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The bHLH Transcription Factor PubHLH66 Improves Salt Tolerance in Daqing Poplar (*Populus ussuriensis*)

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Abstract: Elevated salinity negatively impacts plant growth and yield, presenting substantial challenges to agricultural and forestry productivity. The bHLH transcription factor family is vital for plants to cope with various abiotic stresses. However, it remains uncertain whether bHLH transcription factors can regulate salt stress in *Populus ussuriensis*. In the following study, a salt-induced bHLH transcription factor *PubHLH66* was identified from *P. ussuriensis*. PubHLH66 has a typical and conserved bHLH domain. Subcellular localization and yeast two-hybrid (Y2H) assays confirmed that it is a nucleus-localized transactivator and the activation region is located at the N-terminus. *PubHLH66-OE* and *PubHLH66-SRDX* transgenic *P. ussuriensis* were obtained through *Agrobacterium*-mediated leaf disc transformation. Morphological and physiological results demonstrated that *PubHLH66-OE* enhanced salinity tolerance, as indicated by reduced electrolyte leakage (EL), malondialdehyde (MDA), and H_2O_2 levels, along with increased proline contents and activities of peroxidase (POD) and superoxide dismutase (SOD). In contrast, *PuHLH66-SRDX* poplar showed decreased salt tolerance. Quantitative real-time PCR (RT-qPCR) confirmed that *PubHLH66* enhanced salt tolerance by regulating the expression of genes such as *PuSOD*, *PuPOD*, and *PuP5CS*, resulting in reduced reactive oxygen species (ROS) accumulation and an improved osmotic potential. Thus, *PubHLH66* could be a candidate gene for molecular breeding to enhance salt tolerance in plants. These results laid a foundation for exploring the mechanisms of salt tolerance in *P. ussuriensis*, facilitating the development of more salt-tolerant trees to combat the increasing issue of soil salinization globally.

Keywords: salt resistance; basic helix–loop–helix; gene function; reactive oxygen species (ROS) scavenging; proline

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1. Introduction

At the global level, abiotic stresses (i.e., extreme temperature, salinity, drought, and heavy metal pollution) severely affect the agriculture and forestry distribution, growth, and productivity. Salt stress is among the most challenging environmental stressors that plants encounter, particularly in dry and semi-dry areas [1,2]. Due to improper irrigation practices or the use of salt-contaminated water, the problem of secondary salinization is becoming more widespread. High salt concentrations not only induce water stress in plants but also reduce the nutrient absorption efficiency [3], ultimately suppressing photosynthesis [4] and reducing growth rates [5] and the scavenging of superoxide anion radical molecules [1,6]. As a result, enhancing salt tolerance in crops and woody plants has become a significant challenge in modern agriculture. In response to these difficulties, plants have developed intricate mechanisms for salt tolerance encompassing physiological and biochemical processes, as well as developmental and morphological adaptations [7].

Consequently, identifying key genes related to salt tolerance and elucidating their regulatory mechanisms is essential for developing salt-tolerant varieties that can effectively withstand salt stress.

Salt stress induces oxidative stress by increasing ROS levels, which play a role in various signaling pathways. Low concentrations of ROS are vital for various physiological processes, but excessive ROS production can cause significant damage to cellular structures, potentially resulting in cell death [8,9]. Plants employ a range of endogenous mechanisms to combat oxidative stress induced by challenging environmental conditions. These strategies encompass the activation of oxidative defense systems and the accumulation of osmolytes, which collectively enhance their resilience to stress. Plants utilize both enzymatic and non-enzymatic antioxidant mechanisms to manage the damage caused by ROS. Essential enzymatic antioxidant systems, including peroxidase (POD) and superoxide dismutase (SOD), function to scavenge reactive oxygen species (ROS) and inhibit lipid peroxidation [10,11]. Several research efforts have indicated that increased activity of antioxidant enzymes alleviates the detrimental effects of salt stress on various plant species, such as poplar (*Populus alba* × *P. glandulosa*) [12], rice (*Oryza sativa*) [13], rapeseed (*Brassica napus*) [3], *Reaumuria trigyna* [14], birch (*Betula platyphylla*) [15], and rose (*Rosa chinensis*) [16]. Additionally, proline functions as a non-enzymatic antioxidant within the oxidative defense system, playing a vital role in the neutralization of ROS. It also acts as an osmolyte help to stabilize cellular structures and maintain the osmotic balance under saline stress conditions [17].

The basic helix–loop–helix (bHLH) gene family represents one of the largest and most varied groups of transcription factors (TF) found in plants [18]. The basic domain is roughly 15 amino acids long at the N-terminus of a 60-amino acid region, enabling the transcription factor to bind to E-box (5′-CANNTG-3′), G-box (5′-CACGTG-3′) and T/G-box (5′-CACGTT-3′) elements, which contribute to plant stress tolerance [19,20]. bHLH family genes with diverse functions in plants have been identified and extensively documented, especially their involvement in responses during development, for example, root development [21], flowering regulation [22,23], leaf senescence [24], secondary growth [25], secondary metabolism [26], and seed germination [27,28]. Additionally, specific bHLH proteins, particularly those linked to hormone metabolism, are vital for plant defenses against pests and pathogens [29].

Moreover, bHLH proteins are engaged in responses to abiotic stresses, such as *TabHLH1* in wheat (*Triticum aestivum*) [30], *bHLH041* in cucumber (*Cucumis sativus*) [31], *VvbHLH1* in grape (*Vitis vinifera*) [32], *OsbHLH148* in rice (*O. sativa*) [33], *bHLH55* in maize (*Zea mays*) [34], and *MdbHLH3* in apple (*Malus domestica*) [35]. The *AmDEL* in Antirrhinum (*Antirrhinum majus*) could enhance tolerance to drought and salt stress [36]. TabHLH1-mediated accumulation of osmolytes and maintenance of cellular ROS homeostasis play roles in enhancing tolerance to drought and salt stress [30]. Overexpression of *VvbHLH1* elevated proline levels, lowered malondialdehyde levels, and mitigated membrane injury, which in turn conferred cold stress tolerance in Arabidopsis [32]. Overexpression of *MdbHLH4* in *Arabidopsis thaliana* and *M. domestica* negatively impacted plant cold tolerance by inhibiting *MdCBF1/3* expression while increasing *MdCAX3L-2* expression levels [37]. These studies clearly indicate that the bHLH genes in different species may exhibit functional differentiation. While these studies provide a solid foundation for understanding bHLH functions in plants, more in-depth research is needed to fully elucidate the biological and regulatory roles of many of these transcription factors in poplar.

Poplar is one of the most widely cultivated economic forest trees worldwide and a key timber species in China that is noted for its cold tolerance, adaptability, resilience, and small genome size [38]. It also serves as a model organism for studying the physiological and molecular mechanisms of stress resistance in woody plants [39]. *Populus ussuriensis* is predominantly found in the northeastern part of China and exists mainly in a wild state [40]. It has the characteristics of being easily propagated vegetatively and transformed genetically, making it a valuable genetic resource for selective breeding. Transcription

factors involved in responses to drought [40], zinc (Zn) [41], phosphorus, or nitrate [42,43] have been studied in *P. ussuriensis*, but their effects on salt stress have yet to be explored.

Our research identified that the bHLH TF named *PubHLH66* was highly induced under salt stress; however, the function of *PubHLH66* in the salt tolerance of poplar is still unclear. *PubHLH66*-overexpressing transgenic *P. ussuriensis* was shown to exhibit increased salt tolerance through the promotion of ROS scavenging, while the opposite results were obtained for *PubHLH66-SRDX* lines. Interestingly, when challenged with salt stress, an elevation in the expression levels of genes associated with POD, SOD, and proline biosynthesis was detected in overexpressing transgenic poplars. These findings establish a foundational basis for further investigation into the roles of bHLH transcription factors (TFs) in poplar, as well as in other significant plant species.

2. Materials and Methods

2.1. Plant Materials and Stress Treatment

Populus ussuriensis was utilized for gene cloning, expression analysis, and genetic transformation. Sterile poplar seedlings grown on 1/2 Murashige and Skoog (MS) (Phytotech, Trenton, NJ, USA) medium supplemented with 2% sucrose (Sangon Biotech, Shanghai, China) and 0.3% Gelrite (Duchefa, Haarlem, The Netherlands). The cultivation conditions were a tissue culture room with a 16 h/8 h light/dark photoperiod and a supplemental light intensity of 70 μmol m^{-2} s^{-1} at 24 °C. Two-week-old in vitro poplar seedlings were subjected to 150 mM NaCl (Sigma, Louis, MI, USA). The treatments lasted for 0, 6, 12, 24, 48, or 72 h. Each treatment included three biological replicates. Roots, stems and leaves were promptly frozen in liquid nitrogen for RNA extraction.

For soil culture, 1-month-old in vitro poplar seedlings were transplanted and grown individually in pot containing autoclaved peat soil in the greenhouse (24 °C, 16 h of light and 8 h of darkness, 60% humidity level) in Shenyang, China, located at 123°27′ N and 41°40′ E. For salt stress, uniform wild type (WT) and transgenic *P. ussuriensis* in 11 cm pots filled with a uniform dry weight of soil were equally watered before the salt tolerance experiment, in which they were treated with a 200 mM NaCl aqueous solution for 7 consecutive days.

2.2. RNA Extraction, cDNA Synthesis and RT-qPCR Analysis

RNA extraction was performed with the cetyltrimethylammonium bromide (CTAB) method with minor modifications [44]. To summarize, samples were finely ground into a fine powder using a mortar and liquid nitrogen and then combined with extraction buffer at 65 °C for 5 min. Subsequently, the mixture was centrifuged at 10,000× *g* for 5 min at 4 °C, and the supernatants were carefully transferred to new tubes. To achieve further purification of the extracts, the supernatants underwent two rounds of extraction with an equal volume of chloroform (Keshi, Chengdu, China) and saturated phenol (Solarbio, Beijing, China). The supernatants were subsequently combined with an equal volume of 8 M LiCl (Sigma, Louis, MI, USA) and absolute ethanol (Hengxing, Tianjin, China). The mixture was allowed to stand for 20 min to facilitate RNA precipitation. DNase I (ThermoFisher Scientific, Waltham, MA, USA) was then used to remove DNA contamination. Complementary DNA (cDNA) was synthesized using the HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). RT-qPCR was performed using the ChamQ Universal SYBR gPCR Master Mix with a Light Cycler 480 (Roche Applied Science, Penzberg, Germany). The $2^{-\Delta\Delta CT}$ method was used to determine relative expression [41], with *PuActin* serving as reference gene. Each sample was analyzed in three biological replicates, each consisting of three technical replicates. The primers utilized for this experiment are provided in Table S1.

2.3. Phylogenetic Analysis

Homologs of PubHLH66 from multiple species were identified using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 3 January 2024)). Multiple

alignments were performed using the ClustalX V1.83 program [45]. Phylogenetic construction of PubHLH66 was generated using MEGA 7.0 software utilizing the neighbor-joining (NJ) method with 1000 bootstrap replicates [46].

2.4. Transcriptional Activation Assay and Subcellular Localization Analysis

Transcriptional activation assays of the PubHLH66 protein were performed. The full-length coding sequence (CDS) of *PubHLH66* and two truncated versions of *PubHLH66* were cloned into the pGBKT7 vector. The pGBKT7-PubHLH66, pGBKT7-PubHLH66^{N241} and pGBKT7-PubHLH66^{C214} plasmids, along with the pGBKT7 vector (negative control), were transferred into yeast cells (Clontech, Dalian, China) for the yeast two-hybrid (Y2H) assay. Yeast was cultivated for 3–5 days at 30 °C on synthetic dropout (SD)/-Trp medium containing X-α-Gal or on triple dropout supplements (SD/-Trp/-Ade/-His) to select for positive clones. Images were taken with a digital camera (Canon, EOS 750D, Tokyo, Japan). Each assay was conducted in triplicate.

For the subcellular localization analysis, the CDS sequence of *PubHLH66* lacking the termination codon was inserted into the pBI121 vector (35S::GFP) that contains a green fluorescent protein (GFP) driven by the CaMV (cauliflower mosaic virus) 35S promoter. The respective 35S::*PubHLH66*::GFP fusion vector and the empty vector 35S::GFP (control) were transiently transformed into epithelial cells of the onions via particle bombardment. Observations of GFP fluorescence and fluorescence images were carried out using an inverted metallurgic microscope (Axio Vert. A1, Zeiss, Oberkochen, Germany). All primers used in this study are listed in Table S1.

2.5. Vector Construction and Plant Transformation

35S::*PubHLH66*::GFP was overexpression construct. For the dominant suppression construct, the CDS of *PubHLH66* without termination codon was linked to a 27 bp DNA sequence encoding the SUPERMAN repression domain X (SRDX) and cloned into pBI121 [47]. All recombinant plasmids were transferred into *Agrobacterium tumefaciens* strain EHA105.

The *A. tumefaciens*-mediated leaf disc method was used to transfer *P. ussuriensis* [48]. Briefly, excised leaves from one-month-old tissue culture seedlings of *P. ussurience* were immersed in the bacterial suspension for 20 min. After this treatment, the explants were transferred to a selection medium containing 50 mg L^{-1} of kanamycin (Phytotech, Trenton, NJ, USA) and 200 mg L^{-1} of cefotaxime (Solarbio, Beijing, China) until resistant shoots emerged. The kanamycin -resistant shoots were then excised and placed on 1/2 MS medium supplemented with 50 mg L^{-1} of kanamycin to facilitate rooting. Genomic DNA and total RNA were extracted from the putative transgenic poplar for PCR and RT-qPCR analyses to verify the *PubHLH66-OE* and *PubHLH66-SRDX* lines. Eleven *PubHLH66-OE* lines and ten dominant suppression lines (*PubHLH66-SRDX*) in *P. ussuriensis* were generated. The transgenic statuses of these lines were verified through PCR and RT-qPCR (Figure S1A–D). Furthermore, OE3 and OE7, or SRDX1 and SRDX3 lines with the most elevated expression levels for each were chosen for more detailed analyses. Primers utilized for vector construction are shown in Table S1.

2.6. Measurement of Physiological Indices Related to Stress Tolerance

Electrolyte leakage (EL) and the malondialdehyde (MDA) content are critical indicators for assessing cell membrane damage and lipid peroxidation in tissues [41]. EL and the MDA content were measured accordance with the methodology previously described [41]. For the EL analysis, leaves were placed in 50 mL tubes containing 40 mL of deionized water. Following a 15 min vacuum treatment and shaking at 120 rpm for 2 h, the initial conductivity (C1) was measured. After boiling for 15 min and cooling, C2 and deionized water (C0) were measured again. The EL was evaluated using the following equation: % ion leakage = $[(C1 - C0)/(C2 - C0)] \times 100$.

The hydrogen peroxide (H_2O_2, A007-1-1) content, proline content (A107-1-1), and superoxide dismutase (SOD, A001-1) and peroxidase (POD, A084-3-1) activities were mea-

sured using customized kits (Nanjing Jiancheng, Nanjing, China). Histochemical staining with 3,3′-diaminobenzidine (DAB, Solarbio, Beijing, China) and nitro blue tetrazolium (NBT, Solarbio, Beijing, China) was performed to visualize the in situ accumulation of H_2O_2 and O_2^-, respectively [40]. Three biology replicates were executed, each with three technical replicates, ensuring a robust statistical analysis.

2.7. Expression Patterns of Related Genes Regulated by PubHLH66

RT-qPCR was conducted to assess the expression levels of stress resistance genes in *PubHLH66-OE*, *PubHLH66-SRDX*, and WT poplar with or without salt stress. Each experimental group included at least three biological replicates, with three technical replicates conducted for each. Table S1 contains the primers used in this experiment.

2.8. Statistical Analysis

All data were expressed as the means ± standard deviations (SDs) of three biological replicates, and statistical significance was determined using one-way analysis of variance (ANOVA) followed by Student's *t*-test, conducted with GraphPad Prism 8.

3. Results

3.1. Sequence Analysis and Gene Expression Pattern of PubHLH66

PubHLH66 from *P. ussuriness* was cloned, consisting of 1368 base pairs and encoding 455 amino acids. This protein has a relative molecular mass of 47.87 kDa and was uploaded in the NCBI GenBank database as PQ439370. Multiple sequence alignment results showed that PubHLH66 possesses a typical bHLH domain at the C-terminus (Figure 1A). The result indicated that *PubHLH66* is classified as a member of the bHLH gene family, which underscores its potential involvement in development and responses to environmental stimuli.

The phylogenetic analysis revealed that PubHLH66 is classified within subfamily 5. It is closely related to the Citrange CtbHLH66 (its amino acid sequence shares 62.65%), apple MdbHLH66 (55.56%), rice OsbHLH66 (43.04%), and Arabidopsis AtbHLH66 (41.76%) genes (Figure 1B). Therefore, we designated the poplar bHLH gene as *PubHLH66*.

Furthermore, under NaCl stress conditions, the expression of *PubHLH66* was markedly elevated (Figure 1C). In roots, it reached a 10.06-fold increase at 48 h, while in stems, its expression rose by 6.28-fold at 24 h. In leaves, the expression peaked at 19.94 fold after 12 h (Figure 1C). These findings indicate that the *PubHLH66* gene can be significantly induced by NaCl treatment.

3.2. PubHLH66 Is a Nucleus-Localized Transactivator

To confirm the subcellular localization of PubHLH66, 35S::PubHLH66-GFP and 35S::GFP (control) were transiently introduced into onion epidermal cells (Figure 2). Fluorescence signals were observed under a fluorescence inverted microscope. The results showed that the empty vector demonstrated fluorescence distributed across the entire cell. In contrast, 35S::PubHLH66-GFP displayed fluorescence signals only in the nucleus. Furthermore, the green fluorescent signal of PubHLH66-GFP perfectly overlapped with DAPI (nuclear marker) indicating that the PubHLH66 protein, a transcription factor, is localized in the nucleus (Figure 2A).

To investigate the domain of PubHLH66 responsible for its transcriptional activity, the CDS of *PubHLH66* was fused with the GAL4 DNA-binding domain sequence of pGBKT7. Additionally, two truncated versions were created: one containing the first 241 N-terminal amino acid residues (designated as pGBKT7-PubHLH66^{N241}) and another comprising the C-terminal 214 amino acid residues (referred to as pGBKT7-PubHLH66^{C214}). These constructs were then transformed into the yeast strain Y2HGold, respectively. All constructs were able to grow on SD/-Trp plates (Figure 2B). But only pGBKT7-PubHLH66 and PubHLH66^{N241} showed good growth on SD/-Trp/-His/-Ade medium and X-α-gal was activated (Figure 2B). These findings confirmed that PubHLH66 is a nucleus-localized transactivator and the activation region is located at the N-terminus.

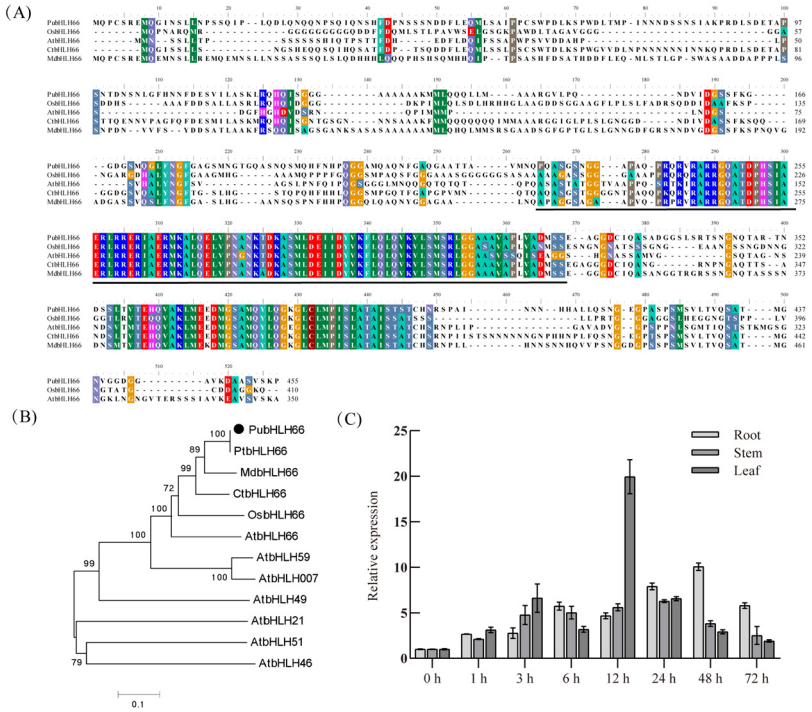

Figure 1. Sequence analysis and gene expression pattern of *PubHLH66*. (**A**) Multiple sequence alignments and analysis of PubHLH66 with homologous bHLH proteins from different plant species. The accession numbers corresponding to these proteins in NCBI ID are OsbHLH66 (XP_015627343), AtbHLH66 (BAD44153), MdbHLH66 (XP_028964276), and CtbHLH66 (XP_006473971). (**B**) Analysis of the phylogenetic tree constructed using the neighbor-joining (NJ) method, with a bootstrap test performed using 1000 iterations in MEGA. The black dot represents PubHLH66. The scale bar represents 0.1 substitutions per site. (**C**) Relative expression level of *PubHLH66* in the root, stem and leaves of plants under 150 mM NaCl stress determined using RT-qPCR. Error bars represent the variability among three biological replicates. The *x*-axis represents the time points following treatment with 150 mM NaCl.

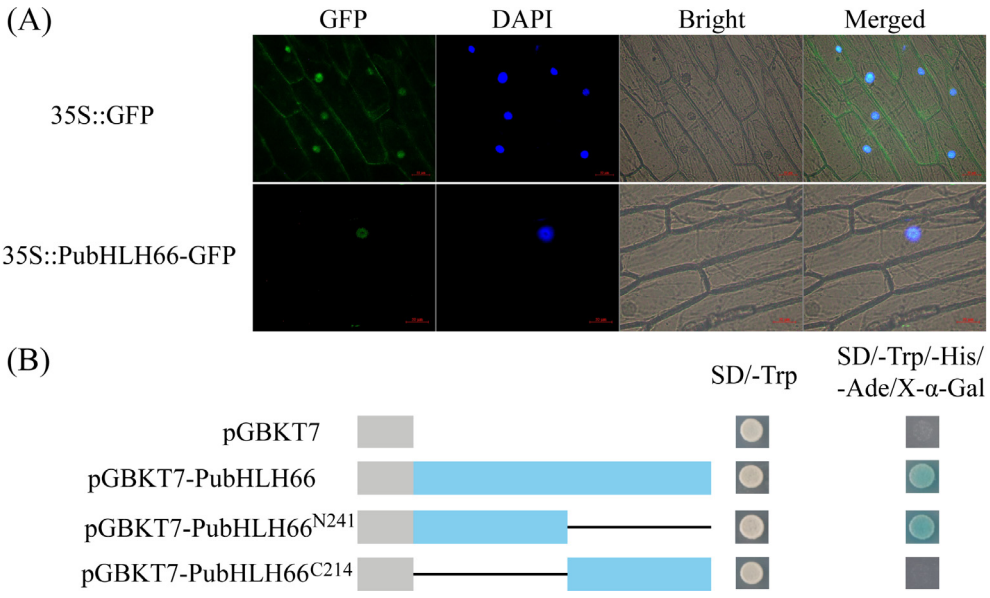

Figure 2. Subcellular localization and transactivation activity of PubHLH66. (**A**) Subcellular localization of PubHLH66. The 35S::GFP (control) and 35S::PubHLH66-GFP translational fusion constructs were transiently introduced into onion epidermal cells, and DAPI was utilized as a marker for the

nucleus. Bar = 50 μm. (**B**) pGBKT7-PubHLH66, pGBKT7-PubHLH66^{N241}, pGBKT7-PubHLH66^{C214}, and pGBKT7 (negative control) were transformed in the Y2H Gold yeast strain. Yeast transformants were cultured in either SD/-Trp or SD/-Trp/-His/-Ade/X-α-Gal media. LacZ activity was measured in the presence of X-α-Gal with pGBKT7. The gray bars represent BD, and blue bars represent gene segment of PubHLH66.

3.3. PubHLH66 Enhances Salt Tolerance in P. ussuriensis

In order to analyze the potential biological function of *PubHLH66* in poplar, plants of similar size, including *PubHLH66-OE* lines, *PubHLH66-SRDX* lines, and WT plants, in soil were used. To assess salt tolerance, 30-day-old WT and *PubHLH66* transgenic plants were subjected to a 200 mM NaCl treatment (Figure 3). In this study, the transgenic lines did not differ compared with WT under stress-free conditions. After exposure to salt stress, the *PubHLH66-OE* lines displayed higher chlorophyll contents, dry weights (DWs), and survival rates compared to WT plants (Figure 3A–D). In contrast, the *PubHLH66-SRDX* lines showed greater leaf wilting, along with reduced chlorophyll contents, dry weights, and survival rates (Figure 3A–D). The results indicated that PubHLH66 could enhance salt tolerance in transgenic poplar.

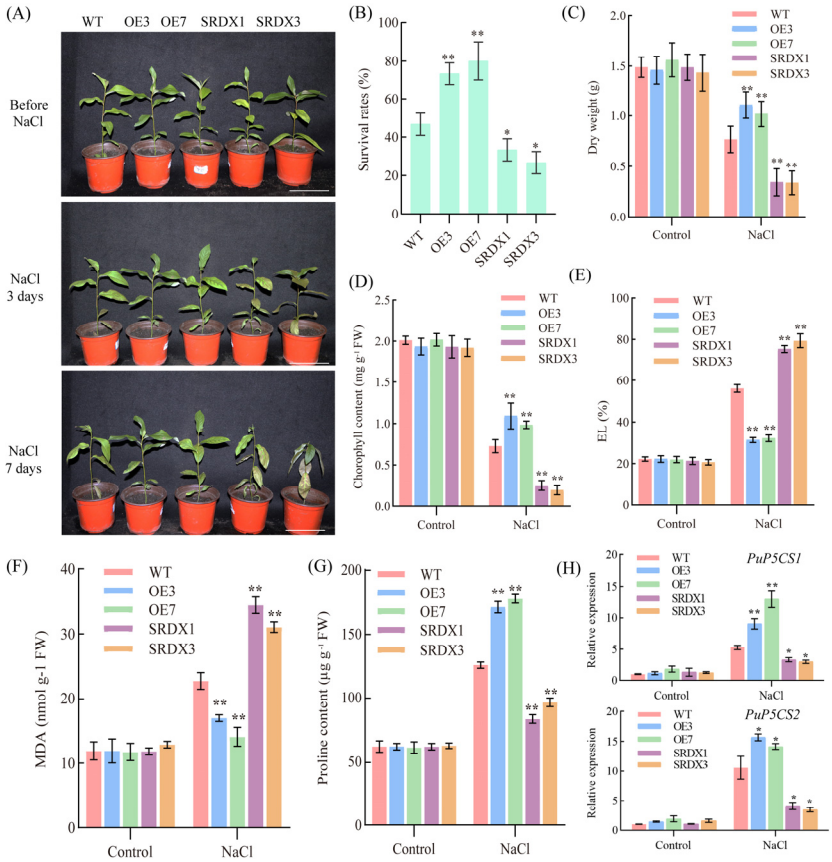

Figure 3. Salt tolerance analysis of the *PubHLH66* transgenic *P. ussuriensis*. (**A**) Phenotype of *PubHLH66* transgenic and WT poplars during salinity treatments. Bars, 10 cm. (**B**) Survival rates, (**C**) dry weights, (**D**) chlorophyll contents, (**E**) electrolyte leakage (EL), (**F**) malondialdehyde (MDA) contents, and (**G**) proline contents of the poplars after growth under normal and NaCl stress conditions for 7 d. (**H**) The expression pattern of *PuP5CS1* and *PuP5CS2*. WT and *PubHLH66* transgenic *P. ussuriensis* were subjected to 150 mM NaCl for 24 h. The WT line was used as a control and set to 1. The *PuActin* gene served as a housekeeping gene. Asterisks (* $p < 0.05$ and ** $p < 0.01$) indicate significant differences determined using Student's *t*-test compared to WT plants.

To further investigate the function of *PubHLH66*, we measured several physiological indicators. Under normal growth conditions, EL and MDA levels in WT and *PubHLH66* transgenic lines were similar, with no significant differences observed (Figure 3E,F). However, following exposure to salt stress, both EL and MDA levels rose in the WT and *PubHLH66* transgenic lines. The EL of the OE3 (25.7%) and OE7 (27.13%) lines were significantly lower than that in the WT line (59.5%), while the SRDX lines exhibited greater increases of 21.8% and 24.3%, respectively, compared with the WT line (Figure 3E). Notably, the overexpressing transgenic poplars exhibited a significantly reduced MDA content compared to WT plants, showing reductions of 25.2% and 37.8%, respectively (Figure 3F). These results suggest that *PubHLH66* enhances salt tolerance in poplar, likely by reducing cell membrane damage and minimizing lipid peroxidation.

3.4. Proline Biosynthesis Is Regulated by PubHLH66

Proline is essential for maintaining the ion balance in plants, aiding in the reduction of salt stress toxicity and improving overall tolerance [49]. The proline levels were assessed in both WT and *PubHLH66* transgenic lines. After comparing WT plants and *PubHLH66* transgenic plants, it was observed that all lines exhibited similar proline levels under normal conditions (Figure 3G). Following salt stress, proline accumulation increased in all lines. Notably, the OE3 and OE7 lines showed significantly higher proline levels, exhibiting levels that were 36% and 41.1% higher than that in WT poplar, respectively. Meanwhile, the SRDX lines displayed considerably lower proline contents by about with 33.7% and 23.4% compared to wild type (WT) poplar (Figure 3G).

Next, two proline biosynthesis-related genes, *PuP5CS1* and *PuP5CS2* (for D-1-pyrroline-5-carboxylate synthetase), were examined in the leaves of the WT, *PubHLH66-OE* and *PubHLH66-SRDX* lines. The expression levels of *PuP5CS1* and *PuP5CS2* were similar in all studied lines under normal conditions (Figure 3H). However, under salt stress, the OE3 and OE7 lines exhibited a significant increase in the expression of both *PuP5CS1* and *PuP5CS2*, while the SRDX lines showed a notable decrease in comparison to the wild type (WT) poplars (Figure 3H). These findings indicate that *PubHLH66* is involved in the regulation of proline biosynthesis when plants are subjected to salt stress.

3.5. Overexpression PubHLH66 Reduces Oxidative Damage in P. ussuriensis

To explore how salt stress influences the production of ROS, we conducted an analysis of O_2^- and H_2O_2 accumulation in the leaves of the WT and transgenic lines. For this purpose, we employed NBT staining to detect O^{2-} and DAB staining for H_2O_2. Under control conditions, DAB and NBT staining results showed no significant variations among *PubHLH66-OE*, *PubHLH66-SRDX*, and WT plants (Figure 4A,B). However, after being subjected to salt stress, the staining intensity of OE lines was noticeably lighter, while SRDX lines exhibited a deeper staining intensity (Figure 4A,B). The results indicated that *PubHLH66-OE* transgenic lines exhibited lower levels of ROS accumulation following salt treatment. The staining results are consistent with the observed decrease in H_2O_2 levels and the elevated activities of SOD and POD in the overexpressing transgenic lines under salt stress when compared to the wild type (WT) plant lines (Figure 4C–E). These findings suggest that *PubHLH66* enhances resistance to salinity in poplar by boosting ROS scavenging through increased antioxidant enzyme activity.

3.6. PubHLH66 Can Upregulate the Expression of Antioxidant-Related Genes

To determine if *PubHLH66* controls salt tolerance in poplar through the ROS pathway, we investigated two *PuPOD* genes and two *PuSOD* genes homologous to well-characterized Arabidopsis genes known for their SOD and POD activities using RT-qPCR (Figure 5). Under regular growth conditions, the expression levels of these genes did not show significant differences between the transgenic and WT plants (Figure 5A–D). However, under salt stress, the expression levels of POD and SOD-related genes in the OE transgenic lines increased significantly and were reduced markedly in the SRDX lines compared with

the WT plants (Figure 5A–D). These findings suggest that PubHLH66 contributes to improved salt tolerance in overexpressing lines by regulating the expression of *POD* and *SOD*-related genes.

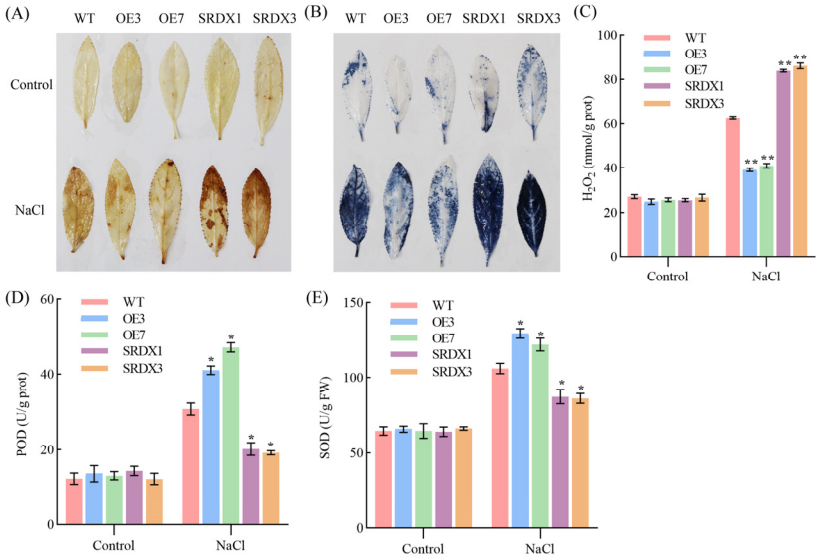

Figure 4. Analysis of the antioxidant capacity of *PubHLH66*. (**A**,**B**) DAB and NBT staining. Poplar leaves subjected to NaCl treatment were infiltrated with DAB (**A**) for hydrogen peroxide (H_2O_2) detection and with NBT (**B**) for superoxide (O_2^-) detection. (**C**) H_2O_2 content assay (**D**,**E**) measurement of POD and SOD activities in the poplars after growth under normal and NaCl stress conditions for 7 d. Data are presented as the means and SDs of three independent experiments. Asterisks (*) represent $p < 0.05$ and (**) $p < 0.01$.

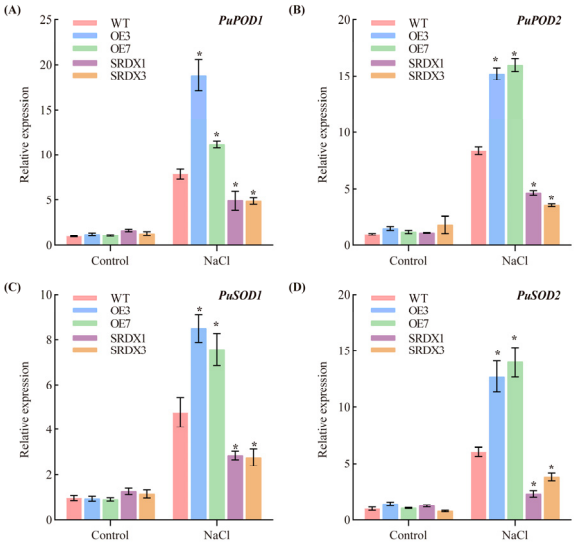

Figure 5. PubHLH66 regulates the expression of *PuPOD*s and *PuSOD*s. (**A**,**B**) The expression pattern of *PuPOD*s, (**C**,**D**) the expression pattern of *PuSOD* genes after exposure to 150 mM NaCl for 24 h. Wild type (WT) plants were utilized as a control and normalized to 1, while the *PuActin* gene served as the internal control. The asterisk (*) represents $p < 0.05$.

4. Discussion

Salinization affects 20% of the world's cultivated land, which represents a major environmental challenge that negatively impacts plant growth, agricultural productivity, and afforestation efforts globally [50]. Poplar is a perennial woody plant of considerable economic and ecological importance due to its rapid growth and substantial biomass

production [51]. bHLH transcription factors are critical for mediating stress responses. So far, many bHLH transcription factors have been shown to be critical for mediating plant responses to salt stress [10,52–54]. Nevertheless, the function of bHLH TFs in salt tolerance within *Populus ussuriensis* still not well understood.

4.1. PubHLH66 Enhances Salt Tolerance

Gene expression profiles can reveal insights into potential biological functions. It is suggested that genes activated by specific abiotic stressors may contribute positively to tolerance to that stress. In this study, we detected a salt-induced bHLH transcription factor in *P. ussuriensis* that was tightly grouped with its homologous proteins Citrange CtbHLH66, apple MdbHLH66, rice OsbHLH66, and Arabidopsis AtbHLH66, and therefore was named PubHLH66 (Figure 1B). We successfully generated transgenic *P. ussuriensis* with both *PubHLH66-OE* and *PubHLH66-SRDX*. Morphological and physiological assessments provided evidence that this gene positively influences stress tolerance in *P. ussuriensis*. Overexpressing *PubHLH66* exhibited enhanced salt tolerance with higher biomass, chlorophyll content, survival rate, and POD and SOD activities, and lower MDA content sand EL, whereas *PubHLH66-SRDX* lines showed reduced tolerance. Importantly, *PubHLH66* did not affect the growth and development of the transgenic lines. Overall, these findings imply that *PubHLH66* may serve as a valuable candidate gene for the development of new poplar varieties with enhanced salinity stress resistance.

4.2. Overexpression of PubHLH66 Decreased ROS Levels to Enhance Stress Tolerance

The excessive accumulation of reactive oxygen species (ROS) in plants can cause oxidative stress, resulting in damage to cell membranes and lipids. To mitigate this, plants have developed mechanisms to detoxify ROS and maintain redox homeostasis; SOD and POD are key enzymes involved in the clearance of ROS [55,56]. For instance, overexpression of *PagbHLH35* enhances salt tolerance by increasing the activities of POD and SOD, which are crucial for ROS scavenging and protecting plant cells from oxidative damage [52]. Overexpression of *PavbHLH106* in tobacco (*Nicotiana benthamiana*) enhances cold resistance by promoting ROS scavenging through the increased activity of antioxidant enzymes (POD and SOD) [57]. *MdbHLH160* also elevated the enzyme activity and upregulated *MdSOD1*, which in turn helped to lower excessive ROS levels [58]. In agreement with these reports, *PubHLH66-OE* lines displayed increased SOD and POD activities in response to NaCl treatment (Figure 4D,E). These findings indicate that the overexpressing transgenic lines experience less oxidative damage to their membrane lipids compared to WT plants. Additionally, histological staining using DAB and NBT demonstrated a decrease in ROS levels in OE lines and increase in ROS levels in SRDX lines under salt stress (Figure 4A,B). These findings further corroborate the role of *PubHLH66* in improving tolerance to oxidative stress. *PubHLH66* is involved in enhancing salt stress, which helps to reduce oxidative damage to cell membranes by enhancing the ability to scavenge ROS.

4.3. PubHLH66 Controls the Biosynthesis of Proline

Proline serves as an important osmolyte that accumulates in significant amounts in response to environmental stressors, facilitating osmotic adjustment in organisms. It plays a crucial role in cellular adaptation and survival under adverse conditions [59]. The *P5CS* gene encodes a crucial rate-limiting enzyme that plays a vital role in proline biosynthesis, which is encoded by two closely related *P5CS* genes (*P5CS1* and *P5CS2*) [60]. *CabHLH035* increases salt tolerance in pepper, which directly binds to the promoters of the *CaP5CS* gene and positively regulates their expression to enhance proline accumulation [61]. In this study, *PubHLH66-OE* lines exhibited a significantly higher proline content and an upregulation of *PuP5CS1* and *PuP5CS2*, while they were downregulated in *PubHLH66-SRDX* lines compared to WT plants (Figure 3G,H). Therefore, it can be suggested that *PubHLH66* positively regulates the expression of the *P5CS* genes and proline accumulation in plants, helping to

maintain the osmotic balance between the intracellular and extracellular environments and thereby enhancing plant tolerance to salt stress.

4.4. PubHLH66 May Regulate Target Genes Through Binding to the G-Box or E-Box

TFs bind interact with specific cis-acting elements located upstream of stress resistance genes, thereby regulating their expression and enhancing plant adaptability to environmental stresses [62]. Previous research has demonstrated that bHLH TFs can activate downstream gene expression by binding to the E-box (CANNTG) or T/G-box (CACGTT) elements found in the promoter regions of target genes [63]. For instance, bHLH106 combines the functions of various genes via their G-box elements to enhance salt tolerance in Arabidopsis [64]. MdbHLH160 enhances activity of SOD enzyme activity during drought conditions by directly upregulating the expression of *MdSOD1* by binding to the E-box (CACGTG) and T/G-box (CACGTT) and interacting with the MdSOD1 protein in the nucleus, thereby alleviating oxidative stress damage in the nucleus under drought conditions and thus mitigating oxidative stress damage under drought conditions [58]. These findings suggest that bHLH transcription factors play pivotal roles in modulating the expression of downstream antioxidant genes by interacting with E-box or T/G-box (CACGTT) motifs in their promoters, thereby enhancing the stress resistance of transgenic plants. In our study, we observed a significant upregulation of the proline synthesis-related genes *PuP5CSs*, *PuPOD*s and *PuSOD*s in the *PubHLH66-OE* lines and downregulation in *PubHLH66-SRDX* lines (Figures 3G,H and 5A–D). Moreover, the prediction of cis-acting elements shows that the promoters of the *PuP5CS1*, *PuSOD* and *PuPOD* genes contain multiple E-box motifs, with the *PuP5CS2* promoter also containing G-box motifs. Based on these observations, we speculate that PubHLH66 regulates the expression of antioxidant enzyme genes through its binding to E-box or G-box elements, thereby contributing to salt tolerance.

5. Conclusions

PubHLH66 is a nucleus-localized transactivator and induced by salt stress, has been successfully cloned, and responds to salt stress by regulating the expression of genes responsive to abiotic stress, such as *PuP5CS*s, *PuSOD*s, and *PuPOD*s, leading to increased proline levels and reduced ROS accumulation. This highlights the importance of *PubHLH66* in orchestrating antioxidant responses and maintaining membrane integrity under saline stress conditions. These results will contribute to lay the groundwork for developing high salt-tolerant and superior new varieties of poplar, and it is expected that these salt-tolerant cultivars will effectively utilize and improve saline–alkali soils, thereby expanding the potential planting range of poplar.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15112051/s1: Table S1: Primer sequences used in the analysis of *PubHLH66*; Figure S1: PCR and RT-qPCR were used to confirm the *PubHLH66-OE* and *PubHLH66-SRDX* lines.

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