Article

BpTCP3 Transcription Factor Improves Salt Tolerance of *Betula platyphylla* by Reducing Reactive Oxygen Species Damage

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Abstract: The plant-specific transcription factors TEOSINTE BRANCHED1/CYCLO IDEAL/PROLIFERATING CELL FACTOR1 (*TCP*) act as developmental regulators that have many roles in the growth and development processes throughout the entire life span of plants. *TCP* transcription factors are responsive to endogenous and environmental signals, such as salt stress. However, studies on the role of the *TCP* genes in salt stress response have rarely focused on woody plants, especially forest trees. In this study, the *BpTCP3* gene, a *CYC/TB1* subfamily member, isolated from *Betula platyphylla* Sukaczew, was significantly influenced by salt stress. The β -glucuronidase (*GUS*) staining analysis of transgenic *B. platyphylla* harboring the *BpTCP3* promoter fused to the reporter gene *GUS* (*pBpTCP3::GUS*) further confirmed that the *BpTCP3* gene acts a positive regulatory position in salt stress. Under salt stress, we found that the *BpTCP3* overexpressed lines had increased relative/absolute high growth but decreased salt damage index, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) levels versus wild-type (WT) plants. Conversely, the *BpTCP3* suppressed lines exhibited sensitivity to salt stress. These results indicate that the *BpTCP3* transcription factor improves the salt tolerance of *B. platyphylla* by reducing reactive oxygen species damage, which provides useful clues for the functions of the *CYC/TB1* subfamily gene in the salt stress response of *B. platyphylla*.

Keywords: *Betula platyphylla*; *CYC/TB1* subfamily gene; *BpTCP3* promoter; salt stress response

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1. Introduction

Environmental challenges, such as drought, saline–alkali soil, extreme temperature, and flooding stress, have serious effects on the growth and development of plants. Plants have developed a series of defense mechanisms, including gene expression regulation, physiological adaptations, and biochemical changes to cope with and survive these environmental challenges [1–3]. Transcription factors containing the bHLH basic domain play critical roles in several types of environmental stresses [4]. The transcriptional regulation of stress-responsive genes regulated by these transcription factors is mediated by dynamic changes in hormone biosynthesis [5].

Members of *TCP* transcription factors are a small family of plant-specific transcription factors, which contain a 59-amino acid basic helix–loop–helix (bHLH) motif that classifies the *TCP* family into two groups, known as Class I (or PCF or TCP-P) and Class II (or TCP-C) [6,7]. The bHLH motif is also involved in nuclear targeting, DNA ligature, and protein interaction [6,8]. Martin-Trillo et al. proposed that the role of the *TCP* protein as a transcriptional activator or repressor does not seem to be determined by the type of *TCP* domain but rather depends on its interaction with other proteins [9]. Moreover, van Es et al. proposed from the comprehensive phenotypic analysis of *TCP* mutant plants that the complex functions of Class I and Class II *TCPs* in plant growth depend on the developmental stage or species-specific differences instead of full genetic redundancy [10].

The plant-specific *TCP* gene family not only plays a role in developmental processes throughout the plant's entire life but also interacts with endogenous (circadian rhythm and

plant hormones) and environmental (pathogens, light, temperature, nutrients, salt, and drought stress) signals to ensure plant suitability and health. Many important developmental processes are mediated by *TCP* family members through different mechanisms, such as embryonic development [11], leaf morphogenesis [12], bud dormancy [13], petal development [14], shoot growth [15], and senescence [16]. In *Prunus persica* (L.) Batsch, a perennial deciduous tree, *PpTCP20*, promotes peach flower bud dormancy by negatively regulating the expression of dormancy-associated genes *PpDAM5* and *PpDAM6* and interacts with *PpABF2* to form heterodimers [17]. In *Arabidopsis*, *AtTCP3* controls the morphology of shoot lateral organs by negatively regulating the expression of a boundary-specific gene *CUC*, resulting in the fusion of cotyledons and defects in the formation of shoots [18]. In addition, by directly binding to the promoter of *VND7*, *TCP4* activates the expression of *VND7* and thus can promote the secondary cell wall biosynthesis of the xylem [19].

The *TCP* family members have been reported to play various roles in several aspects of plant development by interacting with endogenous and environmental factors to cope with and survive environmental challenges. For instance, Guan et al. reported that interaction of *AtTCP20* and *NLP* constituted a molecular link between nitrate signaling and the mitotic cyclin *CYCB1;1*, as well as root meristem growth, which constitutes a pivotal regulatory mechanism for plant to well meet the challenges of living environment [20]. In addition, in *Populus tomentosa* Carrière, the *miR319a*-targeted *TCP19* regulates trichome formation in leaves by repressing the expression of trichome marker genes and by coordinating with GA signaling, which results in the improvement of insect defense ability [21]. In hybrid aspen, *TCP18/BRC1*, which is targeted and regulated by *SVL*, acts as a negative regulator of axillary bud outgrowth under the control of temperature signals to suppress bud break and to ensure that plants survive the cold early in the spring [22]. In *Arabidopsis*, Class I *TCP* family members refer to fine-tuning plant immunity by interacting with *SRFR1*, a negative regulator specifically targeting effector-triggered immunity [23].

Betula platyphylla (Birch) is an important economic and shelterbelt construction species of China. This is a broad-leaved deciduous hardwood tree that is widely distributed in temperate and subarctic regions and belongs to the genus *Betula* in the family *Betulaceae* [24]. Birches are widely used in the production of paper, furniture, and plywood thanks to the advantages of fast growth, good wood properties, and drought and cold resistance [25]. Birch, however, also has a low tolerance to salt stress [26]. Most of the future afforestation sites in North China are arid and semi-arid areas with a high degree of salinization. In addition, birch is also the main landscaping tree species in northern China. However, snow melting causes soil salinity, which affects the growth of *B. platyphylla*. Based on discussion of the salt-tolerant function of genes, salt-tolerant birch will be cultivated by genetic engineering, which holds great significance for afforestation and landscaping in salinized areas. To date, few studies on the role of *TCP* family genes in the salt stress response have focused on woody plants in general and on forest trees in particular. A previous study found that *BpTCP7*, a CIN subclass gene of the *TCP* family from *B. platyphylla*, enhances tolerance to salt stress when overexpressed in transgenic plants [27]. To more widely understand the role of *CYC/TB1* subclass *TCP* genes in the salt stress response of *B. platyphylla*, we speculated that the only two *CYC/TB1* subclass members of *B. platyphylla* may play the same roles in salt stress because of their highly homologous [28] and similar expression patterns in wild-type (WT) *B. platyphylla* under salt treatment (this study). Therefore, the *BpTCP3* gene, one of the *CYC/TB1* subclass genes of *B. platyphylla*, was selected for further study. According to the analysis of the response of the *BpTCP3* gene to salt stress, the function of the *BpTCP3* gene in the process of salt stress was further explored through transgenic birch lines, which provided insight for further unmasking the function of *CYC/TB1* subclass genes. Salt-tolerant transgenic birch lines were screened to establish a theoretical foundation for genetics and breeding of birch.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The leaves and full-sib family seeds from *B. platyphylla* 10-year-old clonal seedlings at the Birch Breeding Base (Northeast Forestry University of Harbin, China) were collected. The leaves were used as clone material for cloning the *BpTCP3* gene and its promoter. Some seeds were used as transgenic receptor materials, and the other seeds were used to study the expression of *BpTCP3* and *BpTCP12* gene after germination. The seeds were sown and cultured in Petri dishes containing double-distilled water. When two cotyledons appeared, seedlings with similar height were transferred to woody plant medium (WPM) [29] agar plates supplemented with 0.4 M sodium chloride (NaCl) [30,31] for 0 (control), 2, 4, 6, and 24 h maintained at 25 ± 2 °C with a 16 h light/8 h dark cycle and a light intensity of 1000–1500 lx. All tissues of the seedlings were harvested. At least 10 seedlings were mixed for each sample, and three biological replications were collected for analyzing the expression of the *BpTCP3* and *BpTCP12* genes in WT *B. platyphylla* under salt treatment using quantitative real-time polymerase chain reaction (RT-qPCR).

2.2. Bioinformatics Analysis

The open reading frames (ORF) of the *BpTCP3* and *BpTCP12* genes were determined using the ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>, accessed on 1 October 2021) and BLASTX (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 1 October 2021) from the National Center for Biotechnology Information program (NCBI). The molecular weight (Mw) and isoelectric point (PI) were calculated by the ExPASy program (<https://web.expasy.org/protparam/>, accessed on 1 October 2021). The Batch CD-Search online tools of NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 1 October 2021) were used to predict the conservative domain. The bHLH domains in the *BpTCP3* and *BpTCP12* sequences (Figure 1A,B) and other CYC/TB1 subfamily proteins sequences from *Arabidopsis thaliana* (L.) Heynh., *Oryza sativa* L., *Zea mays* L., *Phyllostachys heterocycle* (Carrière) Matsum., *Eucalyptus camaldulensis* Dehnh., and *Populus euphratica* Olivier were downloaded from the Plant Transcription Factor Database (<http://planttfdb.gao-lab.org/>, accessed on 1 October 2021); they were concatenated and aligned using ClustalW (BioEdit 7.0.9.0 software, Ibis Biosciences, Carlsbad, CA, USA). The phylogenetic tree was constructed by MEGA (Version 7.0, Mega Limited, Tokyo, Japan) using the neighbor-joining method with 1000 bootstrap replicates. The PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 1 October 2021) [32] and PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>, accessed on 1 October 2021) [33] database were used to analyze the enrichment of the specific cis-acting elements in the *BpTCP3* promoter.

2.3. Gene Cloning and Vector Construction

The PCR amplification technique was used to obtain the promoter fragment of the 1795-bp upstream sequence of the *BpTCP3* gene. Total DNA was isolated from leaves of 10-year-old birch using a Plant Genomic DNA Kit (TIANGEN, Beijing, China). The specific primers (ProBpTCP3) containing *Hind*III and *Xba*I sites were designed according to a 1795 bp sequence upstream of the ATG translation start codon of *BpTCP3* in the birch genome database (Table 1). PCR amplification was completed in a 20 µL solution consisting of 0.4 µg DNA template, 0.6 µL 10 µM forward primer, 0.6 µL 10 µM reverse primer, 2 µL 2 mM dNTPs, 2 µL 10 × PCR Buffer, 0.4 µL KOD-Plus-Neo Polymerase (1 U/µL) (TOYOBO), and double-distilled H₂O up to 20 µL. The PCR cycling conditions were as follows: 94 °C for 4 min; 30 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis, and the expected 1795-bp sequence was purified with the Universal DNA Purification Kit (TIANGEN). Then, the *BpTCP3* promoter sequence was cloned into the pBI101 binary vector containing the *GUS* reporter gene by *Hind*III/*Xba*I sites (pBI101-*pBpTCP3*:*GUS*).

Figure 1. Multiple sequence alignment and phylogenetic tree of *B. platyphylla* (Bp) and other species (*Arabidopsis thaliana*/At, *Oryza sativa*/Os, *Zea mays*/Zm, *Phyllostachys heterocycla*/Ph, *Eucalyptus camaldulensis*/Ec, and *Populus euphratica*/Pe). (A) Multiple sequence alignment. (B) Phylogenetic tree. The phylogenetic tree was drawn by MEGA 7.0 with the neighbor-joining (NJ) method, using the following parameters—bootstrap values (1000 replicates) and the Poisson model. Different species are shown in different colors. The * represent conserved amino acids and black point are shown variable amino acids.

Table 1. Primer sequences.

Primer Name	Forward Primer (5'→3')	Reverse Primer (5'→3')
ProBpTCP3	5'-CCAAGCTTCCCATCAACACCTGTGAAATGC-3' (<i>Hind</i> III)	5'-GCTCTAGAGGGTTGGTCTGAATAAGAGATGG-3' (<i>Xba</i> I)
PBI101	5'-ACAGGAAACAGCTATGACCATGATTACG-3'	5'-TACAGGACGTAACATAAGGGACTGACC-3'
BpTCP3-PCR	5'-ATGTATTCCTCATCAAATAGTAAC-3'	5'-TCAGGCCTCCCATGCCTTGC-3'
BpTCP3-qPCR	5'-GGGATCGGAGAATGAGACTTTTCG-3'	5'-CGTAATAAATTGCAACGTCATCGACG-3'
GUS-qPCR	5'-CTCTATGAACGTGTCGTCACAGC-3'	5'-CGAGCATCTCTTCAGCGTAAGG-3'
BpTubulin	5'-GCACTGGCCTCCAAGGAT-3'	5'-TGGGTCGCTCAATGTCAAGG-3'

To construct the overexpression vector (pGWB2-35S::BpTCP3), the 885-bp CDS of the *BpTCP3* gene was constructed into the plant expression vector pGWB2 under the CaMV 35S promoter using the extracted cDNA from the leaves of *B. platyphylla* as a template with a pair of gene-specific primers, *BpTCP3*-PCR-F and *BpTCP3*-PCR-R (Table 1), by the gateway cloning system. Gateway relevant reagents and vectors were provided by Invitrogen (Carlsbad, CA, USA). For the construction of the suppressed expression vector (pGWB2-35S::BpTCP3-SRDX), the stop codon of *BpTCP3* was replaced by the plant-specific repression domain EAR [34] at the 3'-end and then shuttled into the pGWB2 vector.

Finally, these three combined vectors, pGWB2-35S::BpTCP3, pGWB2-35S::BpTCP3-SRDX, and pBI101-pBpTCP3::GUS, were transferred into GV3101 cells by electroporation.

2.4. Genetic Transformation of *B. platyphylla*

Seeds were agro-transformed using *Agrobacterium*-mediated transformation of zygotic embryos as previously described in Li et al. [35,36]. Birch zygotic embryos were soaked in *Agrobacterium tumefaciens* for 5 min and incubated on co-cultivation medium (WPM + 2.0 mg/L benzyladenine [BA] + 0.2 mg/L naphthalene acetic acid [NAA] + 200 mg/L cefotaxime) in the dark at 25 °C for 2–3 days. The zygotic embryos were then incubated in a selection medium (WPM + 2.0 mg/L BA + 0.2 mg/L NAA + 80 mg/L kana-mycin or 50 mg/L hygromycin + 200 mg/L cefotaxime) for 20 days. During the cultivation period, the selection medium was replaced until the resistant callus emerged. When resistant callus grew resistant buds, they were transferred to a differential medium (WPM + 0.8 mg/L BA + 0.5 mg/L gibberellic acid (GA₃) + 80 mg/L kana-mycin or 50 mg/L hygromycin + 200 mg/L cefotaxime) to differentiate the resistant shoots. Parts of the shoots and leaves of resistant shoots were transferred to a second selection medium (WPM + 1.0 mg/L BA + 80 mg/L kana-mycin or 50 mg/L hygromycin + 200 mg/L cefotaxime) to differentiate the new resistant callus. The new resistant callus grew into the resistant shoots in the differential medium described above. After the resistant shoots were cultured in rooting medium (WPM + 0.4 mg/L indole-3-Butyric acid [IBA]) for 25 days, the rooted seedlings were transferred to the mixture of peat soil, vermiculite, and perlite (5:3:2 v/v). The plants were cultured at 25 ± 2 °C with a 16 h light/8 h dark cycle and a light intensity of 1000–1500 lx.

Finally, the *BpTCP3* promoter was detected by PCR amplification using the extracted DNA from *pBpTCP3::GUS* transgenic lines as a template with plasmid pBI101-*pBpTCP3::GUS* as a positive control and plasmid pBI101 as a negative control; the gene-specific primers (*BpTCP3*-PCR) were used as upstream primers, and the pBI101 vector primers were used as downstream primers (Table 1). The PCR reactions were conducted using rTaq DNA polymerase (TaKaRa, Dalian, China) according to the manufacturer's protocol. The transgenic lines 35S::BpTCP3 and 35S::BpTCP3-SRDX were verified by PCR amplification and RT-qPCR using the extracted DNA or total RNA from leaves of *BpTCP3* transgenic lines as a template with *BpTCP3*-PCR or *BpTCP3*-qPCR as primers (Table 1).

2.5. Analysis of the Activity of the *GUS* Reporter Gene

To evaluate the activity of the *GUS* reporter gene in *pBpTCP3::GUS* transgenic *B. platyphylla* under salt treatment, the tissue-cultured rooting seedlings of the *pBpTCP3::GUS* transgenic line were spread on a medium containing 0.4 M NaCl [30,31] and treated for 0 (control), 2, 4, 6, and 24 h. The seedlings were incubated for GUS staining, which was performed according to the protocol described by Jefferson [37]. The sixth to eighth functional leaves of the remaining seedlings were collected and used for total RNA extraction. At least three seedlings were mixed for each sample, and three biological replications were established to analyze the expression of the *GUS* gene in the *pBpTCP3::GUS* transgenic line under salt treatment using RT-qPCR. The primer *GUS*-qPCR (Table 1) was used to amplify the *GUS* gene.

2.6. RT-qPCR

Total RNA was extracted from the tissue described above using the Universal Plant Total RNA Extraction Kit (Bioteke, Beijing, China). cDNA was synthesized from 1 µg RNA using a ReverT Ace[®] qPCR RT Kit (Toyobo, Osaka, Japan) using primer *BpTubulin* as an internal reference gene. RT-qPCR was conducted using the SYBR[®] Green PCR master mix-Plus (Toyobo, Osaka, Japan, volume 20 µL) and reaction program (95 °C, 30 s; 95 °C, 15 s; 60 °C, 1 min, 40 cycles; and 95 °C, 30 s) running on the ABI 7500 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Each reaction was repeated three

times. The RT-qPCR results were calculated by the $2^{-\Delta\Delta CT}$ method [38]. The primers for RT-qPCR are listed in Table 1.

2.7. Growth Measurement and Physiological Traits of *BpTCP3* Transgenic *B. platyphylla* under Salt Stress

In order to analyze the physiological traits of transgenic lines and the WT in response to salt stress, four-month-old plants were sprayed with a solution of 0.4% [26] NaCl at 18:00 every 2 days for 12 d, and the sixth to eighth functional leaves were harvested before salt treatment (0 days) and 4, 8, and 12 days after salt treatment for hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content assay. The H_2O_2 content was determined by an H_2O_2 assay kit (Nanjing Jiancheng Bioengineering Institute, A064-1), and the MDA content was determined by a plant MDA assay kit (Nanjing Jiancheng Bioengineering Institute, A003-1). At least three seedlings were mixed for each sample, and three biological replications were collected to analyze each experiment. Then, water was applied again for one month, and the plant heights of *BpTCP3* transgenic lines and the WT were measured. At least 15 seedlings per strain were used in the experiment. The height growth (cm) = seedling height after rehydrated in water (cm) – seedling height before salt stress (cm). The relative height growth = height growth (cm)/seedling height before salt stress (cm). The plant salt injury index was proposed to evaluate salt tolerance [26,39]. The classification standard of plant salt damage was as follows: Grade 0: no symptoms of salt damage; Grade 1: mild salt damage, with a few leaf blades, leaf tips, leaf margins, or leaf veins that turned yellow; Grade 2: moderate salt damage, yellowing of leaf tip and edge; Grade 3: severe salt damage, most of the leaf blade, leaf tip, leaf edge, and veins turned yellow; Grade 4: extremely serious salt damage, the leaves were scorched and fell off, followed by plant death. The salt damage index (d) was calculated as $(d) = \Sigma (\text{salt damage series} \times \text{number of plants with the corresponding salt damage level}) / (\text{maximum salt damage level} \times \text{total number of plants tested})$.

2.8. Statistical Analysis

All data analysis and mapping were performed by using SPSS 25.0 (IBM Corp., Armonk, NY, USA) and Microsoft Office Excel (Microsoft, Redmond, WA, USA).

3. Results

3.1. Structural Identification and Phylogeny of the CYC/TB1 Subclass TCP Genes in *B. platyphylla*

In a previous study, a total of 15 potential members of the TCP family with the bHLH basic domain were exposed to *B. platyphylla*. Therefore, we focused on the two genes belonging to the CYC/TB1 subclass of the class II TCP family of *B. platyphylla*, *BpTCP3* and *BpTCP12*. The sequences analysis of the two *BpTCPs* showed that they localized in the nucleus. However, their gene structures varied greatly, such as the boundary of the untranslated region (UTR), ORFs and protein length, PI, and Mw (Table 2).

Table 2. Sequence feature analysis of the 2 *BpTCPs* in *B. platyphylla*.

Gene	5'UTR (bp)	3'UTR (bp)	ORF Length	Protein Length (aa)	Molecular Weight (Mw/kD)	Isoelectric Point (pI)	Subcellular Localization
<i>BpTCP3</i>	20	179	885	294	33.29	9.38	Nucleus
<i>BpTCP12</i>	210	41	1200	399	44.13	7.65	Nucleus

To identify whether *BpTCP3* and *BpTCP12* belonged to the CYC/TB1 clade, multiple sequence alignment was performed between the TCP family CYC/TB1 subclass genes in *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Phyllostachys heterocycla*, *Eucalyptus camaldulensis*, *Populus euphratica*, and *B. platyphylla*. The results are shown in Figure 1A; the CYC/TB1 proteins contain a conserved bHLH domain, characterized by basic, helix I, loop, and helix II regions, known as the TCP domain. The basic domain of CYC/TB1 contains 21 amino acids.

Lysine (Lys, K) and arginine (Arg, R) related to nuclear localization are highly enriched in the basic domain. The helix regions contain multiple leucine (Leu, L), serine (Ser, S), and threonine (Thr, T) residues; the latter two residues are potential sites of phosphorylation and are in conserved positions. In the loop domain, Aspartic acid (Asp, D), Leu (L), alanine (Ala, A), and Ser (S) are highly conserved. In addition, *CYC/TB1* proteins have an additional R domain rich in Arg (R), Lys (K), and glutamic acid (Glu, E) [6].

Furthermore, the phylogenetic tree of *CYC/TB1* protein of birch and other species (including *Arabidopsis thaliana*, *Phyllostachys heterocycla*, *Oryza sativa*, *Eucalyptus camaldulensis*, *Zea mays*, and *Populus euphratica*) was constructed to study their phylogenetic relationships. The phylogenetic tree shows that the similarity between *BpTCP3* and *EcTCP3* from *Eucalyptus camaldulensis* and between *BpTCP12* and *PeTCP1* and *PeTCP24* from *Populus euphratica* was 96%. For the whole of the phylogenetic tree, the *CYC/TB1* proteins from woody plants, including birch, *Populus*, and *Eucalyptus*, gathered on one side of the phylogenetic tree, whereas model plants and gramineous plants gathered on the other side of the phylogenetic tree (Figure 1B).

3.2. The *BpTCP3* Promoter Contains Multiple Hormones and Stress-Response Elements

We obtained the promoter fragment of the 1795-bp upstream sequence of the *BpTCP3* gene according to the PCR amplification technique. Promoter cis-regulatory element prediction showed that the promoter regions of *BpTCP3* enriched eukaryotic necessary cis-acting elements, such as TATA and CAAT boxes (Figure 2). The *BpTCP3* promoter also contained several plant hormone-response elements, including one gibberellin-response element, one auxin-response element, two salicylic acid-response elements, four jasmonic acid-response elements, and four abscisic acid-response elements. In addition, the *BpTCP3* promoter contained multiple adversity stress-response elements, including three MYB binding sites involved in regulation of stress and one drought-induced regulatory element. Other elements in the *BpTCP3* promoter and their putative functions are shown in Table 3. The presence of these response elements indicated that the obtained promoter fragment was in accordance with the basic characteristics of the eukaryotic gene promoter and that the expressions of the *BpTCP3* genes of *B. platyphylla* may be regulated by these elements and may respond to a variety of plant hormones and adversity stresses.

Table 3. Putative cis-acting elements in the *BpTCP3* gene promoter inferred by Plant CARE.

Element	Element Sequence	Function	Number
TATA-box	ATTATA; TATAA; TATATA; TATA; ATATAT; TATACA; TACAAAA	Promoter core element	14
CAAT-box	CAAT; CAAAT; CCCAATTT; CAACCAACTCC	Promoter enhancer conserved elements	30
MYBCORE	CNGTTR;	MYB binding site, involved in regulation of stress	3
MBS	CAACTG	MYB binding zone, drought-induced regulatory element	1
GARE-motif	TCTGTTG	Gibberellin-response element	1
TGA-element	AACGAC	Auxin-response element	1
TCA-element	CCATCTTTTT	Salicylic-acid response element	1
CGTCA-motif	CGTCA	Jasmonic acid-response element	2
TGACG-motif	TGACG	Jasmonic acid-response element	2
ABRE	ACGTG; CACGTG	Absciscic acid-response element	4

Table 3. Cont.

Element	Element Sequence	Function	Number
CAT-box	GCCACT	Cis-acting regulatory element related to specific expression of meristems	2
CCGTCC-box	CCGTCC	Cis-acting regulatory element related to specific activation of meristems	1
Skn-1_motif	GTCAT	Cis-acting regulatory elements related to specific expression of endosperm	2
MRE	AACCTAA	MYB binding site involved in light responsiveness	1
ARE	AAACCA	Anaerobic response element	1
GC-motif	CCCCCG	Anaerobic response element	1
O2-site	GATGACATGG; GATGATGTGG	Cis-acting regulatory element involved in the regulation of protein metabolism	2
Box 4	ATTAAT	Light-responsive element	2
G-Box	CACGTG	Light-responsive element	1

(+) 1795 CCCATCAACA CCTGTGAAAT GCTGTAAAGAT TGAATTGAAAC ATAAATACCT TAAACAAAAA GGTTTTAAAG GGTGTAGAGT
 (-) 1795 GGTAGTTGA GGACACTTTA CGACATTCCTA ACCTAAGTTT TATTATATGA ATTTGTGTTT CCAAAATCTT CCACCTCTCA
 (+) 1715 TTAATAATTA TAAATTCACCA ACTAAAGCCG CTGAAAAAGA AATCCTTTGTC TACATAAAGC ATCCCAATTT TTCTGCTCTC
 (-) 1715 AATTATTAAAT ATT TACTGCTGT TGAATTCGGC TACTTTTTCT TTAGGAACAG ATGTTATTTC TAGGGTTAAA AAGAGCAGAG
 (+) 1635 TCAGGAAAT CTAGCAT TAA TCCACAAAA AGGCTGGAAG ACAACGTACG GAAACAAAAA AAAAAAAA ATCATAGATA
 (-) 1635 AGTCCTTTTA GATCGTAGTT AAGGTGTTTT GCCCACCCTT TGTGTCATGC CTTTGTTTTT TTTTTTTTTT TAGTATCTAT
 (+) 1555 TGCTTTTTTT TTTTCTTCT TATTTCACCC ACATATGGGC TTTGGGTACA GACAACGAGC AGGATGAGAA CTCACCAAGG
 (-) 1555 ACGAAAAAAA AAAAAGAAAG ATAAAGTTGG TGTATACCCG AAACCCATGT CTGTTGCTCG TCTACTCTT GAGTGTTC
 (+) 1475 AAATGAAAGC ACTTAGATTA AAAATGCTCT TTAGGTTTTT TTTTTTTTCC CTCTCTTTTG TGTAGGCTCT CATTATCAGC
 (-) 1475 TTTACTTTTC TGAATCTAAT TTTTACAGAA AATCCAAAAA AAAAAAAGGG GAAGAAAAAC ACATCCGAGA GTAATAGTTCG
 (+) 1395 CACTCAGTT GATGGTACAT TCACCTCTCC TTTTCCTTCA TAGTTATCTT CTCCTTGAAA GCAACCTCAG AACAAACCCA
 (-) 1395 GTGAGTCAA TACCATGTA AGTGAAGGG AAAAGGAAGT ATCAATAGAA GAGGAACCTT CGTTGGAGTC TTGTTTGGGT
 (+) 1315 GCTTTGCCAA GGTGGTCCCT GTAATTATGC TAGTCTAAAG TGGTCGGGCT CAGTTTTATG GAGAGAGAGA GAGAGAGAGA
 (-) 1315 CGAAACGGTT CCACCAAGGA CATTATATCG ATCAGATTTT ACCAGCCCGA GTCAAAATAC CTCTCTCTCT CTCTCTCTCT
 (+) 1235 GAGAGAGAGA GAAATGGTTT GATATGTGTA GAGGACGGCA TGTATATATG GAAAAATATG TGGGTGAAAG AGGGCTTTTA
 (-) 1235 CTCTCTCTCT CTTTACCAAA CTATACAACT CTCTGCGGCT ACATATATAC CTCTTTATCA ACCCAATTTA TCCCGAAGAT
 (+) 1155 TGTGGTATG GGCATTTCC CATTTTGAAT TAAGTATAGA TGGCATTGTT TAGCTAGGCG ACTTTTGGTA TTGGCTACGT
 (-) 1155 TAAACCTAC CCTGATAAGG GTAAAACTTT ATTCTACTCT ACCGTAAAC ATGCAACCCG TGAACACCTT AACGATGCA
 (+) 1075 TTTTGGAGAA GGTCTATGTA TGTGGCAGTG GCAAGTACAA GCAACCAACA CTCTTTCTTG AATATGTTG TGAAGAGAGG
 (-) 1075 AAAACCTCTT CCAGATACAT ACACCGTCAC CGTTATGTTT CGTGTGGTTT GAGAAAGAAC TGTATACAC ACTCTCTCTC
 (+) 0995 AGAGAGAGAG AAAAGCTAAA CAGATAAGCA CGCATGGGGA GGGAAACCCG TGAACATTCT ACAAGAATT GATAGATAGA
 (-) 0995 TCTCTCTCTC TTTTGAATTT GTCTATTTGT GGTACCCCTT CGGTATAGAG TGTTTCTT TATCTATCT
 (+) 0915 CATGGGGGAG AGAGAGAGAG AGAGATGGGA TTTCTCTCTT TTTTGAAGG CGACATATTT TTAATTAATT GCAGAGAGAG
 (-) 0915 GTACCCCTCT TCTCTCTCTC TCTCTACCTT AAAGAGGAAA AAAAACTTTC GGTGTATAAA AATTATTAAT TTTCTCTC
 (+) 0835 CATCAGTCAA AGGGAGCAAT GCGTCTCATG GCAGGCTCAC AGTGCATGCC CCGTTTGAAT GAAATAATTA TTGCTCTCT
 (-) 0835 GTAGTCAGTT TCCCTCGTAA CAGAGTACG CGTCCGAGTG TCACGTACGG GGGAAACTCA CTTTATTAAT TAAAGGAAGA
 (+) 0755 TGTCTAGCGT TAGAAACAGC TGAAGTAGG TTAAGTAGTG TGTATATAGC CTCCCAAAAA ACAGCAACAG CCGTGAATTA
 (-) 0755 GACAATCCCA ATCTTTGTG ATGCACATCC AATTTTCAGC ACAATATCTG AAGGTTTTTT TGTGCGTCTC GCACATTGTT
 (+) 0675 TAAATTAATTA CCACTCGGAG ACACATGCAC TAGGAGGGAT GAATATGGAA TCCATGCAGC ATCTTGGCTG AGTGCAGTGC
 (-) 0675 TAAATTAATTA GGTGAGCGTG TGTGTACGTG ATCTCTCCCTA CTATACCTTT AGGTATCTG TAGAACCAGC TCACGTACAG
 (+) 0595 AGTGAAGTAG GGGCTTCTTC AGAAATTTCT AACAAATATC AAGCCCTATC CTGTAATCTT CAGTAATATC AGTAATTAAT
 (-) 0595 TCACTCACTC CCGGAAGAGG TCTTTAAGAG TTGTTTATAG TGTGGAGTAG GAGCTTTAAT GCAATTAATG TCAATGTTAA
 (+) 0515 TCTGTTGGT ATTTTCTCTT CCACTCATCA CCCCTCTCTA AAACACAAAC ACAATTAATA CACAAATAT ACATATTAAT TATCATCAAT
 (-) 0515 AGAGCAACCA TAAAGGATA GGTGAGTAG TTTTAGGGTT TATAGCGAGA AACCTGAGTT GATGACAAAT GTGTTTTGTC
 (+) 0435 GAAAAAGATTA AGAAAAAGAG GCCTCTTTGT CTGTAATAAA TACCCCTTCT TGGGTCTCTT TTTCTCTCTC ACCCTCTCTT
 (-) 0435 CTTTTCTAAT TCTTTTCTCT CCGGAGAAACA GACGTTAATT ATGGGGAAGA ACCCAGAGA AAAAGGAGAG TGGGAGAGAA
 (+) 0355 TATTATCTC TCTCTCTCTA TCCCATGGC CGTGTACAG TATTCTAGCC ATGCTGTCTT TCTCAACCTC TAGTCTTTCT
 (-) 0355 ATAGTAGAG AGAGAGAGTT AGGGGTACCG GCAGCATGTC ATAGATGCGG TACGACAGAA AGAGTTGGAG ATCAGAAAGA
 (+) 0275 TCTCTCTCTC TCTCTCTCTC TCTCTCTCTA AAACACAAAC ACAATTAATA CACAAATAT ACATATTAAT TATCATCAAT
 (-) 0275 AAGGAGAGAG AGAGAGAGAG AGAGGAGAT TTTGTGTTG TGTGTTTAT GTGTTTTATA TSTATGTTAT ATAGTAGTTG
 (+) 0195 TATGATCTCT CATCATCAAC CTGTCTTCT CAAGCTCTGG TGGAAAAAGG CTGTCTTTCT CAAGTCCAGG TTTTTCACCA
 (-) 0195 TTACGTATCA GTAGTAGTGG GACGAGAAAG GTTCGAGACC ACCTTTTCGC GACAGAAAGA GTTCAGGTCC AAAAAGGTGT
 (+) 0115 CCGTCAGAAA TGAATTAGAA TAGAATTGAA GCCCGCCCAAT GTCTATCTCTA AACCTAGCTA GGGTTGGTGG ATTGCTTTTC
 (-) 0115 GGAGCTCTTT ACTTAATCTT ATCTATTAAT CGGCGCGG TGTGAGTAG TGTGATGAT TGGATGATG CCAACCAAC TAAAGGAGG
 (+) 0035 CCGGCTGAAA TGAATTAAGT AAAAAAATG +3
 (-) 0035 GGGCCAGGTT AAGTTAGTG TTTTTTTTTT TTTTTTAC +3

■ Gibberellin-response element ■ Auxin-response element ■ Salicylic acid-response element
■ Jasmonic acid-response element ■ Abscisic acid-response element ■ Promoter core cis-acting element
■ Stress response element ■ Drought response element ■ Promoter enhancer conserved element

Figure 2. *BpTCP3* gene promoter sequence denoting the cis-elements predicted by the Plant-CARE databases.

3.3. The *BpTCP3* Gene Participates in Salt Treatment Response

To analyze the expression pattern of the *CYC/TB1* subfamily members under salt treatment, nontransgenic birch seedlings that sprouted two cotyledons in hydroponic solution were subjected to 0-, 2-, 4-, 6-, 12-, and 24-h salt treatment in WPM agar plates supplemented with 0.4 M NaCl or WPM agar plates without NaCl as a control (0 h). As shown in Figure 3, during salt treatment, compared with 0 h, the expression of the *BpTCP3* and *BpTCP12* genes at 6 and 12 h significantly decreased, whereas the expression levels at 4 and 24 h significantly increased. At the same time, the expression levels at 2 h did not change significantly. The results confirmed that the expressions of the *CYC/TB1* genes were influenced by salt stress. In other words, the expression profiles of the *BpTCP3* and *BpTCP12* genes were similar under salt stress. We speculated that the *BpTCP3* and *BpTCP12* genes may play the same role in salt stress. Therefore, one gene, *BpTCP3*, was selected for further study.

Figure 3. The expression pattern of the *BpTCP3* gene in WT *B. platyphylla* under salt treatment. (A) Phenotypic observation of non-transgenic birch seedlings under NaCl treatment. (B) RT-qPCR analysis of *BpTCP3* gene expression in nontransgenic birch seedlings under salt stress. (C) RT-qPCR analysis of *BpTCP12* gene expression in non-transgenic birch seedlings under salt stress. Error bars are shown for three replicates of the RT-qPCR assay. Different letters indicate a statistically significant difference compared with 0 h (data were analyzed by one-way ANOVA and a multiple comparison by Tukey's test; $p \leq 0.05$).

To study the characteristics of the *BpTCP3* promoter, we first constructed the *pBpTCP3::GUS*, and then generated *pBpTCP3::GUS* transgenic lines through *Agrobacterium tumefaciens*-mediated genetic transformation. We obtained two independent kanamycin-resistant *pBpTCP3::GUS* transgenic lines, which originally regenerated from different calli; all callus lines were kept separate to ensure that regenerated plants were truly independent. PCR analysis showed that the two lines produced a 1795 bp band similar to the positive control, while the negative control did not produce a band (Figure 4E). These results confirmed that the promoter sequence of the *BpTCP3* gene had been successfully transferred into the genome of birch.

Figure 4. Generation and validation of *pBpTCP3::GUS* transgenic *B. platyphylla*. (A) Seeds from *B. platyphylla* infected with the *pBpTCP3::GUS*-engineered bacteria. (B) Resistant calli. (C) The differentiation culture containing resistant calli. (D) The cultivation of rooted plantlets. (E) PCR amplification analysis of the *BpTCP3* promoter in *pBpTCP3::GUS* transgenic *B. platyphylla*. M DNA Marker DL5000, 1 positive control (*pBpTCP3::GUS* vector plasmid), 2 negative control (pBI101 vector plasmid), 3 H₂O control (double-distilled water), 4–5 *pBpTCP3::GUS* transgenic lines.

The *pBpTCP3::GUS* transgenic rooted plantlets were exposed to 0.4 M NaCl for 0 (control), 2, 4, 6, and 24 h, and analysis of seedlings regarding GUS histochemical activity and *GUS* gene expression was performed. Compared with 0 h, the promoter of *BpTCP3* gene activity was significantly enhanced under salt treatment at 2 and 24 h as evidenced by the stronger staining of plantlets, particularly the roots and leaves (Figure 5A). Moreover, RT-qPCR analysis of the *pBpTCP3::GUS* transgenic lines revealed that the expression of the *GUS* gene was also significantly increased during salt treatment at 2 h and 24 h compared with 0 h (Figure 5B). Based on these results, we can infer that the activity of the promoter of the *BpTCP3* gene was induced by salt treatment. Combined with the results of Figure 3, we further confirmed that the *BpTCP3* gene participated in the salt stress response.

Figure 5. Promoter activity of the *BpTCP3* gene under salt treatment. (A) GUS histochemical assay results for *pBpTCP3::GUS* transgenic birch plantlets treated with NaCl. (B) Expression of the *GUS* gene in *pBpTCP3::GUS* transgenic birch plantlets under salt treatment. Error bars are shown for three replicates of the RT-qPCR assay. Different letters indicate a statistically significant difference compared with 0 h (data were analyzed by one-way ANOVA and a multiple comparison by Tukey's test; $p \leq 0.05$).

3.4. *S::BpTCP3* and *35S::BpTCP3-SRDX* Transgenic *B. platyphylla* Were Obtained

To further study the potential function of the *BpTCP3* gene in salt stress, we constructed an overexpression vector (*35S::BpTCP3*) and a suppressed expression vector (*35S::BpTCP3-SRDX*) of *BpTCP3*. The two recombinant vectors were transferred into WT *B. platyphylla* zygotic embryo by *Agrobacterium-mediated* method. Zygotic embryos were cultivated on the selective medium containing hygromycin for 20 days, and a resistant callus was produced from the cut sites (Figure 6B,G). The resistant calli were proliferated and differentiated to obtain resistant plants (Figure 6C–E,H–J). The 885-bp ORF sequence of the *BpTCP3* gene in the overexpression lines and the 921-bp chimeric *BpTCP3* repressor in the suppressed expression lines were amplified by PCR. Because the *BpTCP3* gene contained introns, all the overexpression lines (OX) and inhibitory expression lines (SR) of the *BpTCP3* gene can amplify two bands, while the WT plants and positive control can only amplify one band. These results preliminarily proved that the exogenous *BpTCP3* and *BpTCP3-SRDX* sequences had been inserted into the genome of *B. platyphylla* (Figure 6K,L).

The transgenic lines and WT plants were further tested by RT-qPCR. The results showed that the expression level of *BpTCP3* in all transgenic lines was higher than that in WT. The highest expression levels of *BpTCP3* were found in OX-2, OX-3, SR-4, SR-5, and SR-7, respectively (Figure 6M). Therefore, OX-2, OX-3, SR-5, and SR-7 were selected for further analysis.

Figure 6. Generation and validation of 35S::BpTCP3 and 35S::BpTCP3-SRDX transgenic *B. platyphylla*. (A–E) Generation of 35S::BpTCP3 transgenic lines, including seeds from *B. platyphylla* infected with the 35S::BpTCP3-engineered bacteria, the resistant calli, the differentiation culture containing resistant calli, and the cultivation of rooted plantlets. (F–J) Generation of 35S::BpTCP3-SRDX transgenic lines. (K) PCR amplification analysis of the *BpTCP3* gene in 35S::BpTCP3 transgenic *B. platyphylla*. M DNA Marker DL2000, 1 positive control (35S::BpTCP3 vector plasmid), 2 negative control (WT birch), 3 H₂O control, 4–8 35S::BpTCP3 transgenic lines. (L) PCR amplification analysis of the chimeric *BpTCP3* repressor in 35S::BpTCP3-SRDX transgenic *B. platyphylla*. M DNA Marker DL2000, 1 positive control (35S::BpTCP3-SRDX vector plasmid), 2 negative control (WT birch), 3 H₂O control, 4–11 35S::BpTCP3-SRDX transgenic lines. (M) RT-qPCR analysis of *BpTCP3* expression in different transgenic lines. Different letters indicate significant differences among different bars.

3.5. Salt Treatments Affected the Growth of BpTCP3 Transgenic Lines

To explore the response of transgenic birch to salt stress, four-month-old transgenic lines and WT after transplanting were irrigated with 0.4% NaCl solution, and the plant salt injury index was measured at 12 days (Figure 7). Then, plants were watered again for one month, after which the plant height growth of the *BpTCP3* transgenic lines and the WT was measured. The results revealed that values of the indices reflecting the salt stress damage were as follows: the *BpTCP3*-inhibition lines > WT > *BpTCP3*-overexpression lines. Both the absolute growth and the relative growth before salt stress and after rehydration were compared (Table 4). Before salt treatment, the seedling height of the *BpTCP3* transgenic lines and WT plants were not visibly different. After salt stress treatment and water application to restore the normal growth of birch, the overexpression lines were taller than the WT, whereas the suppressed expression lines were shorter than the WT seedlings. In addition, the growth height and relative growth height of the overexpression lines were greater than those of the WT, whereas the suppressed expression lines were shorter than those of the WT. In summary, under NaCl stress, the overexpression line showed resistance to salt compared with the WT, whereas suppressed expression lines showed sensitivity. These data imply that *BpTCP3* plays an essential role in salt stress.

Figure 7. Phenotype observation of *BpTCP3* transgenic lines and WT birch after salt stress. **(A)** Plant phenotypes after salt treatment at 12 d. **(B)** The plant salt injury index after salt treatment at 12 d.

Table 4. Plant height growth of the *BpTCP3* transgenic lines and WT plants under salt stress.

Line	Seedling Height before Salt Stress (cm)	Seedling Height after Rehydrated in Water (cm)	Absolute Height Growth (cm)	Relative Height Growth
WT1	26.60 ± 2.39 a	33.12 ± 2.31 b	6.52 ± 0.57 b	0.24 ± 0.036 b
WT2	26.42 ± 2.40 a	33.33 ± 2.80 b	6.91 ± 0.85 b	0.26 ± 0.037 b
OX-2	26.61 ± 1.93 a	35.91 ± 1.74 a	9.30 ± 0.85 a	0.35 ± 0.049 a
OX-3	26.60 ± 1.54 a	36.16 ± 2.15 a	9.56 ± 1.34 a	0.36 ± 0.053 a
SR-5	26.43 ± 1.62 a	31.45 ± 1.90 c	5.02 ± 0.64 c	0.19 ± 0.024 c
SR-7	26.54 ± 1.86 a	31.00 ± 1.75 c	4.47 ± 0.65 c	0.17 ± 0.031 c

Data indicate means ± STDEV (data were analyzed by one-way ANOVA and multiple comparisons using Tukey's test at $p \leq 0.05$). Values labeled with different letters in the same column indicate significant differences among different lines.

3.6. The *BpTCP3* Gene Reduced Reactive Oxygen Species Damage and Improved the Salt Resistance of *B. platyphylla*

Under abiotic stress, excessive H_2O_2 produced in plants will cause cell membrane damage [40]. In this study, the H_2O_2 and MDA contents were measured to detect the concentration of reactive oxygen species (ROS) and the degree of cell membrane damage in the *BpTCP3* transgenic lines and WT seedlings under salt stress (Figure 8). With the increase in the NaCl stress treatment time, the levels of H_2O_2 and MDA of each strain increased

greatly. In addition, the contents of H_2O_2 and MDA in each strain were not visibly different under NaCl stress treatment for 0 d. By contrast, the accumulation of H_2O_2 and MDA was obviously lower in the *BpTCP3* overexpression lines but was significantly higher in the *BpTCP3* suppressed expression lines than in the WT under NaCl stress treatment for 4, 8, and 12 d. These results suggest that the *BpTCP3* gene can improve plant resistance through a decrease in the accumulation level of ROS in plants under salt stress.

Figure 8. Physiological indicators of *BpTCP3* transgenic lines and WT birch under salt stress. **(A)** Analysis of the MDA level. **(B)** Analysis of the H_2O_2 level. The error bars represent the standard deviation, and letters indicate significant differences between transgenic lines and WT1 (data were analyzed by one-way ANOVA and a multiple comparison by Tukey's test; $p \leq 0.05$).

4. Discussion

Many cis-acting elements associated with biotic and abiotic stress were found in the promoter of the *BpTCP3*, implicating the possible role of the *BpTCP3* gene in biotic or abiotic stress responses (Figure 2). The transcription level of the *BpTCP3* gene changed to varying degrees at multiple time points of salt treatment (Figure 3), suggesting that the *BpTCP3* gene can respond to salt stress. The results of the GUS staining analysis in *pBpTCP3::GUS* lines under salt treatment further revealed that the *BpTCP3* gene played an active regulatory role in the salt stress response pathway (Figure 5). In addition, transgenic birch overexpressing *35S::BpTCP3* displayed a reduced salt damage index and increased relative/absolute high growth compared with WT plants below salt treatment. In contrast, the repressed *BpTCP3* plantlets displayed a higher salt damage index and decreased relative/absolute high growth, as well as high sensitivity to salt stress (Figure 7 and Table 4). Interestingly, the *BpTCP7* gene of Class II genes significantly enhanced the salt tolerance of *B. platyphylla* [27].

These facts indicate that the salt-impacted *BpTCP3* gene plays an essential role in the salt endurance of *B. platyphylla*.

Plants finely regulate complex physiological processes and metabolic pathways under continuous salt stress to ensure plant fitness and health. Under salt stress, the ROS, such as singlet oxygen, superoxide, hydroxyl radical, and H_2O_2 , were enriched in plants, resulting in oxidative damage in cellular components, interruption of vital cellular functions of plants, nutrient imbalance, and an impaired ability to detoxify ROS [41]. Generally, the content of H_2O_2 and MDA has been used to analyze stress tolerance related to transcription factors in plants, as H_2O_2 and MDA can be vital biochemical markers of ROS production and cell membrane damage, respectively [42,43]. In this investigation, under salt stress, the levels of H_2O_2 and MDA in leaves of transgenic lines overexpressing *35S::BpTCP3* were lower than that of WT, while *BpTCP3*-inhibition displayed severe ROS damage (Figure 8). These results indicate that the enhanced salt stress tolerance of the transgenic *B. platyphylla* overexpressing *BpTCP3* could be owing to their low ROS levels. That is to say, the *BpTCP3* gene can increase the salt endurance of *B. platyphylla* by reducing ROS accumulation.

In this study, plenty of cis-acting elements related to hormones were found in the promoter region of *BpTCP3*, such as gibberellin, auxin, salicylic, jasmonic acid, and abscisic acid-response elements (Figure 2, Table 3). A previous study from our laboratory revealed that *BpTCP3* was involved in these hormone responses (unpublished data). *TCP* has been extensively investigated to regulate hormone responses depending on how *TCP* works in coordination with other genes. For instance, *AtTCP3* associates with *R2R3-MYB* family proteins to promote flavonoid biosynthesis and thus affects the auxin response in *Arabidopsis thaliana* [44]. *AtTCP15* and related proteins mediate the development of the gynoecium through their involvement in auxin and cytokinin responses [45]. Noh et al. provided evidence that *AtTCP20* and *TCX8* delay plant senescence through their participation in jasmonic acid biosynthesis [46]. Previous studies reported that plants adapt to salt stress by regulating the dynamic changes in hormone biosynthesis [41] (e.g., ABA plays an essential position in salt stress signal transduction of *B. platyphylla* [47] and *Populus euphratica* [5]). The transcription factor ultimately may affect the level of salt sensitivity of plants through mediated hormone responses [48]. In addition, research has indicated that *OsTCP19* appears to act as an important node between the salt stress response and ABA signal pathway [49]. These previous studies and the results of our study together suggest a role for *BpTCP3* in salt stress may by mediated hormonal response in *B. platyphylla*.

Many studies have shown that *TCP* family members play essential roles in organ morphogenesis. For example, *AtTCP4* is an important regulator of flower organ development in *Arabidopsis* [14], and *AtTCP3/4* can promote shoot organogenesis via regulating the expression of a negative cytokinin response regulator, *AtARR16* [15]. *AtTCP17*, *AtTCP5*, and *AtTCP13* influence the hormone metabolic pathway and thus affect the length of hypocotyl by regulating the expression of the light-sensitive signal factor *PIF* [50]. Here, three cis-regulatory elements that are related to meristem-specific expression were found in the promoter region of *BpTCP3*. It might imply the potential role of *BpTCP3* in the organ morphogenesis of plants. In addition, a previous study indicated *TCP* family members were involved in the drought stress response of *B. platyphylla* through hormone pathways [27]. Herein, a large number of cis-acting elements related to hormones and one drought-induced regulatory element were found in the promoter region of *BpTCP3*, which hints that *BpTCP3* may participate in the drought stress response of *B. platyphylla* through the hormone pathway.

The research on *TCP* genes has focused mainly on model species and major cereal crops. Encouragingly, more and more *TCP* family members from different species have been studied [27,51]. Regarding *TCP* genes, biological and molecular mechanism studies have focused mainly on plant development, but little is known about salt stress. More recently, it was shown that a few *TCP* family members of nonmodel species were involved in plant salt tolerance. For instance, for the *PtTCP10* gene, a Class I *TCP* member of moso bamboo, overexpressed *PtTCP10* was found to improve salt endurance of trans-

genic Arabidopsis by increasing primary root length at the vegetative growth stage [52]. In *Vigna unguiculata*, a drought- and heat-tolerant legume crop, the transgenic lines that overexpressed *VuTCP9* displayed higher tolerance to salinity stress through regulation at physiological and biochemical levels, as well as phenotypic alterations [53]. Overexpressing *BpTCP7*, a Class II TCP member of *B. platyphylla*, helps transgenic plants to obtain strong resistance to salt stress [27]. In this study, the *BpTCP3* gene improved the salt tolerance of *B. platyphylla* through a decrease in the accumulation of ROS in plants in salt stress. In conclusion, our results may provide some helpful information for the studies of homologous gene function in the future.

5. Conclusions

In this study, we principally focused on the role of *BpTCP3*, a CYC/TB1 subclass of the TCP gene family of *B. platyphylla*. We found that the *BpTCP3* gene can be influenced by salt stress, and its promoter sequence had many cis-acting elements involved in stress. The GUS staining analysis in *pBpTCP3::GUS* birch lines further confirmed that the *BpTCP3* gene plays a positive regulatory role under salt stress. To further reveal the role of *BpTCP3* in salt stress, the overexpressing 35S::*BpTCP3* birch displayed stronger salt resistance than the WT plants. In contrast, the repressed *BpTCP3* birch exhibited a higher salt sensitivity. The overexpression birch lines had the lowest amount of ROS and the lowest degree of cell membrane damage. These results suggest that the *BpTCP3* transcription factor improved salt tolerance of *B. platyphylla* by reducing ROS damage, which provides a valuable reference for salt stress function of the *BpTCP3* gene in birch and extends the function of the CYC/TB1 subclass of *B. platyphylla*.

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