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Overexpression of BplERD15 enhances drought tolerance in Betula platyphylla Suk

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Overexpression of *BplERD15* Enhances Drought Tolerance in *Betula platyphylla* Suk.

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Abstract: In this study, we report the cloning and functional characterization of an early responsive gene, *BplERD15*, from *Betula platyphylla* Suk to dehydration. *BplERD15* is located in the same branch as *Morus indica* Linnaeus *ERD15* and *Arabidopsis* Heynh *ERD15* in the phylogenetic tree built with ERD family protein sequences. The tissue-specific expression patterns of *BplERD15* were characterized using qRT-PCR and the results showed that the transcript levels of *BplERD15* in six tissues were ranked from the highest to the lowest levels as the following: mature leaves (ML) > young leaves (YL) > roots (R) > buds (B) > young stems (YS) > mature stems (MS). Multiple drought experiments were simulated by adding various osmotica including polyethylene glycol, mannitol, and NaCl to the growth media to decrease their water potentials, and the results showed that the expression of *BplERD15* could be induced to 12, 9, and 10 folds, respectively, within a 48 h period. However, the expression level of *BplERD15* was inhibited by the plant hormone abscisic acid in the early response and then restored to the level of control. The *BplERD15* overexpression (OE) transgenic birch lines were developed and they did not exhibit any phenotypic anomalies and growth deficiency under normal condition. Under drought condition, *BplERD15-OE1*, *3*, and *4* all displayed some drought tolerant characteristics and survived from the drought while the wild type (WT) plants withered and then died. Analysis showed that all *BplERD15-OE* lines had significant lower electrolyte leakage levels as compared to WT. Our study suggests that *BplERD15* is a drought-responsive gene that can reduce mortality under stress condition.

Keywords: *Betula platyphylla*; early responsive to dehydration gene; drought stress; transgenic lines

1. Introduction

Drought stress is a severe environmental condition where plants are subjected to dehydration, resulting in loss in plant biomass productivity [1]. Due to wide-spreading and high-frequent occurrence, the loss caused by drought in crop yield is usually so high that it may exceed losses caused by all other environmental factors together [2,3]. When a drought occurs, various cellular signals are perceived and then conveyed through multiple pathways, for example, ionic and osmotic steady-state signaling pathways, damage control and repair response pathways, and growth regulation pathways [4]. Through these pathways, a series of physiological and biochemical reactions are activated or enhanced to produce gene products and various metabolites that can repair or prevent damages of cellular apparatuses, resulting in the survival in drought condition. In this process, the products of the drought-inducible genes can be largely classified into two categories: (1) stress tolerance proteins, which include chaperones, late embryogenesis abundant (LEA) proteins, osmotins, key enzymes for osmolyte biosynthesis, water channel proteins, and proline transporters, as well as detoxification

enzymes [5]; (2) The other category comprises regulatory proteins, for example, transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules such as calmodulin-binding protein [5]. These drought responsive genes in general contain ABRE (ABA-responsive element) and DRE (dehydration-responsive element)/CRT (C-RepeaT) [6,7]. ABRE and DRE/CRT are *cis*-acting elements that function in dehydration-responsive gene promoters in ABA-dependent and ABA-independent manners, respectively [5,6]. Based on these elements, drought responsive genes can also be classified into ABA-dependent and ABA-independent pathways. ABA-independent pathways do not respond to change of ABA. These genes include *ERD1*, which was reported to be induced 1 h after dehydration treatment [8]. It encodes a Clp protease regulatory subunit [9]. Promoter analysis of the *ERD1* gene revealed that there is an ABRE-like *cis*-acting element that shares similarity to ABRE motif but does not respond to ABA.

To date, 16 early response to the dehydration (ERD) genes have been annotated in *Arabidopsis Thaliana* (L.) Heynh. These genes come from different gene subfamilies and have both same and distinct functions. Among these genes, *ERD2*, *ERD8*, and *ERD16* [10] were identical to those of heat shock protein (HSP) cognates, and their expression are affected by dehydration stress, but not ABA. *AtERD6* expression can be induced not only by dehydration but also by cold treatment [11]. In addition, *ERD10* and *ERD14* [12] are very similar to class II LEA proteins which are ABA-inducible. Application of ABA indeed induces both *ERD10* and *ERD14*. The *ERD10* [13] mutant shows reduced stress tolerance. In addition, *SpERD15* in wild tomato *Solanum pennellii* [14] enhances soluble sugar and proline accumulation in transgenic plants, thereby increasing plant drought resistance. Moreover, *VaERD15* [15] and *ZmERD4* [16] were transferred into *A. thaliana*, and the transgenic lines showed enhanced tolerance to freezing, drought and salt stresses, suggesting divergence in their responses to various stresses and functions in stress tolerance.

White birch, also known as Manchurian birch, Siberian silver birch, Japanese or Asian white birch (*Betula platyphylla* Sukaczev) [17], can on fertile soil grow 27 m in height and 50 cm in diameter, with a growth life of 120 years [18], and they are widely distributed in Japan, North Korea, Russia, China, and Mongolia [19]. In birch, the genes involved in abiotic stress have been reported. For example, overexpression of *BpERF2* or *BpMYB102* can significantly improve the tolerance to drought stress [20]; *BpNAC012* positively regulates abiotic stress responses [21]; *BpERF11* negatively regulates birch salt and osmotic tolerance [22]; *BplMYB46* expression was induced by NaCl, ABA, and mannitol [23]. Recently, *BpERF13* is reported to enhance the cold tolerance when it is overexpressed in transgenic birch lines [24]. These results indicate that birch may have developed a wide-spectrum of stress-responsive programs during evolution. Various abiotic stresses can impose constraints on metabolism, thereby resulting in some physiological and morphological. For example, *Prunus sargentii* Rehder and *Larix kaempferi*, which have roughly the same geographical distribution as white birch, exhibit reduced leaf areas and shorter branches but increased leaf mass area as well as decreased photosynthesis rate and electron transfer rate (*J*max) for both species under drought stress [25].

In this study, we cloned an ERD gene, *BplERD15*, from *B. platyphylla*, which was induced in several simulated stresses by PEG, mannitol and NaCl. Our results showed that overexpression of *BplERD15* improved drought tolerance of transgenic birch lines and enabled them survive from the dehydration treatment.

2. Materials and Methods

2.1. Cloning BplERD15 and Phylogenetic Analysis of ERD Genes

We used *BpeERD15* sequence from *B. pendula* whose genome has been sequenced to design a pair of primers for amplifying *BplERD15* from a cDNA library constructed with mRNAs from *B. platyphylla* leaves. The primer sequences used are shown in Table S1. *BplERD15* PCR products were sequenced and translated into protein sequence using BioEdit software [26]. We downloaded 16 *A. thaliana* ERD genes from TAIR [27] based on the gene identifiers provided in the earlier publication [28] and

several other *ERD15* genes from other species, which include *SpERD15* from *S. pennellii* [14], *MiERD15* from *Morus indica* [29], *GmERD15* from *Glycine max* [30] and *VaERD15* from *Vitis amurensis* Rupr [15]. Following that, we built these genes into a phylogenetic tree using the neighbor-joining statistical method and the Poisson model in Mega X software with 1000 of bootstrap replications.

2.2. Cloning and Tissue-Specific Expression of BplERD15

The analysis of tissue-specific expression patterns of *BplERD15* was performed using qRT-PCR. The samples were collected from multiple tissues including buds, young leaves, mature leaves, young stems, mature stems, and roots of *B. platyphylla* and frozen immediately into liquid nitrogen. The leaves from the first to third stem nodes were referred to as young leaves (YL), while the leaves of the fourth to sixth stem nodes were referred to as mature leaves (ML). Accordingly, the stems of the first to third stem nodes were referred to as young stems (YS), and the fourth to sixth stem nodes were referred to as mature stems (MS). Cetyltrimethylammonium bromide (CTAB)-based protocol [31] was used to extract RNA, which was reversely transcribed into cDNA. The cDNA acquired was then used for qRT-PCR with the Toyo Spinning Kit (TOYOBO SYBR qPCR Mix, QPS-201). The amplification conditions were as follows: 95 °C for 30 s, which was followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s, finally 95 °C for 15 s, 60 °C for 60 s, 95 °C for 30 s. Ubiquitin gene was chosen to be the internal reference. There were three biological replicates.

2.3. Analysis of the Expression of BplERD15 in Wild-Type B. Platyphylla

Drought experiments were simulated by adding various osmotica including polyethylene glycol (PEG) [32], mannitol, and NaCl to the growth media to decrease their water potentials. Two-month-old wild-type birch seedlings were irrigated with solutions containing 20% PEG6000, 200 mM Mannitol, or 200 mM NaCl. The aforementioned tissues (Roots, YS, MS, YL ML and Buds) were harvested at six time points: 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h from the seedlings subject to different treatments. In order to test if *BplERD15* was responsive to ABA, two-month-old birch seedlings were sprayed with a 100 μM solution of ABA and incubated for 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h. The materials we harvested were immediately frozen into liquid nitrogen. RNA was extracted from these samples and used for qRT-PCR to obtain the mRNA abundances of *BplERD15* in different tissues in three biological replicates. Student's *t*-test was used to test the significance of the difference between a treatment and wild-type.

2.4. Plant Transformation

We designed primers with adaptors that contain specific restriction sites, and used birch cDNA as a template for PCR amplification of *BplERD15*; the PCR products were cloned into the binary vector called pROK2 upon a double-enzyme digestion of PCR products and vector sequence. The binary vector harboring *BplERD15* was then transformed into *Agrobacterium* strain EHA105 by the freeze-thaw method [33]. The *B. platyphylla* transgenic lines were developed by the leaf disc method [22] with minor changes. First the transformed Agrobacterium stain EHA105 was cultured at 28 °C for overnight until the OD fell into the range 0.6–0.8. The vigorous birch leaves from cultured *B. platyphylla* plants were cut and soaked in the bacterium culture for 8 to 10 min. Then, the leaves were taken out and placed on a sterile paper to allow the excessive culture to be absorbed. The leaves were then transferred onto the culture plates containing the WPM medium with 0.8 mg/L 6-BA + 0.02 mg/L NAA + 2% (*w*/*v*) sucrose, pH 5.8–6.0. The leaves were cultured in the dark for three days before they were transferred onto the culture with WPM media containing kanamycin (50 mg/L) and timentin (400 mg/L). Calli were first seen in about two months. When seedlings grew to about 1 cm high, they were cut into segments, each inserted into tissue culture bottles containing 1/2 MS+ 0.02 mg/L NAA + 2% (*w*/*v*) sucrose + 400 mg/L timentin + 50 mg/L kanamycin; pH 5.8–6.0. When the seedlings grew large, DNA was extracted with Tiangen DNA extraction kit (TIANGEN, Beijing, China). The transformants were examined with PCR and transgene-specific primers. The expression levels of *BplERD15* in different transgenic birch lines were analyzed by qRT-PCR. The primer sequences for PCR and qTR-PCR are shown in Table S1.

2.5. Drought Tolerance Assays of BplERD15 Overexpression Transgenic Lines

Three-month-old *B. platyphylla* transgenic lines were grown in a greenhouse under 16 h light/8 h dark and 25 °C. Before the drought experiment was performed, all plants were fully irrigated. After 15 days, the plants were subjected to dehydration. The photos were taken three days later after the rehydration we initiated.

2.6. Measurement of Electrolyte Leakage

Three-month-old transgenic lines with the highest expression of *BplERD15* were selected and subjected to drought stress for 10 d together with WT plants. The leaves were harvested and used for measuring electrolyte leakage as described earlier [34]. Briefly, the equal sections from the leaf of each sample were harvested and placed into a clean beaker; 30 mL of deionized water was added and left under vacuum for 15 min. The electrolyte leakage was measured and defined as S1. The leaves were then heated to 90 °C and kept for 20 min before they were cooled down to room temperature. The electrolyte leakage was measured again and defined to be S2. The electrolyte leakage (EL) was calculated with the formula: EL = (S1/S2) × 100%.

2.7. Statistical Analysis

The Student's *t*-test was used to examine the differences between transgenic lines and WT plants, and the difference before and after stress treatment. The threshold for statistically significant differences was set to $p < 0.05$.

3. Results

3.1. ERD Phylogenetic Analysis

The ORF (open reading frame) of *BplERD15* is 480 bp long and thus encodes a protein with 159 amino acids (Figure 1a). With this protein sequence, we used 16 ERD protein sequences from *A. thaliana*, and several other *ERD15* protein sequences from other plant species. We then built a phylogenetic tree (Figure 1b). We found that *BplERD15*, *BpeERD15*, and *MiERD15* had the closest distance and were clustered together. The multiple alignment analysis (Figure 1c) showed that *BplERD15* shared 100% and 50.56% similarity to *BpeERD15* from *B. pendula* and *MiERD155* from *M. indica*, respectively. In addition, *BplERD15* shared 45.98% and 44.31% similarity with *SpERD15* from *S. pennellii* and *AtERD15* from *A. thaliana*, respectively.

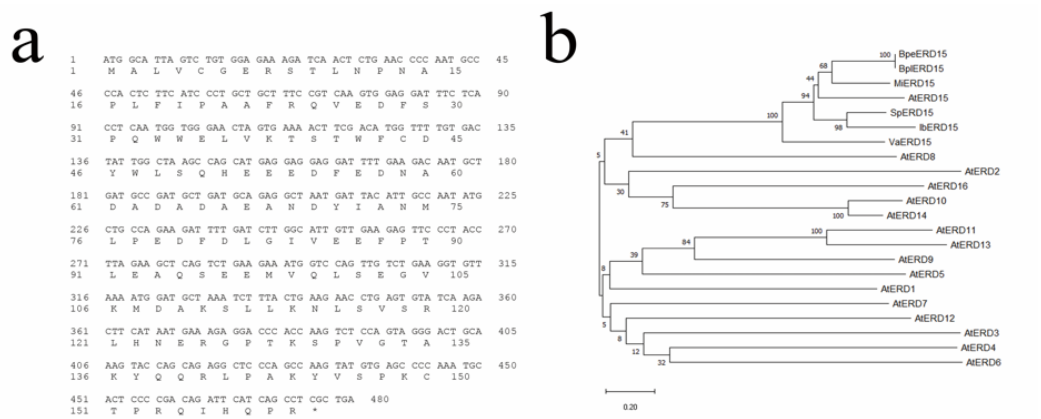

Figure 1. *Cont.*

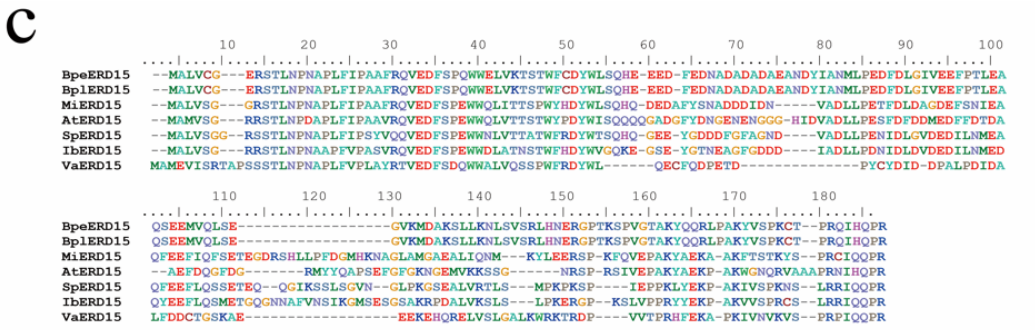

Figure 1. *Betula platyphylla Suk.* early response to the dehydration (*BplERD15*) gene and protein sequence analysis. (**a**): *BplERD15* gene coding sequence and predicted amino acid sequence. (**b**): Phylogenetic analysis of ERD proteins Arabidopsis thaliana, *BplERD15* and other *ERD15* proteins from plant species. The phylogenetic tree was constructed using MEGA X. (**c**): Sequence alignment of *BplERD15* with other *ERD15* from plant species.

3.2. Tissue-Specificity and Drought Stress Response of BplERD15 in WT Plants

The analysis of tissue-specific expression patterns of *BplERD15* was performed using qRT-PCR, and the results are shown in Figure 2a. The transcript levels of *BplERD15* in six tissues are ranked from the highest to the lowest in the following sequence: mature leaves (ML) > young leaves (YL) > roots (R) > buds (B) > young stems (YS) > mature stems (MS). In addition, the WT plants were subjected to drought treatment, and the results are shown in Figure 2b–d. It is obvious that under different drought stress conditions, *BplERD15* positively and differentially responded to PEG, Mannitol, and NaCl stresses. *BplERD15* transcript level was progressively enhanced by PEG treatment, with a slight decrease at the 24 h, and eventually reached its maximal level (12 folds) at the 48 h (Figure 2b).

Under mannitol stress, *BplERD15* transcript level was steadily up-regulated from 0 to 12 h period, and increased up to 9-fold as compared to 0 h (Figure 2c), and after that, it declined all the way to 48 h. Under salt stress, *BplERD15* transcript level peaked at 3 h where it had a more than 9-fold increase (Figure 2d). Though the transcript level of *BplERD15* started to decrease after 3 h, it remains higher than that of 0 h. We also applied ABA treatment. At the first time, the expression level of *BplERD15* was significantly down-regulated at 1 h and started to increased but was still significantly down-regulated at 3 h (Figure 2e). In subsequent time points, the expression level of *BplERD15* was not significant compared to that of wild-type.

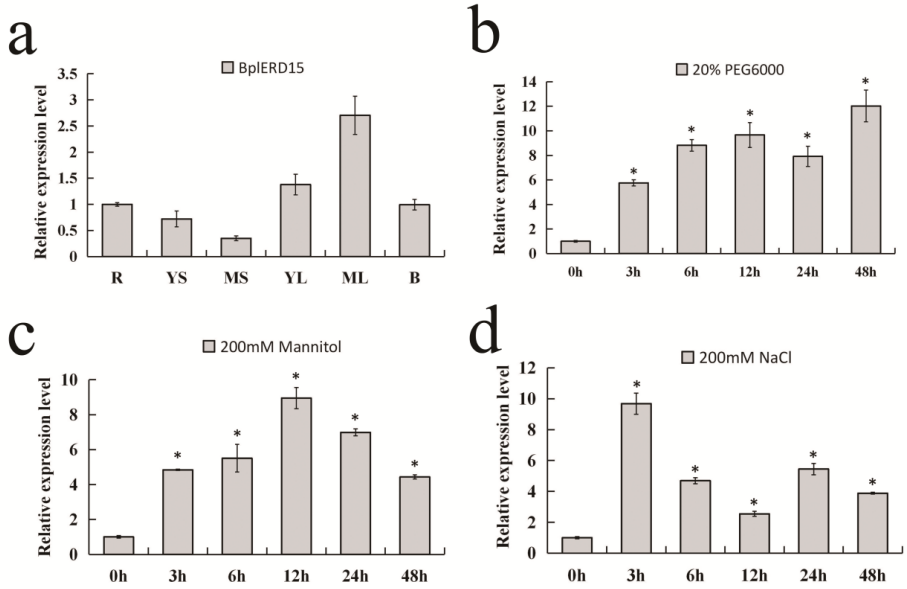

Figure 2. *Cont.*

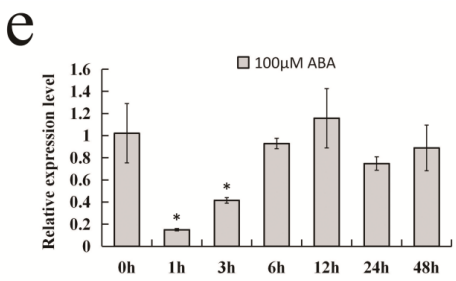

Figure 2. Expression characteristics of the *BplERD15* gene. (**a**): Tissue-specific expression of *BplERD15*, young leaves (YL); mature leaves (ML); young stems (YS); mature stems (MS); roots (R); bus (B). (**b**–**e**): Relative expression of *BplERD15* under different treatment. (**b**): 20% PEG6000; (**c**): 200 mM Mannitol; (**d**): 200 mM NaCl; (**e**): 100 μM ABA. Asterisks indicate significant differences in different tissues and significant differences under different treatment. Three biological replicates were utilized. Error bars represent standard deviations (Student's *t*-test, $p < 0.05$).

3.3. Overexpression of BplERD15 in Transgenic Birch Lines

In this study, five independent overexpression lines of *BplERD15* were generated and validated by PCR (Figure 3a) and the expression levels of *BplERD15* in these lines were quantified with qRT-PCR (Figure 3b). It is obvious that the expression levels of *BplERD15* in OE1, OE3, OE4, and OE5 were significantly higher than that of wild-type. The expression level of *BplERD15* in OE2 was not significantly different from that of wild-type. For all analyses conducted hereafter, we used three transgenic lines, OE1, OE3, and OE4, which had the highest expression.

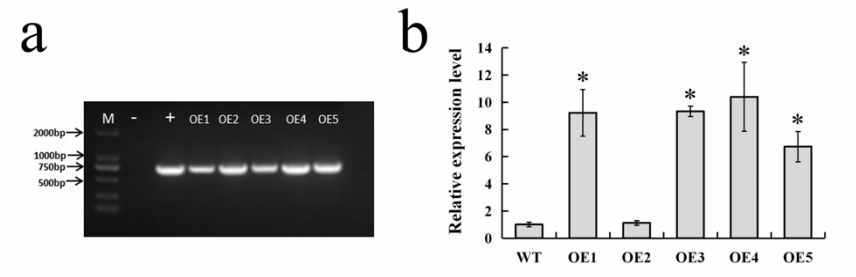

Figure 3. Identification and validation of *BplERD15* transgenic lines. (**a**): Identification *BplERD15* transgene in the genomic DNA from different transgenic lines using PCR with specifically designed primer pair; (**b**): qRT-PCR detection of cDNA from different transgenic lines. The relative expression in other transgenic lines was normalized by that in the wild-type, which was set as 1. Asterisks indicate significant differences to the wild type and *BplERD15* overexpression lines. Three biological replicates were utilized. Error bars represent standard deviations (Student's *t*-test, $p < 0.05$).

3.4. Overexpression of BplERD15 Confers Enhanced Drought Tolerance

The transgenic lines of *BplERD15* and WT plants were grown in a greenhouse until they were three-months old, which is when they were used for the drought stress experiment. Both transgenic lines and WT were well irrigated before the drought experiment was initiated. The transgenic lines were then subjected to dehydration with a duration of 15 days. The plants were fully rehydrated for three days, and they were photographed, as shown in Figure 4a. It is obvious that the wild-type plants showed a severe wilting symptom while all three *BplERD15-OE* lines survived from the extended drought treatment.

We measured the electrolyte leakage in the leaves of all three *BplERD15* transgenic lines with WT plants as comparison. It was found that the transgenic lines had significantly lower electrolyte leakage than WT plants (Figure 4b).

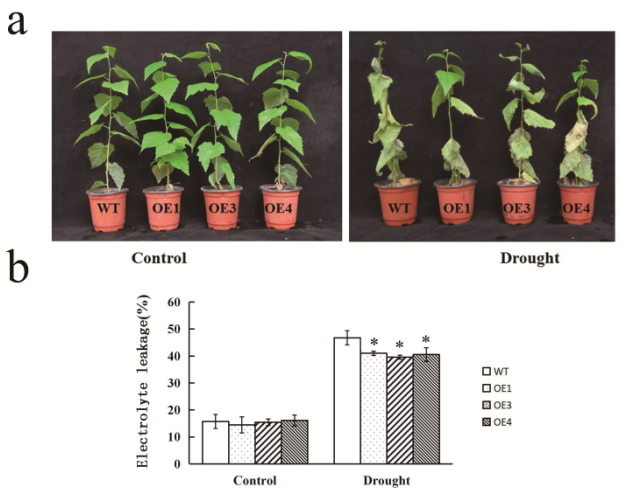

Figure 4. Overexpression of *BplERD15* conferred enhanced drought tolerance in its transgenic plants. (**a**): Control: plants being well watered were the control; drought treatment: three-month-old plants were dehydrated for 15 d and then rehydrated for 3 d; (**b**): Electrolyte leakage. Asterisks indicate significant differences to the wild type and *BplERD15* overexpression lines. Three biological replicates were utilized. Error bars represent standard deviations. (Student's *t*-test, $p < 0.05$).

4. Discussions

Several studies have shown that ERD genes play important roles in various abiotic stresses that include but are not limited to salt [35], drought [36], and freezing [13], as well as protein metabolic processes [37]. For example, *ZmERD3* gene expression is induced by abiotic stress treatments (such as PEG, NaCl, ABA, and low temperature) [35]. Owing to the inhibition of ABA signaling, the overexpression of *ERD15* in Arabidopsis leads to reduced tolerance to drought stress [36]. Compared with wild-type plants, the *ERD10* mutant has reduced tolerance to cold stress [13]. *ERD1*, also referred to as ClpD, is an ATP-dependent chaperone. *ERD1* functions as a component in the plant plastid Clp machinery, which comprises a hetero-oligomeric ClpPRT proteolytic core, ATP-dependent chaperones ClpC and ClpD, and an adaptor protein, and plays crucial roles in maintaining protein homeostasis [37]. In this study, we constructed a phylogenetic tree with the *BplERD15* and *BpeERD15* protein sequences of *B. pendula*, together with 16 ERD protein sequences of *A. thaliana* and four other *ERD15* protein sequences from other plant species. The distances among *BplERD15*, *BpeERD15*, and *MiERD15* were the shortest. A previous study has shown that *MiERD15* can be induced by drought stress, ABA treatment, salinity, and temperature extremes [29], which indicates that *BplERD15* may be an effector of multiple stresses and ABA too. In our study, *BplERD15* was found to be a positive regulator of drought, but its expression was induced by several osmotica that include PEG, mannitol, and NaCl. Surprisingly, the expression level of *BplERD15* was inhibited under ABA treatment. *AtERD15* is a negative regulator of abscisic acid responses in *A. thaliana* [38]. An overexpression of *AtERD15* reduces ABA sensitivity and drought tolerance in *A. thaliana*. The wild *S. pennellii* (*SpERD15*) was most closely related to *AtERD15* (Figure 1b). Transgenic lines overexpressing *SpERD15* manifested stress tolerance to dehydration, salinity, and cold. They exhibited an accumulation of soluble sugars and proline, and a limited lipid peroxidation [14]. Overexpression transgenic lines of *VaERD15* from Chinese wild *V. amurensis* showed robust cold tolerance [15]. The *ERD15* from sweet potato (*Ipomoea batatas* (L.) Lam.), *IbERD15*, has been reported to play an important role in the response to drought stress [39].

Drought stress affects phenotypical traits such as plant height, root length, leaf area, plant biomass, and root stomata area [40]. In addition, drought stress can result in considerable structural alterations in mitochondria, chloroplast, and vacuole [41]. Plants usually survive drought stress through a series of physiological [42], cellular [41], and molecular adaptation mechanisms [5,43]. The physiological adaptation is usually accompanied with significant changes in oxidative and antioxidant metabolism, and an escalation of proline content and scavenging capacity of reactive oxygen species (ROS) through

transgenic approach always leads to augmented stress tolerance [44,45]. Sometimes stress can increase the levels of some metabolites such as glucose, proline, and corilagin [46]. As reported, chloroplastically localized Os3BGlu6 significantly affects cellular ABA pools, which changes drought tolerance in rice [47]. Since the expression level of *BplERD15* in the leaves was the highest, we speculate that it may contribute to the accumulation of soluble osmotic compounds and limit membrane peroxidation to improve the drought stress tolerance. In addition, plants under drought and salt stress share some common signaling transduction pathways [48], indicating the existence of common effector genes in response to both stresses [49,50]. *BplERD15* may be such a gene because it could be induced by both osmotica and salt (Figure 2c,d), implying that it may be located downstream of a common signaling transduction pathway [51]. The *AtERD15* in *A. thaliana* has been recently reported to be a negative regulator of ABA but it was induced by ABA and salicylic acid (SA), as well as by wounding and pathogenic infection [38]. ABA plays an important role in the drought stress and the plants that are subjected to drought release a large amount of ABA [52]. ABA reduces the stomatal conductance and alter many physiological processes, resulting in a progressive decrease of the net photosynthetic rate (*Pn*) and stomatal conductance (G_s) under drought stress. Application of ABA enhances the expression of some members of the same ERD group (*ERD10* and *14*) [12] but have no effect on others (*ERD2*, *8*, and *16*) [10].

In response to dehydration, significant physiological changes such as electrolyte leakage can occur [53]. Overexpression of some drought stress tolerance genes can counteract such a change. For example, overexpression of *BpERF2* or *BpMYB102* in birch significantly reduced the electrolyte leakage, and thereby increased the tolerance to drought stress [20]. We found that the electrolyte leakages were all significantly lower in the three transgenic lines overexpressing *BplERD15* than in wild-type (Figure 4b), suggesting that the *BplERD15* gene plays a determining role in the greater survival rates of transgenic lines under drought stress treatment.

5. Conclusions

BplERD15 is a positive regulator of drought stress response and tolerance. Tissue-specific expression analysis indicates that it has the highest expression level in mature leaves and the second highest expression in young leaves. Transgenic birch lines overexpressing *BplERD15* showed significantly improved drought tolerance. *BplERD15* could be induced by other osmotica, suggesting that it could be used as a wide-spectrum regulator for enhancing stress tolerance in transgenic plants. This study provides some functional basis of *BplERD15* and we believe it is instrumental for genetic engineering of plants for enhanced stress tolerance to both drought and other abiotic stresses. Our findings indicate that *BplERD15* is a common effector to multiple osmotica in addition to drought and thus future research should focus on characterizing its upstream signal pathways so that we could use it precisely in fighting for various stresses.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/9/978/s1, Table S1: Primer pairs used in this study for gene cloning, vector construction, transgenic line validation, qRT-PCR.

Author Contributions: Conceptualization, J.J.; investigation, validation, writing-original draft, K.L.; writing—review and editing, H.W. and J.J. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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