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The *Agropyron mongolicum* bHLH Gene *AmbHLH148* Positively Involved in Transgenic *Nicotiana benthamiana* Adaptive Response to Drought Stress

Xuefeng Zhang [1], Yanhong Ma [1,*], Bobo Fan [1], Fengcheng Sun [2], Yongqing Zhai [1], Yan Zhao [3], Lizhen Nie [2], Yongyu Fang [2], Zhuo Yu [1] and Bingjie Qi [1,*]

[1] Agricultural College, Inner Mongolia Agricultural University, Hohhot 010018, China
[2] Inner Mongolia Academy of Agricultural & Animal Husbandry Sciences, Hohhot 010031, China
[3] College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010018, China
* Correspondence: mayanhong80@imau.edu.cn (Y.M.); qibj@imau.edu.cn (B.Q.)

Abstract: While bHLH transcription factors have been linked to the regulation of various abiotic stressors, research on drought-related bHLH proteins and their molecular processes in *Agropyron mongolicum* has remained limited. In this study, a bHLH gene from *A. mongolicum*, designated as *AmbHLH148*, was successfully cloned and isolated. *AmbHLH148* was exclusively localized within the nucleus. Additionally, qRT-PCR analysis demonstrated a significant upregulation of *AmbHLH148* in response to drought stress. When transferred into tobacco (*Nicotiana benthamiana*), the heterologous expression of *AmbHLH148* led to enhanced drought tolerance. Under drought stress conditions, *AmbHLH148*-OE transgenic tobacco plants exhibited increased activities of antioxidant defense enzymes, such as SOD (superoxide dismutase), POD (peroxidase), and CAT (catalase). These enzymes efficiently mitigated the accumulation of reactive oxygen species (ROS) compared to wild-type plants. Furthermore, *AmbHLH148*-OE transgenic tobacco showed elevated levels of PRO (proline) and reduced MDA (malondialdehyde) content, contributing to enhanced stability in the plant's cell membrane system during drought stress. In summary, this study underscores that the overexpression of *AmbHLH148* in transgenic tobacco acts as a positive regulator under drought stress by enhancing the plant's antioxidant capacity. These findings shed light on the molecular mechanisms involved in bHLH transcription factors' role in drought resistance, contributing to the discovery and utilization of drought-resistant genes in *A. mongolicum* for enhancing crop drought resistance.

Keywords: *Agropyron mongolicum* Keng; bHLH transcription factor; *AmbHLH148*; drought resistance; antioxidant activity

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1. Introduction

Drought stands as one of the main factors causing damage to plant growth and reducing crop yields [1–3]. To adapt to drought stress, plants have developed a series of defense response mechanisms to cope with drought stress, including changing root structure, reducing water loss, improved water use efficiency, accumulating compatible solutes, and improving antioxidant capacity [4]. Plant resistance is activated by modulating the activity of several transcriptional regulators, including DNA-binding transcription factors and their associated regulatory proteins. These factors reprogram transcription in plant cells to activate defense signals [5–7]. In *Arabidopsis thaliana*, drought-induced genes can be divided into two categories [8]. The first category consists of functional proteins, such as Aquaporin, detoxification enzymes, key osmolyte biosynthetic enzymes, and protease. The second category encompasses regulatory proteins, including transcription factors (TF), protein kinases, and protein phosphatases. The basic helix–loop–helix (bHLH) protein family ranks among the largest transcription factor (TF) families and is prevalent

in various plant species [9]. These bHLH transcription factors have been identified in plants like *Arabidopsis thaliana*, *Capsicum annuum*, *Oryza sativa*, *Solanum tuberosum*, *Cucumis sativus*, and *Arachis hypogaea* [10–15]. bHLH transcription factors play important roles in various processes of plant growth and development. For example, bHLH-TFs are involved in processes such as the growth and development process of plants [16–22], secondary metabolism [23–27], fruit color regulation [28], the response to adversity stress [29–31], the maintenance of iron steady state [32–35], and the regulation of hormone signaling [36,37].

As the discovery of bHLH transcription factors continues to expand, mounting evidence supports their significant role in plant responses to abiotic stresses. For example, the overexpression of *EcbHLH57* from millet (*Eleusine coracana*) in tobacco significantly improved tolerance to both salinity and drought stress [38]. *PebHLH35* from *Populus euphratica*, when expressed in transgenic *Arabidopsis thaliana*, bolstered drought resistance by regulating stomatal development and photosynthesis [39]. In *Arabidopsis thaliana*, the overexpression of *bHLH122* in transgenic plants induced heightened resistance to drought, NaCl, and osmotic stress [40]. In wheat, the *TabHLH1* gene increased drought tolerance by regulating the ABA pathway [41]. Similarly, the overexpression of *OsbHLH148* in rice led to increased drought tolerance through the regulation of the JA pathway [42]. Additionally, the transgenic expression of *VvbHLH1* in grapes not only significantly increased flavonoid accumulation but also enhanced drought tolerance in Arabidopsis [43]. Collectively, these studies underscore the pivotal role of bHLH transcription factors in enhancing drought tolerance across diverse plant species, offering valuable insights into potential strategies for enhancing drought resistance in crops.

A. mongolicum Keng diploid (2n = 14) perennial grass is a common forage crop in the deserts and grasslands of China [44]. *A. mongolicum* is a valuable forage grass resource that holds significant ecological value for its attributes of drought resistance, cold resistance, salt tolerance, and disease and insect resistance. It is often cultivated to improve grassland, support soil and water conservation, and establish windbreak forests [45,46]. Some of its exceptional genetic resources for resistance have been harnessed for the genetic modification of grain crops such as wheat and barley [47]. Recent research on *A. mongolicum* has concentrated on genetic diversity, remote hybridization, and double breeding. For instance, the *MwLEA1* gene, crucial for drought tolerance, was effectively extracted from *A. mongolicum* using the homologous sequence of wheat [48]. Genes associated with drought tolerance have also been discovered. Ao et al. employed RT-PCR and RACE techniques to extract the *MwAP2/EREBP* genes from *A. mongolicum*, suggesting their involvement in the physiological process linked to drought tolerance [49]. However, until now, there have been no studies exploring the drought-responsive bHLH family genes in *A. mongolicum*. In a previous investigation that leveraged transcriptome sequencing data obtained from *A. mongolicum* during different drought treatment periods (NCBI, PRJNA742257 [50]), 23 *bHLH* genes were identified through bioinformatics screening. Among them, *A. mongolicum's AmbHLH148* exhibited significant upregulation in response to drought stress [51]. To understand its potential role in drought tolerance, *AmbHLH148* was heterologously inserted into tobacco plants. This research offers valuable insights into the function of *AmbHLH148* in drought tolerance and offers genetic resources that could enhance drought resistance in other crops.

2. Materials and Methods

2.1. Experimental Materials and Growth Condition

A. mongolicum seeds with full seeds were carefully selected by peeling off the seed coat, followed by thorough seed sterilization. In detail, the seeds were put into a 2 mL centrifuge tube and washed with 1.5 mL of sterilized ddH_2O for 30 s. This washing procedure was repeated 5 times, and the liquid was then discarded. Subsequently, the seeds underwent a 30 s wash with 1.5 mL of 75% ethanol, and the ethanol was then discarded. The seeds were washed again with 1.5 mL of sterilized ddH_2O for 30 s, and this process was repeated 3 times before discarding the liquid. A solution comprising sterilized ddH_2O and sodium hypochlorite at a 1:1.3 ratio was used for a 10 min wash, followed by rinsing

with sterilized ddH_2O for 30 s. This rinsing procedure was repeated 5 times, and the liquid was discarded. After these washing steps, the seeds were transferred to an ultra-clean bench, where they were left to dry on sterilized filter paper. The sterilized seeds were then sown in a germination box containing sterilized filter paper, which was moistened with water twice daily at 8:00 a.m. and 8:00 p.m. The germination box was placed inside an artificial climatic chamber (RLD-1000D-4, Ningbo Ledian Instrument Manufacturing Co., Ltd., Ningbo, China), providing a controlled environment with 14 h of light and 10 h of darkness. The temperature was maintained at 25 ± 1 °C, with a relative humidity of 60% and a constant light intensity of 20,000 LX, until germination. Upon successful germination, the seedlings were transplanted into pots measuring 35 cm in length, 27 cm in width, and 10 cm in depth. These pots were filled with 3 L of 1/5 Hoagland's nutrient solution for hydroponic growth.

N. benthamiana L. seeds were evenly sown in pots containing a substrate mixture (comprising nutrient soil, vermiculite, and perlite at a 3:1:1 ratio) within pots measuring 10 cm in diameter and 10 cm in depth. These pots were initially sealed with cling film to create a controlled environment. Following seedling emergence at 4–5 days, small air vents were introduced in the cling film. After approximately 10 days of seedling growth, individual transplanting was conducted. The transplanted seedlings were placed in an artificial climatic chamber (RLD-1000D-4, Ningbo Ledian Instrument Manufacturing Co., Ltd., Ningbo, China) with 14 h of light and 10 h of darkness. The chamber maintained a consistent temperature of 25 ± 1 °C, with a relative humidity of 60% and a light intensity of 20,000 LX. The cling film was removed when the seedlings reached a height of 3–5 cm. For subcellular localization experiments, *N. benthamiana* seedlings were grown for approximately 20 days, a stage deemed suitable for use as test material.

T_1-generation transgenic tobacco seeds were subjected to sterilization procedures before being placed onto 1/2 MS solid medium containing 50 mg/L of kanamycin for positive screening. The transgenic tobacco plants that exhibited positive screening results were subsequently transplanted into pots, each measuring 10 cm in diameter and 10 cm in depth. In each pot, four transgenic plants were placed, and this arrangement was replicated across four pots for each transgenic strain. To serve as a comparative control, a selection of wild-type (WT) tobacco seedlings, closely matching the growth height of the transgenic plants, was chosen and placed in an artificial climate chamber under conditions identical to those described previously. Throughout the growth phase, the plants were watered every two days with 200 mL of water. Additionally, a water-soluble fertilizer, with a concentration of 1 g/L, was supplemented to the plants on a weekly basis.

2.2. Plant Drought Treatment

When the *A. mongolicum* seedlings reached the three-leaf, one-center stage of growth, a 25% PEG-6000 solution was added to the 1/5 Hoagland nutritional solution to induce PEG stress. Subsequent sampling involved the collection of 0.5 g of plant material from each individual. Leaves were collected at specific time intervals, including 0-day (CK), 1-day, 3-day, 5-day, 7-day, and fs24 h (fs24 h indicates the sample collected after 24 h of restoration in 1/5 Hoagland's nutrient solution). These collected leaves were promptly placed in enzyme-free freezing tubes and flash-frozen in liquid nitrogen at −80 °C until further analysis.

In the drought treatment experiment, both the WT and *AmbHLH148*-OE tobacco seedlings, which were cultured to approximately 5 weeks of age, were subjected to a drought treatment by withholding water for a duration of 7 days. Upon completing the 7-day drought treatment, tobacco seedlings were harvested and underwent a thorough wash with distilled water in preparation for subsequent analysis. The experimental procedure encompassed the following steps: (1) the selection of *AmbHLH148*-OE transgenic lines; (2) observation of the phenotype characteristics of the tobacco plants; (3) measurement of the root length and root surface area of tobacco subjected to drought stress; (4) assessment of proline (PRO) and malondialdehyde (MDA) content, the activities of antioxidant en-

zyme superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as gene expression levels using the 3rd leaf (counted from bottom to top).

2.3. Homology Analysis, Expression Analysis and Cloning of the AmbHLH148 Gene

The *AmbHLH148* gene sequence was searched for homology using the blastn tool available on the NCBI. Total RNA was extracted from *A. mongolicum* leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Specific primers were constructed using Premier 5.0 software, based on the full-length Open Reading Frame (ORF) sequence of *AmbHLH148* obtained from *A. mongolicum* transcriptome data (GenBank accession: OR786931). For the reverse transcription reaction, 1 μg of total RNA was utilized, and the cDNA was synthesized using the FastKing RT Kit (With gDNase) (TIANGEN KP116, Beijing, China).

A full-length sequence fragment encompassing the Open Reading Frame (ORF) of the *A. mongolicum AmbHLH148* gene was successfully amplified from *A. mongolicum*. The PCR amplification system comprised of the following components: 1 μL of cDNA template, 1 μL of *AmbHLH148*-F, 1 μL of *AmbHLH148*-R, 10 μL of 2 × Taq PCR Master Mix (Tiangen Co., Beijing, China), and 7 μL of ddH_2O. The PCR amplification program involved an initial denaturation step at 95 °C for 5 min, followed by 35 cycles, each consisting of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. All PCR products were stored at 4 °C for further analysis. The PCR products were detected through 1% agarose gel electrophoresis. Subsequently, they were purified and recovered following the operating instructions provided by the DNA Gel/PCR Purification Miniprep Kit (BIOMIGA, BIOMIGA Medical Technology, San Diego, CA, USA). The recovered products were ligated with a cloning vector (pEASY®-T1 Simple Cloning Vector, Beijing TransGen Biotech Co., Ltd., Beijing, China) and sequenced (BGI, Shenzhen, China).

The *A. mongolicum* seedlings were cultivated for 30 d, and leaves were collected from untreated control (CK) and from seedlings subjected to simulated drought treatments for 1 d, 3 d, 5 d, 7 d, and 24 h of rehydration. The expression of the *AmBHLH148* gene under drought stress conditions was detected using qRT-PCR. The qRT-PCR was performed on a real-time quantitative thermal cycler (FTC-3000P, Funglyn Biotech Inc., Toronto, ON, Canada) using the MonAmpTM SYBR® Green qPCR Mix kit (Mona Biotechnology Co., Ltd., Suzhou, China). The *U6* gene was used as an internal reference gene. The qRT-PCR amplification system consisted of 10 μL of MonAmpTM SYBR® Green qPCR Mix, 0.4 μL of *AmbHLH148*-q-F, 0.4 μL of *AmbHLH148*-q-R, 1.2 μL of cDNA, and 8 μL of nuclease-free water. The reaction program consisted of an initial step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 30 s. The relative expression levels of the genes were quantified using the $2^{-\Delta\Delta CT}$ method [52].

2.4. Subcellular Localization of AmbHLH148

For subcellular localization studies, the pBI121 vector plasmid (Miao Ling Biotechnology Co., Wuhan, China) was digested using *Xhol* and *SalI* restriction endonucleases (Takara, Beijing, China). The pBI121-*AmbHLH148*-EGFP recombinant vector was constructed by ligating the *AmbHLH148* product without a termination codon into the pBI121-EGFP vector fragment. The 25μL enzyme digestion system included 15 μL of pBI121, 1 μL each of *Xhol* and *SalI*, 1.3 μL of 10 × K buffer, and 6.7 μL of ddH_2O. The digestion process was carried out overnight at 37 °C. The 10 μL ligation system included 1 μL of linearized pBI121 vector, 1 μL of *AmbHLH148*, 4 μL of Quick-clone Mix (Takara, China), and 4 μL of ddH_2O. The ligation reaction took place in a water bath at 55 °C for 20 min and 30 °C for an additional 20 min that transformed the ligation products into *E. coli* receptor DH5α cells. Positive transformants were selected on LB agar plates containing 50 μg/mL kanamycin for PCR analysis. The PCR amplification system and procedure were consistent with those described above, and the products were sent for sequencing (BGI, China). The correctly sequenced recombinant vector pBI121-*AmbHLH148*-EGFP and the empty vector pBI121-EGFP were transformed into *Agrobacterium rhizogenes* (GV3101, Shanghai Vidi, Shanghai, China). The

positive bacterial solution was added to 20 mL of LB liquid medium, containing 100 μg/mL kanamycin, 100 μg/mL Rif, and 15 μmol/L As. The culture was incubated overnight at 28 °C with agitation at 200 rpm in a thermostatic oscillator (OD_{600}:0.6). The culture was then centrifuged at 5000 rpm for 5 min to remove the supernatant. The bacterial pellet was resuspended in an equal volume of tobacco-specific infiltration solution (Beijing coolaber Technology Co., Beijing, China; Product No. SL0911) containing 100 μmol/L As, an appropriate amount of $MgCl_2$, and MES. The resuspension was adjusted to OD_{600}:0.6 and the suspension was left at room temperature for 2 h. The plasmid was introduced into tobacco leaves instantly by *Agrobacterium tumefaciens* infection. Tobacco infested with pBI121-*AmbHLH148*-EGFP was incubated in the dark for 1 day and then in a 14 h light/10 h dark cycle for the next 2 days. In contrast, tobacco infested with pBI121-EGFP broth required incubation in the dark for 1 day, followed by an additional day in a 14 h light/10 h dark cycle. Subsequently, the lower epidermis of the transfected tobacco was observed using a laser confocal microscope.

2.5. AmbHLH148 Vector Construction and Genetic Transformation

The pBI121 vector plasmid was digested with *Xhol* and *SalI* restriction endonucleases, and the *AmbHLH148* product was subsequently ligated to the pBI121 vector fragment. The enzymatic digestion system was prepared in 25 μL volumes and consisted of the following components: 15 μL of pBI121, 1 μL of *Xhol* and *SalI*, 1.3 μL of 10 K buffer, and 6.7 μL of ddH_2O. The digestion process was carried out at 37 °C overnight. For the ligation, a 10 μL system was prepared, comprising 1 μL of linearized pBI121 vector, 1 μL of *AmbHLH148*, 4 μL of Quick-clone Mix, and 4 μL of ddH_2O. The ligation reaction was performed in a water bath at 55 °C for 20 min, followed by an additional incubation at 30 °C for another 20 min. The ligated products were subsequently transformed into *E. coli* receptor DH5α cells. Positive transformants were selected by plating on LB agar containing 50 μg/mL kanamycin, and PCR assays were conducted on these colonies. The PCR amplification system and procedure used were consistent with the methods described earlier. The sequences of the correctly assembled recombinant vector were verified through sequencing services provided by BGI, Shenzhen, China. The correctly sequenced recombinant vector was then transferred into GV3101 receptor cells (AC1001, Shanghai Weidi, Shanghai, China). After the identification of positively transformed Agrobacterium, tobacco plants were transformed using the leaf disk method. Leaves from positively transgenic tobacco seedlings were collected for DNA extraction using the Plant Genomic DNA Kit (DP305, Tiangen, Beijing, China). PCR assays were performed using the same PCR amplification system and procedure as described previously. The specific experimental steps for genetic transformation of tobacco were referred to Japelaghi et al. [53]. The primer sequences used in this experiment are shown in Table S1.

2.6. qRT-PCR Analysis

Total RNA was extracted from both drought-stressed and normally watered young tobacco leaves (WT and transgenic lines), and cDNA was synthesized following the previously outlined procedure. qRT-PCR was performed using the MonAmpTM SYBR® Green qPCR Mix kit (Mona Biotechnology Co., Ltd., Suzhou, China) on a Real Time Quantitative Thermal Cycler (FTC-3000P, Funglyn Biotech Inc., Toronto, ON, Canada). Each treatment and control group were subjected to three biological replicates, with each biological replicate including three technical replicates. The *L25* gene was used as the internal reference gene. The qRT-PCR amplification system consisted of 10 μL of MonAmp TM SYBR® Green qPCR Mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, 1.2 μL of cDNA, and 8 μL of nuclease-free water. The amplification program involved an initial denaturation at 95 °C for 30 s, followed by 40 cycles including denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s, and extension at 72 °C for 30 s. The relative expression levels of genes were quantified using the $2^{-\Delta CT}$ method [54].

2.7. Determination of Physiological Indexes

Leaves weighing 0.2 g were collected from the corresponding parts of both WT and transgenic tobacco plants subjected to regular watering and those under 7 days of drought stress. These samples were rapidly frozen with liquid nitrogen and then placed in a −80 °C freezer. The assay encompassed three biological replicates, with each biological replicate consisting of three technical replicates. The activities of SOD (A001-3), POD (A084-3-1), and CAT (A007-1-1), were evaluated using specific test kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Additionally, the content of MDA (A003-1) and PRO (A107-1-1) was determined using test kits. To minimize errors introduced by variations in the external environment, the three biological replicates of each strain were combined at the time of testing. Detailed experimental procedures for each kit assay were followed in accordance with the provided instructions inside the kit.

2.8. Statistical Analysis

Statistical analyses were performed using Microsoft Excel 2019 (Microsoft Corporation, Washington, DC, USA) and SPSS 23 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to test the significance of the samples, with a significance level of 0.05. In order to minimize errors and ensure the reliability of the results, the assay experiments were conducted with three biological replicates and three technical replicates. Standard deviation (STDEV) was used to quantify and calibrate for significant differences among the data sets.

3. Results

3.1. Analysis of AmbHLH148 Gene Expression in A. mongolicum under 25% PEG-6000 Stress

The seedlings of *A. mongolicum* were subjected to a simulated drought stress treatment with 25% PEG-6000, and the expression of the *AmbHLH148* gene was assessed at different time points along the stress gradients (Figure 1).

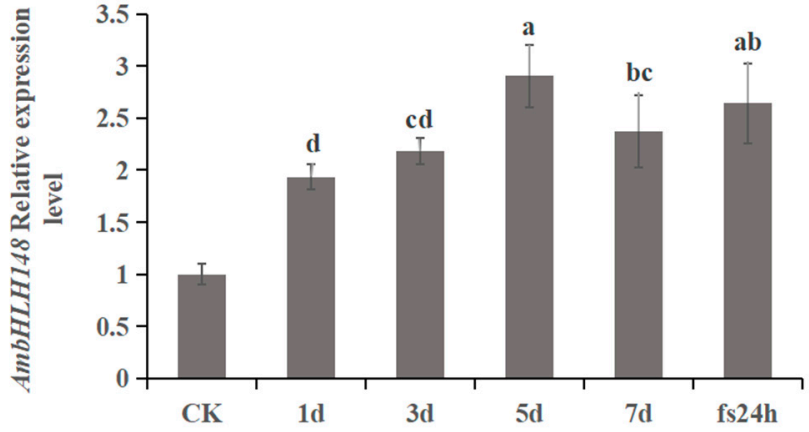

Figure 1. Relative expression of *AmbHLH148* gene. Note: lower case letters in the above graph represent significant differences at the 0.05 level. CK represents the pre-treatment sample, while 1 d, 3 d, 5 d, and 7 d correspond to samples taken at different time points following exposure to the 25% PEG-6000 stress treatment. Additionally, fs24 h indicates the sample collected after 24 h of restoration in 1/5 Hoagland's nutrient solution. The values presented in the above column were calculated using the $2^{-\Delta\Delta CT}$ method. All data are expressed as the mean ± SD.

The expression of the *AmbHLH148* gene exhibited significant variations between the *A. mongolicum* control (CK) and the various drought treatment periods. Importantly, it consistently surpassed the control levels during each treatment period. Notably, the gene expression displayed an overall increasing trend and reached its peak at the 5-day mark of drought stress, where it was approximately 2.9 times higher than the control sample.

These results suggest that the *AmbHLH148* gene in *A. mongolicum* is positively regulated in response to drought stress induced by the 25% PEG-6000 simulated treatment.

3.2. Homology Analysis of the AmbHLH148 Gene and Full-Field Amplification of ORF

The homology of the *A. mongolicum DN27532_c1_g3* gene was examined using the NCBI Blastn tool to compare it with genes from other species. The results showed that the *Aegilops tauschii AtbHLH148* gene (XP_020179773.1) and *AmbHLH148* exhibited the highest similarity, and the similarity was 90.02%. As a consequence of this significant similarity, the gene from *A. mongolicum* was tentatively named *AmbHLH148* (Figure S1).

The full-length ORF sequence of the *AmbHLH148* gene was successfully amplified using the specific primers of *AmbHLH148*-F/R, and the resulting bands were detected through 1% agarose gel electrophoresis (Figure S2). The electrophoresis results revealed that the target band fell within the expected size range of 500–700 bp, which aligned with the projected 513 bp size. The band displayed a clear, well-defined appearance with no signs of smearing or trailing. This distinctive band, presumed to represent the *AmbHLH148* gene, was subsequently purified and recovered. The isolated product was then ligated into the cloning vector, sequenced, and the sequencing results verified the successful acquisition of the *A. mongolicum AmbHLH148* gene sequence.

3.3. Subcellular Localization of AmbHLH148 Gene

To verify the position of *AmbHLH148* in the subcellular, a recombinant vector named pBI121-*AmbHLH148*-EGFP, which carried the green fluorescent protein (EGFP), was constructed. Subsequently, this recombinant vector was injected into tobacco epidermal cells. Observations made through confocal microscopy revealed that the GFP signals originating from the control pBI121-EGFP empty vector were distributed throughout all the cell compartments of the tobacco lower epidermal cells. In contrast, the pBI121-*AmbHLH148*-EGFP fusion protein was exclusively localized in the nucleus (Figure 2).

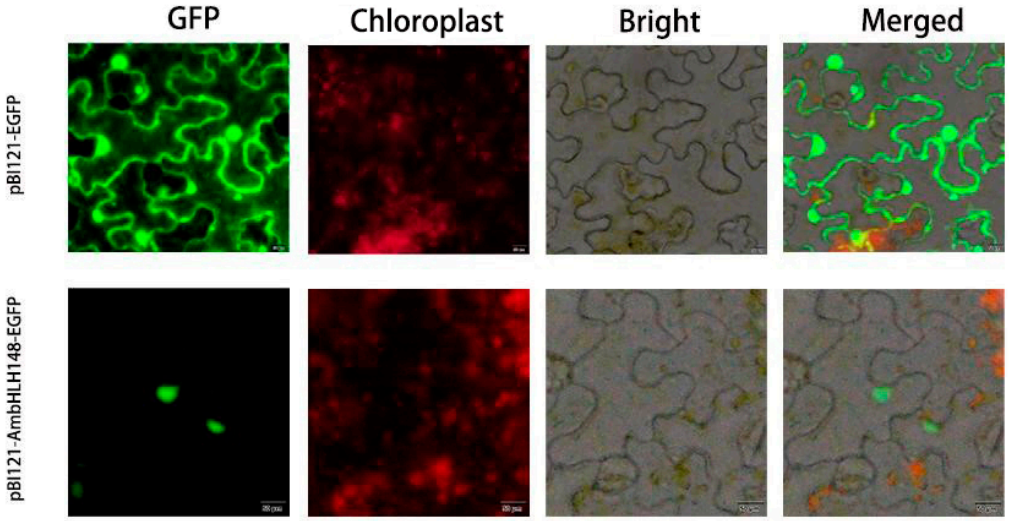

Figure 2. Subcellular localization of *AmbHLH148* in tobacco epidermal cells. The fusion protein (pBI121-*AmbHLH148*-EGFP) and the EGFP-positive control (pBI121-EGFP) were independently transiently expressed in tobacco cells. Scale bar = 50 μm.

3.4. Production and Selection of AmbHLH148 Transgenic Plants

Agrobacterium tumefaciens (GV3101) containing the pBI121-*AmbHLH148* recombinant plasmid was used to transform the sterile young tobacco leaves via the leaf disc method. Transgenic tobacco lines were subsequently obtained through shoot-induced differentiation and rooting culture (Figure S3). Positive transgenic plants were identified via PCR amplification, resulting in the acquisition of 11 *AmbHLH148* overexpressing tobacco transgenic lines,

denoted as OE lines. To investigate the potential role of the *AmbHLH148* gene in enhancing drought tolerance in tobacco, the expression of *AmbHLH148* in all 11 overexpressing tobacco transgenic lines was analyzed. Among these lines, OE6 and OE18 (as shown in Figure S4) were selected for further investigation due to their highest transcript levels.

3.5. Observation of Tobacco Phenotypes under Drought Stress

The natural drought treatment was initiated by discontinuing watering for both *AmbHLH148*-OE and WT tobacco, and the data were gathered during two time points, namely, at the initiation (0 day) and after 7 days of the drought stress period. We closely examined morphological variations between WT and *AmbHLH148*-OE tobacco under normal and drought stress conditions (Figure 3). Under normal growth conditions, there were no significant aboveground phenotypic differences between *AmbHLH148*-OE and WT plants. However, notable disparities emerged below the surface, with *AmbHLH148*-OE plants displaying an increased abundance of lateral roots compared to WT plants. Under drought stress circumstances, WT plants exhibited a high incidence of wilting leaves, including yellowed, wilted leaves near the base of the plant. In contrast, transgenic tobacco plants showed a remarkable reduction in wilted leaves, and they exhibited longer root systems, and greater root surface area compared to the WT plants. This phenomenon indicates that the overexpression of *AmbHLH148* under drought stress conditions may positively regulate the drought tolerance of tobacco by increasing root growth, a speculation that requires further validation.

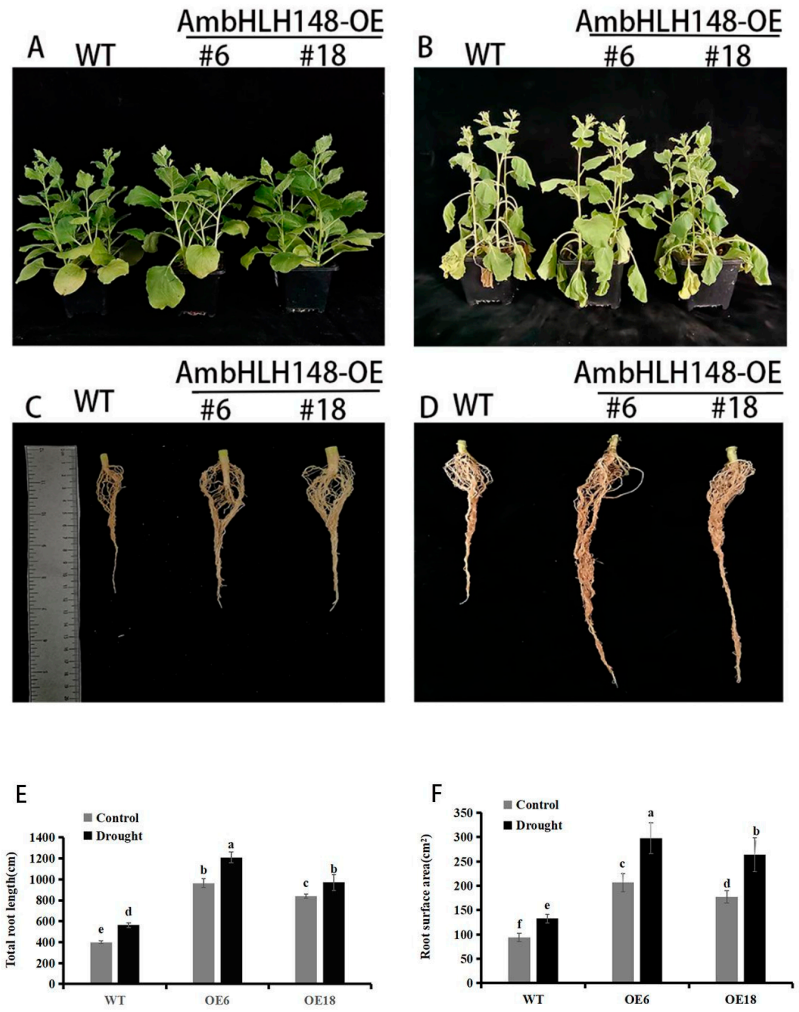

Figure 3. *Cont.*

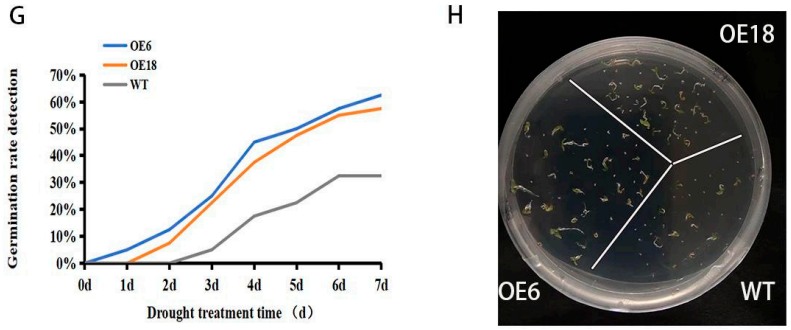

Figure 3. Characterization of *AmbHLH148* transgenic tobacco under drought stress conditions. (**A**): Phenotypes of *WT* and transgenic tobacco under normal watering conditions. (**B**): Phenotypes of *WT* and transgenic tobacco under drought for 7 days. (**C**): Root phenotypes of WT and transgenic tobacco under normal watering conditions. (**D**): Root phenotypes of *WT* and transgenic tobacco under drought for 7 days. (**E**): Root length of WT and transgenic tobacco under normal watering and drought for 7 days. (**F**): Root surface area of WT and transgenic tobacco under normal watering and drought for 7 days. (**G**): Germination of WT and transgenic tobacco under 5% PEG stress for 7 days. (**H**): WT and transgenic tobacco were assayed for germination at 7 days of 5% PEG stress. Note: lower case letters in the above graph represent significant differences at the 0.05 level. All data are expressed as the mean ± SD. (**G**): Three biological replicates were used in the experiment, and the number of seeds sown on each medium was 40.

3.6. AmbHLH148 Affects the Expression of Stress Response Genes

Plants adapt to stress conditions by mobilizing the expression of a large number of stress-related genes. To investigate the expression of *AmbHLH148* and stress-responsive genes to drought stress, a comprehensive analysis of their transcript accumulation levels was conducted. The transcript accumulation of *AmbHLH148* and some classical stress-responsive genes, such as *NtNAC2* [55] and *NtHSP70-8* [56], was detected via qRT-PCR (Figure 4). Both *AmbHLH148*-OE6 and *AmbHLH148*-OE18 tobacco plants exhibited higher transcript levels of accumulation under drought conditions when compared to well-watered conditions (Figure 4A). Moreover, we found that the expression levels of *NtNAC2* and *NtHSP70-8* genes were significantly higher in the overexpressed tobacco (OE6 and OE18) compared to the WT under drought conditions (Figure 4B,C). These results collectively indicate that *AmbHLH148* plays a pivotal role in regulating plant responses to drought stress, and the overexpression of *AmbHLH148*-OE enhanced the expression of stress-responsive genes (*NtNAC2*, *NtHSP70-8*). This coordinated upregulation contributes to the improved drought tolerance observed in tobacco plants.

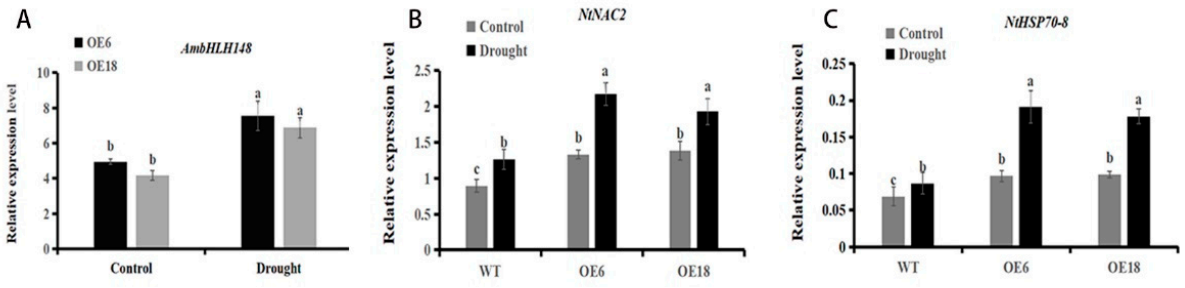

Figure 4. The expression levels of the *AmbHLH148* gene and stress-responsive genes in both control and drought conditions (7 days) in *AmbHLH148*-OE plants. (**A**): Expression levels of the *AmbHLH148* gene in transgenic plants in control and drought for 7 days. (**B**,**C**): Effects of normal watering and drought for 7 days on the expression levels of *NtNAC2* and *NtHSP70-8* genes in wild-type and transgenic plants. In tobacco, the *L25* gene was used as an internal reference gene. Note: lower case letters in the above graph represent significant differences at the 0.05 level. The values presented in the above column were calculated using the $2^{-\Delta CT}$ method. All data are expressed as the mean ± SD.

3.7. Overexpression of AmbHLH148 Enhanced Antioxidant Capacity of Tobacco

In order to examine the involvement of the *AmbHLH148* gene in the antioxidant defense mechanism of transgenic tobacco, we assessed the levels of MDA and PRO, along with the activities of antioxidant enzymes such as SOD, POD, and CAT, in both *AmbHLH148*-OE and WT tobacco plants under conditions of drought stress (Figure 5). At the 7-day mark of drought stress, the SOD activities of *AmbHLH148*-OE tobacco (OE6 and OE18) were 1.09 and 1.06 times higher than those of WT tobacco, respectively. Similarly, the activities of POD in transgenic tobacco were 1.10 and 1.13 times those of WT, respectively. These results indicate that the membrane defense enzymes SOD and POD were more active in the transgenic plants, indicating that *AmbHLH148*-OE tobacco plays a more effective role in stress resistance under drought stress. The activities of CAT in transgenic tobacco were 1.18 and 1.17 times those of WT, respectively. Furthermore, the PRO content of transgenic tobacco was 1.50 and 1.35 times higher than that of WT, respectively. Proline, an important osmoregulator, plays a crucial role in maintaining osmoregulation and scavenging ROS to improve the stability of the cell membrane system when plants are exposed to adverse stress conditions [57]. In addition, the MDA content of WT tobacco was higher than that of transgenic tobacco at 7 days of drought stress, by 0.9 and 0.62 times, respectively. This suggests that the cell membrane of transgenic plants was less damaged under drought stress, while the membrane system of WT plants suffered severe damage. In conclusion, the results above strongly suggest that *AmbHLH148*-OE enhances the antioxidant capacity of tobacco, resulting in greater drought tolerance.

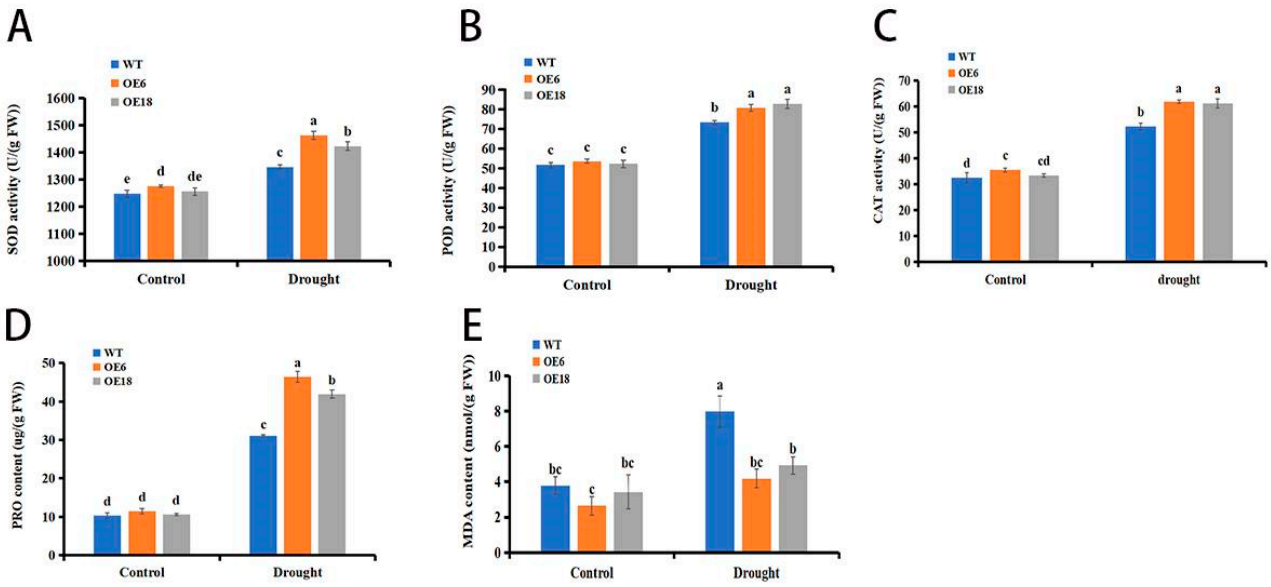

Figure 5. Analysis of the antioxidant capacity of *AmbHLH148*-OE transgenic tobacco under both control and drought conditions. (**A**) SOD activity of WT and transgenic plants. (**B**) POD activity of WT and transgenic plants. (**C**) CAT activity of WT and transgenic plants. (**D**) PRO content of WT and transgenic plants. (**E**) MDA content of WT and transgenic plants. Note: lower case letters in the above graph represent significant differences at the 0.05 level. All data are expressed as the mean ± SD.

4. Discussion

The bHLH protein family, as the second largest transcription factor (TF) family, plays a crucial role in plant responses to drought stress. Cui et al. used *Camellia sinensis* transcriptome data and identified 39 differential *CsbHLH* genes showing various expression under drought stress conditions [58]. Further validation through qRT-PCR of nine selected *CsbHLH* genes, which corroborated the transcriptome data, suggested their responsiveness to drought stress. *Myrothamnus flabellifolia*'s *MfbHLH38* overexpression, on the other hand, was shown to increase drought tolerance in *Arabidopsis thaliana*. Additionally, it increased

Arabidopsis thaliana's sensitivity to mannitol and abscisic acid and increased ABA levels during drought stress [59]. Similarly, in *Malus domestica*, *MdbHLH130* acts as a positive regulator of the water stress response by regulating tobacco stomatal closure and the scavenging of ROS [60]. Moreover, *AtbHLH68* is involved in the regulation of lateral root extension and the response to drought stress by modulating ABA signaling and/or metabolism in a direct or indirect manner [61]. However, there is a dearth of information on the abiotic stress of the *bHLH* gene in *A. mongolicum*. This study bridges this knowledge gap by elucidating the isolation of the *AmbHLH148* gene from *A. mongolicum*. Sequence analysis using the NCBI's blastn tool revealed that *AmbHLH148* exhibits a high degree of similarity with *AtbHLH148* (*Aegilops tauschii*), *TabHLH148-like* (*Triticum aestivum*), and *TdbHLH148-like* (*Triticum dicoccoides*). Nevertheless, existing literature fails to provide insights into the functional characterization of these genes. Building upon previous research where we examined 23 *A. mongolicum bHLH* genes and 9 other *bHLH* genes known for their drought-resistant capabilities, we constructed an evolutionary tree and conducted sequence comparisons. These analyses showcased that *AmbHLH148* from *A. mongolicum* forms a distinct branch with *OsbHLH148* (*O. sativa*), demonstrating a kinship of 41%. Intriguingly, gene expression analysis revealed an upregulation trend of *A. mongolicum bHLH* genes with the duration of drought treatment [51]. In light of these findings, we hypothesized that *AmbHLH148* might be related to drought stress. We then heterologously transformed this gene into tobacco and thoroughly examined its effects on overexpressed tobacco plants following drought stress conditions.

Numerous studies have consistently demonstrated that plant bHLH family proteins are predominantly localized within the nucleus. These nuclear regulatory proteins typically exert their influence by either activating or repressing the transcription of target genes, thereby regulating gene expression. Subcellular localization analyses of several bHLH proteins associated with drought resistance have consistently revealed their concentration within the nucleus. For instance, *AbbHLH122* [62] and *TabHLH49* [63] are noteworthy instances of such nuclear localization. In line with these findings, our study also identified the nuclear localization of the *AmbHLH148* protein.

Plant root growth is closely related to drought tolerance. Studies have shown that plants with longer root systems and larger root surface areas exhibit enhanced tolerance to drought stress [64]. In this study, we selected two *AmbHLH148* transgenic lines, OE6 and OE18, for drought stress experiments using transgenic tobacco. Our observations following the drought treatment revealed that the transgenic tobacco plants showed greater vitality. *AmbHLH148*-OE plants displayed longer roots and a larger root surface area when compared to their wild-type counterparts. This extended root system provides *AmbHLH148*-OE plants with a greater capacity to absorb water and nutrients from the soil, which may positively contribute to drought tolerance.

Furthermore, our results also show that under drought stress, *AmbHLH148*-OE plants had higher SOD, POD, and CAT activities than WT plants. In addition, *AmbHLH148*-OE plants displayed elevated levels of PRO and reduced levels of MDA when contrasted with WT plants, aligning with the results reported by Song et al. [56]. This suggests that overexpression of the *AmbHLH148* gene enhances the antioxidant capacity of tobacco plants, thereby enhancing their drought tolerance. These observations collectively suggest that *AmbHLH148* may be actively involved in osmotic regulation within tobacco plants during drought stress, thus contributing to an overall enhancement in the plant's ability to endure such adverse conditions.

However, the precise mechanisms by which *AmbHLH148* regulates drought stress warrant further investigation. For example, the *OsbHLH148* gene has been shown to enhance drought tolerance in rice by regulating the jasmonic acid metabolic pathway and interacting with the OsJAZ protein [42]. In addition, *OsbHLH148* positively regulates the expression of the *Osr40C1* gene, conferring drought tolerance in rice [65]. In future studies, we aim to further identify the types of signaling and metabolic pathways engaged by *A. mongolicum* through techniques such as the ChIP-seq assay, gel electrophoresis migration

assay (EMSA), yeast two-hybrid technique, and other experimental methods. We will also endeavor to screen potential downstream target genes of *A. mongolicum*, thereby gaining a comprehensive understanding of the regulatory network established by *A. mongolicum* with its downstream target genes and elucidating the regulatory mechanism of *AmbHLH148* in response to drought.

5. Conclusions

AmbHLH148 is evidently a nuclear-localized protein that may be a key regulator during drought stress in plants. These findings contribute to the identification of crucial candidate genes for genetic engineering aimed at enhancing crop drought tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122918/s1, Table S1. Primers sequence and usage. Figure S1. Homology analysis of the *AmbHLH148*. Figure S2. The electrophoresis detection of *AmbHLH148* PCR amplification. Figure S3. The flow of genetic transformation of *AmbHLH148* transgenic tobacco. Figure S4. Relative expression levels of the *AmbHLH148* gene in transgenic plants.

Author Contributions: The study was conceived and designed by Y.M.; X.Z. and B.Q. conducted the experiments and analyzed the data; B.F. and Y.Z. (Yongqing Zhai) analyzed the data; Y.Z. (Yan Zhao) provided the seeds; X.Z. and Y.M. wrote the manuscript. B.Q., F.S., L.N., Y.F. and Z.Y. performed the editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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