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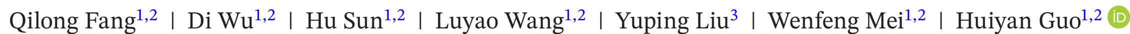
A bHLH Transcription Factor Confers Salinity Stress Tolerance in *Betula platyphylla*



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ABSTRACT

Basic helix–loop–helix (bHLH) proteins comprise a large family of transcription factors that are involved in plant growth and development, as well as responses to various types of environmental stress. *Betula platyphylla* (birch) is a pioneer tree species in secondary forest that plays a key role in maintaining ecosystem stability and forest regeneration, but few bHLHs involved in abiotic stress responses have been unveiled in birch. In this study, nine BpbHLH TFs related to stress responses in the birch genome were identified. Quantitative real-time polymerase chain reaction (RT-PCR) analysis indicated that the expression of these TFs can be induced by salt stress, and the expression of *BpbHLH1* was higher than that of other *BpbHLH* genes. Particle bombardment analysis revealed that BpbHLH1 was localized to the nucleus. Yeast transformation found that BpbHLH1 has transcriptional activation activity. We generated *BpbHLH1*-overexpressing and silencing transgenic birch plants and subjected them to salt stress analysis. *BpbHLH1* can enhance the salt tolerance of birch plants by increasing the reactive oxygen species scavenging ability and inhibiting cell death. Yeast one-hybrid, ß-glucuronidase, and chromatin immunoprecipitation assays revealed that BpbHLH1 can regulate the expression of target genes involved in stress resistance by binding to the E-box-1, E-box-2 and G-box elements in their promoters. The results of this study enhanced our understanding of the salt tolerance conferred by BpbHLH TFs in *B. platyphylla* and identified useful genes for the breeding of novel birch germplasm.

1 | Introduction

Abiotic stress, including extreme temperatures (heat, chilling, and freezing), drought, heavy metals, and salinity, inhibits plant growth and development (Liang et al. 2017; Zhang et al. 2021). Plants have evolved various acclimatization mechanisms to survive under stress conditions, and the expression of specific sets of genes plays a key role in these acclimatization mechanisms (Jia et al. 2023). Transcription factors (TFs) play key roles in regulating the stress tolerance of plants.

The basic helix–loop–helix (bHLH) TF family is one of the largest families of eukaryotic TFs, and bHLH TFs are widespread in plants. The first plant bHLH TF was identified in *Zea mays*, and 162, 122, 167, and 155 bHLH TFs have been identified in *Arabidopsis thaliana*, *Oryza sativa*, *Phaseolus vulgaris*, and *Capsicum annuum*, respectively (Bailey et al. 2003; Kavas et al. 2015; Li et al. 2006; Zhang et al. 2020). Some studies indicate that bHLH TFs can play a role in reproduction, such as in flower development, fruit development, and the biosynthesis of secondary metabolites, such as anthocyanins

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(Farquharson 2016; Li et al. 2022; Yu et al. 2021). In addition, they play key regulatory roles in the responses of plants to stress conditions. For example, the expression of a previously identified bHLH TF in peanut has been shown to be induced by drought stress, and its overexpression can enhance the drought tolerance of transgenic plants in both the seedling and adult stages compared with wild-type plants (Li et al. 2021). Overexpression of *OsbHLH38* in rice increases seedling salt tolerance, and knockout of *OsbHLH38* increases sensitivity to salt stress (Du et al. 2023). Overexpression of *MebHLH18* in cassava can promote low-temperature tolerance by decreasing the low temperature-induced leaf abscission rate (Liao et al. 2023). However, most studies of bHLH TFs have focused on model and crop plants. Although some molecular approaches have been used to reveal the roles of bHLH TFs in plant growth and development, metabolic pathways, and stress responses, much remains to be learned regarding the biological functions and regulatory mechanisms of these intriguing bHLH TFs.

Betula platyphylla (birch) is a broad-leaved deciduous tree with high economic, ornamental, and ecological value (Chen et al. 2021; Ritonga et al. 2021). The global area of saline-alkali land has reached 954 million hectares, with an annual expansion rate of 10% (Yan et al. 2022); this substantially restricts forestry development. There is thus a need to cultivate new varieties of salt-tolerant forest trees for forestry production. A total of 128 BpbHLH TFs of *B. platyphylla* have been identified in a previous study (Zhao et al. 2023). Here, nine BpbHLH TF genes involved in stress responses in the birch genome were identified, and their expression under salt stress was analyzed. Furthermore, the functions and downstream regulatory mechanism of a BpbHLH TF that regulates salt stress tolerance in plants were elucidated. The results of our study have implications for improving the stress tolerance of forest trees.

2 | Materials and Methods

2.1 | Plant Growth and Salt Stress Treatments

The seeds of *B. platyphylla* were collected in the secondary forest in Hengyang (the People's Republic of China), located at 26°8′N and 112°14′ E. The seeds were sown in plastic pots with a mixture of perlite/vermiculite/soil (1:1:3) and subjected to a 16 h/8 h light/dark cycle, an average temperature of 2°C, and 60%–70% relative humidity in a greenhouse. The pots were watered twice daily until seed germination, after which they were watered once daily.

After 2 months of cultivation, healthy birch seedlings approximately 20 cm in. height were treated with 0.2 M of NaCl solution for 0.5, 1, 3, 6, 12, 24, and 48 h in reverse time order. That is, plants with the longest treatment time were treated first, and plants with the shortest treatment time were treated last. The control plants were treated with fresh water for 48 h. The roots of birch seedlings were collected. Three independent biological replicates were conducted, and each replicate comprised six seedlings. All samples were quickly frozen in liquid nitrogen and stored at −80°C.

2.2 | RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

The RNA of each sample was extracted using a Universal Plant RNA Extraction Kit (BioTeke Corporation, China) from 100 mg of birch root samples, and cDNA was synthesized from approximately 1 μg of total RNA using PrimeScript IV First-Strand cDNA Synthesis Mix (TaKaRa, Japan).

PCR reactions were conducted in a volume of 20 μL containing 10 μL of SYBR Green Real-time PCR Master Mix (Novoprotein Scientific Inc, China), 1 μL of cDNA template, 1 μL of the forward primer (10 μM), 1 μL of the reverse primer (10 μM), and the rest with ultrapure H_2O. Ubiquitin (GenBank accession number: FG065618) and tubulin (GenBank accession number: FG067376) were used as the internal controls, with primer sequences listed in Table S1. The thermal cycling conditions were as follows: 95°C for 30 s; 44 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s; and 60°C for 15 s. The relative expression levels of nine *BpbHLH* genes were then calculated using the delta–delta CT method (Livak and Schmittgen 2001). Three biological replicates were conducted.

2.3 | Subcellular Localization and Transactivation Assay of BpbHLH1

The cDNA sequence of *BpbHLH1* was obtained from the birch genome (Wang et al. 2015). The coding sequence (CDS) of *BpbHLH1* without the stop codon fused in-frame to the N-terminus of green fluorescent protein (GFP) was transformed into the pROK2 vector under control of the CaMV 35S promoter (35S:: *BpbHLH1*-GFP). The GFP protein under the 35S promoter was used as a control (35S::GFP). The 35S:: *BpbHLH1*-GFP and 35S:: GFP constructs were separately introduced into onion epidermal cells by particle bombardment (Bio-Rad Laboratories, Inc. Hercules, California, USA). After incubation for 24 h, the transformed onion epidermal cells were stained with DAPI (100 ng/mL) and visualized under an inverted fluorescence microscope (Zeiss, Jena, Germany).

The complete and various truncated versions of the CDS of *BpbHLH1* were PCR amplified and fused in-frame to the GAL4 DNA-binding domain in the pGBKT7 vector to generate the pGBKT7-*BpbHLH1* construct (Clontech laboratories, Inc. Mountain View, California, USA). The pGBKT7-*BpbHLH1* construct was transformed into Y2H Gold Yeast Strain, which were incubated on SD/-Trp or SD/-Trp/-His/-Ade/X-α-gal medium at 30°C for 3–5 days. Primer sequences are shown in Table S2.

2.4 | Vector Construction, Plant Transformation, and Expression of *BpbHLH1*

The full-length CDS of *BpbHLH1* was amplified by PCR and then constructed into the pROKII vector digested using *Sma* I (Takara Bio Inc. Kusatsu, Shiga, Japan) under control of the CaMV 35S promoter for overexpression of *BpbHLH1*. To silence *BpbHLH1*, its truncated CDS with sense and antisense sequences was inserted into the modified pROKII vector

(Figure S1) and digested using *Sma* I in the forward and reverse direction to form an inverted repeat sequence, respectively. All primers and amplicon sizes are shown in Table S3.

To generate overexpressing (OE) and silencing (SE) birch plants, transgenic birch plants were constructed using *Agrobacterium tumefaciens*-mediated transient transformation following the method described in a previous study (Zang et al. 2017), and the pROKII-35S empty vector was transformed into birch plants, which were used as control plants.

Total RNA from each sample was reverse-transcribed into cDNA. The expression levels of *BpbHLH1* in OE and SE birch plants were determined using qRT-PCR, and the expression levels of these genes in pROKII-35S plants were used as the control. The relative expression level of *BpbHLH1* was determined using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were performed.

2.5 | Stress Tolerance and Expression of Target Genes in Transgenic Plants

BpbHLH1-OE and *BpbHLH1*-SE transgenic birch plants were treated with 0.2 M of NaCl for 3 h. The pROKII-35S empty vector transformants were also treated with 0.2 M of NaCl for 3 h. Control plants were treated with water.

The detached leaves of birch plants were infiltrated with DAB (1.0 mg/mL) and NBT (0.5 mg/mL) following a previously described method (Zhang et al. 2011). Evans blue (1.0 mg/mL) staining was performed to detect cell death following a previously described procedure (Kim et al. 2003). SOD and POD activities, H_2O_2 content, and electrolyte leakage were measured following previously described procedures (Liu et al. 2015; Wang et al. 2015). Three independent biological replicates were performed.

Total RNA of the whole plant from OE and SE transgenic birch plants and pROKII-35S plants was extracted and treated with DNase I before being reverse-transcribed into cDNA using a PrimeScript RT reagent Kit (Lab). Real-time PCR was performed on three *BpSOD* genes and three *BpPOD* genes. The primer sequences are listed in Table S4.

2.6 | Plasmid Construction and Yeast One-Hybrid (Y1H) Assays

Some studies have found that bHLH TFs can bind to the E-box (5′-CANNTG-3′) or G-box (5′-CACGTG-3′) DNA motif (a specific member of the E-box family) (Qian et al. 2021). Here, Y1H was conducted to analyze the interaction of BpbHLH1 with these elements. Firstly, the sequences of the three tandem copies of the E-box-1, E-box-2 and G-box were inserted into multiple cloning sites of the pHIS2 plasmid digested by *EcoR* I and *Sac* I to drive *HIS3* expression, and this was used as the reporter construct. The CDS of *BpbHLH1* was cloned into pGADT7-Rec2 digested by *Sma* I as the effector (pGADT7-*BpbHLH1*). All primers used are listed in Table S5.

The reporter and effector constructs were co-transformed into Y187 cells using the Y1H technique. The Y1H system consisted of the following: 0.5 μg of reporter, 0.5 μg of effector, and 5 μL of carrier DNA. The mixture was added to 50 μL of competent Y187 yeast cells. The transformation was performed using Yeast Transformation System 2 (Clontech); the colonies were then plated onto SD/-Trp/-His/ (DDO) and SD/-Trp/-His/-Leu/ (TDO) medium supplemented with 50 mM 3-AT (3-amino-1,2,4-triazole) and incubated at 30°C for 3–5 days.

2.7 | Validation via Transient Expression Assays

The binding ability of the BpbHLH1 factor to specific elements in the promoters of the target genes was validated using the *GUS* reporter gene assay. The truncated promoter including or lacking the E-box-1, E-box-2, or G-box elements was fused with the CaMV35S minimal promoter (46 bp to +1, replacing the 35S promoter) to drive the expression of the *GUS* gene. The primers are listed in Table S6. The pROKII-BpbHLH1 vector under control of the CaMV35S promoter was used as an effector. The effector of pROKII-35S::BpbHLH1 was co-transformed with the reporter into one-month-old birch plants by *A. tumefaciens*-mediated transient expression. The plants were also treated with 0.2 M of NaCl solution for 3 h. Control plants were treated with water. GUS activity was determined. Data were represented as the mean of three biological replicates.

2.8 | Chromatin Immunoprecipitation (ChIP) Analysis

Cis-acting elements in the promoters of the *BpSOD* and *BpPOD* genes were analyzed, and E-box-1(5′-CAATTG-3′), E-box-2 (5′-CATATG-3′) and G-box (5′-CACGTG-3′) elements were found. So, ChIP assay was performed to verify the binding of BpbHLH1 and these elements. The open reading frame (ORF) of *BpbHLH1* was inserted into the pCAMBIA1307 vector downstream of the Flag antibody under control of the CaMV 35S promoter. The p1307-35S:: Flag-BpbHLH1 construct was transformed into *Agrobacterium* EHA105 competent cells and then into 1-month-old birch plants via *A. tumefaciens*-mediated transient transformation, then was also treated with 0.2 M of NaCl solution for 3 h. Control plants were treated with water.

To determine whether BpbHLH1 could bind to specific *cis*-acting elements in the promoters of *SOD* and *POD* genes to regulate their expression in vivo, ChIP analysis was conducted following a previously described method (Zhao et al. 2020) with some modifications. Briefly, transgenic birch plants (1–5 g) transiently expressing *BpbHLH1* were collected and cross-linked with 3% formaldehyde for 10 min at room temperature in a vacuum. The cross-linking was quenched by 2 mol/L glycine for 2 min in a vacuum at room temperature, followed by five washes with deionized water. Tissue was then ground into a fine powder using liquid nitrogen. The purified cross-linked nuclei were sonicated to shear the chromatin into 0.2–0.8 kb of fragments, and 1/10 volume was saved as the input control. One portion of chromatin was immunoprecipitated with Flag antibody (ChIP+). The other portion was immunoprecipitated without antibody as a negative control (ChIP−). The immunoprecipitated complexes were incubated at 65°C for 12 h to

release the DNA fragments. The immunoprecipitated DNA was extracted with chloroform for purification. The DNA fragments containing or lacking E-box-1, E-box-2, or G-box elements in the promoter regions of *BpSOD* and *BpPOD* genes were selected for amplification. The thermal cycling conditions for PCR were as follows: 95°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final incubation at 72°C for 7 min. Enrichment of truncated promoters in the immunoprecipitated samples was determined using quantitative PCR (qPCR). The thermal cycling protocol was as follows: 95°C for 30 s; 40 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s; and 60°C for 15 s. The *tubulin* gene was used as an internal control. Three biological replicates were performed. All the primers used are shown in Table S7.

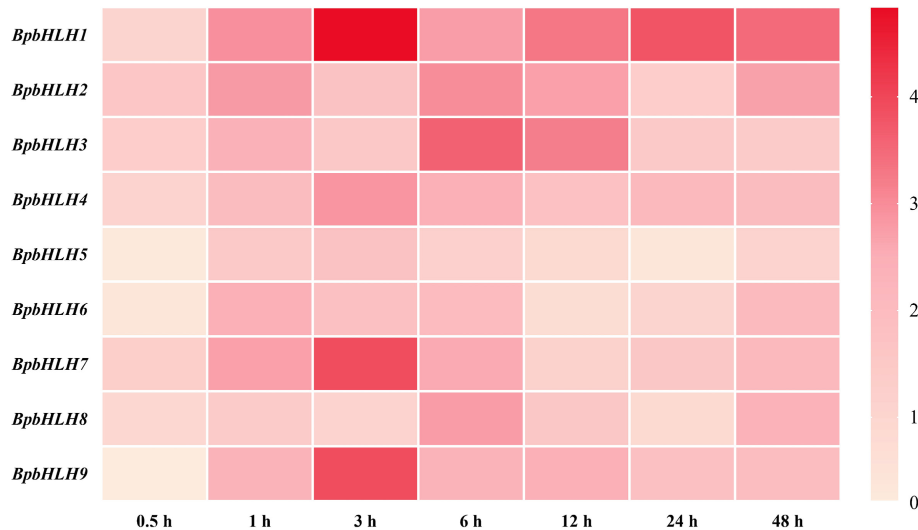

FIGURE 1 | Expression patterns of nine *BpbHLH* genes in response to salt treatment. *Betula platyphylla* seedlings under salt stress treatment. Well-watered plants were used as controls to normalize expression levels at each time point. The expression levels of genes in the roots of birch plants were analyzed via qRT-PCR. Red indicates induction in arrays. The intensity of the red increases as the positive (induction) values increase.

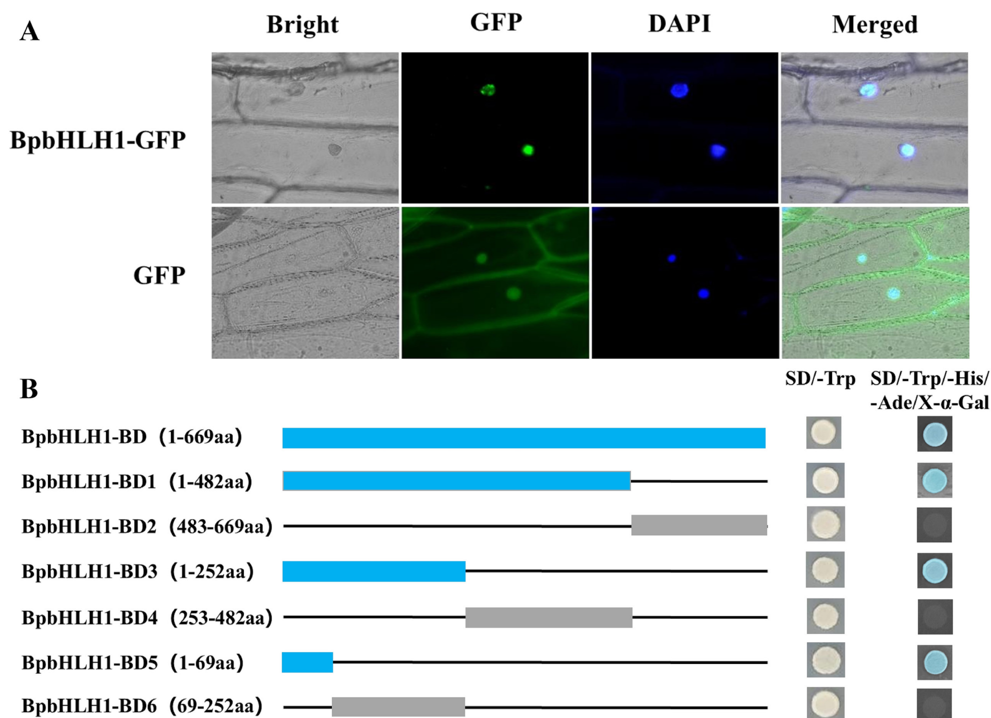

FIGURE 2 | Subcellular localization and transactivation assay of BpbHLH1. (A) Subcellular localization of BpbHLH1. The fusion of *BpbHLH1* with GFP was transiently expressed in onion epidermal cells using the particle bombardment method. The 35S:: GFP construct was used as the control. The transformed cells were cultured on 1/2 Murashige-Skoog (1/2 MS) medium for 24 h and visualized using a confocal microscope at 488 nm. DAPI:DAPI staining of nuclei; GFP:GFP fluorescence detection; Bright: bright field; Merged: the DAPI, GFP, and bright field images merged. (B) The transactivation assay of BpbHLH1. Various truncated sequences of the CDS of BpbHLH1 were fused in-frame, respectively, to the GAL4 DNA-binding domain in pGBKT7 and transformed into Y2HGold Yeast Strain. The transformed cells were plated onto SD/−Trp (growth control) or SD/-Trp/-His/-Ade/X-α-Gal medium.

2.9 | Statistical Analysis

The multiple comparison of one-way analysis of variance was performed using SPSS 22.0 software (IBM, IL, USA). Analysis of variance was used to evaluate the significance of differences between groups. The different capital letters in the same column indicate a difference at the significant level of $p = 0.01$.

3 | Results

3.1 | Expression Analysis of Nine *BpbHLH*s in Response to Salt Stress

Two-month-old birch plants were subjected to stress treatment to clarify changes in the expression of *BpbHLH* genes under salt stress. Nine *BpbHLH* genes involved in regulating stress tolerance were selected based on the results of the phylogenetic tree. The relative expression levels of these genes in the roots of plants subjected to 0.2 M of NaCl treatment and water treatment (control) were analyzed.

The nine *BpbHLH* genes were differentially expressed under salt stress for different salt treatment periods (Figure 1). The expression of all nine genes was upregulated for different salt treatment periods compared with the control treatment. Moreover, the expression of more than half of the genes was highest at 3 h of stress. In addition, the expression of *BpbHLH1* was higher than that of other genes. Therefore, in-depth analyses were conducted on *BpbHLH1*.

3.2 | BpbHLH1 Localizes to the Nucleus and Has Transcriptional Activation

The fusion gene of *BpbHLH1* with GFP was transformed into onion epidermal cells by particle bombardment using 35S::GFP as the control. The green fluorescent signal was uniformly distributed throughout the cell for 35S::GFP, but the fluorescent signal from 35S::BpbHLH1-GFP-transformed cells was detected in the nuclei, which were stained using DAPI (Figure 2A). Our findings revealed that BpbHLH1 is a nuclear protein.

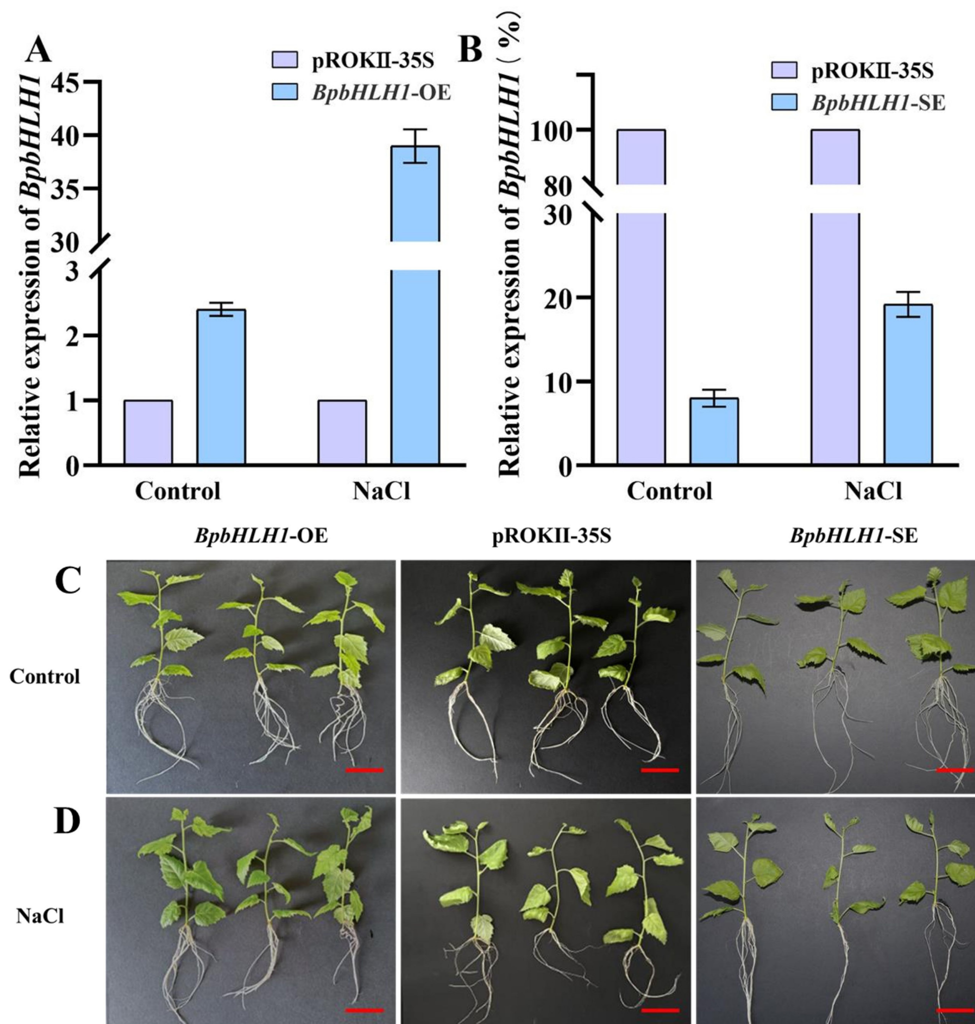

FIGURE 3 | The relative expression level of *BpbHLH1* and the salt stress tolerance in the transgenic birch plants. (A) The relative expression level of *BpbHLH1* in its OE plants. (B) The relative expression level of *BpbHLH1* in its SE plants. Error bars indicate the standard deviation of three biological replicates. (C) The phenotype in *BpbHLH1* OE, SE and pROKII–35S plants under control cioditions. (D) The phenotype in *BpbHLH1* OE, SE, and pROKII–35S plants under salt stress cioditions. OE: overexpressing, SE: silencing. Bar = 3 cm.

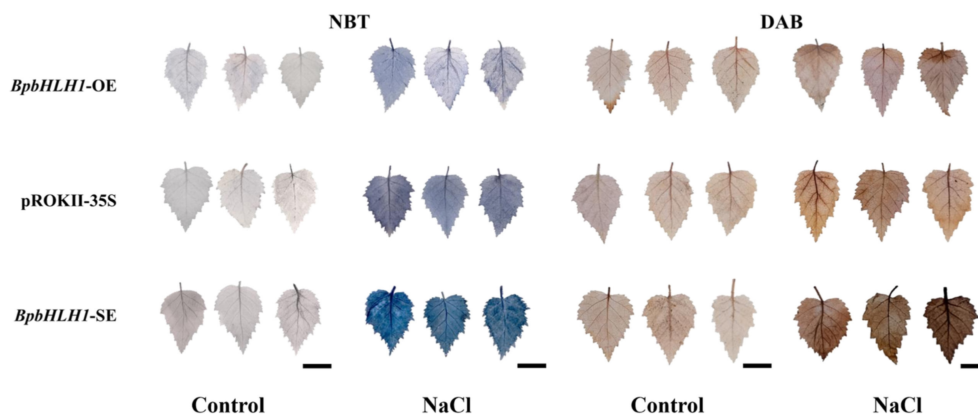

FIGURE 4 | Analysis of ROS accumulation in *BpbHLH1* transgenic birch plants. Following 3 h of 0.2 M of NaCl solution treatment, *BpbHLH1*-OE and -SE transgenic birch plants and control pROKII−35S plants were stained with NBT and DAB to visualize O^{2-} and H_2O_2 concentrations, respectively. Bar = 15 mm.

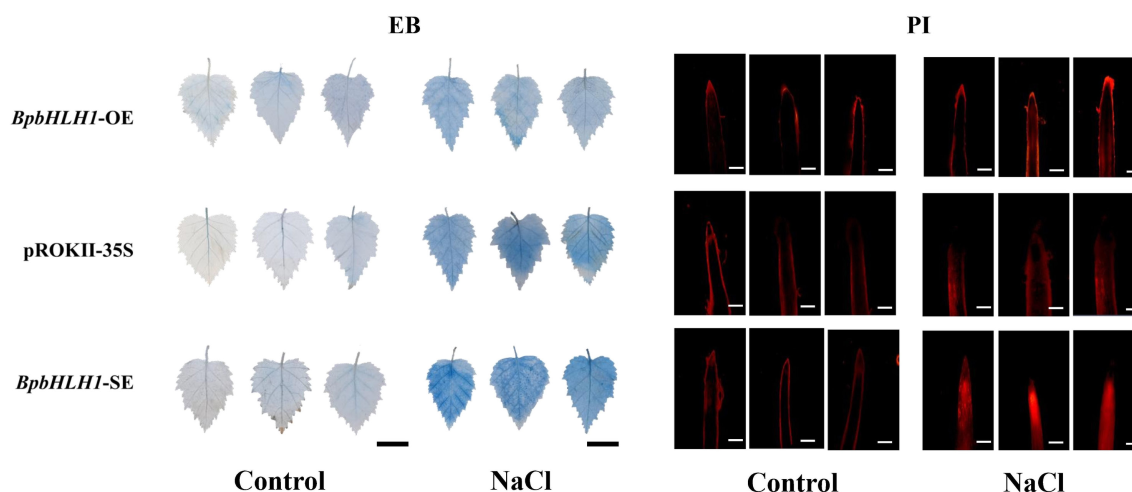

FIGURE 5 | Analysis of the cell membrane damage induced in *BpbHLH1* transgenic birch plants. Following 3 h of 0.2 M of NaCl solution treatment, *BpbHLH1*-OE and *BpbHLH1*-SE transgenic birch plants and control pROKII−35S plants were subjected to Evans blue (bar = 15 mm) and propidium iodide (PI) fluorescence staining to characterize damage to the cell membrane (bar = 50 μm).

To investigate whether BpbHLH1 activated transcription and to identify the activation domain, a series of deletions of BpbHLH1 CDS were fused with the GAL4 DNA-binding domain sequence in pGBKT7 (Clontech). The resulting constructs were, respectively, transformed into yeast cells for transcriptional activation analysis using the yeast two-hybrid system (Y2H). Yeast cells harboring the full CDS of BpbHLH1 grew normally on SD/-Trp/-His/-Ade/X-α-Gal medium, and X-α-Gal was activated (Figure 2B), suggesting that BpbHLH1 is a transcriptional activator. Furthermore, analysis of deletions with truncated CDS of BpbHLH1 suggested that the transcriptional activation domain is located in a region from amino acid 1 to 69 in BpbHLH1 (Figure 2B).

3.3 | Oxidative Stress and Cell Membrane Damage Were Affected in *BpbHLH1* Transgenic Plants

BpbHLH1-OE and *BpbHLH1*-SE transgenic birch plants were obtained using transient *Agrobacterium*-mediated transformation. qRT-PCR was performed to analyze the expression of *BpbHLH1* (Figure 3). The relative expression of *BpbHLH1* was 39-fold higher in *BpbHLH1*-OE plants than in pROKII-35S plants (Figure 3A), and this difference was significant; however, the expression of *BpbHLH1* was significantly lower in SE plants (81%) than in pROKII-35S plants (Figure 3B). These findings suggest that our transient transformation system was effective.

The birch plants, including OE and SE plants of *BpbHLH1* and pROKII-35S plants, were exposed to salt to evaluate their stress tolerance. There was no substantial difference in phenotype among OE, pROKII-35S and SE under control condition (Figure 3C). Under salt treatment, compared with pROKII-35S plants, OE plants displayed less leaf wilting. By contrast, SE plants exhibited more severe leaf rolling and wilting (Figure 3D).

NBT and DAB staining were used to detect O^{2-} and H_2O_2 levels and determine the salt tolerance of *BpbHLH1*-OE and *BpbHLH1*-SE birch plants. The leaves of transgenic plants and pROKII-35S plants were stained using DAB or NBT; the stained leaves of water-treated plants were used as controls. O^{2-} and H_2O_2 levels were significantly lower in the leaves of *BpbHLH1*-OE plants than in pROKII-35S plants under salt stress; by contrast, O^{2-} and H_2O_2 levels were higher in the leaves

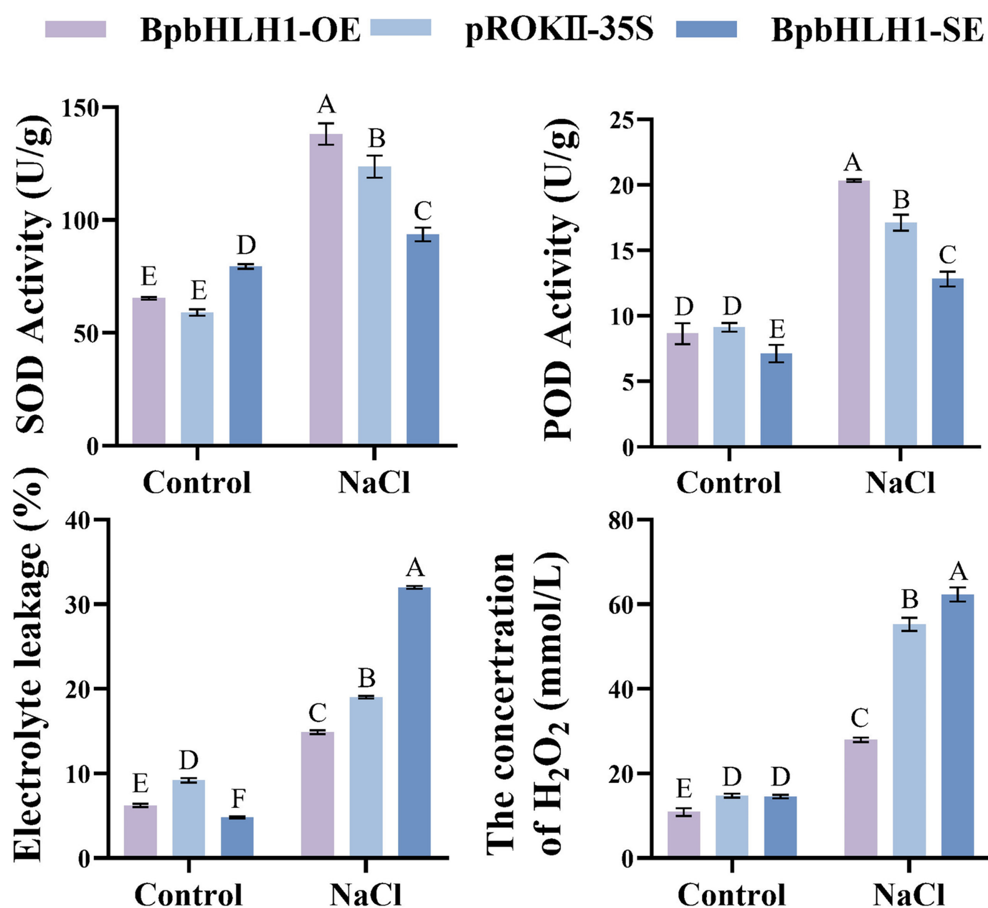

FIGURE 6 | Physiological analysis of *BpbHLH1* transgenic birch plants. SOD and POD activities, electrolyte leakage, and H_2O_2 content of *BpbHLH1*-OE and -SE transgenic birch plants and control pROKII−35S plants following 3h of 0.2M of NaCl solution treatment. The error bars indicate the standard deviation of three biological replicates. The different capital letters in the same column indicate a difference at the significant level of $p = 0.01$.

of *BpbHLH1*-SE plants than in pROK11-35S plants (Figure 4). The O^{2-} and H_2O_2 content was negatively correlated with the reactive oxygen species (ROS) scavenging ability of plants. Thus, our findings indicate that the ROS scavenging ability of OE plants and SE plants was higher and lower than that of control plants, respectively.

Evans blue staining and PI staining were used to detect cell membrane damage. Evans blue stain and PI fluorescence were weaker in *BpbHLH1*-OE plants and stronger in *BpbHLH1*-SE plants than in pROKII-35S plants under salt stress (Figure 5).

3.4 | Physiological Characterization of BpbHLH1 Transgenic Plants

In our study, SOD and POD activities, electrolyte leakage, and the H_2O_2 content were used to evaluate the salt stress resistance of *BpbHLH1* transgenic plants and pROKII-35S transformants (Figure 6). Water treatment was used as a control. SOD and POD play key roles in ROS removal in plants under stress. Our findings suggested that SOD and POD activities were higher in *BpbHLH1*-OE birch plants and lower in *BpbHLH1*-SE birch plants than in pROKII-35S plants under salt stress. Cell death was assessed using the electrolyte leakage rate. The electrolyte leakage rate was lower and higher in *BpbHLH1*-OE birch plants and *BpbHLH1*-SE birch plants than in pROKII-35S plants, respectively, under salt stress. The H_2O_2 content was significantly lower in *BpbHLH1*-OE birch plants and higher in *BpbHLH1*-SE plants than in pROKII-35S plants. These findings suggest that *BpbHLH1* can enhance the ROS scavenging ability and inhibit cell death in birch plants.

3.5 | Changes in the Expression of Target Genes in BpbHLH1 Transgenic Plants

To further analyze whether *BpbHLH1* can affect the expression of target genes, the relative expression levels of the *BpSOD* and *BpPOD* genes in transgenic plants and pROKII-35S plants were measured. Under control treatment, the relative expression levels of the *BpSOD* and *BpPOD* genes were higher in *BpbHLH1*-OE plants and lower in *BpbHLH1*-SE plants than in pROKII-35S plants, but changes were not obvious. Under 0.2M of solution NaCl treatment, their expression levels were significantly increased in the OE plants and obviously reduced in the SE plants compared with the pROKII-35S plants (Figure 7). These results indicated that *BpbHLH1* can enhance the expression levels of *BpSOD* and *BpPOD* genes, especially under salt stress conditions.

3.6 | The Binding of BpbHLH1 to the Specific *cis-Acting* Elements

Previous studies have shown that bHLH TFs can bind to E-box (5′-CANNTG-3′) and G-box elements (5′-CACGTG-3′) (Qian et al. 2021). To determine whether BpbHLH1 binds to these elements, three tandem copies of E-box-1, E-box-2 and G-box elements were cloned into pHIS2, and their interactions with BpbHLH1 were determined using Y1H assays. The results (Figure 8) revealed that yeast cells co-transformed with each effector, and reporter could grow on TDO/3-AT medium, indicating that BpbHLH1 can bind to the E-box-1, E-box-2 and G-box elements.

To further clarify the binding ability of BpbHLH1 and the specific elements in truncated *BpSOD* and *BpPOD* promoters, GUS reporter assays were conducted. The effector of pROKII-35S::BpbHLH1 was co-transformed with each truncated promoter containing or lacking G-box and E-box-1, E-box-2 elements into birch plants (Figure 9A). Truncated promoters containing or lacking G-box and E-box-1, E-box-2 elements, without effector, were used as controls. The results (Figure 9B) suggested that relative GUS activity was approximately 2–10 times higher in transformed plants harboring the G-box and E-box-1, E-box-2 elements than that in lacking the elements plants, under normal conditions. Additionally, the relative GUS activity in plants harboring the elements was significantly higher than that in lacking the elements, under salt

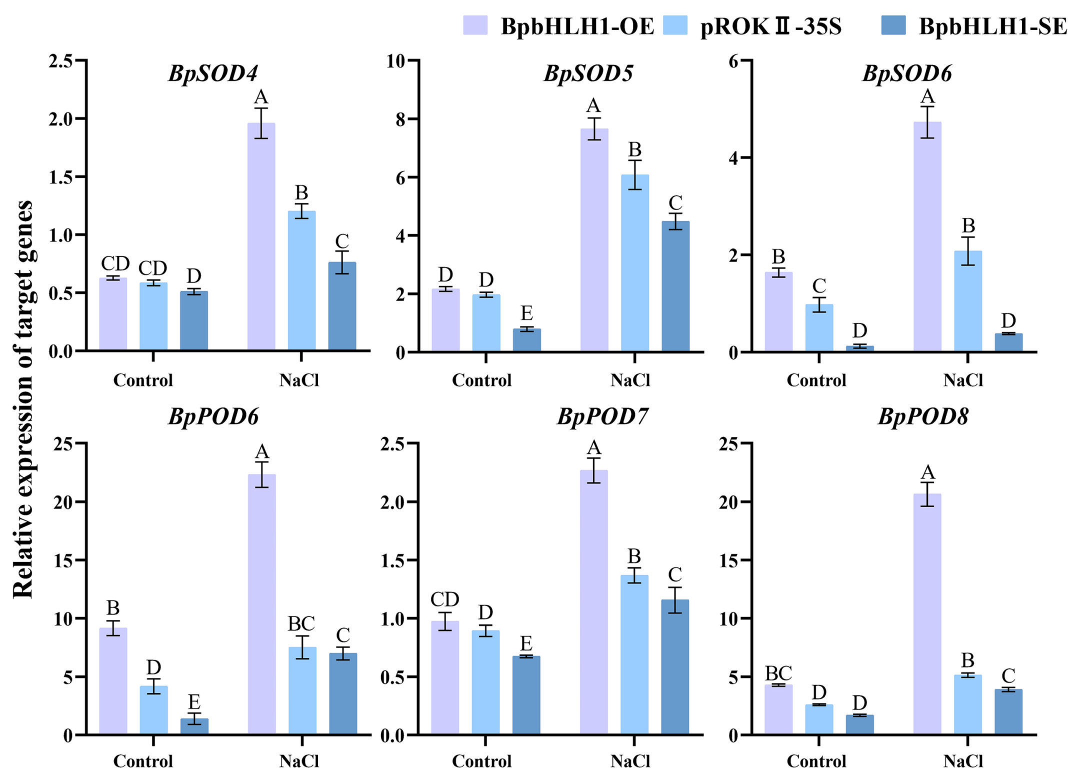

FIGURE 7 | Analysis of the relative expression of target genes in *BpbHLH1* transgenic birch plants. Relative expression of *BpSOD* and *BpPOD* genes in *BpbHLH1*-OE and -SE transgenic birch plants and control pROKII−35S plants under control and salt treatment. Control: the plants were irrigated with water for 3 h. The salt treatment: the plants were treated with 0.2 M of NaCl solution for 3 h. Error bars indicate the standard deviation of three biological replicates. The different capital letters in the same column indicate a difference at the significant level of $p = 0.01$.

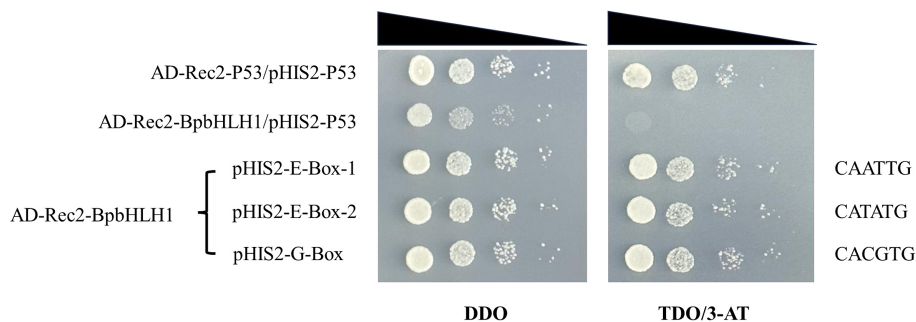

FIGURE 8 | Y1H assays revealing the binding of BpbHLH1 to the E-box-1, E-box-2 and G-box elements. Positive control: pGADT7-Rec2-p53/pHIS2-p53; Negative control: pGADT7-Rec2-BpbHLH1/pHIS2-p53.

stress conditions (Figure 9C). Thus, our findings indicated that BpbHLH1 could bind strongly to the G-box and E-box-1, E-box-2 elements, and the binding affinities are in response to salt stress.

3.7 | Interaction of BpbHLH1 With Specific Elements In Vivo

Our study indicated that BpbHLH1 can regulate the expression of *BpSOD* and *BpPOD* genes according to our qRT-PCR analysis. Thus, the *cis*-acting elements in the *SOD* and *POD* promoters were analyzed, and E-box-1 (5′-CAATTG-3′), E-box-2 (5′-CATATG-3′) and G-box elements (5′-CACGTG-3′) were detected in the promoters of the *BpSOD* and *BpPOD* genes. Therefore, the interaction of BpbHLH1 with the specific *cis*-acting elements in vivo was analyzed using ChIP.

The fragments containing E-box-1, E-box-2 and G-box elements (Figure 10A) in the *BpSOD* and *BpPOD* promoters bound by BpbHLH1 were separately captured by ChIP using Flag antibody. The ChIP-PCR results revealed that the truncated *BpSOD* and *BpPOD* promoters containing the E-box-1, E-box-2 and G-box elements could be enriched by ChIP using Flag antibody (Figure 8B). However, truncated promoters lacking E-box-1, E-box-2 and G-box

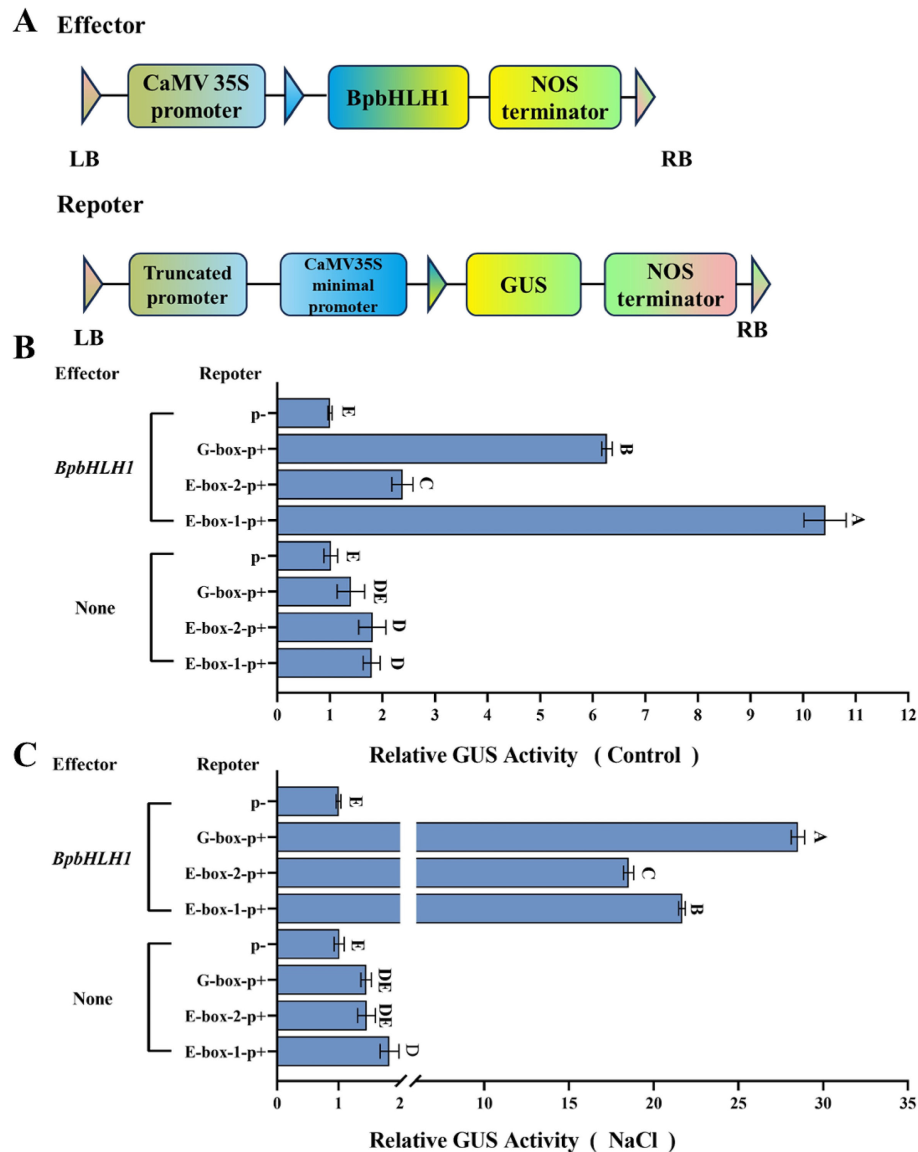

FIGURE 9 | Analysis of the binding of BpbHLH1 to the truncated promoters, including G-box and E-box-1, E-box-2 elements, in birch plants. (A) Schematic representation of BpbHLH1 and truncated promoters including or lacking G-box and E-box-1, E-box-2 elements used for the co-expression of effectors and reporters in birch plants. Effector: pROKII-35S::BpbHLH1. Reporter: G-box p+ or E-box-1 p+, E-box-2 p+, which correspond to the truncated promoter containing G-box or E-box-1, E-box-2 elements, respectively. G-box p- and E-box-1 p-, E-box-2 p- correspond to the truncated promoter lacking G-box or E-box-1, E-box-2 elements, respectively. (B) Transient co-transformation of effector and reporter constructs in birch plants to clarify the binding of BpbHLH1 to G-box and E-box-1, E-box-2 elements, under control conditions. (C) Transient co-transformation of effector and reporter constructs in birch plants to clarify the binding of BpbHLH1 to G-box and E-box-1, E-box-2 elements, under salt stress conditions. GUS activity revealed the binding affinity of BpbHLH1 to the specific *cis*-acting elements. Error bars indicate the standard deviation of three biological replicates. The different capital letters in the same column indicate a difference at the significant level of $p = 0.01$.

elements were not enriched by ChIP with Flag antibody (ChIP+) compared with the positive control input and the negative control (ChIP−) (Figure 10B). ChIP-qPCR analysis revealed that the promoter regions of *BpSOD* and *BpPOD* genes, including the E-box-1, E-box-2 and G-box elements, were significantly enriched in ChIP+ compared with ChIP− (Figure 10C), indicating that BpbHLH1 could specifically bind to the E-box-1, E-box-2 and G-box elements in vivo. Moreover, these truncated promoters including the elements were much more highly enriched under salt stress conditions compared with control conditions (Figure 9D), suggesting that the binding affinities of BpbHLH1 to the promoters of these studied genes are induced by salt stress.

4 | Discussion

4.1 | The Expression of Nine *BpbHLH* Genes Under Salt Treatment

Plants have evolved various strategies to withstand various types of abiotic stress (Ma et al. 2015). Previous studies have shown that *bHLH* genes play key roles in response to abiotic stress (Gu et al. 2021; Qiu et al. 2020). We analyzed the expression of nine *BpbHLH* TF genes clustered with *AtbHLH* TF genes of *Arabidopsis* and involved in stress responses under salt stress. The expression levels of these nine *BpbHLH* genes were upregulated, and their expression patterns under different salt treatment times compared with the control varied, indicating that these genes play key roles in the response to salt stress in birch plants.

4.2 | Salt Stress Tolerance Conferred by *BpbHLH1*

The expression of *BpbHLH1* was higher than that of other *BpbHLH* genes under salt stress. Thus, the stress resistance of *BpbHLH1*-OE and *BpbHLH1*-SE birch plants was studied. Plants often experience various adverse conditions that can lead to the accumulation of ROS (Wang et al. 2005). ROS scavenging is thus an important process for enhancing resistance to various types of stress in plants. Two major ROS species, O^{2-} and H_2O_2, are key molecules that can induce oxidative injuries and play an important role in stress signaling. NBT and DAB staining indicated that ROS accumulation was lower in *BpbHLH1*-OE plants than in pROKII-35S plants under salt stress (Figure 4); this finding was consistent with H_2O_2 levels

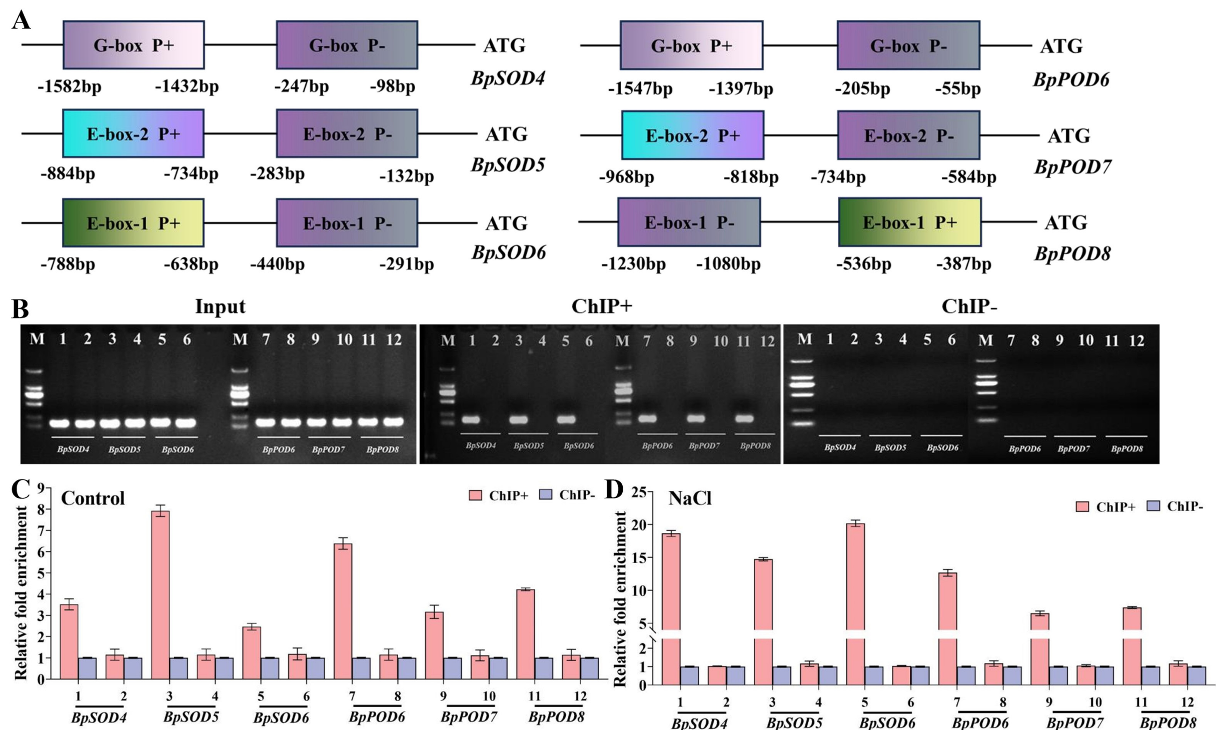

FIGURE 10 | ChIP assays revealing the binding specificity of BpbHLH1 to the G-box and E-box-1, E-box-2 elements. (A) Positions of truncated *BpSOD* and *BpPOD* promoters containing and lacking G-box and E-box-1, E-box-2 elements. G-box p+ and E-box-1, E-box-2 p+: truncated *BpSOD* and *BpPOD* promoters containing G-box and E-box-1, E-box-2 elements, respectively. p-: truncated *BpSOD* and *BpPOD* promoters without G-box and E-box-1, E-box-2 elements. (B) ChIP products obtained from the promoters of *BpSOD* and *BpPOD* analyzed via gel electrophoresis following PCR amplification. M: DL2000 Marker (from top to bottom: 2 kb, 1 kb, 750 bp, 500 bp, 250 bp, and 100 bp). (C) Real-time quantitative PCR analysis showing the enrichment of the promoter sequences of *BpSOD* and *BpPOD* after ChIP, under control conditions. (D) Real-time quantitative PCR analysis showing the enrichment of the promoter sequences of *BpSOD* and *BpPOD* after ChIP, under salt stress conditions. Error bars indicate the standard deviation of three biological replicates. Input, Input DNA (positive control); CHIP+: chromatin immunoprecipitation with anti-GFP antibody; CHIP-: chromatin immunoprecipitation without antibody (negative control). 1: G-box p+ in *SOD4* promoter, 2: G-box p- in *BpSOD4* promoter, 3: E-box-2 p+ in *BpSOD5* promoter, 4: E-box-2 p- in *SOD5* promoter, 5: E-box-1 p+ in *BpSOD6* promoter, 6: E-box-1 p- in *BpSOD6* promoter, 7:G-box p+ in *BpPOD6* promoter, 8: G-box p- in *BpPOD6* promoter, 9: E-box-2 p+ in *BpPOD7* promoter, 10: E-box-2 p- in *BpPOD7* promoter, 11: E-box-1 p+ in *POD8* promoter, 12: E-box-1 p- in *BpPOD8* promoter.

(Figure 6). SOD and POD play key roles in the removal of ROS. SOD and POD activities were higher and significantly lower in *BpbHLH1*-OE and -SE plants, respectively, than in pROKII-35S plants under salt stress (Figure 6). Given that SOD and POD activities in *BpbHLH1*-OE and -SE plants were altered in response to salt stress, we measured the expression of *BpSOD* and *BpPOD* genes. The expression of *BpSOD* and *BpPOD* genes was higher in *BpbHLH1*-OE plants than in pROKII-35S plants under salt stress; however, their expression levels were lower in *BpbHLH1*-SE plants than in pROKII-35S plants (Figure 7). These findings suggest that *BpbHLH1* enhances SOD and POD activities by increasing the expression of *BpSOD* and *BpPOD* genes. Overall, these findings suggest that *BpbHLH1* could increase SOD and POD activities by enhancing the expression of *BpSOD* and *BpPOD* genes, which promotes ROS scavenging. Evans blue staining and PI fluorescence were weaker in *BpbHLH1*-OE plants than in pROKII-35S plants (Figure 5); this finding is consistent with the lower electrolyte leakage rate of *BpbHLH1*-OE plants (Figure 6) compared with pROKII-35S plants. These results indicate that *BpbHLH1* reduced cell death and thus enhanced the resistance of plants to salt stress.

4.3 | BpbHLH1 Directly Binds to Specific Elements in the Promoters of *BpSOD* and *BpPOD* Genes

Abiotic stress limits plant growth and development, and TFs play important roles in abiotic stress responses (Xu et al. 2018). Previous studies suggest that bHLH TFs can bind to E-box and G-box elements (Qian et al. 2021). BpbHLH1 can regulate the expression of *BpSOD* and *BpPOD* genes (Figure 7); analysis of *cis*-acting elements revealed E-box-1, E-box-2 and G-box elements in the promoters of *BpSOD* and *BpPOD*. Previous studies indicate that TFs can regulate the expression of target genes by binding to *cis*-acting elements in their promoters. For example, BpNAC012 can induce the expression of secondary wall-associated downstream genes by directly binding to secondary wall NAC-binding elements (Hu, Zhang, and Yang 2019). The new R2R3-MYB gene *ThRAX2* can regulate the expression of some genes involved in the response to cadmium stress by binding to the CTTCCA element in their promoters in *Tamarix hispida* (Wang et al. 2023). In our study, ChIP assays revealed that BpbHLH1 can specifically bind to the E-box-1, E-box-2 and G-box elements in the promoters of *SOD* and *POD* genes (Figure 10B,C). Analysis of GUS activity revealed strong binding activity of BpbHLH1 to the E-box-1, E-box-2 and G-box elements (Figure 9B), and the relative expression of *BpSOD* and *BpPOD* genes was much higher in *BpbHLH1*-OE birch plants than in control plants (Figure 7), which suggests that BpbHLH1 can directly regulate the expression of *BpSOD* and *BpPOD* genes by binding to the E-box-1, E-box-2 and G-box elements in their promoters. In addition, GUS activity and ChIP under salt stress conditions further demonstrated that binding affinities of BpbHLH1 to the specific elements are induced by salt stress (Figures 9C and 10D).

5 | Conclusions

The expression of nine *bHLH* genes could be induced by salt stress, and the expression of *BpbHLH1* was higher than that of other *BpbHLH* genes. Overexpression of *BpbHLH1* can enhance the salt stress tolerance of birch plants. BpbHLH1 can also regulate the expression of target genes involved in stress tolerance by binding to the E-box-1, E-box-2 and G-box elements in their promoters. Further study found that binding affinities of BpbHLH1 to these specific elements are induced by salt stress. The findings of our study will aid the development of new birch germplasm with high salt tolerance.

Author Contributions

Qilong Fang performed the experiments, analyzed the data, and wrote the manuscript. Di Wu performed the experiments and analyzed the data. Hu Sun performed the experiments and analyzed the data. Luyao Wang performed the experiments. Yuping Liu performed the experiments. Wenfeng Mei performed the experiments. Huiyan Guo designed and managed the research work and improved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as all new data are already contained within this article or in the supplementary material.

Peer Review

The peer review history for this article is available in the Supporting Information for this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.