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

Wang Lirong¹, Zhao Pengshan², Zhao Xin², Wang Xiaopeng³, Ma Xiaofei², Li Yi¹*

¹ College of Forestry Science, Gansu Agricultural University, Lanzhou 730070, China
² Key Laboratory of Stress Physiology and Ecology in Cold and Arid Regions, Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou 730000, China
³ Department of Geography, Teachers' College, Dingxi 743000, China

* Corresponding author. Email: lyy@gsau.edu.cn

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$_2^-$), hydrogen peroxide (H$_2$O$_2$), 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, antioxidant 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: \( \text{RWC} = \frac{\text{FW} - \text{DW}}{\text{SW} - \text{DW}} \) [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 Na2HPO4·12H2O, 5.5 mM NaH2PO4, 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$_2^−$, 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$_2$O$_2$ (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$_2$O$_2$ 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$_2$O$_2$ (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$_2$O$_2$ 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,
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'-TGAGGATATTGCCCCCCTTG-3'), and its stability in B. humilis during osmotic stress was validated applying Ct 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 (Ct) values from Ct values for the gene of interest using the 2−ΔΔCt 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/orfalg.cgi). The molecular mass and isoelectric point (pl) of the proteins were predicted using Protein Calculator v.3.4 (http://procalc.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.

| 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 |
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).

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 \(AtCIPK9\).
The relative transcript levels of \textit{BhCIPK12} were determined by qRT-PCR for the various leaf samples collected. As shown in Fig. 4, the mRNA levels of \textit{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 \textit{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 \textit{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 \textit{B. humilis}, Arabidopsis, grape, and \textit{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 \textit{B. humilis} had a tolerance for PEG-6000 concentrations between 20\% and 30\%, thus providing a reference for future work on drought stress in \textit{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.
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 $O_2^-$ 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 \( \text{BhCIPK12} \) 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 \( B. \text{humilis} \) to PEG-induced osmotic stress at PEG-6000 concentrations between 20% and 30%. In addition, we hypothesized that the highest \( \text{BhCIPK12} \) 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 \( \text{BhCIPK12} \). The expression pattern of \( \text{BhCIPK12} \) was similar to that found in comparable short-term osmosis research. When rice plants were subjected to drought for 24 h, \( \text{OsCIPK12} \) showed an expression pattern that first increased and then decreased \[22\]. However, different expression patterns also have been observed. \( \text{McCIPK12} \) expression did not show obvious trend during osmotic treatment in cassava \[56\]. Altogether, these results suggest that \( \text{BhCIPK12} \) might have an important role in \( B. \text{humilis} \) plants exposed to osmotic stress or drought. However, the expression profile of \( \text{BhCIPK12} \) remains to be more fully characterized, which may lead to a better understanding of the genetic mechanisms that underlie drought stress tolerance in \( B. \text{humilis} \).

In conclusion, in the present study, we inferred that \( B. \text{humilis} \) 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 \( B. \text{humilis} \) 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 \( B. \text{humilis} \) 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 \( B. \text{humilis} \) plants. Thus, these results provide valuable insights into the mechanism(s) mediating drought tolerance in \( B. \text{humilis} \).

References

1. Morte A, Lovisolo C, Schubert A. Effect of drought stress on growth and water relations of the mycorrhizal association \( Helianthemum almeriense-\)Terfezia claveryi. Mycorrhiza. 2000;10:115–119. http://dx.doi.org/10.1007/s005720000066

2. Maarouf HE, Zuil-Fodil Y, Garell M, d’Arcy-Lameta A, Pham-Thi AT. Enzymatic activity and gene expression under water stress of phospholipase D in two cultivars of \( \text{Vigna unguiculata} \) L. Walp. differing in drought tolerance. Plant Mol Biol. 1999;39(6):1257–1265. http://dx.doi.org/10.1023/A:1006165919928

3. Shinozaki K, Yamaguchi-Shinozaki K, Seki M. Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol. 2003;6:410–417. http://dx.doi.org/10.1016/S1369-5266(03)00092-X

4. Hasanuzzaman M, Nahar K, Gill SS, Fujita M. Drought stress responses in plants, oxidative stress, and antioxidant defense. In: Tuteja N, Gill SS, editors. Climate change and plant abiotic stress tolerance. Hoboken: Wiley Online Library Press; 2014. p. 209–250. http://dx.doi.org/10.1002/9783527675265.ch09

5. Hameed A, Goher M, Iqbal N. Drought induced programmed cell death and associated changes in antioxidants, proteases, and lipid peroxidation in wheat leaves. Biol Plant. 2013;57:370–374. http://dx.doi.org/10.1007/s10535-012-0286-9
6. Haugen R, Steffes L, Wolf J, Brown P, Matzner SD, Siemens DH. Evolution of drought tolerance and defense: dependence of tradeoffs on mechanism, environment and defense switching. Oikos. 2008;117(2):231–244. http://dx.doi.org/10.1111/j.2007.0030-1299.16111.x

7. Bai LP, Sui FG, Ti-Da G, Sun ZH, Lu YY, Zhou GS. Effect of soil drought stress on leaf water status, membrane permeability and enzymatic antioxidant system of maize. Pedosphere. 2006;16:326–332. http://dx.doi.org/10.1016/S1002-0160(06)60059-3

8. Gajewska E, Sklodowska M, Slaba M, Mazur J. Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots. Biol Plant. 2006;50:653–659. http://dx.doi.org/10.1007/s1002-006-0102-5

9. Simova-Stoilova L, Demirevska K, Petrova T, Tsenov N, Feller U. Antioxidative protection and proteolytic activity in tolerant and sensitive wheat (Triticum aestivum L.) varieties subjected to long-term field drought. Plant Growth Regul. 2009;58(58):107–117. http://dx.doi.org/10.1007/s10725-008-9356-6

10. Zhang W, Fei W, Pan XJ, Tian, ZG, Zhao XM. Antioxidant enzymes and photosynthetic responses to drought stress of three Canna edulis Cultivars. Korean Journal of Horticultural Science and Technology. 2013;31(6):677–686. http://dx.doi.org/10.7235/hort.2013.13031

11. Bai J, Gong CM, Chen K, Kang HM, Wang G. Examination of antioxidative system’s responses in the different phases of drought stress and during recovery in desert plant Reaumuria soongorica (Pall.) Maxim. J Plant Biol. 2009;52:417–425. http://dx.doi.org/10.1007/s12374-009-9053-7

12. Lepeduš H, Gaća V, Viljevac M, Kovač S, Fulgosi H, Šimić D, et al. Changes in photosynthetic performance and antioxidative strategies during maturation of Norway maple (Acer platanoides L.) leaves. Plant Physiol Bioch. 2011;49:368–376. http://dx.doi.org/10.1016/j.plaphy.2010.12.011

13. Song Y, Ci D, Tian M, Zhang D. Comparison of the physiological effects and transcriptome responses of Populus simonii under different abiotic stresses. Plant Mol Biol. 2014;86:139–156. http://dx.doi.org/10.1007/s11103-014-0218-5

14. Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, et al. Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol. 2007;63:591–608. http://dx.doi.org/10.1007/s11103-009-9111-1

15. Aprile A, Mastrangelo AM, DeLeonardis AMD, Galiba G, Roncaglia E, Ferrari F, et al. Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. BMC Genomics. 2009;10:279. http://dx.doi.org/10.1186/1471-2164-10-279

16. Cohen D, Bogeart-Triboulot MB, Tisserant E, Balzergue S, Martin-Magniette ML, Lelandais G, et al. Comparative transcriptomics of drought responses in Populus: a meta-analysis of genome-wide expression profiling in mature leaves and root apices across two genotypes. BMC Genomics. 2010;11:630. http://dx.doi.org/10.1186/1471-2164-11-630

17. Deeba F, Pandey AK, Ranjan S, Mishra A, Singh R, Sharma YK, et al. Physiological and proteomic responses of cotton (Gossypium herbaceum L.) to drought stress. Plant Physiol Bioch. 2012;53:6–18. http://dx.doi.org/10.1016/j.plaphy.2012.01.002

18. Torres GAM, Stephanie P, Fabienne CM, Christelle M, Caroline H, Christine LB. Identification of novel drought-related mRNAs in common bean roots by differential display RT-PCR. Plant Sci. 2006;171:300–307. http://dx.doi.org/10.1016/j.plantsci.2006.03.008

19. Kavar TMM, Kidri M, Ular-Vozli J, Meglic V. Identification of genes involved in the response of leaves of Phaseolus vulgaris to drought stress. Molecular Breeding. 2008;21:159–172. http://dx.doi.org/10.1007/s11103-007-9116-8

20. Padmalatha KV, Dhandapani G, Kanakachari M, Kumar S, Dass A, Patil DP, et al. Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defense responsive genes. Plant Mol Biol. 2012;78:223–246. http://dx.doi.org/10.1007/s11103-011-9857-y

21. Kolukisaoglu Ü, Weinl S, Blazevic D, Batistic O, Kudla J. Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL–CIPK signaling networks. Plant Physiol. 2004;134:43–58. http://dx.doi.org/10.1104/pp.103.033068

22. Yong X, Yuemin H, Lizhong X. Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. Plant Physiol. 2007;144:1416–1428. http://dx.doi.org/10.1104/pp.107.101295

23. Girdhar KP, Poonam K, Amita P. Distribution and expression in plants. In: Girdhar KP, Poonam K, Amita P, editors. Global comparative analysis of CBL–CIPK gene
families in plants. London: Springer Briefs in Plant Science; 2014. p. 19–23. http://dx.doi.org/10.1007/978-3-319-09078-8_3

24. Zhou GY, Chen GC, Chen ZG, Si Zhen MA, Han YJ. Response of the characteristics of Alpine meadow plant community to disturbance gradient of human along Qinghai–Tibet railway: a case study in the Alpine meadow in Fenghuoshan area. J Glaciol Geocryol. 2006;28:240–248.

25. Liu B, Wenjin LI. Influence of nurse effect on the diversity of desert Reaumuria soongorica community under arid environment in the Loess Plateau. Journal of Arid Land Resources and Environment. 2012;26(10):117–120.

26. Warwick SI, Al-Shehbaz IA, Sauder C, Harris JM. Phylogeny of Braya and Neotorularia (Brassicaceae) based on nuclear ribosomal internal transcribed spacer and chloroplast trnL intron sequences. Can J Bot. 2004;82:376–392. http://dx.doi.org/10.1139/b04-012

27. German DA, Friesen N, Neuffer B, Al-Shehbaz IA, Hurka H. Contribution to ITS phylogeny of the Brassicaceae, with special reference to some Asian taxa. Plant Syst Evol. 2009;283:33–56. http://dx.doi.org/10.1007/s00606-009-0213-5

28. Zhou TY, editor. Brassicaceae. Beijing: Chinese Academy of Sciences; 1987. (Flora of China; vol 33).

29. Jin X, Yin H, Wang W, Qin M, Liao X, Xia L. Identification of Cd-responsive genes of Solanum nigrum seedlings through differential display. Plant Mol Biol Rep. 2009;27:563–569. http://dx.doi.org/10.1007/s11105-009-0098-z

30. Yildiztugay E, Ozfidan-Konakci C, Kucukoduk M. Exogenous nitric oxide (as sodium nitroprusside) ameliorates polyethylene glycol-induced osmotic stress in hydroponically grown maize roots. J Plant Growth Regul. 2014;33:683–696. http://dx.doi.org/10.1007/s00344-014-9417-1

31. Marok MA, Tarrago L, Ksas B, Henri P, Abrous-Belbachir O, Havaux M, et al. A drought-sensitive barley variety displays oxidative stress and strongly increased contents in low-molecular weight antioxidant compounds during water deficit compared to a tolerant variety. J Plant Physiol. 2013;170:633–645. http://dx.doi.org/10.1016/j.jplph.2012.12.008

32. Jung S. Variation in antioxidant metabolism of young and mature leaves of Arabidopsis thaliana subjected to drought. Plant Sci. 2004;166(2):459–466. http://dx.doi.org/10.1016/j.plantsci.2003.10.012

33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

34. H Mete T, Turgay S, Mine I, Fahrettin A, Kabulay U, Ergul S. Effect of quercetine and glutathione on the level of superoxide dismutase, catalase, malonyldialdehyde, blood pressure and neonatal outcome in a rat model of pre-eclampsia induced by NG-nitro-L-arginine-methyl ester. Eur J Obstet Gynecol. 2005;118(2):190–195. http://dx.doi.org/10.1016/j.ejogrb.2004.04.033

35. Garcia A. Hypoxia, reoxygenation and cytosolic manganese superoxide dismutase (cMnSOD) silencing in Litopenaeus vannamei: effects on cMnSOD transcripts, superoxide dismutase activity and superoxide anion production capacity. Dev Comp Immunol. 2010;34(11):1230–1235. http://dx.doi.org/10.1016/j.dci.2010.06.018

36. Wei X, Li D, Liu G. Anti-oxidative responses of Elodea nuttallii (Planch.) H. St. John to short-term iron exposure. Plant Physiol Bioch. 2010;48(10–11):873–878. http://dx.doi.org/10.1016/j.plaphy.2010.08.006

37. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005;139(1):5–17. http://dx.doi.org/10.1104/pp.105.063743

38. Nopo-Olazabal C, Condori J, Nopo-Olazabal L, Medina-Bolivar F. Differential induction of antioxidant stilbenoids in hairy roots of Vitis rotundifolia treated with methyl jasmonate and hydrogen peroxide. Plant Physiol Bioch. 2014;74:50–69. http://dx.doi.org/10.1016/j.plaphy.2013.10.035

39. Hong SY, Seo PJ, Yang MS, Xiang F, Park CM. Exploring valid reference genes for gene expression studies in Brachypodium distachyon by real-time PCR. BMC Plant Biol. 2008;8(1):112. http://dx.doi.org/10.1186/1471-2229-8-112

40. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 2008;8(6):443–447. http://dx.doi.org/10.1186/1471-2229-8-131
41. Nygard AB, Jørgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol Biol. 2007;8(16):67. http://dx.doi.org/10.1186/1471-2199-8-67
42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{ΔΔCT}/ method. Methods. 2001;25:402–408. http://dx.doi.org/10.1006/meth.2001.1262
43. Zhang JY, Cruz de Carvalho MHC, Torres-Jerez I, Kang Y, Allen SN, Huhman DV, et al. Global reprogramming of transcription and metabolism in Medicago truncatula during progressive drought and after re-watering. Plant Cell Environ. 2014;37(11):2553–2576. http://dx.doi.org/10.1111/pce.12328
44. Dhindsa RS. Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in Tortula ruralis. Plant Physiol. 1991;95:648–651. http://dx.doi.org/10.1104/pp.95.2.648
45. Rahman MA, Ren L, Wu W, Yan Y. Proteomic analysis of PEG-induced drought stress responsive protein in TERRF1 overexpressed sugarcane (Saccharum officinarum) leaves. Plant Mol Biol Rep. 2014;33(3):716–730. http://dx.doi.org/10.1007/s11105-014-0784-3
46. Skutnik M, Rychter AM. Differential response of antioxidant systems in leaves and roots of barley subjected to anoxia and post-anoxia. J Plant Physiol. 2009;166:926–937. http://dx.doi.org/10.1016/j.jplph.2008.11.010
47. Hare PD, Cress WA, Van Staden J. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J Exp Bot. 1999;50:413–434. http://dx.doi.org/10.1093/jxb/50.333.413
48. Mostajeran A, Rahimi-Eichi V. Effects of drought stress on growth and yield of rice (Oryza sativa L.) cultivars and accumulation of proline and soluble sugars in sheath and blades of their different ages leaves. Am Eurasian J Agric Environ Sci. 2009;5(2):264–272.
49. Sairam RK, Srivastava GC. Water stress tolerance of wheat (Triticum aestivum L.): variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. Journal of Agronomy and Crop Science. 2001;186:63–70. http://dx.doi.org/10.1046/j.1439-037x.2001.00461.x
50. Selote DS, Khanna-Chopra R. Drought acclimation confers oxidative stress tolerance by inducing co-ordinated antioxidant defense at cellular and subcellular level in leaves of wheat seedlings. Physiol Plant. 2006;127:494–506. http://dx.doi.org/10.1111/j.1399-3054.2006.00678.x
51. Mittler R, Vanderauwera S, Gollery M, van Breusegem F. Reactive oxygen gene network of plants. Trends Plant Sci. 2004;9(10):490–498. http://dx.doi.org/10.1016/j.tplants.2004.08.009
52. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Bioch. 2010;48(12):909–930. http://dx.doi.org/10.1016/j.plaphy.2010.08.016
53. Tattini M, Loreto F, Fini A, Guidi L, Brunetti C, Velikova V, et al. Isoprenoids and phenylpropanoids are part of the antioxidant defense orchestrated daily by drought-stressed Platanus acerifolia plants during Mediterranean summers. New Phytol. 2015;207(3). http://dx.doi.org/10.1111/nph.13380
54. Rossi CF, Abreu OJT, Simone MA, Almeida VR. Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytol. 2004;163(3):563–571. http://dx.doi.org/10.1111/j.1469-8137.2004.01139.x
55. Chen XF, Gu ZM, Liu F, Ma BJ, Zhang HS. Molecular analysis of rice CIPKs involved in biotic and abiotic stress responses. Rice Science. 2010;6:3. http://dx.doi.org/10.1016/S1672-6308(11)60001-2
56. Hu W, Xia ZQ, Yan Y, Ding ZH, Tie WW, Wang LZ, et al. Genome-wide gene phylogeny of CIPK family in cassava and expression analysis of partial drought-induced genes. Front Plant Sci. http://dx.doi.org/10.3389/fpls.2015.00914