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WLR, ZPS, and YHX designed the methods and experiments; WLR, XZ, and SY carried out the laboratory experiments; WLR analyzed the data, interpreted the results, and wrote the paper; ZPS, WXP, and DHR reviewed and edited the manuscript

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No competing interests have been declared.

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ORIGINAL RESEARCH PAPER

Physiological adaptations to osmotic stress and characterization of a polyethylene glycol-responsive gene in *Braya humilis*

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Abstract
Braya humilis (Brassicaceae) is a widely distributed plant in arid and semi-arid regions of northern Asia. This plant is well adapted to extremely arid conditions and is a promising candidate species to discover novel drought tolerance strategies. However, not much information about the mechanism(s) mediating drought resistance in this species is currently available. Therefore, the present study aimed to characterize the physiological traits and expression patterns of a polyethylene glycol (PEG)-responsive gene in *B. humilis* responding to different levels of osmotic stress induced by PEG-6000. Several important physiological parameters were examined, including the levels of relative water content, soluble protein, malondialdehyde, and antioxidant enzyme activity. A tolerance threshold between 20 and 30% PEG-6000 was identified for *B. humilis*. The water status and oxidative damage below this threshold were maintained at a relatively constant level during the 12 h of treatment. However, once the threshold was exceeded, the water status and oxidative damage were obviously affected after treatment for 4 h. The soluble protein results suggest that *B. humilis* maintains a vigorous resistance to osmotic stress and that it may play a greater role in osmotic regulation at late stages of stress. Moreover, superoxide dismutase and catalase may be important at preventing oxidative damage in plants at early stages of stress, while peroxidase may be more involved in some biological processes that resist osmotic stress at the late stage, especially in severely damaged plants. Furthermore, a PEG-responsive gene, *BhCIPK12*, was identified by differential display reverse transcription-polymerase chain reaction (PCR), cloned, and characterized by quantitative real-time PCR. We hypothesized that this gene may play an important role in mediating osmotic stress or drought resistance in plants. Altogether, these results provide valuable insights into the mechanism(s) mediating drought tolerance in *B. humilis*.

Keywords
Braya humilis; drought; polyethylene glycol; physiological response; *BhCIPK12*; expression pattern

Introduction

Numerous studies have demonstrated that drought affects the normal growth and development of plants by altering their water relation or water balance, inhibiting enzymatic activities, and affecting gene expression [1–3]. Drought also represents an oxidative stress to plants by enhancing the accumulation of reactive oxygen species (ROS) in cells, including superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [4]. These ROS can oxidize or peroxidize lipids, proteins, enzymes, pigments, and DNA, thereby further damaging the structure and function of cells, and ultimately inducing cell death [5].

Plants have developed various mechanisms to cope with drought [6]. To alleviate oxidative stress caused by drought, an efficient antioxidant mechanism involving non-enzymatic and enzymatic systems has evolved [7]. The former includes flavonoids, alkaloids, and phenols, whereas the enzymatic antioxidant system involves a series of enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) [4,5]. Antioxidant systems in various plant species have been studied, and antioxidant enzymes that effectively protect plants against oxidative damage induced by various biotic and abiotic stresses, including drought, have been found [4,8]. Drought stress research has indicated that higher antioxidant activities are detected in resistant or tolerant species/genotypes, compared with sensitive ones [9,10]; therefore, antioxidative mechanisms in drought-resistant or -tolerant species/genotypes remain a research focus [11–13]. The three important enzymes in the antioxidant system, SOD, POD, and CAT, have been studied intensively in various species [11–13]. Studies based on physiological, biochemical, and genetic levels show that antioxidant systems are complex and vary depending on species-specific drought-coping mechanisms and strategies [11–13].

Numerous stress-responsive genes have been identified in model plants and crop species through genomic, transcriptome, and proteomic analyses [14–17]. Differential display reverse transcription-polymerase chain reaction (DDRT-PCR) also has been used to identify stress-related genes [18,19]. In general, stress-resistant genes can be divided into two major groups according to their functions. One group plays a crucial role in avoiding cell damage, while the second group is involved in signal transduction and transcription regulation [20]. In particular, calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) constitute an important Ca^{2+} signaling network that copes with various biotic and abiotic stresses [21]. Many *CIPKs*, such as *CIPK1*, *CIPK3*, *CIPK6*, *CIPK16*, *CIPK17*, *CIPK19*, *CIPK25*, *CIPK29*, and *CIPK12*, have been reported to be involved in the responses to drought and osmotic stress [22,23]. However, these studies have mainly focused on model plants, like Arabidopsis and rice [22,23]. *CIPKs* involved in other plant species remain to be identified and characterized. In particular, little information is known regarding the role of *CIPK12*, compared with the other *CIPKs*, in the stress response [23].

Braya humilis (Brassicaceae, Braya) is well adapted to extremely arid conditions [24,25] and is widely distributed in the arctic and alpine regions of northern North America and in arid and semi-arid regions of northern Asia. In China, it expands in the north, including the northwest arid and semi-arid regions [26–28]. Field surveys of *B. humilis* from different regions of China have shown that the species has varied morphological traits, suggesting variability in the genetic resource pool. Therefore, *B. humilis* is a promising candidate species to discover novel drought tolerance strategies. However, to date, little information is available regarding the mechanism(s) mediating drought resistance in this species. In the present study, we explored the possible drought tolerance mechanisms in *B. humilis* by exposing the species to different polyethylene glycol (PEG)-6000 concentrations for various time periods. Our specific objectives in *B. humilis* were as follows: (***i***) to determine the physiological response patterns related to oxidative stress levels and antioxidant systems associated with different osmotic stress levels for various periods of time in *B. humilis* leaves, and (***ii***) to clone a PEG-responsive gene (*BhCIPK12*) isolated by DDRT-PCR from *B. humilis* osmotically stressed leaves and to characterize its expression patterns to different osmotic stress levels for various periods of time. Altogether, these results will provide valuable insights into the drought tolerance mechanisms of *B. humilis*.

Material and methods

Plant materials, growth conditions, and stress treatments

Seeds of *B. humilis* were collected in December 2011 from Huzhu, Qinghai province, China (N 36°57′8.72″, E 102°28′54.94″), and stored at 4°C until use. The seeds were surface-sterilized with a sodium hypochlorite solution (5%, v/v) for 8 min, washed with sterile water three times, and then sown in petri dishes containing Murashige and Skoog medium solidified with 0.8% (w/v) agar [29]. The entire process was conducted under sterile conditions. Cultures were maintained with a 16 h light (intensity, 5400 ±50 Lux)/8 h dark cycle at 23 ±1°C. After one week, the seedlings were planted in individual plastic pots (60 mm high, 55 mm bottom diameter, and 80 mm caliber), which were filled with a mixture of peat moss (19081215/LV/SEEOING/pH 5.5/0–10 mm/300 L; Pindstrup, Beijing, China) and perlite at a 2:1 ratio. The seedlings were placed in a culture room under the same light and temperature conditions with 60 ±1% relative humidity. The pots were carefully irrigated every 2 days with deionized water until the substrates were thoroughly moistened. After 44 days, uniform plants (15 to 18 true leaves) were transferred to floating polyester rafts containing plastic pots filled with 1/2 Hoagland's solution. The plants were aerated twice a day for 3 days.

After a 3-day preculturing period, the plants were carefully removed from the solution, rinsed with deionized water, and treated with various concentrations of PEG-6000 nutrient solution for stress assays. In a pilot trial, the plants were grouped and subjected to 10%, 20%, 30%, or 40% (w/v, g/mL) PEG-6000 nutrient solution (prepared in 1/2 Hoagland's solution) as described by Yildiztugay et al. [30]. Plant phenotypes were observed at 1-h intervals. Leaf wilting and rolling were observed at the 4 h and 12 h timepoints, respectively, for the 30% PEG-6000- and 40% PEG-6000-treated plants. Therefore, these two timepoints were chosen for subsequent experiments. Briefly, the plants were divided into nine groups (12 plants per group). The first group was used as a control, while the other eight groups were exposed to different concentrations of PEG-6000 (10%, 20%, 30%, or 40%) for 4 h or 12 h. The culture conditions were the same as described above. Leaf materials were harvested quickly from the plants, and a subset of each sample was packaged to collect the relative water content (RWC) data. The remaining materials for each sample were flash-frozen in liquid nitrogen and stored at −80°C.

RWC assay

The leaf RWC was determined after harvesting. First, the leaves were weighed to determine their fresh weight (FW). Then, the leaves were subsequently soaked in distilled water for 6 h at room temperature. After blotting to remove excess water, the leaves were weighed to determine their water-saturated weight (SW). Finally, the leaf samples were dried at 80°C for 48 h and the dry weight (DW) for each sample was recorded. The leaf RWC was calculated using the formula: $RWC = (FW - DW) / (SW - DW)$ [31].

Soluble protein concentration assay

Soluble proteins were extracted using a Komin kit (Suzhou, China), and the entire process was conducted at 4°C. In detail, frozen leaves (0.30–0.35 g) were macerated to a fine powder in a mortar using liquid nitrogen. Soluble proteins were extracted by homogenizing the powder in phosphate buffer (45.7 mM $Na_2HPO_412{\cdot}H_2O$, 5.5 mM NaH_2PO_4, pH 7.8; FW of leaves : buffer volume = 0.1 g : 1 mL). Insoluble materials were removed by centrifugation at 8000 *g* for 10 min at 4°C [32]. The soluble solution was sub-packed and used to determine the physiological characteristics. Soluble protein concentrations were subsequently assayed using the Bradford method using bovine serum albumin as the standard [33].

Oxidative damage assay

To estimate the oxidative damage levels, the leaf malondialdehyde (MDA) content determination was carried out according to the manufacturer's protocol (Komin, Suzhou, China), which was based on the thiobarbituric acid (TBA) method [34]. In detail, soluble proteins (0.1 mL; protein extraction as described above) were homogenized into 0.3 mL of a reaction solution containing TBA (0.35 M) and trichloroacetic acid (0.31 mM). The mixture was heated at 95°C for 30 min, immediately cooled in an ice bath, and centrifuged at 10 000 *g* for 10 min at room temperature. The supernatant absorbance at 532 and 600 nm was determined with a spectrophotometer (NanoDrop2000C, Thermo Scientific, Waltham, MA, USA). The MDA content was determined using the following formula: MDA content (nmol/g FW) = 64.5 × (A_{532} – A_{600}).

Antioxidant enzyme assays

The activities of SOD, POD, and CAT were ascertained according to the manufacturer's instructions (Komin, Suzhou, China), with the following respective details.

The SOD activity was determined employing xanthine and xanthine oxidase (XO) to generate $O_2^{\cdot-}$, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red azan dye. The SOD activities were established by the degree of reaction inhibition [35]. The detailed method was as follows: soluble proteins (90 µL; protein extraction as above) were homogenized into 936 µL of a solution composed of 510 µL xanthine (1.5 mM), 6 µL XO (5 U/mL), 180 µL INT (0.45 mM), and 240 µL potassium phosphate buffer (300 mM, pH 7.8). The reaction mixture was exposed to light for 30 min, and then the absorbance was measured at 560 nm using a NanoDrop2000C spectrophotometer. The XO inhibition degree (ID) of the lotus-linking reaction was calculated using the following formula: ID = [A(control) – A(sample)] / [A(control)] × 100%.

One SOD activity unit was defined as the amount of enzyme corresponding to 50% inhibition of the INT reduction. Finally, the SOD activity was calculated using the following formula (protein is abbreviated as "prot" and protein concentration is abbreviated as "Cpr" in formulas): SOD activity (U/mg prot) = (dilution multiples × 11.4 × ID / (1 – ID)) / Cpr.

The POD activity was determined based on the guaiacol method [36]. Soluble proteins (15 µL; protein extraction as described above) was homogenized into a reaction mixture, containing 135 µL guaiacol (20 mM), 130 µL H_2O_2 (22 mM), and 520 µL acetic acid buffer (0.1 mM, pH 5.4), in a 1.055 mL volume. The reaction mixture absorbance change per min at 470 nm was determined. One POD activity unit was defined as an absorbance change of 0.01 units per min at 470 nm. The POD activity was calculated according to the following formula: POD activity (U/mg prot) = ΔA_{470} × 7133 / Cpr.

The CAT activity was determined based on monitoring the absorbance of H_2O_2 at 240 nm [36]. The method involved homogenizing 35 µL of soluble protein (protein extraction as described above) into 1 mL of solution composed of 49.75 µL H_2O_2 (88.2 mM) and 950.25 sodium phosphate buffer, (0.1 M, pH 7.0). The reaction mixture absorbance change per min at 240 nm was determined. One unit was defined as the amount of protein in milligrams that causes the degradation of 1 nmol H_2O_2 per min at 25°C. The CAT activity was calculated using the following formula: CAT activity (U/mg protein) = ΔA_{240} × 678 / Cpr.

DDRT-PCR, gene clone, and quantitative real-time PCR (qRT-PCR) analyses

Because an obvious difference in phenotype was observed in plants treated with 20% or 30% PEG-6000 solution for 4 h, the total RNA from these leaf samples was subjected to DDRT-PCR. Briefly, the total RNA was isolated using an RNAprep Pure Plant Kit (Tiangen, Beijing, China) and a DNase treatment step. The resulting cDNAs were synthesized using a reverse transcription system kit (Thermofisher Scientific,

Waltham, MA, USA). Three 3′-oligo (dT) anchored primers (AP1–3) and seven 5′-arbitrary primers (RP1–7), described previously by Xu et al. [29], were used for PCR amplification. Each sample contained 12.5 μL of Taq plus master mix (Tiangen, Beijing, China), 9.9 μL of double-distilled water, 0.5 μL of anchored primer (10 ng/μL), 0.5 μL of arbitrary primers (10 ng/μL), and 1.6 μL of template DNA. PCR amplification included a denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 34–50°C for 30 s, and 72°C for 45 s, followed by a final extension step at 72°C for 10 min. PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. All samples were analyzed in duplicate to avoid false positives in differential display banding patterns [29]. Using a gel extraction kit (Omega Biotech, Norcross, GA, USA), differentially expressed cDNA bands were excised from the agarose gel and were purified and ligated into the pGEM-T Easy vector (Promega Corp, Madison, WI, USA). Following the transformation of the ligated vector into *Escherichia coli* cells (Trans5a; Transgen, Beijing, China), six individual white clones were selected and cultured. Isolated DNA samples were bidirectionally sequenced using SP6 and T7 primers (Majorbio, Shanghai, China).

Quantitative RT-PCR was performed using a Stratagene Mx3000P detection system (Agilent Technologies, Santa Clara, CA, USA) and a Thermo SYBR Green RT-PCR Kit (Thermofisher Scientific, Waltham, MA, USA), as described by each manufacturer, respectively. Briefly, 1 μL of cDNA (a 1:5 dilution of the original synthesized cDNA) served as the template in each 20 μL reaction containing 1 μL (10 ng/μL) of each primer, 10 μL of SYBR Green RT-PCR Master Mix (Thermofisher Scientific, Waltham, MA, USA), and 7 μL of double-distilled water. The specificity of the amplified transcripts was verified by monitoring the melting curves that were generated for each run. *BhCIPK12* primers (forward, 5′-ATCCCAACCATGACACCAGT-3′; reverse, 5′-TCTTTCTGTCCCATCCCAAC-3′) were designed according to the sequences obtained from the DDRT-PCR analyses. *Actin 7* (U27811 in Genebank, AT5G09810 in TAIR) [37–39], 134 bp in length, was amplified as an internal control (forward, 5′-ATCCCAACCATGACACCAGT-3′; reverse, 5′-TGAGGATATTCAGCCCCTTG-3′), and its stability in *B. humilis* during osmotic stress was validated applying C_T values on geNorm v.3.5 software (http://medgen.ugent.be/~jvdesomp/genorm/) [40,41]. The latter primers were designed based on unpublished transcriptome data in our laboratory. The PCR amplification program included a denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and then a final extension step at 72°C for 5 min. Each sample was assayed three times. The mRNA transcriptional levels were calculated by subtracting the mean actin 7 cycle threshold (C_T) values from C_T values for the gene of interest using the $2^{-\Delta\Delta C_T}$ method [42].

Statistical analysis

All physiological characteristics are presented as the mean ± standard error (*SE*) of three replicates and analyzed by one-way analysis of variance with Tukey's honestly significant difference test as the post-hoc analysis using SPSS statistics 17.0 (USA) software. Significant differences were set as $p < 0.05$.

Multiple sequence alignments were assembled using MEGA 6.0 (USA) software following nucleotide sequence screening and identification using the BLAST NCBI platform (http://www.ncbi.nlm.nih.gov, BLAST, USA). The open reading frame (ORF) of the gene was determined using the Open Reading Frame Finder from NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The molecular mass and isoelectric point (pI) of the proteins were predicted using Protein Calculator v.3.4 (http://protcalc.sourceforge.net, USA). A BLAST search analysis was performed using the cDNA sequence differentially expressed as a query and sequences obtained for *AtCIPKs* in order to reveal the phylogenetic relationships of *BhCIPK12* with its homologs. A phylogenetic tree was generated using MEGA 6.0 (USA) software. Neighbor joining gaps in the sequences were treated as missing data. Bootstrap values from 1000 iterations were applied.

Results

Changes in phenotype in response to different osmotic stress treatments

Braya humilis plants were treated with 10%, 20%, 30%, or 40% solutions of PEG-6000. The phenotypes of the leaves changed as time lapsed, and different visible symptoms at various timepoints are shown in Tab. 1. The leaves of plants treated with 10% or 20% PEG were upstanding and hard at the 12 h timepoint, while obvious wilting was observed in plants treated with 30% or 40% PEG for 4 h (Tab. 1). At the 12 h timepoint, leaf wilting was aggravated, and some leaves were curly in the 30% or 40% PEG-treated plants. In addition, some leaves appeared withered in the tip and purple on the back in a small portion of the 40% PEG-treated plants (Tab. 1).

Physiological parameters in response to different osmotic stress treatments

The RWC levels were found to correspond well with the phenotypes observed. The RWC levels decreased along with progressive osmotic stress for the sets of plants treated for 4 h or 12 h. A notable decrease ($p < 0.05$) was achieved following treatment with 30% or 40% PEG just at the 4 h timepoint (Fig. 1a), whereas the RWC of the 10% or 20% PEG-treated plants remained at a relatively constant level during the 12 h of treatment. For the convenience of the discussion that follows, the plants treated with 30% or 40% PEG for 4 h were characterized as mildly damaged, while the plants treated with the same concentrations of PEG for 12 h were characterized as severely damaged.

Soluble protein concentration assays were performed to detect the metabolic levels of plants subjected to different osmotic treatments. Slight increases in soluble protein concentrations were exhibited at 4 h of treatment (Fig. 1b). At 12 h, significantly higher ($p < 0.05$) soluble protein levels were observed in all treatment groups, compared with the control, and the levels of soluble protein had increased with the increase of PEG concentration to 30% and subsequently decreased following treatment with 40% PEG (Fig. 1b).

MDA content assays were applied to detect the oxidative damage levels in plants subjected to different osmotic treatments. The MDA results were in accordance with the phenotype and RWC described above. The MDA content tended to increase as the PEG concentration increased and was significantly higher ($p < 0.05$) in the plants treated with 30% or 40% PEG, compared with the plants treated with 10% or 20% PEG, independent of time (Fig. 1c).

To examine the response of antioxidant enzymes of *B. humilis* to various osmotic stress conditions, the enzyme activities of SOD, POD, and CAT were assayed

Tab. 1 Visible symptoms of *Braya humilis* following treatment for 4 h or 12 h with various concentrations of polyethylene glycol (PEG)-6000 solution.

Culture time (h)	PEG concentration (%)	Visible symptoms
4	10	Leaves were upstanding and hard
4	20	Leaves were upstanding and hard
4	30	Leaves were wilted and drooped
4	40	Leaves were wilted and drooped
12	10	Leaves were upstanding and hard
12	20	Leaves were soft and approximately 80% of leaves were upstanding
12	30	Leaves were wilted, and approximately 20% of leaves were curly
12	40	Leaves were wilted, and approximately 30% of leaves were curly, withered in the tip, or purple on the back

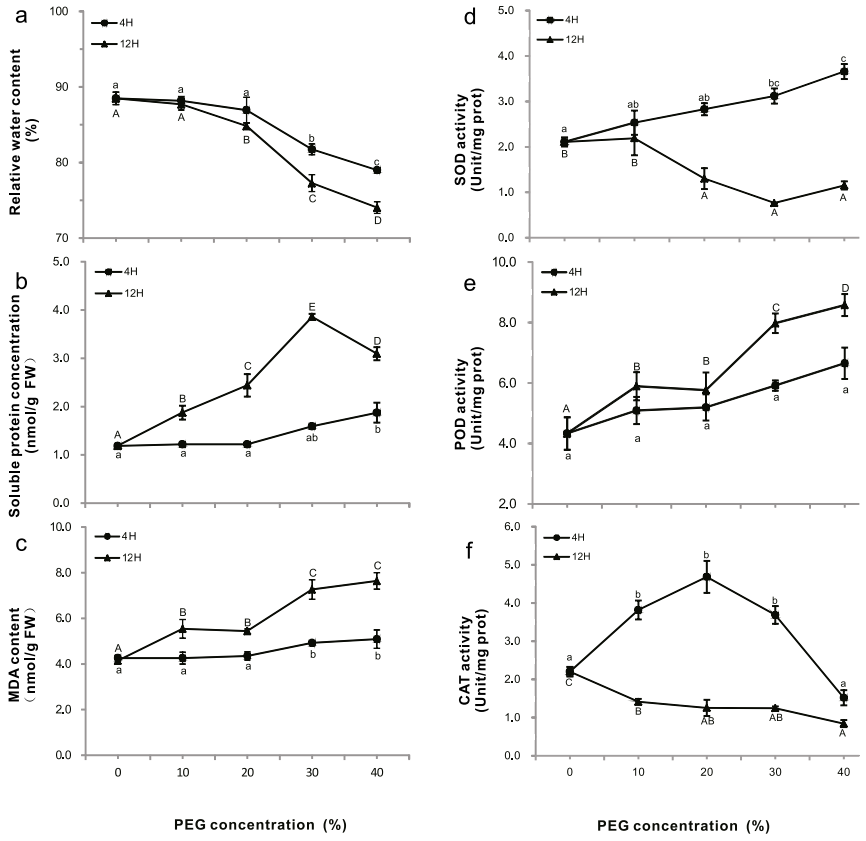

Fig. 1 Physiological characteristics of PEG-treated *Braya humilis* leaves. *Braya humilis* seedlings were treated with various concentrations of PEG-6000 solution for 4 h or 12 h, and then (**a**) the relative water content (RWC), (**b**) soluble protein concentration, (**c**) malonaldehyde (MDA) content, and levels of (**d**) superoxide dismutase (SOD), (**e**) peroxidase (POD), and (**f**) catalase (CAT) were assayed. Bars represent the mean ±*SE* of at least three replicates. Capital letters denote significant values for the 4-h treatment data, and lowercases letters denote significant values for the 12-h treatment data ($p < 0.05$).

(Fig. 1d–f). In general, higher levels of these enzyme activities were observed during the course of osmotic stress. However, different response patterns were detected. For example, after 4 h of treatment with 10%, 20%, 30%, and 40% PEG, respectively, the levels of SOD and POD activity increased accordingly, with marked increases ($p < 0.05$) in SOD activity levels but only slight increases in POD activity levels. The CAT activity levels first increased and then decreased with the increase of PEG concentration at the 4 h timepoint. Markedly higher levels ($p < 0.05$) of CAT activity were observed for the 10%, 20%, and 30% treatments at the 4 h timepoint, compared with the controls. At the 12 h timepoint, decreasing trends were observed for both the SOD and CAT activity levels, whereas the POD activity levels increased with increasing PEG concentrations, which were markedly increased with 30% or 40% PEG treatment ($p < 0.05$). Of note, the POD activity values in the sets of plants treated for 12 h were all higher than those treated for 4 h, and the opposite case was observed for SOD and CAT activities (Fig. 1d–f).

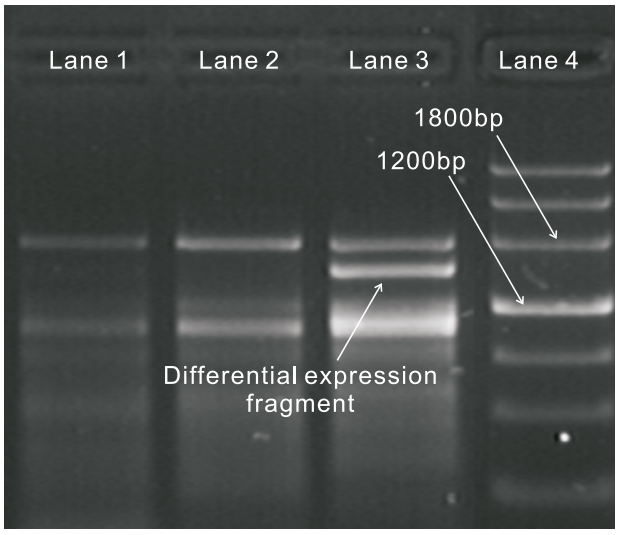

Fig. 2 Differentially displayed cDNA fragments as detected by differential display reverse transcription-polymerase chain reaction (DDRT-PCR). Lane 1 – control plant sample; lane 2 – 20% PEG-6000/4-h sample; lane 3 – 30% PEG-6000/4-h sample; lane 4 – DNA ladder control. The differential display fragment of interest is labeled.

Isolation and characterization of the PEG-responsive gene, *BhCIPK12*

A differential display cDNA fragment was amplified from *B. humilis* leaves treated with 30% PEG-6000 for 4 h using a 5′-arbitrary primer (RP4, AAGAGCCCGT) and a 3′-oligo (dT) primer (AP2, AAGCTTTTTTTTTTC) at an annealing temperature of 36°C [29]. The fragment identified was approximately 1600 bp in length, and it was not present in the control or 20% PEG/4 h samples that were also analyzed (Fig. 2). Sequencing and BLAST analysis results revealed that the fragment shared the highest sequence homology (92%) with *CIPK12* in *Arabidopsis thaliana* and *Arabidopsis lyrata*. Given that Arabidopsis and *B. humilis* belong to the Brassicaceae family and that the *AtCIPK* family has been well studied, a phylogenetic tree was generated using the nucleotide sequences of 25 *AtCIPKs* and *BhCIPK12* to study the phylogenetic relationship among *BhCIPK12* and *AtCIPKs* (Fig. 3). *BhCIPK12* was found to be most closely related to *AtCIPK12* and was more distant from the other *AtCIPKs*, especially *AtCIPK3* and *AtCIPK9*.

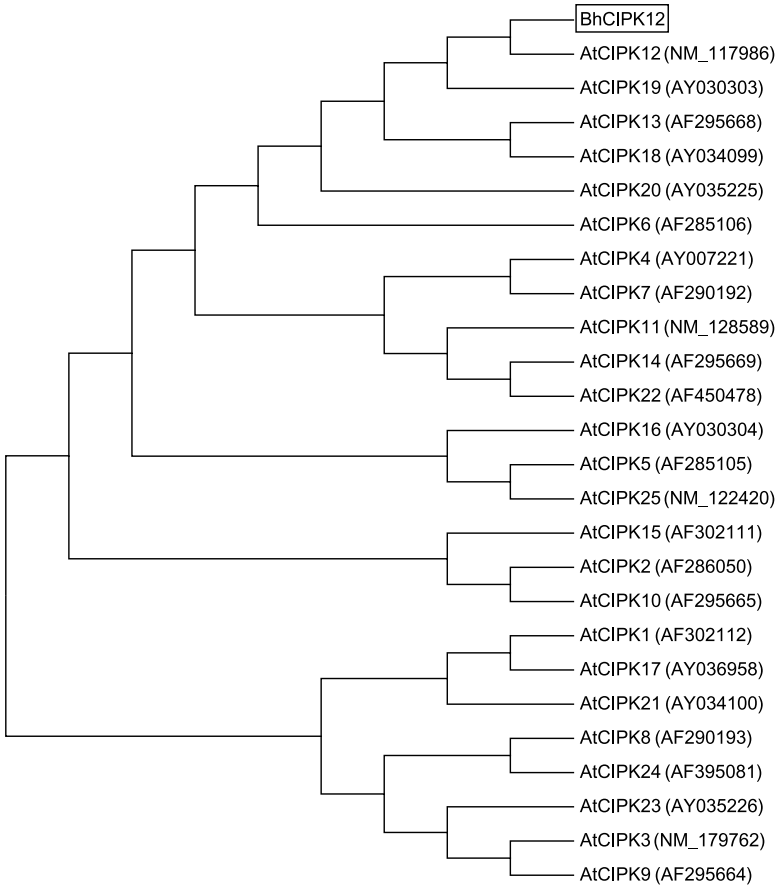

Fig. 3 A phylogenetic tree was generated that shows the relationship among *BhCIPK12* and *AtCIPKs* genes. GenBank accession numbers are listed in parentheses.

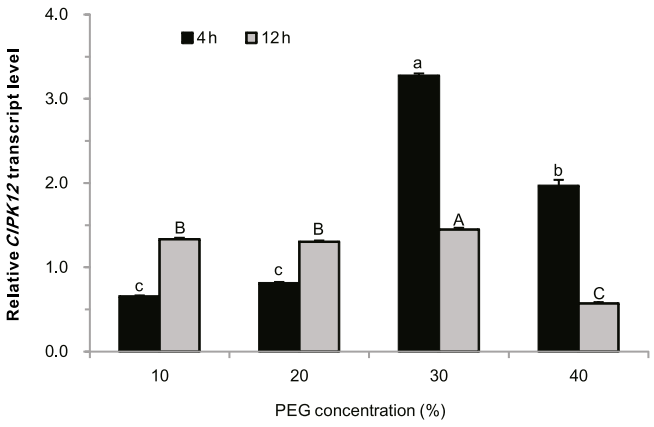

Fig. 4 Relative transcript levels of *BhCIPK12* in *B. humilis* leaves. Total RNA was extracted from seedlings treated with different concentrations of PEG solution for 4 h and 12 h. Quantitative real-time (qRT-PCR) was used to detect the relative transcript levels of *BhCIPK12* in plants that underwent different treatments. Bars represent the mean ±*SE* of at least three replicates. Capital letters denote significant values for the 4-h treatment data, and lowercases letters denote significant values for the 12-h treatment data ($p < 0.05$).

The relative transcript levels of *BhCIPK12* were determined by qRT-PCR for the various leaf samples collected. As shown in Fig. 4, the mRNA levels of *BhCIPK12* were significantly higher in the plants treated with 30% or 40% PEG for 4 h, compared with the plants treated with 10% or 20% PEG for 4 h. In contrast, there were no obvious changes in the *BhCIPK12* mRNA levels for three of the four 12-h samples.

The coding sequence and ORF were 1060 bp in length and 490 amino acids, respectively (Fig. 5a). The predicted molecular mass was approximately 54.99 kDa, and the pI was 7.66. CIPKs have a highly conserved domain structure that includes an N-terminal kinase domain with an activation loop and a regulatory C-terminal domain that includes an NAF domain and an adjacent PPI domain [21]. According to the putative amino acid sequence identified for BhCIPK12, the activation loop contains 30 amino acid residues and is located between conserved DFG and APE motifs (Fig. 5b). The NAF motif (from NAF to LFD), consisting of 19 amino acid residues, is found in the C-terminal regulatory domain. The PPI motif within the C-terminus contains 32 amino acid residues (from GEG to VRK). Comparison of the amino acid sequences of *B. humilis*, Arabidopsis, grape, and *Populus euphratica* (data from NCBI, http://www.ncbi.nlm.nih.gov/protein/?term=cipk12) revealed 20 highly conserved sites in the activation loop, nine in the NAF motif, and nine in the PPI motif (italic bold in the motif boxes in Fig. 5a).

Discussion

In the present study, no obvious changes in water status were observed in the plants treated with 10% or 20% PEG within 12 h. However, loss of water was evident for the plants treated with 30% or 40% PEG just at the 4 h timepoint (Tab. 1, Fig. 1a). These results are consistent with previous studies [1,7]. Generally, plants achieve a plateau in water status when they are exposed to drought within certain limits of stress severity or within a certain period of time. However, once the threshold is exceeded, plants begin to lose water rapidly [11,43]. In the present study, we inferred that *B. humilis* had a tolerance for PEG-6000 concentrations between 20% and 30%, thus providing a reference for future work on drought stress in *B. humilis*. In addition, these results provide support for the hypothesis that osmotic pressure can be maintained within certain limits, yet this balance is compromised when osmotic pressure exceeds a given set of conditions.

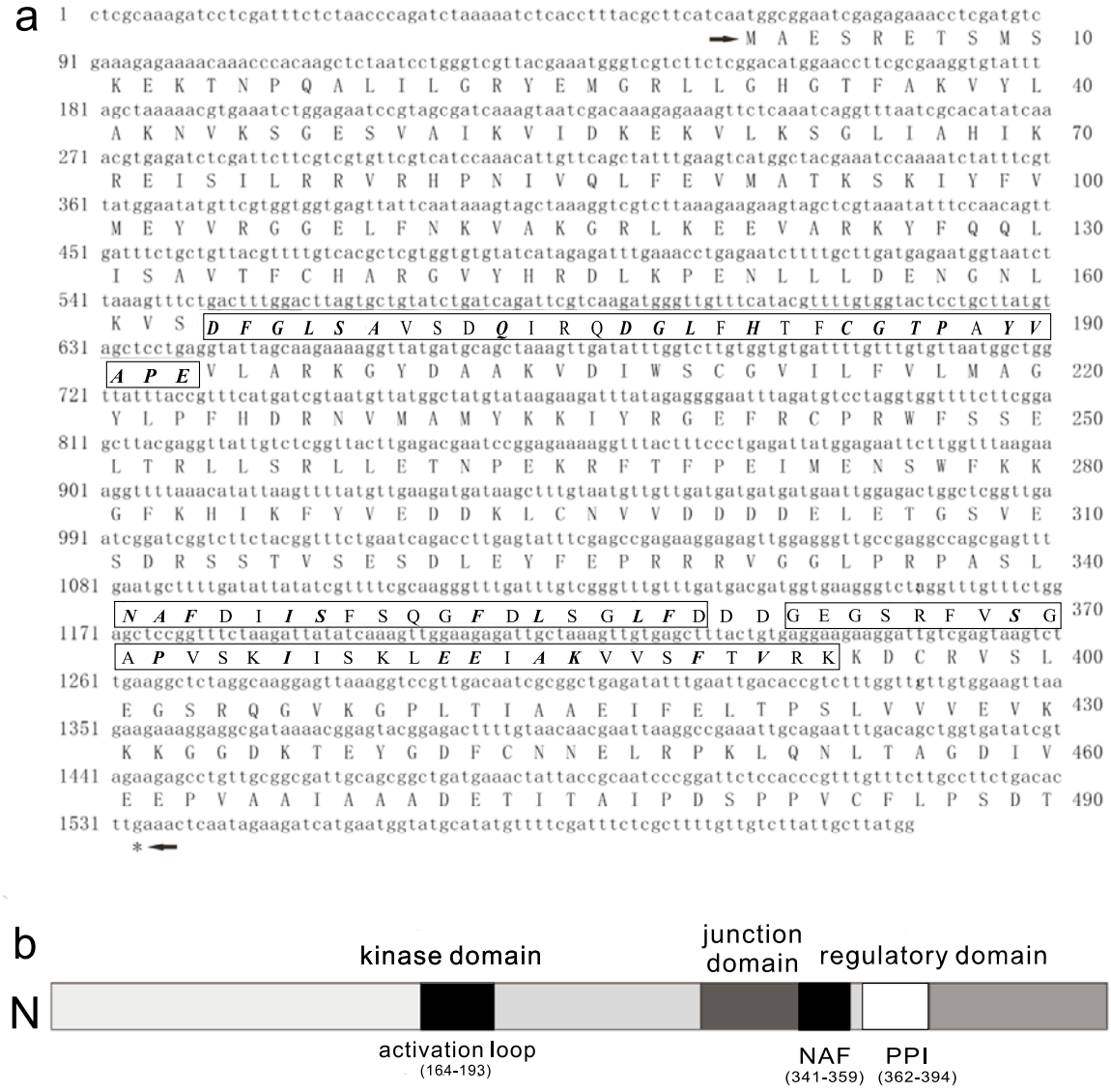

Fig. 5 Nucleotide and deduced amino acid sequences for BhCIPK12. **a** The predicted amino acid sequence is shown below the nucleotide sequence, and the numbering for each is indicated at the right and left of the sequence lines, respectively. The putative N-terminal kinase domain (activation loop), the NAF domain, and the PPI domain are boxed. Highly conserved amino acid sites of the domains are shown in italicized bold font. **b** The overall domain structure of BhCIPK12. The putative activation loop, the NAF domain, and the PPI domain are represented by a shaded box, a blank box, and a white box, respectively.

Soluble proteins have been shown to play an important role in mediating the adaptation of plants to osmotic stress and in osmotic regulation. Correspondingly, most studies have shown that concentrations of soluble proteins increase when plants are exposed to drought conditions [44,45]. Moreover, the accumulation of antioxidant enzymes and the synthesis of drought-resistant proteins can increase soluble protein levels in plants [45]. In the present study, the level of soluble proteins first increased and then peaked following treatment with 30% PEG for 12 h, yet decreased following treatment with 40% PEG for 12 h. These discrepant results are similar to those observed by Skutnik and Rychter [46]. The first increase in soluble protein levels in the plants treated with 10%, 20%, and 30% PEG for 12 h indicated that *B. humilis* maintains a vigorous metabolic level to increase resistance to osmotic stress at the late stage. Accordingly, the decrease in soluble protein levels in the plants treated with 40% PEG for 12 h might reflect a decrease in resistance to osmotic stress, possibly related to the lower levels of antioxidase activities such as SOD and CAT at the late stage of stress or when stress was aggravated. An additional consideration is that soluble proteins may not constitute the primary osmotic regulators, rather, proline and soluble

sugars might play important roles in the osmotic adjustments, which take place in response to early stress or mild damage [47,48]. In addition, soluble proteins might function when plants are at the late stage of stress or seriously damaged.

MDA is a product of lipid peroxidation and is considered a marker of oxidative damage in plants [5]. Many studies have shown that MDA levels increase when plants subjected to drought stress [49,50]. In the present study, the MDA levels were significantly higher ($p < 0.05$) in plants treated with 30% or 40% PEG, compared with those treated with 10% or 20% PEG, independent of time (Fig. 1c). These results also corresponded with the mild versus severe plant damage observed with the 10%/20% PEG and 30%/40% PEG treatments, respectively. In combination with the antioxidant enzyme levels that were detected, it appears that SOD and CAT, potentially in combination with other antioxidant enzymes or mechanisms [31], provided a good protective effect from oxidative damage at the early stage of stress [38,51]. Both a high POD activity and a strong membrane lipid peroxidation were detected in severely damaged plants, indicating a possible relationship between them. An explanation is that the increased POD activity results from high ROS levels which might be as a signal [52].

Antioxidant enzymes play an important role in protecting cells from oxidative damage caused by various environmental stresses [3,8]. In the present study, the levels of SOD and CAT activities were higher at the early stage of stress, while the POD activity levels were higher at the late stage of stress, especially in plants that suffered serious damage (Fig. 1d–f). Similarly, higher SOD and CAT levels resulting from mild damage or at the early stage of stress have also been reported in *Platanus acerifolia* [53] and *Populus simonii* [13]. The rapid drought response for SOD and CAT in these species might be explained by a mechanism in which the two antioxidant enzymes are primarily expressed in plant protective systems against oxidative bursts in early stages of stress [38,51]. When ROS increase, SOD acts as the first line of defense and catalyzes $O_2^{\cdot-}$ detoxification to a relatively stable and electrically neutral H_2O_2 [51,52]. Subsequently, CAT and other antioxidants further convert H_2O_2 into H_2O and O_2 [51,52]. Higher levels of POD activity at the late stage of stress, especially in severely damaged plants, might function in protecting plants against further oxidative damage [51,52]. Apart from ROS scavenging, high levels of POD activity might be involved in other physiological processes at the late stage of stress, such as the biosynthesis of cell-wall components and lignification [11,54]. However, a lack of congruency was observed in *Reaumuria soogorica*, maize, and other species [11,30]. The contradictory data in terms of signal transmission, gene transcriptional expression, and other complex physiological activities possibly varied due to differences in species/genotypes, tissues/organs, growth and developmental stages, as well as the degrees of stress severity and exposure time [11–13,30]. In general, these results suggested that *B. humilis* employs an effective antioxidative mechanism in response to a wide range of drought stresses. However, additional studies are needed to confirm and further develop these findings.

The CBL/CIPK signaling network has been extensively investigated in Arabidopsis, rice, and other model plant species, and the expression of *CIPKs* has been found to be stimulus-specific [21] and many *CIPKs* that are associated with drought or osmotic stresses have also been isolated [21–23]. In the present study, *BhCIPK12* expression was induced by PEG, and a similar observation was made in rice [55]. Chen et al. also have reported that *OsCIPK12* improved tolerance to drought and is associated with the ABA signaling pathway [55]. Thus, *BhCIPK12* may contribute to osmotic or drought tolerance, and it is possible that other stimuli may induce the expression of *BhCIPK12* in *B. humilis*.

It is important to note that a recent study mostly focused on stimulus-induced and tissue-specific expression profiles [23], Furthermore, the differential expression of many genes is detected during different stress time courses and under various stress conditions [3,43]. However, very little data are available regarding the expression of *CIPK* homologs in response to various drought levels, especially for *CIPK12*. In the present study, the *BhCIPK12* mRNA expression levels in response to various degrees of osmotic stress were evaluated. Based on the phenotypic and RWC results, we hypothesized that the expression levels of *BhCIPK12* would first increase and then decrease with a reduction in water status and that the marked increase in expression would be

stimulated by a sharp water loss in the leaves. We found that a significant increase of *BhCIPK12* expression occurred in plants treated with 30% PEG for 4 h, compared to those treated with 20% PEG for 4 h. These results might be due to the tolerance of *B. humilis* to PEG-induced osmotic stress at PEG-6000 concentrations between 20% and 30%. In addition, we hypothesized that the highest *BhCIPK12* expression might occur when plants were treated with approximately 30% PEG. Since gene expression is regulated by a series of physiological and biochemical events, changes in expression levels are not necessarily synchronous with phenotypes or one physiological parameter [43]. Therefore, further studies are needed to confirm the exact PEG treatment concentration and time for the highest expression of *BhCIPK12*. The expression pattern of *BhCIPK12* was similar to that found in comparable short-term osmosis research. When rice plants were subjected to drought for 24 h, *OsCIPK12* showed an expression pattern that first increased and then decreased [22]. However, different expression patterns also have been observed. *MeCIPK12* expression did not show obvious trend during osmotic treatment in cassava [56]. Altogether, these results suggest that *BhCIPK12* might have an important role in *B. humilis* plants exposed to osmotic stress or drought. However, the expression profile of *BhCIPK12* remains to be more fully characterized, which may lead to a better understanding of the genetic mechanisms that underlie drought stress tolerance in *B. humilis*.

In conclusion, in the present study, we inferred that *B. humilis* has a tolerance of PEG-induced osmotic stress between concentrations of 20% and 30% PEG-6000. The water status and oxidative damage in plants below this threshold were maintained at a relatively constant level during the 12 h of treatment. However, when this threshold was exceeded, the water status and levels of oxidative damage were obviously affected at 4 h timepoint. Accordingly, the soluble protein results suggest that *B. humilis* maintains a vigorous metabolic level to resist osmotic stress at the late stage, and it might function in osmotic regulation at the late stage of stress or in seriously damaged plants, rather than at the early stage of stress or in mildly damaged plants. Regarding antioxidase enzymes, SOD and CAT antioxidase enzymes might be involved in preventing or reducing oxidative damage in *B. humilis* at the early stage of stress, whereas POD might contribute more to some biological processes that resist osmotic stress at the late stage or in severely damaged plants. Furthermore, we hypothesized that the PEG-responsive gene identified by DDRT-PCR might play an important role in mediating osmotic stress or drought resistance in *B. humilis* plants. Thus, these results provide valuable insights into the mechanism(s) mediating drought tolerance in *B. humilis*.

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