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A B-Box Transcription Factor CoBBX24 from *Camellia oleifera* Delays Leaf Senescence and Enhances Drought Tolerance in *Arabidopsis*

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Abstract: Plants face various biotic and abiotic stress factors during their growth and development, among which, drought is a serious adverse factor that affects yield and quality in agriculture and forestry. Several transcription factors are involved in regulating plant responses to drought stress. In this study, the B-box (BBX) transcription factor *CoBBX24* was cloned from *Camellia oleifera*. This gene encodes a 241-amino-acid polypeptide containing two B-box domains at the N-terminus. A phylogenetic analysis revealed that CoBBX24 and CsBBX24 from *Camellia sinensis* are in the same branch, with their amino acid sequences being identical by 96.96%. CoBBX24 was localized to the nucleus and acted as a transcriptional activator. The overexpression of *CoBBX24* in *Arabidopsis* heightened its drought tolerance along with a relatively high survival rate, and the rate of water loss in the OX-*CoBBX24* lines was observably lower than that of the wild-type. Compared to the wild-type, the root lengths of the OX-*CoBBX24* lines were significantly inhibited with abscisic acid. Leaf senescence was delayed in the OX-*CoBBX24* lines treated with abscisic acid. The expression of genes related to leaf senescence and chlorophyll breakdown (e.g., *SAG12*, *SAG29*, *NYC1*, *NYE1*, and *NYE2*) was downregulated in the OX-*CoBBX24* lines. This study indicated that *CoBBX24* positively regulates the drought tolerance in *Arabidopsis* through delayed leaf senescence.

Keywords: *Camellia oleifera*; *CoBBX24*; drought; abscisic acid; chlorophyll degradation

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1. Introduction

Leaves are the main photosynthetic organs for energy production and nutrient assimilation during plant growth and development [1]. Leaf senescence is the last developmental stage of plant growth and is accompanied by the yellowing of leaves owing to a loss of chlorophyll [2]. In addition to genetic factors, leaf senescence is regulated by environmental factors and phytohormones. For example, darkness, drought, salt stress, and high temperatures can induce leaf senescence [3–6]. Phytohormones, such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and ethylene, are positive regulators of leaf senescence induction [2,7–9]. ABA plays a positive role in leaf senescence, with exogenous ABA promoting it [2]. Senescence-associated genes (SAGs) are upregulated during ABA-induced senescence [10]. ABF4, an ABA-responsive element binding factor (ABF), induces *SAG29* expression by directly binding to its promoter [11]. The expression of *ABF3/4* increases in senescing leaves and induces the expression of *SAG12* and *SAG29* [11–13]. SAG29 is a key molecule involved in environmental stress responses during senescence [14]. NON-YELLOWING (NYE), an Mg-dechelatase, catabolizes the first step of chlorophyll *a* breakdown [15]. The functional loss of *NYE1* and *NYE2* results in an almost complete retention of chlorophyll during leaf senescence and produces green seeds in *Arabidopsis* [16]. NON-YELLOW COLORING 1 (NYC1) is a chlorophyll *b* reductase that

mediates the breakdown of chlorophyll *b* [17]. The transcript and protein levels of NYC1 are low in green leaves, but increase during darkness-induced senescence [18].

Plants develop complex drought mechanisms by interacting with the external environment to respond to drought stress. Plant responses to drought stress comprise morphological, physiological, and biochemical mechanisms [19,20]. After sensing water-scarcity signals, plants induce the expression of drought-regulated and functional genes [21]. These genes include transcription and signaling factors and stress-inducing, protein-related, osmoregulatory, and antioxidant metabolism-related genes [20,22]. Many transcription factors are related to drought tolerance in plants, including the bZIP, DREB, ABF, MYC, MYB, NAC, WRKY, ERF, bHLH, and BBX proteins [22,23]. The mechanism of BBX family involvement in the drought response of plants has received much attention in recent years.

BBX proteins are a kind of zinc-finger transcription factor comprising one or two B-box domains at the N-terminus; some members have a CONSTANS, CO-like, and TOC1 (CCT) domain at the C-terminus [24,25]. BBX proteins are key regulators of plant growth and developmental processes, involved in seedling morphogenesis, the photoperiodic regulation of flower formation, shading response, anthocyanin accumulation, and responses to biological and abiotic stresses [7,26–29]. Several BBX transcription factors play critical roles in the drought stress response of plants [30,31]. The rice BBX gene, *Ghd2*, confers drought sensitivity by upregulating senescence-associated genes (SAGs) in transgenic lines [32]. The heterologous expression of sweet potato *IbBBX28* in *Arabidopsis* decreases the activities of the antioxidant enzymes, such as superoxide dismutase, peroxidase, and catalase, in *IbBBX28*-OX lines, indicating that *IbBBX28* negatively regulates drought tolerance [30]. *CmBBX22* mediates the ABA response to regulate the drought tolerance in chrysanthemum [33]. BBX plays a pivotal role in chlorophyll breakdown and leaf senescence [34,35]. In apples, *MdBBX22* negatively regulates chlorophyll breakdown and leaf senescence by downregulating the transcript levels of *MdNYE1* and *MdNYC1* [34]. MdBBX37 interacts with MdbHLH93 to promote the expression of *MdSAG18* and subsequently induce leaf senescence [35].

Previous reports have shown that AtBBX24 negatively regulates the photomorphogenesis of the UV-B responses in *Arabidopsis* through interacting with COP1 and HY5 [36]. In addition, BBX24 plays an important role in abiotic stress. It has been reported that AtBBX24 enhances drought and salinity tolerance through ABA signaling in *Arabidopsis* seeds [37]. The chrysanthemum *CmBBX24* confers drought and freezing tolerance by influencing the genes that mediate stress responses and GA biosynthesis [38]. In conclusion, the findings of these studies suggest that *BBX* genes are involved in regulating the abiotic stress responses in plants, especially the response to drought.

Oil tea tree (*Camellia oleifera* Abel.), a woody oil tree, is an ornamental and edible plant. They are mainly planted in hilly areas, and their growing environment limits their access to water. As a result, studies on the drought tolerance of oil tea tree and the identification of drought-tolerant genes may help in generating new drought-tolerant species, which have theoretical and practical significance for promoting the development of agriculture and forestry. In this study, we cloned a *BBX* gene that responds to drought stress and found that the ectopic expression of *CoBBX24* enhanced the drought tolerance in *Arabidopsis* by regulating the expression of SAGs and chlorophyll catabolic genes (CCGs).

2. Materials and Methods

2.1. Plant Materials and Treatments

In this study, two-year-old oil tea trees (*C. oleifera* Abel. cv. 'changlin 4') were used. Uniform plants were selected, and the third fully expanded leaf from the top was collected and stored at −80 °C after treatment with liquid nitrogen. For the drought treatment, plants in soil were watered well, followed by the withdrawal of water for 15 d. The control group was cultured normally.

Col-0 wild-type (WT) ecotype *Arabidopsis* was used for genetic transformation. *Arabidopsis* seeds were sown in 1/2 MS medium, imbibed at 4 °C for three days in the dark,

and then cultured at 22/18 °C for 10 d within a light incubator (day/night, 14/10 h). The seedlings were transferred to a planter (peat soil:vermiculite, 1:2) and grown for two weeks. Water was provided before the drought treatment, and then we stopped watering the four-week-old plants for the subsequent 15 d. After re-watering for seven days, the survival rate of the plants was determined. To detect the water loss in the OX-*CoBBX24* transgenic lines and WT plants, whole rosettes of 21-day-old plants were cut from the base and placed at room temperature to make them lose water naturally, then weighed at 0, 2, 4, 6, 8, 10, and 12 h to calculate the percentage of their weight reduction in 0 h. The experiment was repeated three times, with each replicate consisting of ten plants per genotype.

2.2. RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The total RNA was extracted from the *Arabidopsis* or oil tea tree tissues using a StarSpin Plant RNA Kit (GenStar, Beijing, China) and reverse-transcribed using HiScript III RT SuperMix (Vazyme, Nanjing, China). qPCR was used to analyze the expression of genes using an SYBR qPCR Master Mix (Vazyme, Nanjing, China). *Actin2* and *Tub3α* were served as the reference genes for normalization in the *Arabidopsis* and oil tea trees, severally. The reaction mixture contained 1 μL of cDNA, 10 μL of 2× ChamQ Universal SYBR qPCR master mix, and 4 pmol of each primer. Fold changes in expression were computed using the $2^{-\Delta\Delta Ct}$ method [39]. The experiment included three biological replicates. Each sample was run in three technical replicates with the following parameters: 95 °C for 3 min, 40 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. All the primers are listed in Supplemental Table S1.

2.3. Isolation and Sequence Analysis of CoBBX24

The open reading frame (ORF) of *CoBBX24* from the oil tea trees was cloned into a pMD19-T (TaKaRa, Tokyo, Japan) vector using PCR with the primers *CoBBX24*-ORF-F/R (Table S1). The *CoBBX24* sequence was aligned with nine homologous sequences from other species by the means of the DNAMAN version 6.0 software, and a phylogenetic tree was constructed using the MEGA 5 software based on the neighbor-joining method and 1000 bootstrap replicates.

2.4. Subcellular Localization Analysis of CoBBX24

The ORF of *CoBBX24* was amplified using primers containing *Eco*RI and *Not*I sites. The amplicons and pORE-R4-35SAA vector were digested using *Eco*RI and *Not*I, and the products were ligated using a T4 DNA ligase (TaKaRa, Tokyo, Japan) to generate pORE-R4-35SAA-*CoBBX24* fusions [40]. The p35S::D53-RFP construct, as a nuclear marker, was co-transformed with pORE-R4-35SAA-*CoBBX24* (35S::GFP-CoBBX24) [41]. *Agrobacterium tumefaciens* strain GV3101 carrying these constructs was injected into *Nicotiana benthamiana* leaves for transient expression. The infiltrated leaves were observed after 72 h using a ZEISS LSM 780 microsystem (ZEISS, Oberkochen, Germany).

2.5. Transactivation Activity Analysis of CoBBX24

To examine the transcriptional activity of CoBBX24, the ORF of *CoBBX24* was amplified using primers containing *Eco*RI and *Bam*HI sites. Both amplicons and the pGBKT7 vector were digested using *Eco*RI and *Bam*HI, and the products were ligated using a T4 DNA ligase (TaKaRa, Tokyo, Japan) to generate pGBKT7-*CoBBX24* fusions. pCL1 and pGBKT7 were used as positive and negative controls, respectively (pCL1 is a positive-control plasmid that encodes the full-length, wild-type GAL4 protein). Plasmids were introduced into the Y2H Gold strain (Coolaber, Beijing, China). The experimental method was according to Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual. pGBKT7-*CoBBX24* or pGBKT7 transformants were cultivated on the medium lack of Tryptophan (Trp) amino acid, whereas pCL1 was cultivated on the medium lack of Leucine (Leu) amino acid for three days at 30 °C. The selected clones were then transferred to the medium lack of

Histidine (His) and Adenine (Ade), with or without X-α-Gal, to observe the cell growth and blue appearance.

2.6. Transformation of Arabidopsis

Arabidopsis transformants were generated by introducing pORE-R4-35SAA-*CoBBX24* into the Col-0 ecotype using the floral dip way. Transgenic plants were chosen via the germination of seeds on 1/2 MS medium containing 10 mg/L of kanamycin. The screened plants with true leaves were transplanted into soil, and the genome of the *Arabidopsis* leaves was extracted after two weeks of growth. The primers CoBBX24-F/R were used to analyze the transfer of *CoBBX24*. Semiquantitative PCR detected the expression of *CoBBX24* in the transformants of the T3 generation using the primers RT-CoBBX24-F/R.

2.7. ABA Treatment of Transgenic Arabidopsis

For the ABA treatments, *Arabidopsis* seeds were sown on 1/2 MS plates for four days and then transplanted to media containing 3 μM of ABA. Root length was surveyed after the sixth day. Detached leaves were taken from four-week-old plants and incubated in water or 20 mL of 100 μM ABA solution (Solarbio, Beijing, China) under dark conditions. After two days of treatment, the leaves were transferred to clean plates for photography. For the ABA treatment of attached leaves, four-week-old plants were sprayed on their leaves with 100 μM of ABA, and samples were collected after 24 h [11].

2.8. Determination of Chlorophyll Content

The leaves from four-week-old *Arabidopsis* plants in soil were detached and soaked in a 100 μM ABA solution for two days. The fresh weights of the samples were determined, and their chlorophyll was extracted with 95% ethanol for 48 h in the dark and measured spectrophotometrically at 663 and 645 nm [37]. The chlorophyll concentration was calculated as described previously [11].

2.9. Statistical Analysis

The data were analyzed using SPSS v.20 software (SPSS, Inc., Chicago, IL, USA). Student's *t*-test (* $p < 0.05$; ** $p < 0.01$) was used to decide striking differences in the results of the water loss assays in *Arabidopsis*, and Duncan's multiple-range test ($p < 0.05$) was used to analyze striking differences in the other results.

3. Results

3.1. Cloning and Sequence Analysis of CoBBX24

The BBX transcription factor gene, *CoBBX24*, isolated from oil tea trees, encodes a 241-amino-acid protein with a calculated molecular weight of 26.83 kDa. A phylogenetic analysis revealed that CoBBX24 was highly similar to CsBBX24 from *Camellia sinensis* (Figure 1A). Phylogenetic tree clustering showed that CoBBX24 was grouped with AtBBX24 in *Arabidopsis* (Figure S1). A BLASTP search showed that CoBBX24 was similar to the BBX proteins from several plant species and contained the characteristics of the structure group IV members of the BBX family. A sequence analysis demonstrated that CoBBX24 had two conserved B-box domains in the N-terminus, but no CCT domain in the C-terminus (Figure 1B).

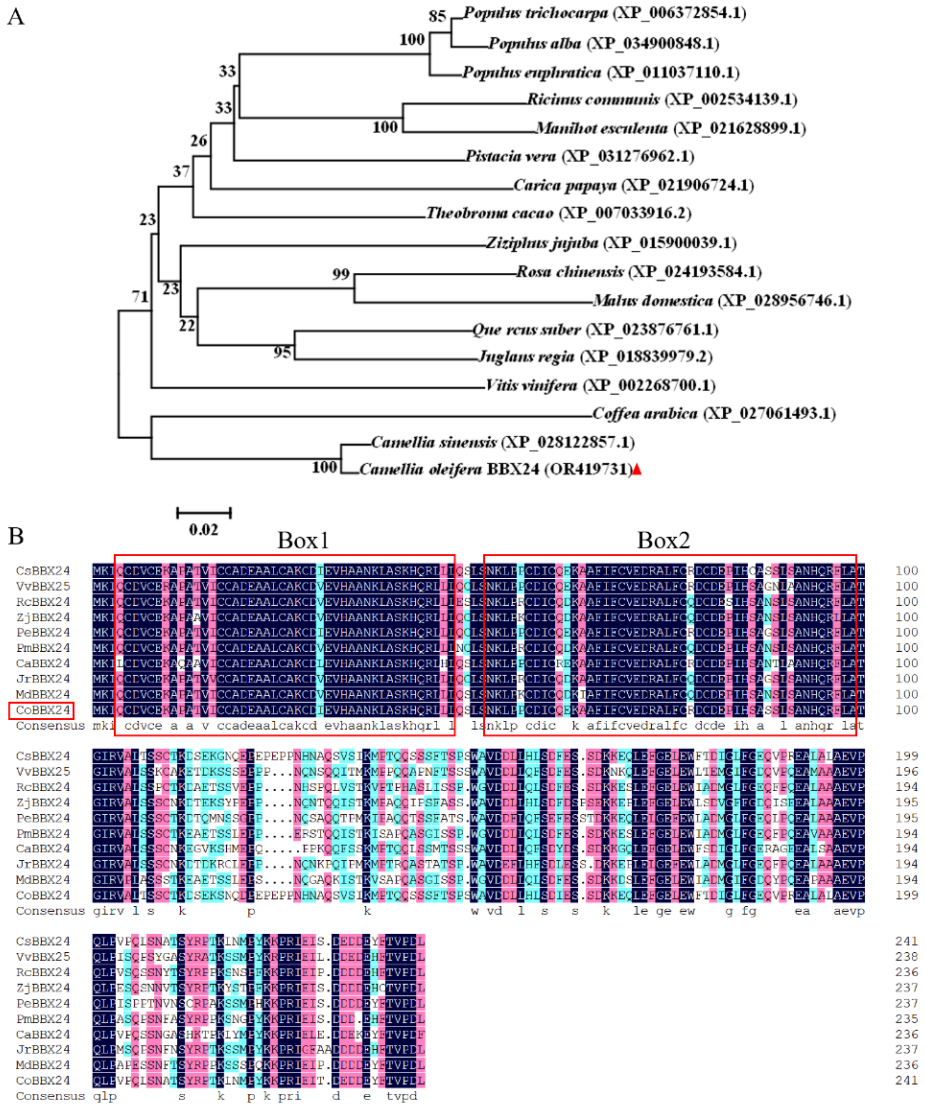

Figure 1. Phylogenetic characteristics and structural domains of CoBBX24. (**A**) Phylogenetic evaluation of CoBBX24 and other plant BBX proteins. The phylogenetic trees were derived using the neighbor-joining (NJ) method with a bootstrap value of 1000 replicates. Bootstrap values indicate the divergence of each branch, with the scale representing the branch length. Red triangle indicate the protein sequence from *Camellia oleifera*. (**B**) Alignment of the deduced polypeptide sequences of CoBBX22 with those of other plant BBXs. Red lines indicate the conserved B1 and B2 B-box domains. The accession numbers of the proteins are: CsBBX24 (XP_028122857.1), VvBBX25 (XP_028122857.1), RcBBX24 (XP_024193584.1), ZjBBX24 (XP_015900039.1), PeBBX24 (XP_015900039.1), PmBBX24 (XP_008222603.1), CaBBX24 (XP_027061493.1), JrBBX24 (XP_018839979.2), and MdBBX24 (XP_028956746.1).

3.2. Subcellular Localization, Transcriptional Activation, and Transcriptional Profiling of CoBBX24

We determined the fluorescence signals of the CoBBX24 fused with green fluorescent protein (35S::GFP-CoBBX24) following its transient expression in the leaves of *N. benthamiana*. The green fluorescent signals of the 35S::GFP-CoBBX24 fusion protein were detected in the nucleus and colocalized with the sites of the deposition of the red fluorescent signal of the positive nuclear marker protein (35S::D53-RFP) (Figure 2C). This indicated that CoBBX24 is localized in the nucleus, similar to other transcription factors.

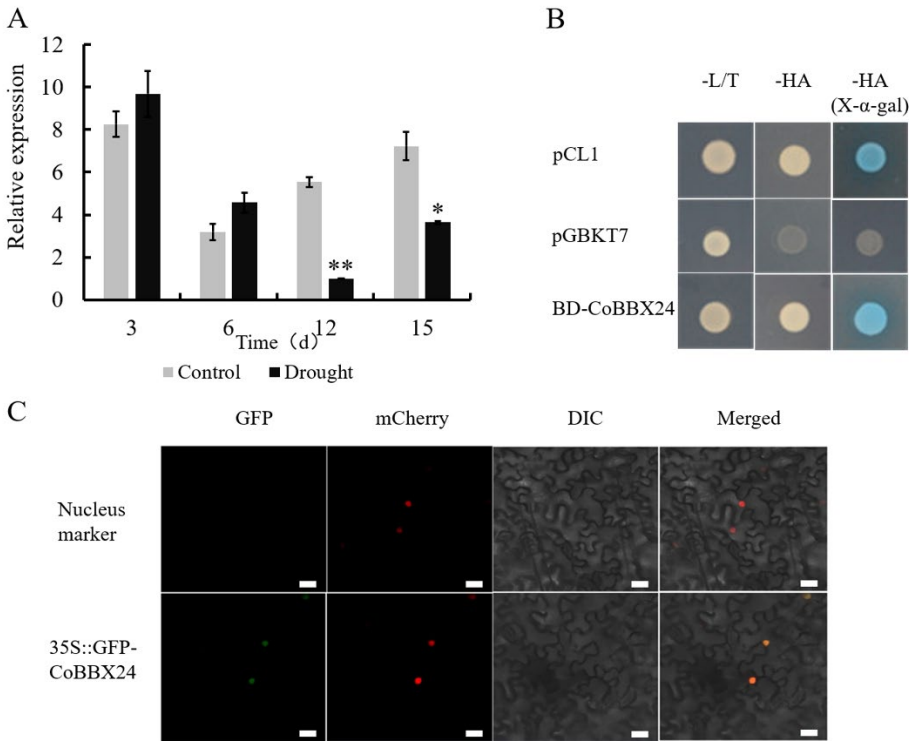

Figure 2. Transcriptional profiling of *CoBBX24* under drought stress, transactivation analysis, and subcellular localization of CoBBX24. (**A**) Relative expression of *CoBBX24* under drought. *Camellia oleifera Tub3α* was used as the reference gene for normalization. Error bars indicate the standard deviation (SD); n = 3. Significant differences were determined by Duncan's test (* $p < 0.05$, ** $p < 0.01$). (**B**) Transactivation activity analysis of the CoBBX24 protein in yeast cells. pCL1 served as a positive control, and pGBKT7 served as a negative control. (**C**) Subcellular localization of CoBBX24 in tobacco (*N. benthamiana*) cells. 35S::D53-RFP was used as a nuclear marker. Bars: 20 μm.

Next, we implemented a transactivation assay using yeast cells. Sequences encoding the ORF of *CoBBX24* were inserted into the expression vector pGBKT7, and the construct was transformed into the yeast Y2H Gold strain. The yeast cells harboring the positive control pCL1 and pGBKT7-CoBBX24 construct grew well on SD/-His-Ade medium and turned blue on the SD/-Ade-His medium supplemented with X-α-gal; however, the yeast cells containing the negative control pGBKT7 were unable to grow on the SD/-His-Ade medium. These findings imply that CoBBX24 acts as a transcriptional activator in yeast cells (Figure 2B).

qRT-PCR was performed to investigate the transcriptional profile of *CoBBX24* in oil tea trees. The expression levels of *CoBBX24* were decreased at the 3 and 12 h points (Figure S2A), which is consistent with natural drought in soil for a long time. After 12 d of drought stress, the expression of the *CoBBX24* transcript demonstrated a 5.6-fold decrease relative to the untreated plants (Figure 2A). The expression levels of *CoBBX24* were decreased at the 6 and 12 h points with ABA treatment (Figure S2B).

3.3. Overexpression of CoBBX24 Confers Drought Tolerance in Arabidopsis

To study the function of *CoBBX24* in *Arabidopsis*, three homozygous T3 lines with ectopic expression (OX-*CoBBX24*-1#, 3#, and 4#) were selected for a phenotypic analysis. The levels of *CoBBX24* expression in each transgenic line were detected (Figure S3). The OX-*CoBBX24* lines exhibited an improved drought tolerance. Under drought conditions, almost all the OX-*CoBBX24* lines demonstrated mild damage following vigorous growth, whereas most WT plants died (Figure 3A). In one experiment, the survival rate of the WT seedlings was 25%, and those of the OX-*CoBBX24*-1#, 3#, and 4# lines were 52.78%, 83.33%,

and 72.22%, respectively (Figure 3B). The water loss rate of the leaves was obviously lower in the OX-*CoBBX24* lines than that in the WT plants (Figure 3C). The findings imply that *CoBBX24* increases the drought tolerance of transgenic plants.

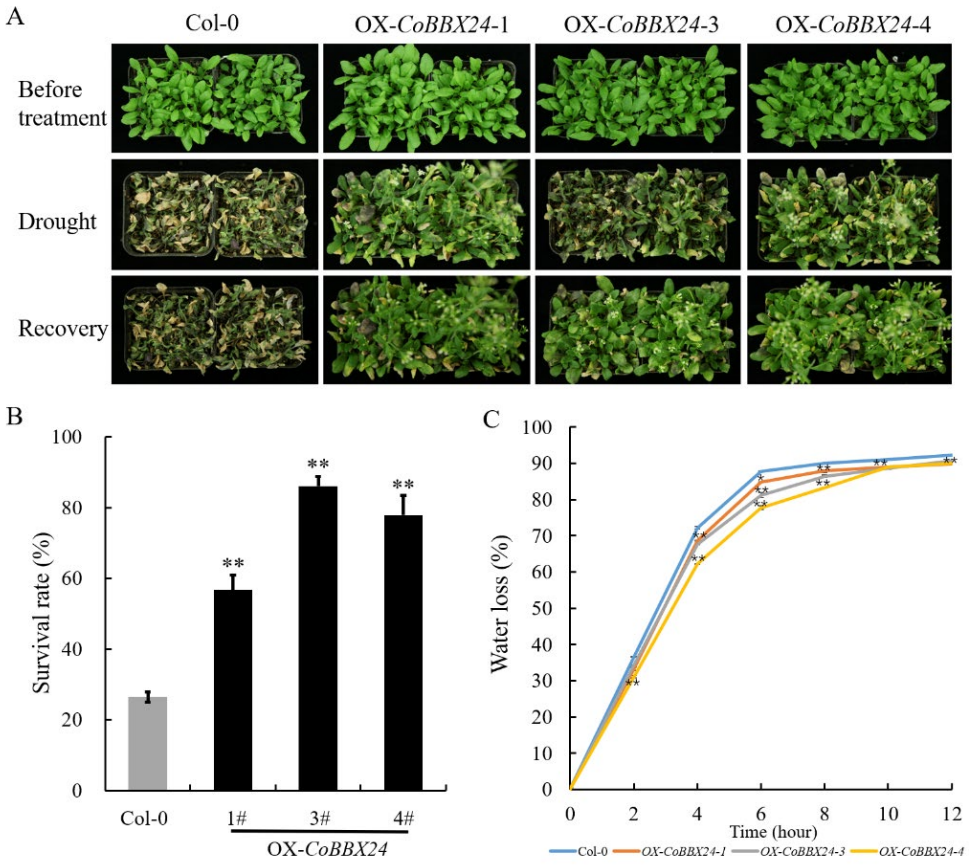

Figure 3. Overexpression of *CoBBX24* enhanced the tolerance of transgenic *Arabidopsis* under drought stress. (**A**) Phenotypes of 3-week-old *CoBBX24* transgenic and WT plants withheld water for 15 d followed by recovery for 7 d with regular watering. Three independent assays were performed with similar findings. (**B**) Survival rates of *CoBBX24* transgenic and WT plants after 7 d of re-watering following a 15 d drought treatment. Three independent experiments were performed, a total of 118 plants were counted for each genotype. Significant differences were determined by Duncan's test ($p < 0.01$). (**C**) Water loss from detached leaves of *CoBBX24* transgenic lines and WT plants. The data are presented as means ± SD of three replicates. * represents a significant difference compared with WT; * $p < 0.05$, ** $p < 0.01$ in the Student's test.

3.4. Overexpression of CoBBX24 in Arabidopsis Improves ABA Sensitivity

ABA is a stress hormone that plays a critical role in drought stress responses. To assess whether the drought tolerance of the overexpression lines was related to ABA, the main root lengths of the WT and OX-*CoBBX24* plants were measured after outside ABA treatment. No striking difference in root lengths was observed between the WT and overexpression plants when the medium was free of exogenous ABA (Figure 4A). Growth on a medium containing 3 μM of ABA resulted in a more severe inhibition of root growth in the OX-*CoBBX24* plants compared to the WT plants (Figure 4B). The seed germination rate and proportion of green cotyledon in the OX-*CoBBX24* plants were lower than those in the WT plants under ABA treatment, while 55% of the WT cotyledons turned green, the proportion for the three OE lines was much lower, and the rates of green cotyledon of the OX-*CoBBX24* plants were 25%, 30%, and 40% (Figure S4), indicating that the overexpression of *CoBBX24* in *Arabidopsis* increases ABA sensitivity.

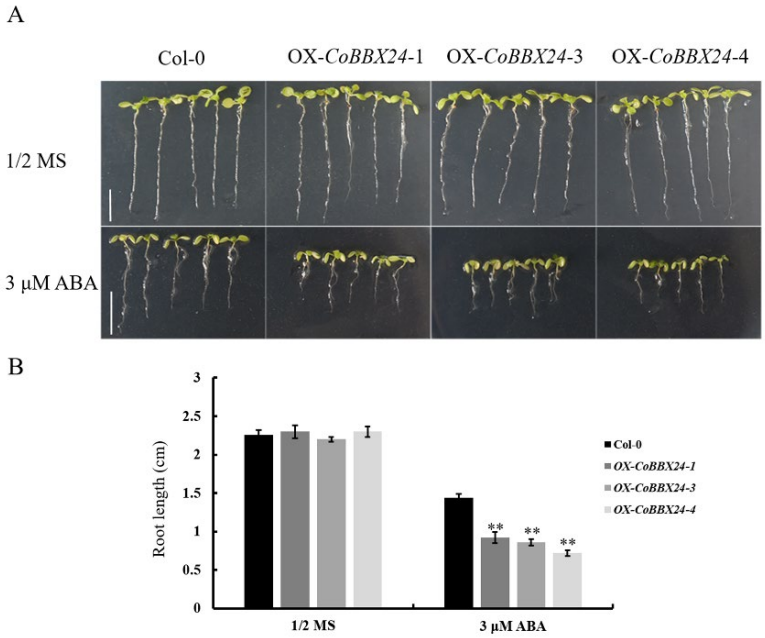

Figure 4. The constitutive expression of *CoBBX24* in *A. thaliana* enhanced root length sensitivity to ABA treatment. (**A**) Seedlings grown on 1/2 MS plates for 4 d followed by transfer to media containing 3 μM ABA; Bars: 1 cm. (**B**) The root length measured on the 6th d post-transfer. Significant differences were determined by Duncan's test (** $p < 0.01$).

3.5. Overexpression of CoBBX24 Reduces ABA-Induced Leaf Senescence in Arabidopsis

ABA is a key regulator of the drought stress response in plants and an active regulator of leaf senescence [42,43]. Exogenously applied ABA can facilitate chlorophyll breakdown [44]. To explore the phenotypic effects of ABA treatment on OX-*CoBBX24* and WT *Arabidopsis* plants, detached leaves were treated with 100 μM of ABA after being maintained for 2 d under dark conditions. The leaves of the OX-*CoBBX24* lines were maintained green, whereas the WT leaves turned significantly yellow (Figure 5A). In compliance with these visible phenotypes, the chlorophyll content in the OX-*CoBBX24* leaves was higher than that in the WT leaves (Figure 5B).

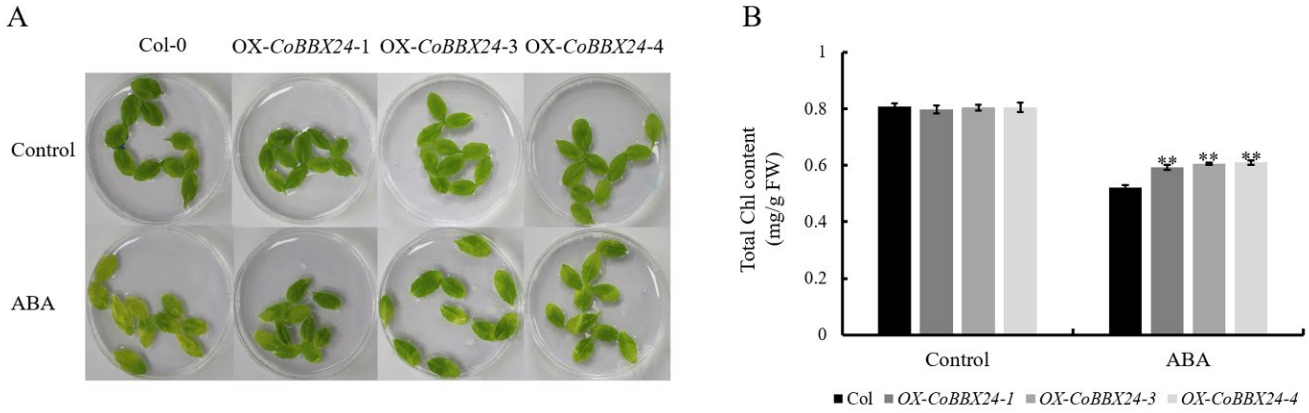

Figure 5. The phenotypic effect of ABA treatment on *CoBBX24ox* and WT *Arabidopsis* plants. (**A**) Detached leaves of 4-week-old transgenic plants after maintaining them for 2 d under dark conditions. (**B**) The chlorophyll content of the leaves shown in (**A**); significant differences were determined by Duncan's test (** $p < 0.01$).

3.6. CoBBX24 Regulates the Transcription of SAGs and CCGs in Arabidopsis

We observed that the transgenic leaves exhibited a delayed senescence phenotype under ABA treatment. Given that the products of *SAG12*, *SAG29*, *NYE1*, *NYE2*, and *NYC1* were labels of leaf senescence, we examined the transcript levels of these genes in the OX-*CoBBX24* lines and WT plants using qRT-PCR. The transcript levels of these genes were downregulated in the transgenic plants with ABA treatment (Figure 6), which is consistent with the observation that the leaves showed delayed senescence in the OX-*CoBBX24* lines. The transcription factor ABF4 can directly induce *SAG29* expression [11]. The abundance of the *ABF4* transcript was downregulated in the transgenic plants. Our findings indicate that *CoBBX24* negatively regulates the transcript levels of *ABF4*, *SAG12*, *SAG29*, *NYE1*, *NYE2*, and *NYC1*, delaying chlorophyll breakdown and leaf senescence in *Arabidopsis*.

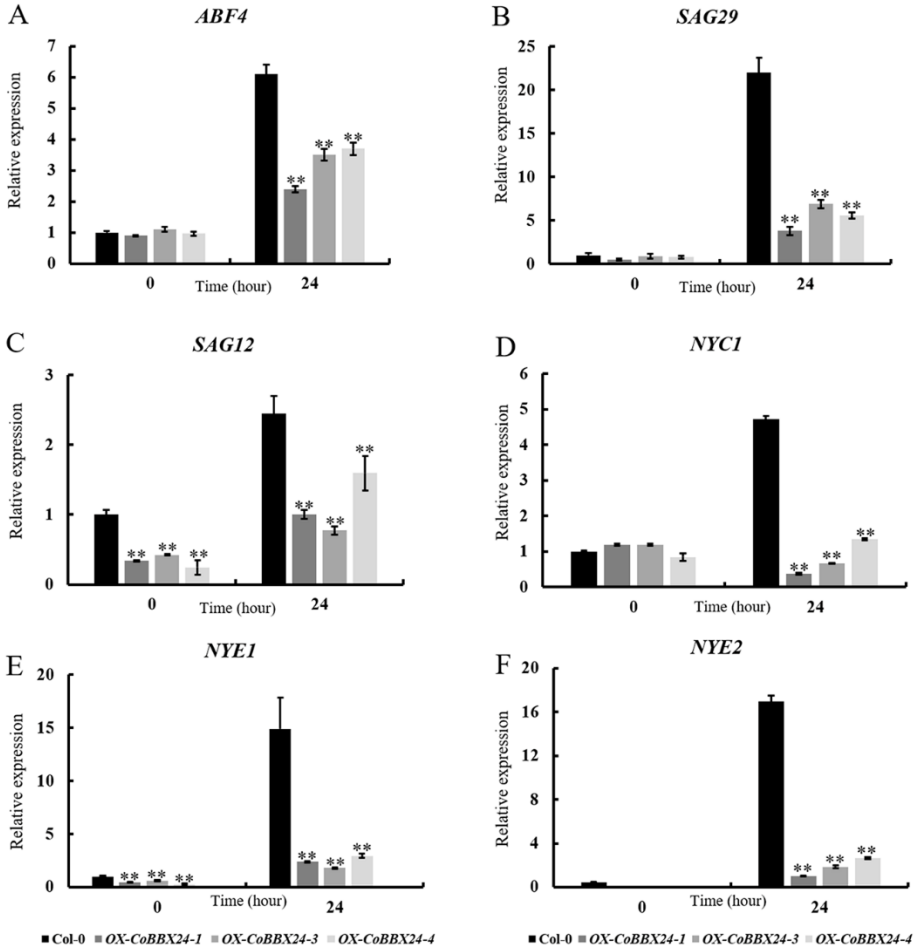

Figure 6. qRT-PCR assay revealed the expression of (**A**) *AtABF4*, (**B**) *AtSAG29*, (**C**) *AtSAG12*, (**D**) *AtNYC1*, (**E**) *AtNYE1*, and (**F**) *AtNYE2* in *CoBBX24ox* and WT plants under ABA treatment. The *Arabidopsis Actin2* gene was used as the reference gene for normalization. Error bars indicate the SD; n = 3. Significant differences were determined by Duncan's test (** $p < 0.01$).

4. Discussion

BBX proteins are a kind of zinc-finger transcription factor comprising one or two B-box domains, with some members also having a CCT domain. In *Arabidopsis*, the B-box family consists of 32 proteins and is divided into five subgroups according to their structural domains [45,46]. CoBBX24 contains two B-box domains belonging to the fourth subgroup. BBX24 was originally cloned from *Arabidopsis* as a Salt-Tolerant Protein (STO) [47]. It fully complements the phenotype of the yeast mutant and enhances the salt tolerance of WT yeast [47]. In *Arabidopsis*, the overexpression of *BBX24* (*STO*) enhances salt tolerance

compared to that observed in WT [48]. Transgenic chrysanthemum lines with a suppressed expression of *CmBBX24* (Cm*BBX24*-RNAi) demonstrate a decreased tolerance to drought stress and reduced transcript levels of the genes related to carbohydrate metabolism and soluble substances [38]. In this study, we observed that the ectopic expression of *CoBBX24* enhanced the drought tolerance in *Arabidopsis* by regulating the expressions of SAGs and CCGs. In our previous report, the heterologous expression of *CmBBX22* in *Arabidopsis* led to delayed leaf senescence and an improved drought tolerance [33]. BBX22 and BBX24 are members of the fourth subfamily, and these findings indicate a conserved role for BBX genes in plant drought responses. Interestingly, the *CoBBX24* expression was down-regulated in the oil tea trees with drought stress, which was consistent with previous reports. The amounts of *SsBBX24* transcript decreased in the leaves of 2-week-old phytotron-grown, water-deprived *S. sogarandinum* plants [49]. The expression of *AtBBX24* decreased in *Arabidopsis* seeds is strongly repressed by PEG stress [37]. It is very interesting that the BBX24 expression was down-regulated, but it positively regulates the drought tolerance in plants.

Leaf senescence is the last developmental stage of plant growth, and a feature of leaf senescence is chloroplast breakdown [50]. CCGs, including *NYC1*, *NYE1/SGR1*, *NYE2/SGR2*, and *PHEOPHORBIDE a OXYGENASE* (*PaO*), play key roles in regulating chlorophyll breakdown [51]. Drought-induced leaf senescence happens gradually, and previous studies have shown that some genes are involved in senescence and also play crucial roles in stress response [52,53]. The overexpression of *GhTZF1* enhanced drought tolerance and delayed drought-induced leaf senescence through regulating the expression of antioxidant genes and SAGs in transgenic *Arabidopsis* [54]. The overexpression of *GhWRKY91* delayed leaf senescence and improved drought tolerance in transgenic *Arabidopsis* [55]. *NtNAC028* loss-of-function tobacco plants showed delayed leaf senescence and an increased tolerance to drought stress [56]. It has been hypothesized that these genes integrate different signaling pathways and play important roles between stress responses and senescence. Kim et al. (2018) reported that transcription factors critically contribute to leaf senescence [51]. Several transcription factors, including NAC, NAP, bHLH, MYC, and BBX, are involved in leaf senescence through regulating the expression of SAGs [57–60]. In this study, we identified the role of *CoBBX24* in leaf senescence and found that the plants overexpressing *CoBBX24* had relatively low rates of ABA-induced leaf senescence and chlorophyll degradation, consistent with the relatively high tolerance of plants overexpressing *CoBBX24* to drought stress. MdBBX22 interacted with MdABI5 in apples to suppress the transcriptional activity of *MdNYE1* and *MdNYC1*, thereby negatively regulating chlorophyll degradation and leaf senescence [7]. It is speculated that CoBBX24 may have a similar function, but additional research is needed to obtain a greater understanding of the molecular mechanisms of CoBBX24 in drought response.

ABA plays an active role in regulating leaf senescence. ABFs are critically involved in initiating the ABA response [61]. ABF4 promotes chlorophyll breakdown and leaf senescence by increasing the expressions of CCGs and SAGs in *Arabidopsis* through an ABA-dependent pathway [11]. Exogenous ABA promotes the transcription of *NYC1*, *NYE1*, and *PaO*, implying that multiple genes are involved in regulating chlorophyll breakdown during ABA-induced leaf senescence [58]. *SAGs* are upregulated during ABA-induced senescence, and SAG29 is a molecular link that integrates environmental stress responses with the senescence process [10,14]. The transcript levels of *ABF4*, *SAG12*, *SAG29*, *NYE1*, *NYE2*, and *NYC1* were decreased in the OX-*CoBBX24* plants under ABA treatment (Figure 6), which was consistent with the observation of delayed leaf senescence in the OX-*CoBBX24* lines. *DREB2A* is one of the main regulators of drought, and the expression level of *DREB2A* in the OX-*CoBBX24* plants was significantly higher than that in the WT plants after the drought treatment (Figure S5). The expression of the *CoDREB2A* transcript in the oil tea trees also increased after 12 d of drought stress (Figure S2C). However, the mechanism of *CoBBX24* repressed under drought stress remains to be explored.

ABA is a stress-related signaling molecule that regulates stress responses, including drought stress in higher plants [62]. The response to drought stress during the vegetative growth period correlates with the sensitivity of seedlings to ABA. During germination and seedling growth, the constitutive expression of *VvNAC17* in *Arabidopsis* enhances its sensitivity to ABA, which heightens its stomatal closure and reduces its water loss, increasing its drought tolerance [63]. The constitutive expression of *TaNTL1* in *Arabidopsis* enhances its drought tolerance, and the germination of transgenic seeds was hypersensitive to ABA [64]. However, the relationship between ABA sensitivity in seedlings and drought response during the vegetative growth stage is not always relevant. In *Arabidopsis*, the *abo3* mutant is hypersensitive to ABA during seedling establishment and seedling growth; however, this mutant is less drought-tolerant than the WT [65]. In the present study, the root growth of the OX-*CoBBX24* lines was inhibited more severely than that of the WT plants (Figure 4B), and the proportion of green cotyledon in the OX-*CoBBX24* plants was lower than that of the WT on a medium containing 0.2 μM of ABA. (Figure S4). These findings indicate that *CoBBX24* regulates ABA signaling during seedling growth.

Overall, *CoBBX24* plays a pivotal role in drought stress responses by regulating ABA signaling, SAGs, and CCGs. The downregulation of *ABF4, SAG12, SAG29, NYE1, NYE2*, and *NYC1* in OX-*CoBBX24* lines delays chlorophyll breakdown and leaf senescence. This functional characterization of *CoBBX24* provides new insights into drought stress responses and their underlying regulatory networks.

5. Conclusions

In this study, we investigated the function of a *BBX* gene that responds to drought stress and found that the ectopic expression of *CoBBX24* enhanced the drought tolerance in *Arabidopsis* by regulating the expressions of SAGs and CCGs. Our results suggest that *CoBBX24* plays an important role in drought tolerance and ABA-induced leaf senescence, identifying an excellent drought tolerance gene for the molecular breeding of oil tea trees. This functional characterization of *CoBBX24* provides a new point of view on drought stress responses and their underlying regulatory networks.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9090991/s1, Figure S1: The phylogenetic tree of the *Arabidopsis* BBX family and CoBBX24; Figure S2: Transcriptional profiling of *CoBBX24* and *CoDREB2A* under drought stress or ABA treatment; Figure S3: RT-PCR analysis of *CoBBX24* expression in WT and transgenic lines; Figure S4: Overexpression of *CoBBX24* in *Arabidopsis* enhanced seedling sensitivity to ABA treatment; Figure S5: qRT-PCR assay to examine the expression of genes in *CoBBX24ox* and WT plants under drought treatment; Table S1: List of primers used in this study.

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