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A novel *Medicago truncatula* HD-Zip gene, *MtHB2*, is involved in abiotic stress responses

Shiyong Song[a,b,1], Ying Chen[a,b,1], Mingui Zhao[a], Wen-Hao Zhang[a,*]

[a] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, PR China
[b] Graduate University of the Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

The HD-Zip proteins are important transcription factors participating in numerous physiological processes in plants. In this study, we identified a homeobox-leucine zipper gene from *Medicago truncatula*, designated *MtHB2* by monitoring the expression profile of *M. truncatula* exposed to low temperature. A fusion protein of MtHB2 with green fluorescent protein showed localization of MtHB2 in the nucleus. To evaluate the role of *MtHB2* in response to abiotic stresses, transgenic *Arabidopsis* plants were generated by expressing *MtHB2*, and the effects of abiotic stress on the transgenic and wild-type *Arabidopsis* plants were studied. Transgenic plants generated that constitutively expressed *MtHB2* were more sensitive to drought, salt and freezing stresses than wild-type plants. The physiological mechanisms underlying the reduced tolerance of the transgenic plants to drought, salt and freezing stresses were investigated. Expression of *MtHB2* in *Arabidopsis* resulted in the transgenic plants accumulating less amounts of Pro and soluble sugars and greater amounts of malondialdehyde (MDA) and H_2O_2 than their wild-type counterparts treated with and without abiotic stresses. The reduced accumulation of Pro and soluble sugars may account for the lower osmolality in the transgenic plants, thus rendering the osmo-regulation of the transgenic plants less effective, while the higher levels of MDA and H_2O_2 in the transgenic plants made the transgenic plants more susceptible to oxidative damage under the conditions of abiotic stress. These findings demonstrate that *MtHB2* encodes a novel stress-responsive HD transcription factor that may play a negative role in regulation of abiotic stress response mechanisms.

1. Introduction

Plants are frequently exposed to adverse environments. To cope with environmental stress, plants have evolved sophisticated mechanisms to respond and adapt to those stresses such as low temperature, drought and high salinity. Exposure of plants to the adverse environments often alters the expression of numerous genes, resulting in changes in molecular, cellular and physiological processes (Thomashow, 1999; Shinozaki et al., 2003). As important regulatory proteins, transcription factors, which regulate expression of many functional genes, play significant roles in the response and adaptation of plants to various types of abiotic stress (Nakashima et al., 2009). A number of transcription factors such as CBF, DREB, bZIP, NAC and MYB have been demonstrated to play important roles in responses of plants to abiotic stress (see reviews Seki et al., 2003; Shinozaki et al., 2003). In addition, there is emerging evidence demonstrating that the homeodomain-leucine zipper (HD-Zip) proteins are also involved in plant response to abiotic stress (Harris et al., 2011).

The HD-Zip proteins that contain homeodomain (HD) and leucine zipper (LZ) domains are unique to plants, and are divided into four subfamilies (HD-Zip I, II III and IV) based on their sequence conservation, structural features and functions (Ariel et al., 2007; Harris et al., 2011). The HD domain is involved in the specific binding to DNA by its helix III, while the LZ domain has the ability to dimerize, which is necessary for binding to the target sequence CAAT N ATTG (Sessa et al., 1997; Palena et al., 1999). In addition to the HD and LZ domains, subfamily II of HD-Zip protein (HD-Zip II) also contains a CPSCE domain, which has been suggested to be involved in sensing redox state (Tron et al., 2002). There are 17 and 10 genes encoding HD-Zip I and HD-Zip II proteins in the *Arabidopsis* genome, respectively (Henriksson et al., 2005; Ciarbelli et al., 2008). Several studies have demonstrated that HD-Zip I and HD-Zip II proteins play a regulatory role in response and adaptation of plants to environmental changes and the phytohormone network (see review of Harris et al., 2011). For example, it has been reported that *Arabidopsis* ATHB2 is involved in regulation of flowering (Steindler et al., 1999) and shade avoidance (Ohgishi et al., 2001). In a recent study, Sorin et al. (2009) reported that ATHB4

* Corresponding author. Tel.: +86 10 6283 6697; fax: +86 10 6259 2430.
E-mail address: whzhang@ibcas.ac.cn (W.-H. Zhang).
[1] These authors contribute equally to this work.

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modulates shade avoidance syndrome responses by controlling auxin, brassinosteroid and gibberellin molecular and/or physiological responsiveness. Huang et al. (2008) reported that the transcripts of *HAT2* (Homeobox from *Arabidopsis thaliana* 2) and *HAT22* are up-regulated under drought conditions. Furthermore, the expression level of *CpHB1* and *CpHB2*, which belong to HD-Zip II subfamily, is induced by water deficit in *Craterostigma plantagineum* (Deng et al., 2002). In addition to the involvement in abiotic stress, a recent report also revealed that a sunflower HD-Zip II transcription factor (HAHB10) also participates in the induction of flowering and in the control of phytohormone-mediated responses to biotic stress (Dezar et al., 2011). Most studies on the functional characterization of HD-Zip I and HD-Zip II proteins have been conducted in *Arabidopsis* so far. In a recent study, Ariel et al. (2010) reported that a *M. truncatula* HD-Zip I transcription factor MtHB1 regulates an adaptive developmental response to minimize the root surface exposed to adverse environmental stress. In contrast, there has been no report on the identification and functional characterization of HD-Zip II proteins in *M. truncatula*.

In the present study, we identified a *M. truncatula* gene, *MtHB2*, encoding a putative transcriptional factor, and functionally characterized the role of *MtHB2* in response to cold, drought and salt stress by expressing *MtHB2* in *Arabidopsis*. Our results reveal that overexpressing *MtHB2* in *Arabidopsis* rendered the transgenic plants less tolerant to abiotic stresses such as cold, drought and salinity. We further demonstrate that the altered accumulation of soluble sugars, Pro, malondialdehyde (MDA) and H_2O_2 may account for the reduced tolerance of the transgenic plants to cold, drought and salinity.

2. Materials and methods

2.1. Plant growth and stress treatments

M. truncatula ('Jemalong A17') seedlings were grown in a greenhouse at 27/23 °C 14 h light/10 h dark. For the treatment of salt and drought stress, 21-day-old seedlings grown in hydroponic solution with 1/2 MS were transferred to the hydroponic solution supplemented with 200 mM NaCl or 15% PEG 6000 for 5 and 10 h, and leaves were sampled for analysis of gene expression. For cold stress, 21-day-old seedlings grown in pots filled with vermiculite watered with 1/2 MS solution were transferred to a growth chamber at 2–5 °C and sampled at 0, 2, 5, 10, 24, 36 and 72 h. For analysis of known stress-responsive genes, 4-week-old seedlings of wild-type (Col-0) and transgenic *Arabidopsis* grown in pots filled vermiculite were treated with water (control), NaCl (200 mM), 15% PEG6000 and cold (4 °C), and harvested after 24 h treatment. In each case, the treated seedlings were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Plasmid construction and transgenic plants generation

The full-length cDNA of *MtHB2* was amplified from *M. truncatula* by RT-PCR using the primers *MtHB2*-FL-F (5′-AGCAGGGATCCATGGGTCTTAATGATCAAGATTC-3′; *Bam* H I site underlined) and *MtHB2*-FL-R (5′-ATGCTGAGCTCTTAACATGCTG CAGAAGGATTG-3′; *Sac* I site underlined). The sequencing confirmed PCR fragment was directionally cloned into a pSN1301 vector with a *GUS*-fused fragment driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter to create the pSN1301-*MtHB2* construct.

For *Arabidopsis* transformation, the pSN1301-*MtHB2* construct was introduced into *Agrobacterium* strain GV3101, and transferred into *Arabidopsis* wild-type plants (Col-0) by using floral dip transformation (Zhang et al., 2006). Positive transgenic lines were firstly screened on hygromycin plates and then identified through genome PCR, and T_3 homozygous transgenic lines were selected for evaluating their sensitivity to abiotic stress.

2.3. Phylogenetic tree construction of MtHB2

Phylogenetic analysis was performed to understand the relationship between MtHB2 and other HD-Zip II members from other plant species. A maximum likelihood tree was constructed with the ClustalX 2 software with the putative amino acid sequences.

2.4. Subcellular localization of the MtHB2 protein

To construct the vector for transient expression analysis, the coding sequences of *MtHB2* were first amplified using primer pairs *MtHB2-GFP*-F (5′-TCGTGAGCTCATGGGTCTTAATGATC-3′; *Sac* I site underlined) and *MtHB2-GFP*-R (5′-CTTGGATCCACATGCTGCAGAAGGA-3′; *Bam* H I site underlined), the PCR products were initially cloned into the pGEM-T-easy vector and confirmed by sequencing. It was then digested with *Sac* I and *Bam* H I and ligated into the pCM1205-*GFP* vector driven by the CaMV 35S promoter for construction of a 35S: *MtHB2-GFP* fusion construct. The construct was delivered into tobacco cells by an *Agrobacterium*-mediated transient transformation method as described by Imogen et al. (2006). After 40 h, GFP fluorescence was observed under a laser scanning confocal microscope.

2.5. Determination of stress tolerance

The survival experiments were performed by growing plants in pots. For salt and drought survival experiments, 4-week-old seedlings grown in soil were exposed to salt and drought stress by irrigating with 200 mM NaCl for 14 days and withholding water for 15 days, respectively. After the treatments, plants were transferred to normal growth conditions. The survival rate was determined by scoring the seedlings that failed to grow after recovery from treatments with salt or drought stress. For cold treatment, 4-week-old seedlings were cold acclimated at 4 °C for 4 day, and transferred to -7 °C for 9 h, thawed at 4 °C overnight, then transferred to normal growth conditions. Survival of the seedlings was recorded visually (plants with green leaves) 7 day later. All the stress tolerance experiments were repeated at least three times, and data were calculated from the results of three independent experiments. Plants were considered dead if all the leaves were brown and no re-growth was observed after transferring to the control growth conditions.

2.6. Determination of water loss rate

For measurements of water loss, mature leaves from 4-week-old WT and the transgenic plants were weighed immediately. The leaves were then kept on filter paper in an illumination incubator under the conditions of humidity 35–40% and temperature of 27 °C for varying periods (0, 0.5, 1, 2, 3, 4, 5 and 6 h) and weighed again. The percentage loss of fresh weight was calculated relative to the initial plant weights.

2.7. Determination of osmolality

Leaves of *Arabidopsis* wild-type and transgenic lines were excised from plants and put into 1.5 mL tubes, and then the tubes were frozen in liquid nitrogen. The frozen leaves were thawed at room temperature to disrupt the cell structure and centrifuged at a speed of 50,000 × *g* for 3 min to extract the cell sap as described by Zhang et al. (1996). The osmolality of cell sap of leaves was determined using a VAPRO 5520 vapor pressure osmometer. Three replicates for each treatment were used to determine osmolality.

2.8. Determination of Pro and soluble sugar contents

Pro content in leaves of *Arabidopsis* wild-type and transgenic lines was determined following the protocols described by Bates et al. (1973). Briefly, leaves were harvested, weighted and extracted in 3% sulfosalicyclic acid. An aliquot of each extract (2 mL) was incubated with 2 mL ninhydrin reagent (2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, 40% 6 M phosphoric acid) and 2 mL of glacial acetic acid at 100 °C for 40 min, and the reaction terminated in an ice bath. Toluene (5 mL) was added, followed by vortexing and incubation at 23 °C for 24 h. The absorbance was measured spectrophotoetrically at wavelength of 525 nm using a spectrophotometer (SmartSpec™ Plus, BioRad).

Total soluble sugar content was measured as described previously (Bailey, 1958). Briefly, about 0.1 g leaves were homogenized in 5 mL of double-distilled water, then boiled in a water bath at 100 °C for 30 min. One milliliter of extract was incubated with 5 mL anthrone reagent at 95 °C for 15 min, and then the reaction was terminated in an ice bath. The absorbance was measured at wavelength of 620 nm using a spectrophotometer (SmartSpec™ Plus, BioRad).

2.9. Determination of H_2O_2 and MDA contents

Hydrogen peroxide was measured as described by Alexieva et al. (2001) with some modification. Briefly, the leaves were grounded with 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 20 min at 4 °C. The reaction mixture consisted of 1 mL of the extracted supernatant, 1 mL of potassium phosphate buffer and 2 mL of 1 M KI. The absorbance was measured at 390 nm after reaction 1 h.

For the measurement of MDA, the leaves were homogenized in 5 mL of 10% TCA solution and centrifuged at 4 °C, then the supernatant was added to 0.6% thiobarbituric acid in 10% TCA. The mixture was incubated in boiling water for 15 min, and then stopped by ice bath. The reaction product was centrifuged and the absorbance of the supernatant was measured at wavelength of 450, 532 and 600 nm. MDA contents were calculated by the formula: [6.45 × (A532–A600)–0.56 × A450]/fresh weight (Zhang et al., 2009).

2.10. RNA isolation and quantitative PCR

RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR) were carried out as described by Sun et al. (2010). An actin gene was used as internal control to quantify the relative transcript level. The reaction primers utilized were as follows: for *MtHB2* (5′-CCAAAGGAAATAACTA-3′ and 5′-TAACAACACTATGAGG-3′), for *AtP5CS1* (5′-CTCGCTTAGTTATGACGC-3′ and 5′-CTCCTTTCC-ACCCTTTA-3′), for *AtProDH* (5′-ATCTTACCGTTTACCCG-3′ and 5′-TCACCGAAGCGTCCATA-3′), *MtActin* gene (accession no. AC184161) and *AtActin* (accession no. X16280) were used as internal control with primers (5′-ACGAGCGTTTCAGATG-3′ and 5′-ACCT-CCGATCCAGACA-3′) and (5′-CCACATGCTATTCTGCGTTTGGACC-3′ and 5′-CATCCCTTACGATTTCACGCTCTGC-3′), respectively. Each sample was run in three independent experiments, the relative expression levels were analyzed by using the comparative C*t* method as described by Livak and Schmittgen (2001).

2.11. Statistical analysis

The analysis of variance was conducted between different treatments. The significant differences between transgenic plants and their counterpart wild-type plants under control and treatments were evaluated by LSD multiple range tests ($P < 0.05$) using the SAS statistical software.

3. Results

3.1. Isolation and characterization of MtHB2 gene

Expression profiles of *M. truncatula* under cold stress at 4 °C for 5 h were monitored by cDNA microarray (GeneChip® Medicago Genome Array, Affymetrix). A gene encoding a HD-Zip protein (MtHB2, GeneBank: BT051548.1) that was up-regulated by the cold treatment was identified from the cold-stress DNA microarray data. The gene was 813 bp in length and encoded a putative protein of 269 amino acids with 3 exons and 2 introns. Sequence analyses revealed that the MtHB2 protein contained a homeodomain, leucine-zipper and CPSCE domain, and that MtHB2 belonged to HD-Zip II subfamily (Fig. 1A). As shown in Fig. 1B, MtHB2 had highly conserved HD and LZ domains when compared with other HD-Zip II proteins in *Arabidopsis*, rice and tomato.

3.2. Subcellular localization of the MtHB2 protein

To determine the subcellular localization of the MtHB2 protein, an *MtHB2-GFP* fusion construct driven by the CaMV 35S promoter was introduced into tobacco cells using an Agrobacterium-mediated transient transformation method, and the tobacco cells were observed under a laser confocal microscope. As shown in Fig. 2, the GFP signal was observed in the cytomembrane, cytoplasm and nucleus in the cells expressing the control *GFP* gene, while the fluorescence signal was observed in the nucleus exclusively in the *MtHB2-GFP* fusion gene, suggesting that MtHB2 is a nuclear-localized protein.

3.3. Expression patterns of MtHB2

To verify the microarray data, the effect of cold treatment on *MtHB2* at the transcriptional level was studied by real-time PCR. There was a time-dependent increase in *MtHB2* transcript upon exposure to cold treatment at 4 °C (Fig. 3A). The cold-treatment induction of the *MtHB2* transcript peaked at 12 h of the cold treatment, and it gradually declined thereafter (Fig. 3A). In addition to cold stress, the expression of *MtHB2* was also up-regulated by treatments with PEG and salt stress (Fig. 3B). Furthermore, the expression of *MtHB2* was found in leaves, roots, stems, flowers and pods with the expression level being the highest in pods and the lowest in flowers (Fig. 3C).

3.4. Arabidopsis plants expressing MtHB2 were more sensitive to cold stress

To functionally characterize *MtHB2*, we overexpressed *MtHB2* in transgenic *Arabidopsis* under control of a CaMV 35S promoter. Several independent transgenic lines were obtained and confirmed by PCR (data not shown). Six transgenic lines were randomly chosen to determine expression of *MtHB2* by RT-qPCR, and the relative expression levels of *MtHB2* in the transgenic lines were shown in Fig. 4. Two independent transgenic lines (L2 and L18) were used for further physiological studies throughout this paper.

Given that expression of *MtHB2* was rapidly induced by cold stress, we compared the tolerance of *Arabidopsis* seedlings expressing *MtHB2* to freezing stress with that of wild-type (WT) *Arabidopsis* seedlings. After *Arabidopsis* seedlings of 4-week-old of both expressing *MtHB2* and WT were exposed to −7 °C for 9 h following cold acclimation at 4 °C for 4 days, WT plants exhibited less flagged and whitened leaves than the two transgenic lines (Fig. 5A), suggesting that expression of *MtHB2* renders the transgenic plants more sensitive to cold stress. Accordingly, the transgenic lines displayed much lower survival rate than WT plants following 9 h cold treatment at −7 °C and recovery for 7 days. For instance, the

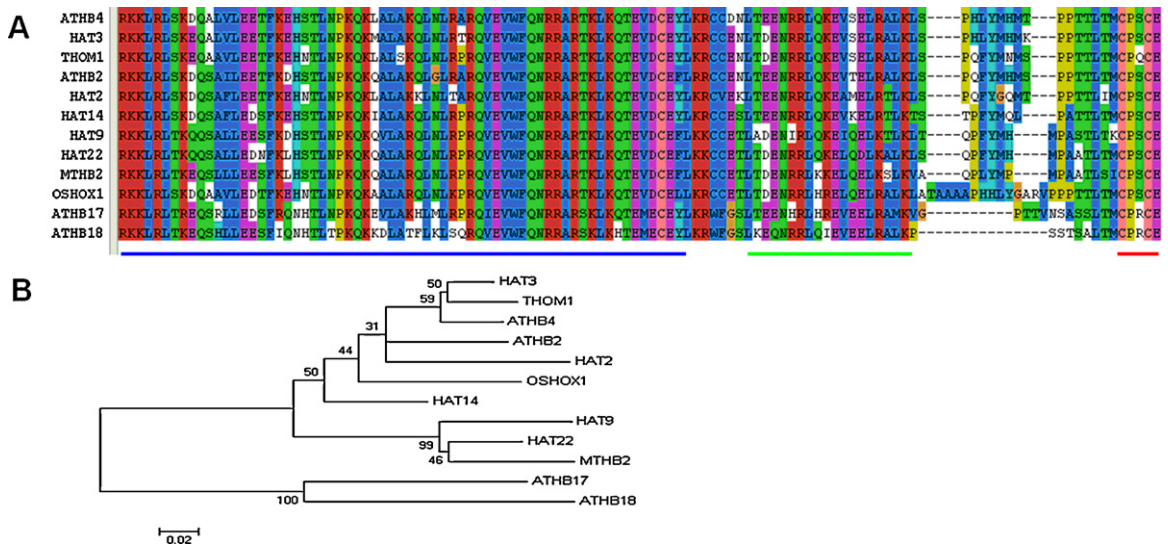

Fig. 1. Alignment of the high conservation of HD and LZ domains sequence of MtHB2 and other known HD-Zip II subfamily proteins (A). Alignments were performed using the ClustalX 2 software. HD, LZ and CPSCE domain was underlined by blue, green and red, respectively. (B) Phylogenetic tree of MtHB2 and other HD-zip II subfamily proteins was constructed by MEGA5. The corresponding ID of ATHB2, ATHB4, ATHB17, ATHB18, HAT2, HAT3, HAT9, HAT14, HAT22, OSHOX1, THOM1 are AT4G16780, AT2G44910, AT2G01430, AT1G70920, AT5G47370, AT3G60390, AT2G22800, AT5G06710, AT4G37790, OSJNBb0015I11.18, CAA62608, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

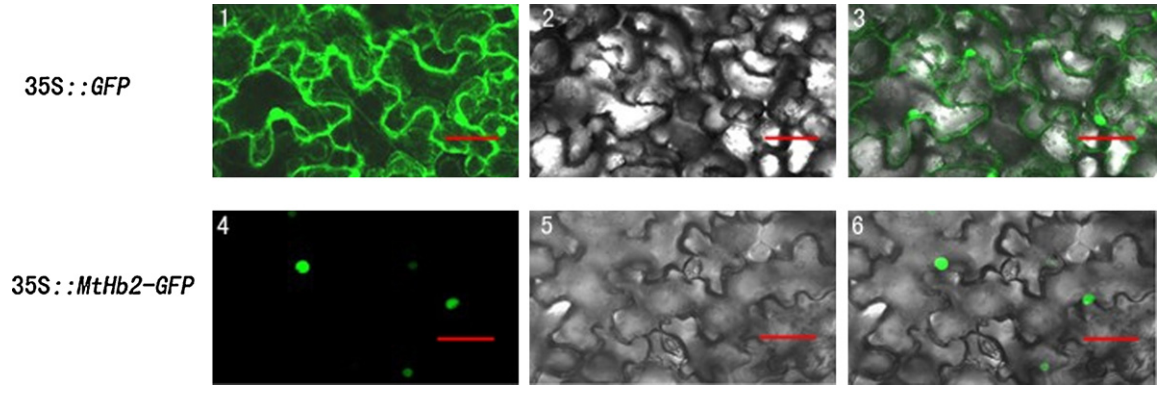

Fig. 2. Nuclear localization of HB2. Green fluorescent protein (GFP) and GFP fusion with HB2 (HB2-GFP) were transiently expressed in tobacco epidermal cells as described in the materials and methods. Images were taken in the dark field for green fluorescence (1, 4), while the outline of the cell (2, 5) and the combination (3, 6) were photographed in a bright field. Bars represent 50 μm.

survival rate for WT, L2 and L18 was found to be 81.9%, 32.4%, 27.3%, respectively (Fig. 5B).

3.5. Transgenic Arabidopsis plants expressing MtHB2 were less tolerant to salt and osmotic stress

In addition to the cold stress, the involvement of *MtHB2* in salt and drought stress was also examined by studying the effect of salt stress on survival rate of WT and the transgenic lines. The transgenic lines were more sensitive to salt stress than WT plants, as evidenced by the lower survival rate of the two transgenic lines than that of WT plants in response to treatment with NaCl (Fig. 6A). The sensitivity of WT and transgenic plants to 200 mM NaCl was also evaluated by measuring the survival rate. After recovery from the salt treatment, the transgenic plants showed less survival rate than WT plants (Fig. 6B). Similar to salt stress, the transgenic plants were more sensitive to drought stress as judged by the lower survival rate for the transgenic plants than WT plants following the 15 days withholding water and recovering for 7 days after watering (Fig. 7). For instance, 73.6% of WT plants survived following the drought treatment, while only 16.6% of transgenic plants survived following the identical drought treatment (Fig. 7B). The ability of water retention is often used as an indicator of drought tolerance in plants (Clarke et al., 1989; Dhanda and Sethi, 1998). We found that the two transgenic lines exhibited higher water loss rate than WT following the drought treatment (Fig. 7C). These results indicate that the expression of *MtHB2* in *Arabidopsis* renders the transgenic plants more sensitive to drought stress than WT plants.

3.6. MtHB2 reduced the accumulation of Pro and soluble sugars

To explore the physiological mechanism that may be responsible for reduced tolerance of the transgenic plants to cold (4 °C for 5 days), drought (withholding water for 10 days) and salt stress (200 mM NaCl for 10 days) than WT plants, Pro and soluble sugars in WT and the transgenic plants grown in the non-stressed, control and the stressed conditions were determined. As shown in Fig. 8, the transgenic plants exhibited lower contents of Pro and soluble sugars than WT plants under both control and stressed conditions (Fig. 8A and B). The transcripts for *P5CS1* (Δ1-pyrroline-5-carboxylate synthetase 1) and *ProDH* (proline dehydrogenase), which are two key enzymes responsible for accumulation of Pro,

Table 1
Osmolalities in leaves of wild-type and transgenic *Arabidopsis* plants overexpressing *MtHB2* under control and different treatments (cold, drought and salt). Data are mean ± SE of four replicates. Means with different letters within a column are significantly different ($P < 0.05$) with regard to treatments. Osmolality was expressed as mOsmol kg^{-1}. WT, L2 and L18 represent wild-type, transgenic lines, respectively.

Treatments	Control	Cold	Drought	Salt
WT	393.8 ± 5.0a	628.6 ± 6.6a	576.9 ± 4.2a	852.2 ± 8.0a
L2	328.0 ± 3.7b	563.0 ± 17.7b	464.8 ± 12.1b	784.7 ± 23.7b
L18	320.6 ± 3.30b	566.34 ± 18.15b	516.3 ± 0.9b	657.8 ± 24.1c

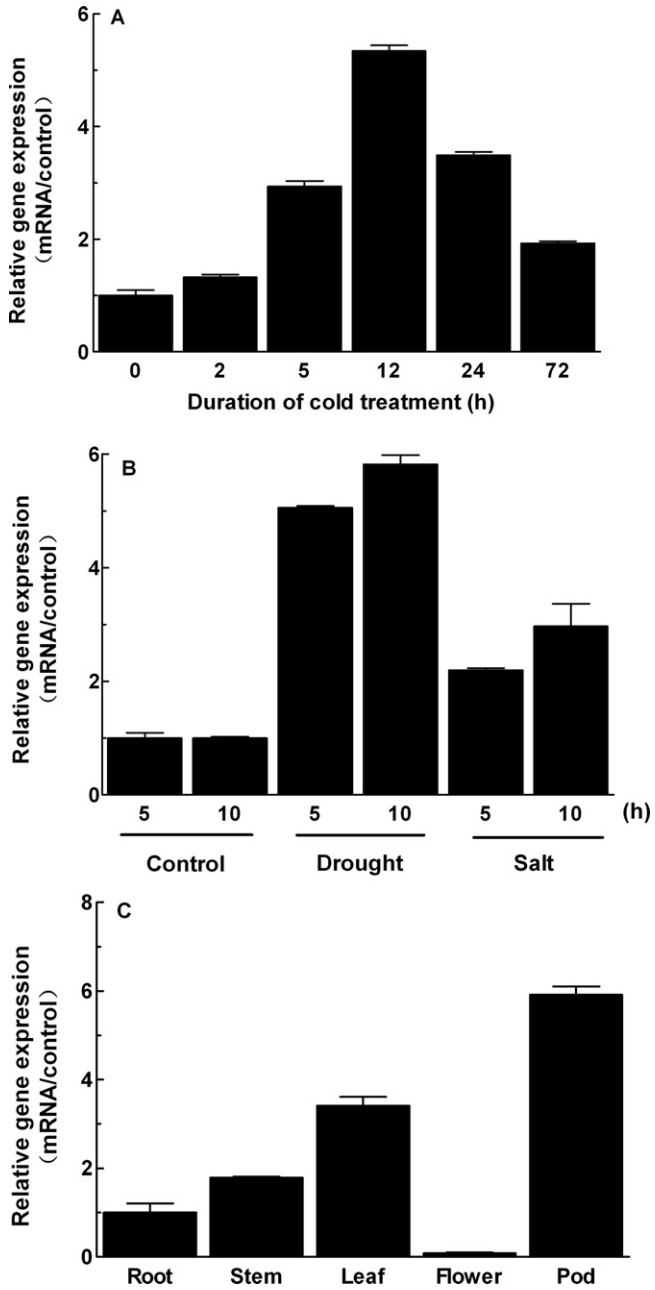

Fig. 3. Quantitative polymerase chain reaction (PCR) analysis of *MtHB2* expression in response to cold treatment (4 °C) (A), salt stress (200 mM NaCl) and PEG-induced drought stress (15% PEG 6000) for 5 and 10 h (B) and in different organs (C). Data are mean ± SE for three biological replicates.

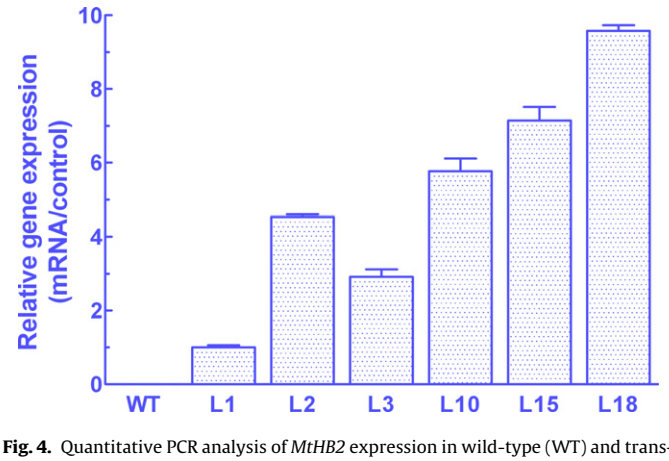

Fig. 4. Quantitative PCR analysis of *MtHB2* expression in wild-type (WT) and transgenic plants (L1, L2, L3, L10, L15 and L18). Data are mean ± SE with three biological replicates.

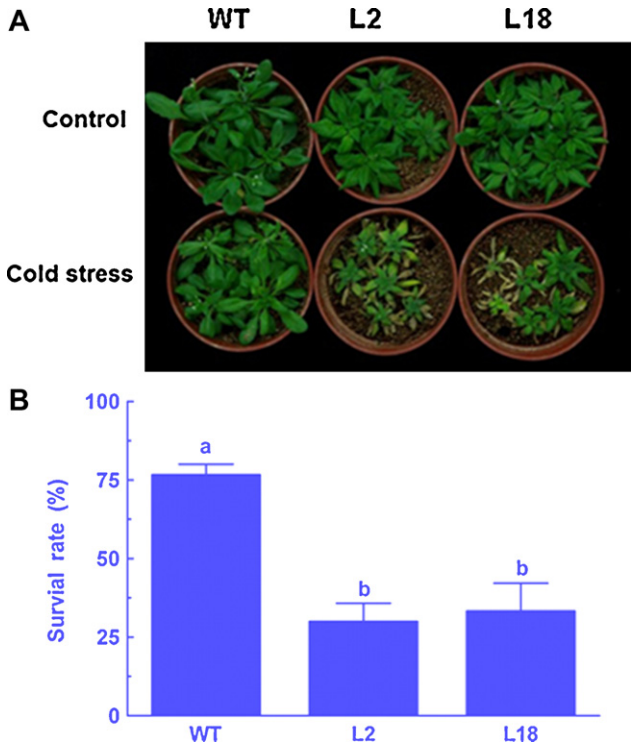

Fig. 5. Comparison of *Arabidopsis* wild-type and transgenic plants in response to cold stress. WT (Col-0) and transgenic lines (L2 and L18) before and after exposure to −7 °C for 9 h (A). Survival rates of WT and transgenic plants after recovery from the freezing regime (−7 °C for 9 h) for 7 days (B). Data are mean ± SE with three replicates. Each replicate contained more than 8 plants. Different letters shown in the error bars mean significant differences among control and treatments at $P < 0.05$.

were monitored under both non-stressed and stressed conditions for the transgenic and WT plants. Under non-stressed and stressed conditions, *P5CS1* transcript levels in the transgenic plants were lower than in WT plants (Fig. 8C). In contrast, the transcripts of *ProDH* were significantly higher in the transgenic lines than in WT plants under both control and stressed conditions (Fig. 8D). In addition, the transgenic plants expressing *MtHB2* had lower osmolality than WT plants under both control and stressed conditions (Table 1).

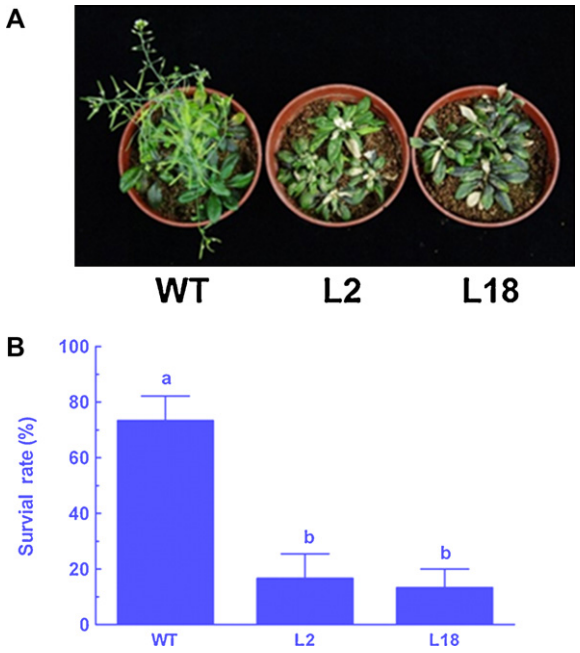

Fig. 6. Effect of salt stress on *Arabidopsis* WT and transgenic plants. Phenotypes of WT, transgenic seedlings grown in normal and 1/2 MS medium containing 125 mM NaCl during post-germination stage (A). Effect of NaCl on relative root length of WT, L2 and L18 seedlings (B). Data are mean ± SE with 10 seedlings for each treatment with three replicates. Survival rates of WT and transgenic lines (L2 and L18) after treated with 200 mM NaCl for 15 days and recovering for 7 days (C, D). The survival rate data are mean ± SE for 15 seedlings for each treatment with three replicates. Different letters shown in the error bars mean significant differences among control and treatments at $P < 0.05$.

3.7. Transgenic plants displayed enhanced accumulation of MDA and H_2O_2

Plants suffering from abiotic stress often exhibit symptoms of oxidative stress as evidenced by enhanced accumulation of reactive oxygen species (ROS) and MDA (Chinta et al., 2001; Verslues et al., 2007). To investigate whether the transgenic plants differ from WT plants in their sensitivity to oxidative stress resulting from drought and salt stress, H_2O_2 contents in WT and transgenic plants grown under control and stressed conditions were measured. The two transgenic lines had higher H_2O_2 contents than WT plants under both control and stressed conditions (Fig. 9A). In addition to H_2O_2, higher contents of MDA in the transgenic plants than in WT plants were also found under both control and stressed conditions (Fig. 9B).

4. Discussion

HD-Zip is a plant-specific protein family that has been divided into four sub-groups (I–IV) according to their conservation of sequence, structural features and functions (Ariel et al., 2007; Harris et al., 2011). Several studies have demonstrated that HD-Zip II proteins are involved in the regulation of developmental processes and flowering response (Schena et al., 1993; Steindler et al., 1999; Sorin et al., 2009; Rueda et al., 2005; Dezar et al., 2011). There has been little information on the role of HD-Zip II proteins in the response of plants to abiotic stress. In the present study, we indentified a novel HD-Zip gene (*MtHB2*) from the legume model plant *M. truncatula* and functionally characterized the gene in the response to cold, drought and salt stress by generating *Arabidopsis* plants expressing *MtHB2*. One important finding in the present study is that expression of *MtHB2* in *Arabidopsis* rendered the transgenic plants more sensitive to salt, cold and drought stress, suggesting

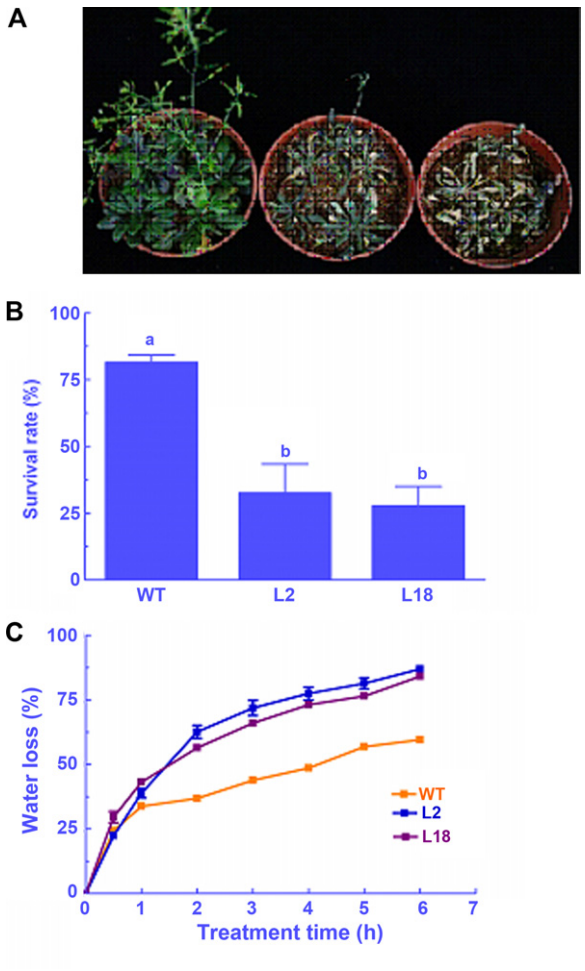

Fig. 7. Comparison of tolerance of *MtHB2*-overexpressing transgenic *Arabidopsis* and wild-type plants to drought stress. Survival rates of WT and transgenic lines (L2 and L18) after withholding water for 15 days and recovering for 7 days (A, B). Survival rate data are mean ± SE with approximately 15 seedlings for each treatment with three replicates. Water loss rates for WT, L2 and L18 were determined at 0, 0.5, 1, 2, 3, 4, 5 and 6 h after drought treatment (C). Data are mean ± SE with three replicates. Different letters shown in the error bars mean significant differences among control and treatments at $P < 0.05$.

that *MtBH2* is likely to negatively regulate the response mechanism to abiotic stress. Previous studies reported that HD-Zip II proteins function as negative regulators to modulate physiological processes in plants (Ohgishi et al., 2001; Ciarbelli et al., 2008). Therefore, the observed phenotypes of *Arabidopsis* seedlings expressing *MtHB2* may result from suppression of the HD-Zip proteins in *Arabidopsis*. To test this possibility, we compared the expression of these genes (*ATHB2*:*ATHB4*, *HAT1*, *2*, *3*, *9*, *14*, *17* and *22*) in the leaves of transgenic lines with that in WT plants. Expression of the 9 genes was not found to be suppressed in the transgenic lines expressing *MtHB2* compared to those in WT plants (Supplemental Fig. 1). Therefore, the altered sensitivity of the transgenic plants expressing *MtHB2* is unlikely to be accounted for by the suppression of expression of genes encoding HD-Zip II proteins in *Arabidopsis*.

We further explored the physiological mechanism underlying the reduced tolerance of *Arabidopsis* seedlings expressing *MtHB2*. Our results revealed that expressing *MtHB2* led to disruption of synthesis of Pro and soluble sugars and increases in accumulation of ROS and MDA, thus rendering the transgenic plants expressing *MtHB2* less effective in osmo-regulation and more susceptible to oxidative damage under stressed conditions. Rapid up-regulation of *MtHB2* expression by cold, drought and salt stress implies that

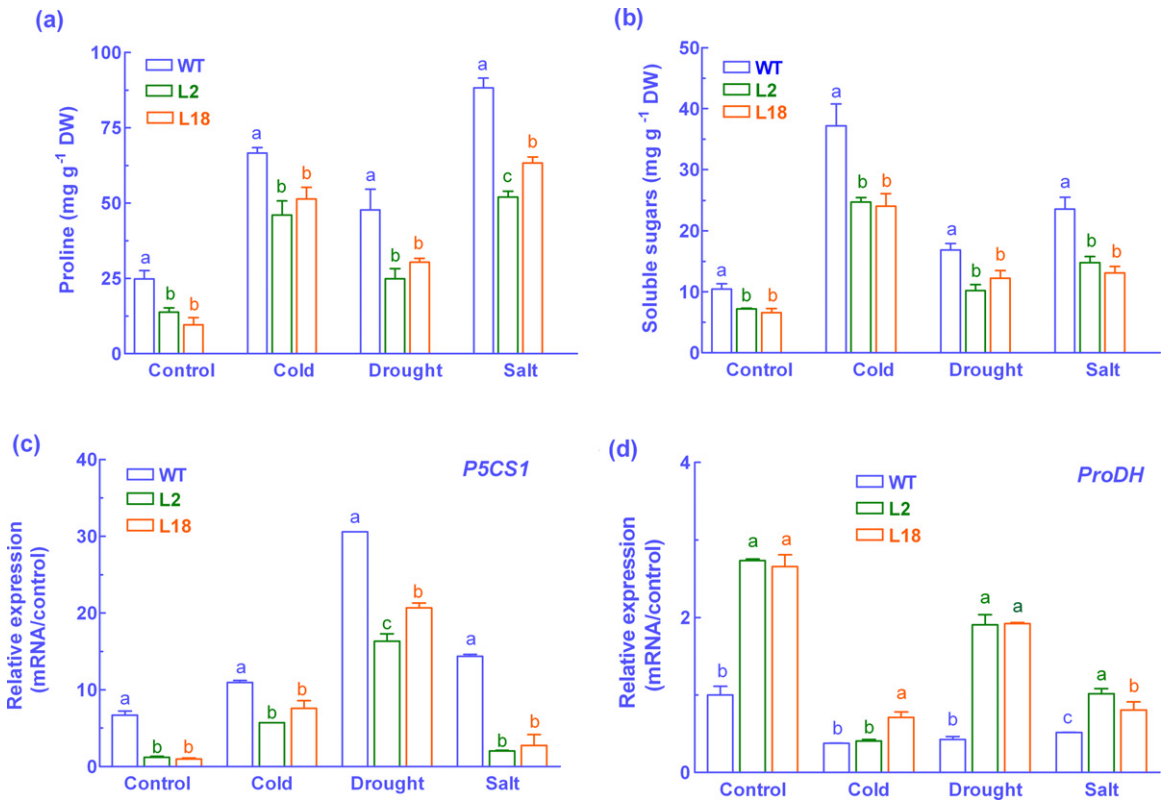

Fig. 8. Effect of cold (4 °C for 5 days), drought (withholding water for 10 days) and NaCl (200 mM for 10 days) on Pro and sugar contents of WT and two transgenic lines (L2 and L18) *Arabidopsis* seedlings (A, B) and the expression levels of genes encoding proline synthetase (*P5CS1*) (C) and dehydrogenase (*ProDH*) (D). Data are mean ± SE with three replicates. Different letters shown in the error bars mean significant differences among control and treatments at $P < 0.05$.

MtHB2 may be involved in response to these stresses. Plants have evolved various mechanisms to adapt to abiotic stress such as cold, drought and salinity. Accumulation of free Pro and soluble sugars to facilitate osmo-regulation under abiotic stress has been widely observed in plants (Liu and Zhu, 1997; Gilmour et al., 2000). The accumulated Pro can also act as a molecular chaperone to stabilize the structure of proteins and play a significant role in the antioxidant system (Székely et al., 2008). In the present study, we found that expressing *MtHB2* disrupted biosynthesis of Pro and soluble sugars under both control and stressed conditions. This may account for the reduced accumulation of Pro and soluble sugars in the transgenic plants than WT plants (Fig. 8). The lower contents of Pro and soluble sugars in the transgenic plants would account for the lower osmolality in the transgenic plants than in WT plants (Table 1). The lower osmolality in turn would contribute to a higher water potential in the transgenic plants than WT plants, thus making them less effective at retaining water, particularly under drought, salt and cold stress. Water loss rate is an important parameter to reflect plant water status and has been used as an indicator of drought tolerance (Clarke et al., 1989; Dhanda and Sethi, 1998). In the present study, we found that the transgenic plants showed higher water loss rate than WT plants (cf. Fig. 7C). This result may be explained by the lower osmolality due to reduced accumulation of Pro and soluble sugars.

Several reports demonstrated that overexpression of some transcriptional factors leads to enhanced accumulation of Pro due to up-regulation of Pro synthase (Kishor et al., 1995; Xiang et al., 2008), thus conferring tolerance to abiotic stress. To elucidate

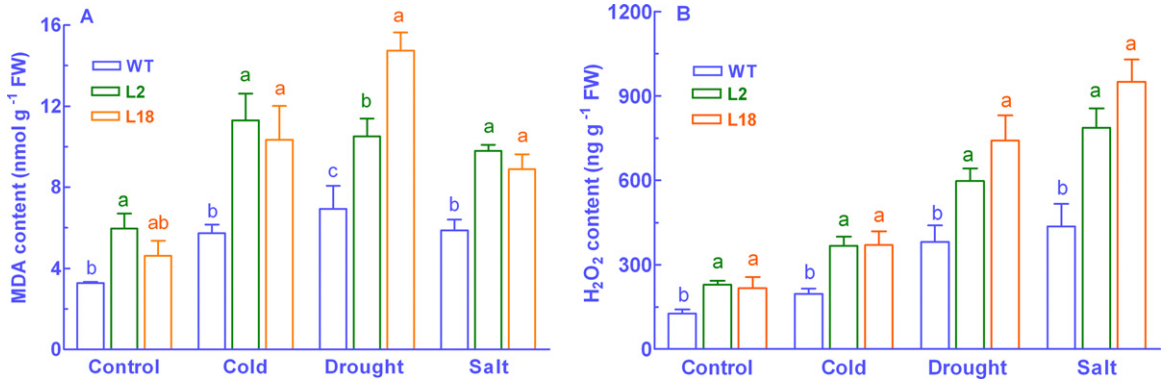

Fig. 9. Comparison of H_2O_2 and MDA contents between *Arabidopsis* wild-type and transgenic lines (L2 and L18) grown in control and stressed conditions. Cold stress was achieved by exposing WT and transgenic plants to 4 °C for 5 days; drought stress by withholding water for 10 days and salt stress by treating WT and transgenic plants with 200 mM NaCl for 10 days). Data are mean ± SE with three replicates. Different letters shown in the error bars mean significant differences among control and treatments at $P < 0.05$.

the molecular mechanisms underlying the enhanced sensitivity of transgenic plants to abiotic stress, we monitored the expression levels of Pro synthase (*P5CS*) and Pro dehydrogenase (*ProDH*) genes. Our results revealed that the transcript of *P5CS*1 in transgenic plants was lower than that in WT plants, while *ProDH* transcript was much high than in WT plants (Fig. 8B). These results may indicate that the expression of *MtHB2* impairs Pro synthesis and promotes Pro degradation, thus resulting in less accumulation of Pro in transgenic plants. HD-ZipII proteins are able to bind to the pseudopalindromic sequence CAAT N ATTG (Johannesson et al., 2001; Ariel et al., 2007). We analyzed the promoters of P5CS and ProDH and found that P5CS contained the core recognition motif, CAAT N ATTG in their 2 kb promoter regions. Further analyses revealed that there were 3 and 2 AAT N ATT motifs in P5CS1 (−495 to −502, −543 to −550 and −998 to −1005) and P5CS2 (−359 to −366 and −616 to −623) promoters, respectively. Therefore, MtHB2 may bind to the recognition sequence in the P5CS promoters to inhibit the expression of P5CS. In contrast, no AAT N ATT motifs were found in the 2 kb promoter region of ProDH. These findings suggest that P5CS1 and P5CS2, but not ProDH, may be direct targets of MtHB2.

Plants suffering from abiotic stresses often display symptoms of oxidative damage as indicated by marked accumulation of reactive oxygen species such as H_2O_2 and malondialdehyde (MDA) (Chinta et al., 2001; Xiong et al., 2002). We found that the amounts of H_2O_2 and MDA in the transgenic plants were significantly greater than in WT plants when challenged by cold, drought and salt stress (Fig. 9), suggesting that the transgenic plants are more susceptible to oxidative stress under the abiotic stresses. Moreover, the increased sensitivity to oxidative stress of the transgenic plants than WT plants may also be related to their impaired capacity to synthesize Pro as Pro can act as an antioxidant to counteract the oxidative stress (Székely et al., 2008).

Dong et al. (2006) reported that a cold-induced gene, *HOS1*, negatively regulates freezing tolerance by repressing the expression of CBFs and their downstream genes. Moreover, previous reports showed that some HD-Zip transcription factors play a negative role in mediation of responses to abiotic stress (Dezar et al., 2011). In the present study, we found that the expression of *MtHB2* in *Arabidopsis* plants rendered them more sensitive to cold, drought and salt stress, suggesting that *MtHB2* plays a negative role in regulation of abiotic stress response mechanisms, probably in a spatially specific manner. Further work to elucidate the regulatory mechanisms underlying the reduced tolerance of transgenic plants expressing *MtHB2* is warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2012.02.001.

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