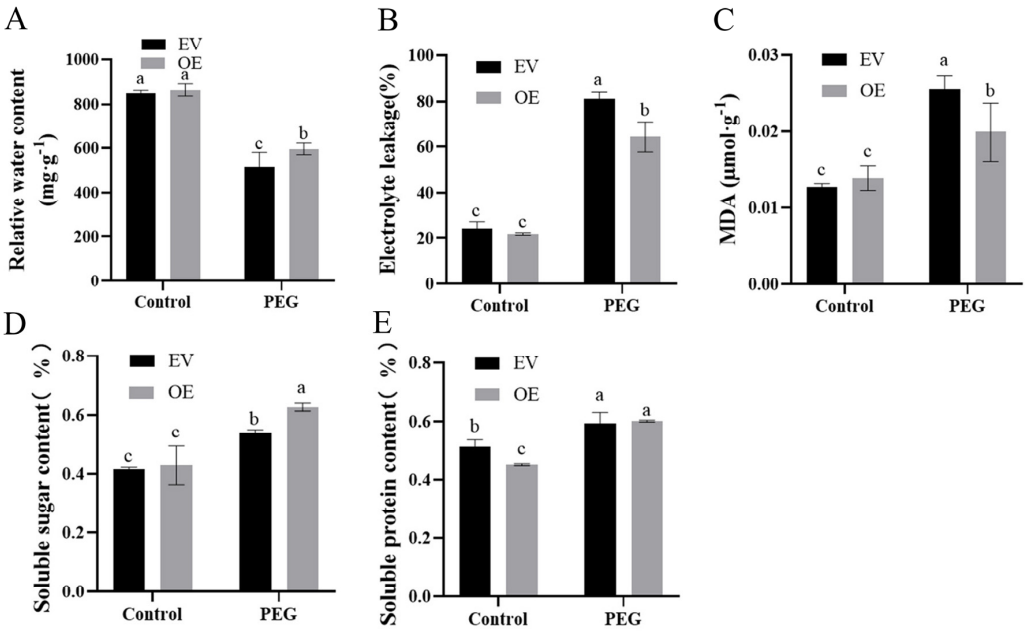

Figure 5. Physiological indexes of EV and *OE-ChaWRKY40* plants before and after drought treatment. (**A**) Relative water content (RWC) of EV and *OE-ChaWRKY40* plants before and after drought treatment. (**B**) Electrolyte leakage. (**C**) Malondialdehyde (MDA) concentrations. (**D**) Soluble sugar content. (**E**) Soluble protein content before and after drought treatment. Duncan's range test was used to test for significance (n = 3; $p < 0.05$). Note: EV: empty vector; OE: overexpression.

3.6. Determination of Reactive Oxygen Species and Antioxidant Enzymes in OE-ChaWRKY40 and EV Plants under Drought Stress

$O_2^{\cdot -}$ and H_2O_2 are two notable reactive oxygen species molecules which accumulate in the injured parts of plants. The findings showed that there were no significant distinctions in the accumulations of $O_2^{\cdot -}$ and H_2O_2 between the EV and the *OE-ChaWRKY40* plants under control conditions. However, the contents of the $O_2^{\cdot -}$ (Figure 6B) and H_2O_2 (Figure 6D) in the EV plants were substantially higher than those in the *OE-ChaWRKY40* plants under drought conditions. At the same time, the histochemical staining method suggested that the EV strains and *OE-ChaWRKY40* plants had slight staining, but no significant difference

was observed under water treatment. We found that the EV and *OE-ChaWRKY40* plants were lightly stained without drought stress using the NBT staining method (Figure 6A). *OE-ChaWRKY40* plants stained less blue than the EV plants in the presence of PEG-6000. The leaf edges of EV plants were stained deeper and more brownish yellow than those of *OE-ChaWRKY40* plants under drought stress (Figure 6C). Additionally, we found that activities of SOD and POD increased by 19.47 $U \cdot g^{-1} \cdot min^{-1}$ and 45.33 $U \cdot g^{-1} \cdot min^{-1}$ in *OE-ChaWRKY40* plants under drought stress while SOD and POD activities increased by 9.94 $U \cdot g^{-1} \cdot min^{-1}$ (Figure 6E) and 24.00 $U \cdot g^{-1} \cdot min^{-1}$ (Figure 6F) in the EV plants. These results suggest that *OE-ChaWRKY40* plants enhanced their ability to eliminate reactive oxygen species by increasing the activity of antioxidant enzymes, thereby reducing plant damage under stress conditions.

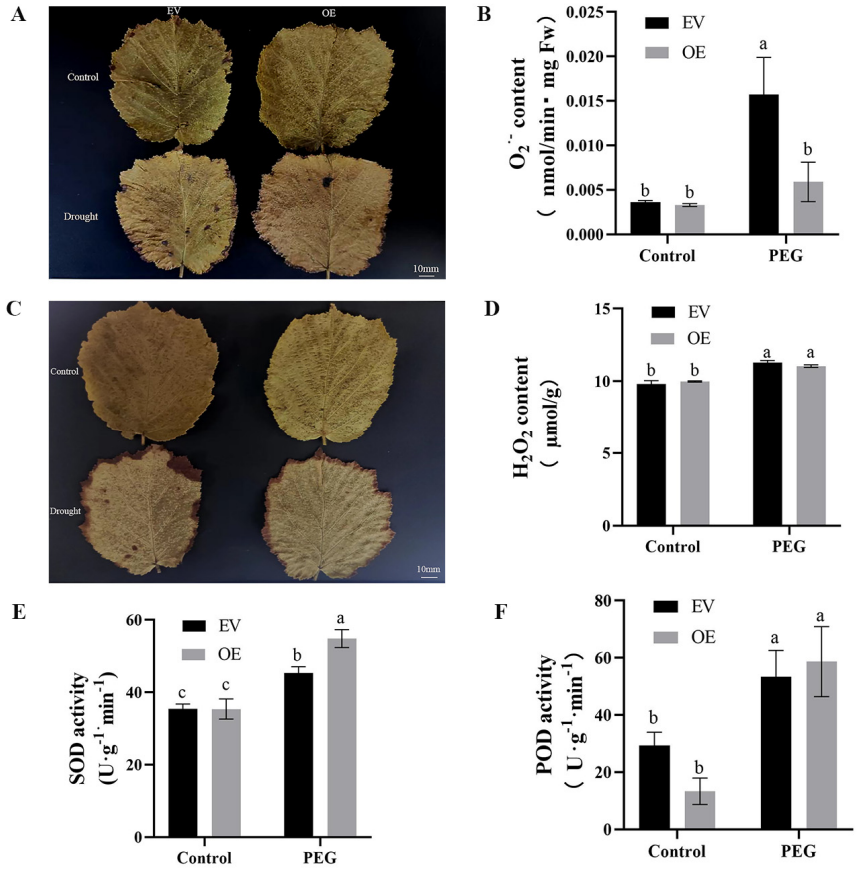

Figure 6. Overexpression of *ChaWRKY40* in 'Dawei' reduced the concentration of H_2O_2 and $O_2^{\cdot-}$ and increased the activity of SOD and POD under drought stress. (**A**) Histochemical staining with 3,30-diaminobenzidine (DAB). (**B**) Superoxide anion content of EV and *OE-ChaWRKY40* plants before and after drought treatment. (**C**) Histochemical staining with nitro blue tetrazolium (NBT). (**D**) H_2O_2 content. (**E**) SOD activity. (**F**) POD activity. Duncan's range test was used to test for significance ($n = 3$; $p < 0.05$). Note: EV: empty vector; OE: overexpression.

3.7. ChaWRKY40 Can Directly Bind to the W-Box of ChaP5CS Promoter

To further explore the effect of *ChaWRKY40* on the proline content, the interactions between *ChaP5CS* and *ChaWRKY40* were identified using the Y1H assay. One W-box action element (TTGACC) was present in the promoters of *ChaP5CS* (Figure 7A). Therefore, *ChaWRKY40* was treated as prey, and promoter sequences with normal and mutant W-box action elements were used to obtain baits (Figure 7B). All yeast cells grew normally on SD/-Leu medium without ABA. Yeast cells co-transformed with the prey and normal W-box action element survived on the medium with an ABA concentration of 300 $ng \cdot mL^{-1}$, while cells co-transformed with the prey and mutant W-box were fully suppressed (Figure 7C).

Together, our molecular studies demonstrated that *ChaWRKY40* acts upstream of *ChaP5CS* and participates in proline synthesis.

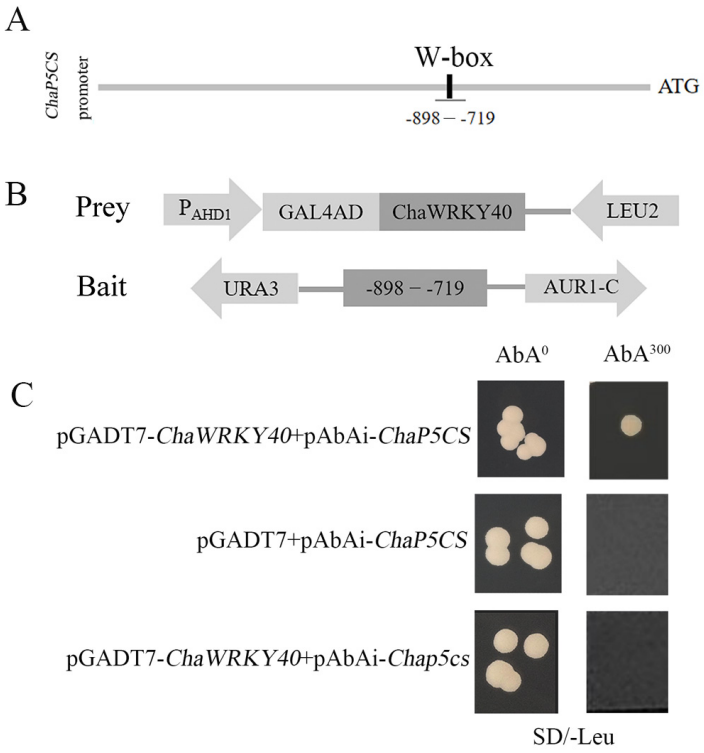

Figure 7. *ChaWRKY40* binds to the promoters of *ChaP5CS*. (**A**) Schematic diagram of the *ChaP5CS* promoter. The black square indicates the position of the W-box element and −898–−719 indicates the promoter fragment that is amplified (**B**) Schematic diagram of Y1H vector construction. (**C**) Results of yeast one-hybrid. Growth after co-conversion of *ChaWRKY40* and *ChaP5CS* on SD/-Leu plates with AbA concentrations of 0 ng·mL^{-1} and 300 ng·mL^{-1}. Growth after co-conversion of pGADT7 and *ChaP5CS* on SD/-Leu plates with AbA concentrations of 0 ng·mL^{-1} and 300 ng·mL^{-1}. Growth after co-conversion of *ChaWRKY40* and *Chap5cs* on SD/-Leu plates with AbA concentrations of 0 ng·mL^{-1} and 300 ng·mL^{-1}.

4. Discussion

WRKY transcription factors, among the largest transcription families, play important roles in plant development, metabolic synthesis, and stress tolerance. One study indicated that *AcWRKY40* might mediate the post-harvest ripening and softening of kiwifruit by specifically regulating the expression of genes related to ethylene biosynthesis [34]. One transgenic experiment showed that *TaWRKY51* has a positive regulatory effect on the growth and development of lateral roots [35]. In grapes, *VvWRKY5* enhances tolerance to white rot through the JAZ-MYC module, which mediates the JA signaling pathway [36]. In apples, *MdWRKY18* and *MdWRKY40* form homodimers or heterodimers that enhance salt tolerance in the callus [37]. Conserved domain prediction suggested that the protein encoded by ChaWRKY40 contains a WRKY conserved domain. These results indicate that *ChaWRKY40* belongs to the WRKY family of transcription factors [38]. Homologous amino acid sequence alignment and phylogenetic tree analysis showed that ChaWRKY40 and AtWRKY40 are closely related. Sequence analysis of the *ChaWRKY40* promoter revealed three elements that responded to drought stress. Therefore, we speculate that *ChaWRKY40* may respond to stress tolerance in hazelnuts.

To ensure normal physiological functions following exposure to osmotic stress, plants typically continuously accumulate these substances. In general, higher levels of osmoregulatory substances indicate stronger drought resistance in plants [39]. Proline is an important osmolyte that is produced in large quantities by plants subjected to stress [40]. The concen-

tration of osmoregulatory substances in the plant body increases, resulting in an increase in osmotic pressure and improvement in the absorption of external water by the plant [41]. The proline content of 100-day-old banana seedlings significantly increases under drought conditions [42]. P5CS, a key enzyme in the proline biosynthesis pathway, is closely related to proline content. Overexpression of P5CS increased the proline content and thus increased osmotic regulatory substances in plants. When plants were under drought stress, higher osmotic pressure was generated than in the control, which improved the water absorption capacity and, thus, improved drought tolerance [43]. Transgenic Arabidopsis plants expressing OE-*SpP5CS* accumulate more proline than wild-type plants under drought stress [44]. In poplar, *PagP5CS1* overexpression enhances drought tolerance by increasing proline synthesis [45]. Transfer of the P5CS gene to potatoes also improves tolerance to drought stress [46]. The results of this study indicate that the proline content and the expression of *ChaP5CS* increased under drought stress. At the same time, they had a positive correlation. In summary, an increase in proline content increased the concentration of osmotic substances in the plant body, further enhancing osmotic pressure, thereby improving the absorption of external water by the plant and improving drought tolerance. WRKY is an important transcription factor that responds to drought stress [47,48] and affects drought tolerance by regulating the expression of stress genes [49] and stomata [50,51]. However, few studies have investigated the responses of hybrid hazelnuts to drought stress. In this study, the expression of *ChaWRKY40* was induced by drought stress and gradually increased with its aggravation. Therefore, we speculated that there was a correlation between *ChaWRKY40*, *ChaP5CS*, and proline.

In tomatoes, one study showed that the transcription levels of *SlbHLH96* in OE-*SlbHLH96*-2 and OE-*SlbHLH96*-17 tomato seedlings were approximately 60 and 55 times higher than those in control plants, respectively [52]. In this study, the expression levels of *ChaWRKY40* in OE-*ChaWRKY40* plants were approximately 36 times higher than those in the control plants after transient overexpression of *ChaWRKY40*. These results indicated that 'Dawei' plants with transient *ChaWRKY40* overexpression were obtained. We found that the proline content and the expression of *ChaP5CS* would increase accordingly. This further confirms the relationship between these three factors.

Transient overexpression of *ChaWRKY40* enhances drought tolerance in hazelnuts. The leaves appeared curled and wilted, but the control plants were affected more severely after 2 days of treatment in the OE and EV plants treated with PEG-6000. This is consistent with the phenomenon observed in transgenic Arabidopsis with *OE-ZmWRKY40* [26] and transgenic tobacco with *OE-MbWRKY2* [53] after losing water for some time. Therefore, it can be observed from the phenotype that *OE-ChaWRKY40* improved the drought tolerance of hybrid hazelnut. Simultaneously, it was found that the proline content and transcriptional levels of *ChaP5CS* in *OE-ChaWRKY40* plants were significantly higher than those in the control under drought stress, which was consistent with the results in apple [54]. Research has suggested that the concentrations of MDA and EL in transgenic *OE-FcWRKY40* tobacco were remarkably lower than those in WT plants under salt stress [25]. In Hippophae rhamnoides, transgenic tobacco (*OE-HrWRKY21*) has lower EL and MDA contents than WT tobacco under drought stress [55]. In this study, we demonstrated that the levels of EL and MDA in the *OE-ChaWRKY40* plants were lower than those in the control plants, whereas the RWC was higher than that in the control plants. This suggests that *OE-ChaWRKY40* strains accumulated more soluble sugars and proteins under drought stress. These results are consistent with those of previous studies [56,57].

$O_2^{\cdot-}$ and H_2O_2 accumulate when plants are subjected to stress. Antioxidant enzymes such as SOD and POD play an important role in scavenging ROS in plants [58]. Strains overexpressing *CmWRKY10* showed increased SOD and POD activity compared to the WT under drought stress, resulting in less ROS accumulation [59]. In transgenic Arabidopsis, the activities of SOD and POD in *OE-PbWRKY40* [60] and *OE-MxWRKY64* [61] were much higher than those in the WT under salt stress. In this research, we detected the accumulation of $O_2^{\cdot-}$ and H_2O_2, and carried out NBT and DAB histochemical staining

to detect them, respectively. These results suggested that the *OE-ChaWRKY40* plants had enhanced antioxidant capacity, and the concentrations of $O_2^{\cdot-}$ and H_2O_2 were lower than the control plants. Similar results were obtained by histochemical staining. This study also found that the SOD and POD activities of *OE-ChaWRKY40* were higher than those of the control. These results further confirmed that the ROS content of *OE-ChaWRKY40* was lower than that of the control plants under drought conditions. In summary, overexpression of *ChaWRKY40* can improve the tolerance of hybrid hazelnuts to drought stress.

WRKY transcription factors, among the largest transcription families, regulate the expression of structural genes by specifically binding to the W-box-acting element in the promoter sequences of structural genes. In apples, *MdWRKY17* regulates chlorophyll levels by directly binding to the W-box of the *MdSUFB* promoter [62]. In wild strawberry, *FvWRKY48* regulates pectin degradation and fruit softening by directly binding to the W-box in the promoter sequence of *FvPLA* [63]. *FvWRKY50* negatively regulates strawberry leaf senescence by directly binding to the W-box in the promoter sequence of *FvSAUR36* [64]. In pears, *PbWRKY40* regulates the expression of *PbVHA-B1* by directly binding to the W-box in the promoter sequence, thereby enhancing tolerance to salt stress and the accumulation of organic acids [65]. In cotton, *GhWRKY21* binds to the W-box of the *GhHAB* promoter [66]. The promoter sequence of *ChaP5CS* was analyzed and revealed a W-box-acting element. To confirm the regulation of *ChaP5CS* by *ChaWRKY40*, we conducted the Y1H assay. These results suggest that *ChaWRKY40* directly binds to the W-box of the *ChaP5CS* promoter sequence. Therefore, it is preliminarily inferred that *ChaWRKY40* may promote proline synthesis by regulating the expression of *ChaP5CS*.

5. Conclusions

Based on these results, we propose a possible functional working model for *ChaWRKY40* that confers drought stress tolerance. In conclusion, *ChaWRKY40* expression is induced under severe drought stress. *ChaWRKY40* activates the expression of *ChaP5CS* and promotes proline accumulation, thus improving drought tolerance of hybrid hazelnuts. From another perspective, *ChaWRKY40* may improve the drought tolerance of hybrid hazelnuts by regulating other stress-responsive genes related to SOD, POD, soluble sugars, and soluble proteins(Figure 8).

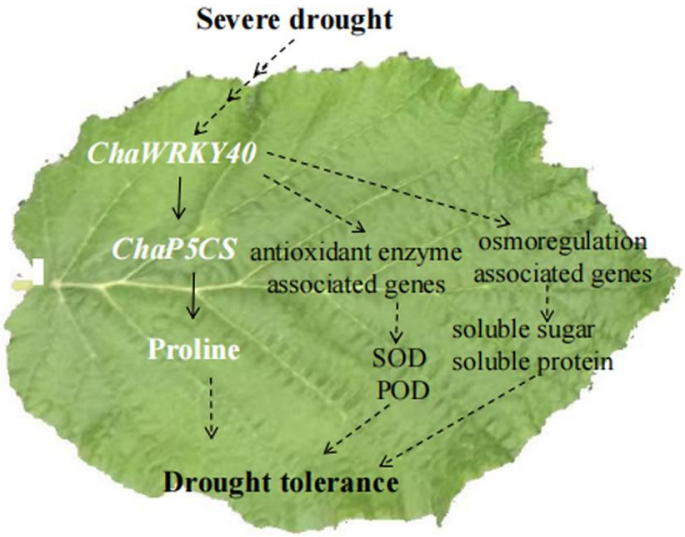

Figure 8. Proposed model for *ChaWRKY40* function under drought stress in hybrid hazelnut. *ChaWRKY40* is induced under severe drought stress. *ChaWRKY40* directly binds to elements in the *ChaP5CS* promoter and increases the accumulation of proline to improve the drought tolerance of hybrid hazelnut. *ChaWRKY40* may enhance drought tolerance by regulating the expression of osmoregulation and antioxidant enzyme-associated genes.

Author Contributions: Conceptualization, P.W. and R.C.; methodology, P.Z. and R.C.; software, R.C. and L.Q.; validation, L.Q. and W.G.; formal analysis, R.C.; investigation, J.L. and R.C.; resources, P.W. and J.L.; data curation, L.Q.; writing—original draft preparation, P.W. and R.C.; writing—review and editing, P.W.; visualization, J.L.; supervision, P.W. and J.L.; project administration, P.W. and P.Z.; funding acquisition, P.W. and P.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Shanxi Agricultural University biological breeding project "hazelnut germplasm resources collection and preservation and new variety breeding" Project No. (YZGC 111).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are available in this article.

Acknowledgments: We would like to thank Zhen Yang from the Chinese Academy of Forestry Sciences for the CDS and promoter sequence of *ChaWRKY40 and ChaP5CS* which were provided for our research.

Conflicts of Interest: The authors declare that no competing interests exist.

Appendix A

Table A1. Primers used in this study.

Primer Name	Primer Sequence (5′–3′)	Tm (°C)	Product Length (bp)
qRT-*ChaWRKY40*-F qRT-*ChaWRKY40*-R	TTCTTCAAGCCCATCTCC GTCTTCCCGACACCTTTG	54	212
qRT-*ChaP5CS*-F qRT-*ChaP5CS*-R	CCCAGAGGCAGCAATAAAC AACAGTGCAAGCCAACGAA	56	281
G-*ChaWRKY40*-F G-*ChaWRKY40*-R	GC*TCTAGA*ATGGAAGCAGCTCATGCCT GG*GGTACC*TTAGGCATGCGTGGAGGAG	62	578
Y1H-*ChaWRKY40*-F Y1H-*ChaWRKY40*-R	CG*GAATTC*ATGGAAGCAGCTCATGCCT CG*GGATCC*TTAGGCATGCGTGGAGGA	60	578
Y1H-*ChaP5CS*-F Y1H-*ChaP5CS*-R	C*GAGCTC*GCTTTCTACAACATTCTAATTTCTA GG*GGTACC*CCCCACACTACTTTTTTCTTATTC	59	180
ChaWRKY40-F *ChaWRKY40*-R	ATGGAAGCAGCTCATGCCT TTAGGCATGCGTGGAGGAG	57	578

Table A2. Water potential of PEG-6000 at different concentrations.

$C_{PEG\text{-}6000}$ ($g \cdot L^{-1}$)	Ψ_{PEG} (bar)
0	0
50	−0.47
150	−3.02
250	−7.73

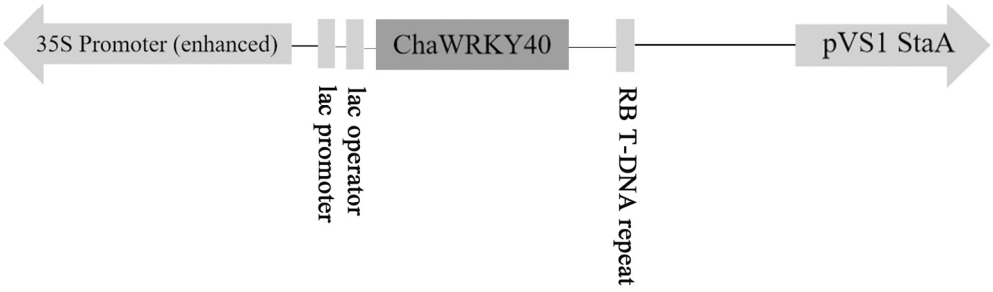

Figure A1. pCAMBIA1300-*ChaWRKY40* carrier construction diagram.

References

1. Ma, Q.; Wang, G.; Liang, W.; Liang, L.; Zhao, T. The research progresses, germplasm utilization and breeding innovation of *Corylus* (hazelnuts) in China: A review. *J. Fruit Sci.* **2013**, *30*, 159–164.
2. Köksal, A.İ.; Artik, N.; Şimşek, A.; Güneş, N. Nutrient composition of hazelnut (*Corylus avellana* L.) varieties cultivated in Turkey. *Food Chem.* **2006**, *99*, 509–515. [CrossRef]
3. Liu, G.; Lin, Y.; Zhang, L.; Feng, B.; Tang, G.; Ren, J.; Wang, Z. Evaluation on nut economic traits of cold resistant *Corylus heterophylla* × *C. avellana*. *J. Northeast. For. Univ.* **2018**, *46*, 36–39.
4. Li, C.; Dong, F.; Wang, G.; Zhang, R.; Liang, L. Study on the tolerance and critical water capacity of shoot shriveling in hybrid hazelnuts. *J. For. Res.* **2010**, *23*, 330–335.
5. Xue, J. *Physiological Mechanism of Shoot Shriveling Resistance in Excellent Lines of Interspecific Hybrid F1 between Corylus heterophylla Fisch. and Corylus avellana L.*; Shanxi Agricultural University: Jinzhong, China, 2015.
6. Wang, F.; Zhang, B.; Chang, B.; Shi, X.; Shi, Y.; Liu, Y.; Ji, L. Study on shoot shrivelling sensitive period of Ping'ou hybrid hazelnut in Taigu Shanxi. *China Fruits* **2022**, *4*, 34–39.
7. Felagari, K.; Bahramnejad, B.; Siosemardeh, A.; Mirzaei, K.; Lei, X.; Zhang, J. A comparison of the physiological traits and gene expression of brassinosteroids signaling under drought conditions in two chickpea cultivars. *Agronomy* **2023**, *13*, 2963. [CrossRef]
8. Ma, Y.; Ma, R.; Cao, Z.; Li, Y. Effects of PEG stress on physiological characteristics of the lespedeza seedlings leaves. *J. Desert Res.* **2012**, *32*, 1662–1668.
9. Li, Z.; Xu, X.; Jiao, J.; Ling, F.; Li, C. Physiological responses and mechanism of drought resistance in leaves of different olive varieties under osmotic stress. *Acta Bot. Boreali Occident. Sin.* **2014**, *34*, 1808–1814.
10. Ghosh, U.K.; Islam, M.N.; Siddiqui, M.N.; Cao, X.; Khan, M.A.R. Proline, a multifaceted signalling molecule in plant responses to abiotic stress: Understanding the physiological mechanisms. *Plant. Biol.* **2022**, *24*, 227–239. [CrossRef]
11. Yang, S.; Zhu, D.; Ren, Y.; Zhu, Y. Change of leaf membrane permeability and some osmotic regulation substances of 3 poplar varieties under drought stress. *Acta Agric. Shanghai* **2016**, *32*, 118–123.
12. Liang, Q.; Han, Y.; Qiao, Y.; Xie, K.; Li, S.; Dong, Y.; Li, S.; Zhang, S. Effects of drought stress on the growth and physiological characteristics of Sect. *Aigeiors* clones. *J. Beijing For. Univ.* **2023**, *45*, 81–89.
13. Ren, R. *Expression Characteristics of the Proline Synthesis Key Gene P5CS1 in Poplar and Functional Verification of Drought Tolerance*; Northwest A&F University: Xianyang, China, 2022.
14. Shang, T. *P5CS1 Cloning and Identification and the Analyze of Proline Metabolic Genes Expression in Citrus*; Huazhong Agricultural University: Wuhan, China, 2020.
15. Yamada, M.; Morishita, H.; Urano, K.; Shiozaki, N.; Yamaguchi-Shinozaki, K.; Shinozaki, K.; Yoshiba, Y. Effects of free proline accumulation in petunias under drought stress. *J. Exp. Bot.* **2005**, *56*, 1975–1981. [CrossRef] [PubMed]
16. Feng, X.; Hu, Y.; Zhang, W.; Xie, R.; Guan, H.; Xiong, H.; Jia, L.; Zhang, X.; Zhou, H.; Zheng, D. Revisiting the role of delta-1-pyrroline-5-carboxylate synthetase in drought-tolerant crop breeding. *Crop J.* **2022**, *10*, 1213–1218. [CrossRef]
17. Zhang, Y.; Yang, X.; Nvsvrot, T.; Huang, L.; Cai, G.; Ding, Y.; Ren, W.; Wang, N. The transcription factor WRKY75 regulates the development of adventitious roots, lateral buds and callus by modulating hydrogen peroxide content in poplar. *J. Exp. Bot.* **2022**, *73*, 1483–1498. [CrossRef] [PubMed]
18. Liu, H.; Gao, J.; Sun, J.; Li, S.; Zhang, B.; Wang, Z.; Zhou, C.; Sulis, D.B.; Wang, J.P.; Chiang, V.L. Dimerization of PtrMYB074 and PtrWRKY19 mediates transcriptional activation of PtrbHLH186 for secondary xylem development in *Populus trichocarpa*. *New Phytol.* **2022**, *234*, 918–933. [CrossRef] [PubMed]
19. Yuan, Y.; Ren, S.; Liu, X.; Su, L.; Wu, Y.; Zhang, W.; Li, Y.; Jiang, Y.; Wang, H.; Fu, R.; et al. SlWRKY35 positively regulates carotenoid biosynthesis by activating the MEP pathway in tomato fruit. *New Phytol.* **2022**, *234*, 164–178. [CrossRef] [PubMed]
20. Li, C.; Wu, J.; Hu, K.; Wei, S.; Sun, H.; Hu, L.; Han, Z.; Yao, G.; Zhang, H. PyWRKY26 and PybHLH3 cotargeted the PyMYB114 promoter to regulate anthocyanin biosynthesis and transport in red-skinned pears. *Hort. Res.* **2020**, *7*, 37. [CrossRef]
21. Jiang, Y.; Tong, S.; Chen, N.; Liu, B.; Bai, Q.; Chen, Y.; Bi, H.; Zhang, Z.; Lou, S.; Tang, H.; et al. The PalWRKY77 transcription factor negatively regulates salt tolerance and abscisic acid signaling in Populus. *Plant J.* **2021**, *105*, 1258–1273. [CrossRef]
22. Dong, Q.; Tian, Y.; Zhang, X.; Duan, D.; Zhang, H.; Yang, K.; Jia, P.; Luan, H.; Guo, S.; Qi, G.; et al. Overexpression of the transcription factor MdWRKY115 improves drought and osmotic stress tolerance by directly binding to the MdRD22 promoter in apple. *Hortic. Plant J.* **2023**, *in press*. [CrossRef]
23. Zhou, R.; Dong, Y.; Liu, X.; Feng, S.; Wang, C.; Ma, X.; Liu, J.; Liang, Q.; Bao, Y.; Xu, S.; et al. JrWRKY21 interacts with JrPTI5L to activate the expression of JrPR5L for resistance to Colletotrichum gloeosporioides in walnut. *Plant J.* **2022**, *111*, 1152–1166. [CrossRef]
24. Yang, Z.; Wang, F.; Wang, J. Sequence and expression pattern analysis of potato StWRKY40 gene. *Mol. Breed.* **2020**, *18*, 7283–7292.
25. Dai, W.; Wang, M.; Gong, X.; Liu, J. The transcription factor FcWRKY40 of *Fortunella crassifolia* functions positively in salt tolerance through modulation of ion homeostasis and proline biosynthesis by directly regulating *SOS2* and *P5CS1* homologs. *New Phytol.* **2018**, *219*, 972–989. [CrossRef] [PubMed]
26. Wang, C.; Ru, J.; Liu, Y.; Yang, J.; Li, M.; Xu, Z.; Fu, J. The maize WRKY transcription factor ZmWRKY40 confers drought resistance in transgenic *Arabidopsis*. *Int. J. Mol. Sci.* **2018**, *19*, 2580. [CrossRef]
27. Liu, Y.; He, L.; Zhao, X.; Liang, N.; Li, X.; Liang, X.; Zhan, Y. Cloning and expression analysis of FmWRKY40 gene in *Fraxinus mandshurica*. *Mol. Breed.* **2017**, *15*, 833–838.

28. Huang, Z.; Wang, J.; Li, Y.; Song, L.; Chen, D.; Liu, L.; Jiang, C. A WRKY protein, MfWRKY40, of resurrection plant *Myrothamnus flabellifolia* plays a positive role in regulating tolerance to drought and salinity stresses of Arabidopsis. *Int. J. Mol. Sci.* **2022**, *23*, 8145. [CrossRef] [PubMed]
29. Liang, C.; Yang, B.; Wei, Y.; Zhang, P.; Wen, P. SA incubation induced accumulation of flavan-3-ols through activated VvANR expression in grape leaves. *Sci. Hortic.* **2021**, *287*, 110296–110396. [CrossRef]
30. Lei, H.; Su, S.; Ma, L.; Ma, Z. Cloning and functional analysis of ChaCBF1, a CBF/DREB1-like transcriptional factor from Corylus heterophylla × C. avellana. *J. Beijing For. Univ.* **2016**, *38*, 69–79.
31. Ding, K.; Wang, L.; Tian, G.; Wang, H.; Li, F.; Pan, Y.; Pang, Z.; Shan, Y. Effect of exogenous uniconazole on antioxidant capacity and osmotic adjustment of potato leaves under drought stress. *J. Nucl. Agric. Sci.* **2024**, *38*, 169–178.
32. Zhang, L.; Fan, J.; Ruan, Y.; Guan, Y. Application of polyethylene glycol in the study of plant osmotic stress physiology. *Plant Physiol. Comm.* **2004**, *40*, 361–364.
33. Michel, B.; Wiggins, O.; Outlaw, W. A guide to establishing water potential of aqueous two-phase solutions (polyethylene glycol plus dextran) by amendment with mannitol. *Plant Physiol.* **1983**, *72*, 60–65. [CrossRef]
34. Gan, Z.; Yuan, X.; Shan, N.; Wan, C.; Chen, C.; Xu, Y.; Xu, Q.; Chen, J. AcWRKY40 mediates ethylene biosynthesis during postharvest ripening in kiwifruit. *Plant Sci.* **2021**, *309*, 110948. [CrossRef] [PubMed]
35. Li, Y.; Zhang, Y.; Li, C.; Chen, X.; Yang, L.; Zhang, J.; Wang, J.; Li, L.; Reynolds, M.P.; Jing, R.; et al. Transcription factor TaWRKY51 is a positive regulator in root architecture and grain yield contributing traits. *Front. Plant Sci.* **2021**, *12*, 734614. [CrossRef] [PubMed]
36. Zhang, Z.; Jiang, C.; Chen, C.; Su, K.; Lin, H.; Zhao, Y.; Guo, Y. VvWRKY5 enhances white rot resistance in grape by promoting the jasmonic acid pathway. *Hortic. Res.* **2023**, *10*, uhad172. [CrossRef]
37. Xu, H.; Yang, G.; Zhang, J.; Zou, Q.; Wang, Y.; Qu, C.; Jiang, S.; Wang, N.; Chen, X. Molecular mechanism of apple MdWRKY18 and MdWRKY40 participating in salt stress. *Sci. Agric. Sin.* **2018**, *51*, 4514–4521.
38. Zhang, G.; Liang, C.; Guo, J.; Zhang, Z.; Zhang, P.; Zhao, Q.; Liang, J.; Wen, P. Clone and expression analysis of VvWRKY26 gene in grape (*Vitis vinifera*). *J. Agric. Biotechnol.* **2023**, *31*, 475–487.
39. Xiao, J. Physiological and biochemical influences of different drought stress on *Robinia pseudoacacia* seedlings. *J. Cent. South Univ. For. Techno.* **2015**, *35*, 23–26.
40. Li, X.; Hua, Z. Study on osmotic regulation ability and cross relationship of *Scutellaria baicalensis* under temperature and water stress. *J. Agric. Sci.* **2023**, *51*, 162–168.
41. Chen, T.; Xu, G.; Liu, S.; Mi, X.; Li, Y. Leaf water status and non-structural carbohydrate dynamics with different crown height levels of *Populus bolleana* Lauche. under drought stress. *Acta Bot. Boreali Occident. Sin.* **2022**, *42*, 462–472.
42. Xu, Y.; Hu, W.; Song, S.; Ye, X.; Ding, Z.; Liu, J.; Wang, Z.; Li, J.; Hou, X.; Xu, B.; et al. MaDREB1F confers cold and drought stress resistance through common regulation of hormone synthesis and protectant metabolite contents in banana. *Hortic. Res.* **2023**, *10*, uhac275. [CrossRef]
43. Li, J. *Analysis of Drought Resistance Function of Sugarcane Δ^1-pyrroline-5-carboxylate synthase Gene (SoP5CS)*; Guangxi University: Nanning, China, 2018.
44. Yang, D.; Ni, R.; Yang, S.; Pu, Y.; Qian, M.; Yang, Y.; Yang, Y. Functional characterization of the Stipa purpurea P5CS gene under drought stress conditions. *Int. J. Mol. Sci.* **2021**, *22*, 9599. [CrossRef]
45. Ren, R.; Wang, H.; Wu, C.; Heng, Q.; Chen, W.; Sun, T.; Zhang, L.; He, H.; Li, X.; Zhang, Y.; et al. Full-length cloning and functional berification of PagP5CS1 from *Populus alba* × *P. glandulosa*. *J. Northeast. For. Univ.* **2023**, *38*, 90–96.
46. Li, K.; Gao, Y.; Wu, J. Study on salt tolerance and drought resistance of potato transgenic P5CS gene 'Dongnong 303'. *Jiangsu Agric. Sci.* **2014**, *42*, 131–133.
47. Liu, Y.; Yang, T.; Lin, Z.; Gu, B.; Xing, C.; Zhao, L.; Dong, H.; Gao, J.; Xie, Z.; Zhang, S.; et al. A WRKY transcription factor PbrWRKY53 from *Pyrus betulaefolia* is involved in drought tolerance and AsA accumulation. *Plant Biotechnol. J.* **2019**, *17*, 1770–1787. [CrossRef] [PubMed]
48. Wang, Y.; Zhang, Y.; Guo, W.; Dai, W.; Zhou, X.; Jiao, Y.; Shen, X. Cloning and function of GsWRKY57 transcription factor gene response to drought stress. *Chin. J. Oil Crop Sci.* **2019**, *41*, 524–530.
49. Zhang, L.; Cheng, J.; Sun, X.; Zhao, T.; Li, M.; Wang, Q.; Li, S.; Xin, H. Overexpression of VaWRKY14 increases drought tolerance in Arabidopsis by modulating the expression of stress-related genes. *Plant Cell Rep.* **2018**, *37*, 1159–1172. [CrossRef] [PubMed]
50. Zhang, L.; Zhang, R.; Ye, X.; Zheng, X.; Tan, B.; Wang, W.; Li, Z.; Li, J.; Cheng, J.; Feng, J. Overexpressing VvWRKY18 from grapevine reduces the drought tolerance in Arabidopsis by increasing leaf stomatal density. *J. Plant Physiol.* **2022**, *275*, 153741. [CrossRef] [PubMed]
51. Wang, D.; Chen, Q.; Chen, W.; Liu, X.; Xia, Y.; Guo, Q.; Jing, D.; Liang, G. A WRKY transcription factor, EjWRKY17, from *Eriobotrya japonica* enhances drought tolerance in transgenic Arabidopsis. *Int. J. Mol. Sci.* **2021**, *22*, 5593. [CrossRef] [PubMed]
52. Liang, Y.; Ma, F.; Li, B.; Guo, C.; Hu, T.; Zhang, M.; Liang, Y.; Zhu, J.; Zhan, X. A bHLH transcription factor, SlbHLH96, promotes drought tolerance in tomato. *Hortic. Res.* **2022**, *9*, uhac198. [CrossRef]
53. Han, D.; Zhang, Z.; Ding, H.; Chai, L.; Liu, W.; Li, H.; Yang, G. Isolation and characterization of MbWRKY2 gene involved in enhanced drought tolerance in transgenic tobacco. *J. Plant Interact.* **2018**, *13*, 163–172. [CrossRef]
54. Duan, D.; Yi, R.; Ma, Y.; Dong, Q.; Mao, K.; Ma, F. Apple WRKY transcription factor MdWRKY56 positively modulates drought stress tolerance. *Environ. Exp. Bot.* **2023**, *212*, 105400. [CrossRef]

55. Wang, Z.; Feng, R.; Zhang, X.; Su, Z.; Wei, J.; Liu, J. Characterization of the *Hippophae rhamnoides* WRKY gene family and functional analysis of the role of the HrWRKY21 gene in resistance to abiotic stresses. *Genome* **2019**, *62*, 689–703. [CrossRef] [PubMed]
56. Gao, H.; Wang, Y.; Xu, P.; Zhang, Z. Overexpression of a WRKY transcription factor TaWRKY2 enhances drought stress tolerance in transgenic wheat. *Front. Plant Sci.* **2018**, *9*, 997. [CrossRef] [PubMed]
57. El-Esawi, M.A.; Al-Ghamdi, A.A.; Ali, H.M.; Ahmad, M. Overexpression of AtWRKY30 transcription factor enhances heat and drought stress tolerance in wheat (*Triticum aestivum* L.). *Genes* **2019**, *10*, 163. [CrossRef] [PubMed]
58. Wang, J.; Chen, G.; Zhang, C. The effects of water stress on soluble protein content, the activity of SOD, POD and CAT of two ecotypes of reeds (*Phragmites communis*). *Acta Bot. Boreali Occident. Sin.* **2002**, *22*, 561–565.
59. Jaffar, M.A.; Song, A.; Faheem, M.; Chen, S.; Jiang, J.; Liu, C.; Fan, Q.; Chen, F. Involvement of CmWRKY10 in drought tolerance of chrysanthemum through the ABA-signaling pathway. *Int. J. Mol. Sci.* **2016**, *17*, 693. [CrossRef]
60. Lin, L.; Yuan, K.; Huang, Y.; Dong, H.; Qiao, Q.; Xing, C.; Huang, X.; Zhang, S. A WRKY transcription factor PbWRKY40 from *Pyrus betulaefolia* functions positively in salt tolerance and modulating organic acid accumulation by regulating PbVHA-B1 expression. *Environ. Exp. Bot.* **2022**, *196*, 104782. [CrossRef]
61. Han, D.; Han, J.; Xu, T.; Li, T.; Yao, C.; Wang, Y.; Luo, D.; Yang, G. Isolation and preliminary functional characterization of MxWRKY64, a new WRKY transcription factor gene from *Malus xiaojinensis* Cheng et Jiang. *In Vitro Cell. Dev. Plant* **2021**, *57*, 202–213. [CrossRef]
62. Shan, D.; Wang, C.; Song, H.; Bai, Y.; Zhang, H.; Hu, Z.; Wang, L.; Shi, K.; Zheng, X.; Yan, T.; et al. The MdMEK2–MdMPK6–MdWRKY17 pathway stabilizes chlorophyll levels by directly regulating MdSUFB in apple under drought stress. *Plant J.* **2021**, *108*, 814–828. [CrossRef]
63. Zhang, W.; Zhao, S.; Gu, S.; Cao, X.; Zhang, Y.; Niu, J.; Liu, L.; Li, A.; Jia, W.; Qi, B.; et al. FvWRKY48 binds to the pectate lyase FvPLA promoter to control fruit softening in *Fragaria vesca*. *Plant Physiol.* **2022**, *189*, 1037–1049. [CrossRef]
64. Chen, Y.; Liu, L.; Feng, Q.; Liu, C.; Bao, Y.; Zhang, N.; Sun, R.; Yin, Z.; Zhong, C.; Wang, Y.; et al. *FvWRKY50* is an important gene that regulates both vegetative growth and reproductive growth in strawberry. *Hortic. Res.* **2023**, *10*, uhad115. [CrossRef]
65. Cong, L.; Qu, Y.; Sha, G.; Zhang, S.; Ma, Y.; Chen, M.; Zhai, R.; Yang, C.; Xu, L.; Wang, Z. *PbWRKY75* promotes anthocyanin synthesis by activating *PbDFR*, *PbUFGT*, and *PbMYB10b* in pear. *Physiol. Plantarum.* **2021**, *173*, 1841–1849. [CrossRef]
66. Wang, J.; Wang, L.; Yan, Y.; Zhang, S.; Li, H.; Gao, Z.; Wang, C.; Guo, X. *GhWRKY21* regulates ABA-mediated drought tolerance by fine-tuning the expression of *GhHAB* in cotton. *Plant Cell Rep.* **2021**, *40*, 2135–2150. [CrossRef] [PubMed]