Tree Physiology 36, 193–207
doi:10.1093/treephys/tpv139

Research paper

A *bHLH* gene from *Tamarix hispida* improves abiotic stress tolerance by enhancing osmotic potential and decreasing reactive oxygen species accumulation

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Received August 23, 2015; accepted December 3, 2015; published online January 19, 2016; handling Editor Chung-Jui Tsai

Basic helix-loop-helix (bHLH) leucine-zipper transcription factors play important roles in abiotic stress responses. However, their specific roles in abiotic stress tolerance are not fully known. Here, we functionally characterized a *bHLH* gene, *ThbHLH1*, from *Tamarix hispida* in abiotic stress tolerance. ThbHLH1 specifically binds to G-box motif with the sequence of 'CACGTG'. Transiently transfected *T. hispida* plantlets with transiently overexpressed *ThbHLH1* and RNAi-silenced *ThbHLH1* were generated for gain- and loss-of-function analysis. Transgenic *Arabidopsis thaliana* lines overexpressing *ThbHLH1* were generated to confirm the gain- and loss-of-function analysis. Overexpression of *ThbHLH1* significantly elevates glycine betaine and proline levels, increases Ca^{2+} concentration and enhances peroxidase (POD) and superoxide dismutase (SOD) activities to decrease reactive oxygen species (ROS) accumulation. Additionally, ThbHLH1 regulates the expression of the genes including *P5CS*, *BADH*, *CaM*, *POD* and *SOD*, to activate the above physiological changes, and also induces the expression of stress tolerance-related genes *LEAs* and *HSPs*. These data suggest that ThbHLH1 induces the expression of stress tolerance-related genes to improve abiotic stress tolerance by increasing osmotic potential, improving ROS scavenging capability and enhancing second messenger in stress signaling cascades.

Keywords: basic helix-loop-helix leucine-zipper, gene expression regulation, transient transformation, yeast one hybrid.

Introduction

The basic helix-loop-helix (bHLH) proteins are widely distributed in eukaryotes and are one of the largest transcription factor (TF) families. The bHLHs are characterized by the bHLH signature domain (Peng et al. 2013), which contains two functionally distinct regions: the N-terminal basic region that contains 13–17 primarily basic amino acids, which functions as a DNA-binding domain, and a C-terminal helix-loop-helix region, which enables homo- and heterodimerization with one or several different partners required for TF function (Feller et al. 2011, Liu et al. 2014). The animal bHLHs have been classified into six groups (Groups A–F) according to phylogenetic analyses of the amino acid sequences (Ledent and Vervoort 2001). In plants, bHLHs comprise the second largest TF family. There are 167, 177 and 99 bHLHs, respectively, identified in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) (Heang and Sassa 2012). Most plant bHLHs have evolved from the ancestral group B class of bHLHs, according to the classification of animal bHLHs. Therefore, Heim et al. (2003) further classified the bHLHs of *Arabidopsis* into 12 subfamilies. Despite the vital roles of these bHLHs, the functions of most bHLHs are poorly understood. For instance, so far, only 19 of 177 bHLHs have been characterized or partially characterized in rice; in *Arabidopsis*, 64 out of 167 genes had been functionally determined or partially determined (Heang and Sassa 2012). This limited research has demonstrated that plant bHLHs serve as key regulatory components in transcriptional regulatory networks

controlling a broad range of growth and developmental signaling pathways and abiotic stress responses (Peng et al. 2013). For instance, bHLHs are involved in gynoecium development (Heisler et al. 2001), light signaling (Leivar et al. 2012), brassinosteroid and abscisic acid (ABA) signaling (Zhang et al. 2009), flavonoid biosynthesis (Baudry et al. 2006), flowering time control (Ito et al. 2012), trichome and root hair differentiation (Zhao et al. 2012), floral organogenesis, nodule vascular patterning and axillary meristem formation (Reymond et al. 2012, Yang et al. 2012). Increasing evidence suggests that bHLHs play important roles in plants in response to abiotic stress. For example, Nakata et al. (2013) showed that a bHLH protein (JAM1) could negatively regulate jasmonic acid (JA) signaling, and plays a pivotal role in fine-tuning of JA-mediated plant stress responses. OsbHLH148 functions as an initial response of JA-regulated gene expression involved in drought tolerance (Seo et al. 2011). Some plant bHLHs are involved in iron homeostasis (Yuan et al. 2008) or serve as key regulators of iron-deficiency responses in plants (Sivitz et al. 2012). Moreover, plants overexpressing bHLHs display enhanced tolerance to salt, drought, freezing and oxidative stress, demonstrating that bHLHs play pivotal roles in mediating abiotic stress responses (Xie et al. 2012, Babitha et al. 2013, Huang et al. 2013, Liu et al. 2014). Despite intensive research efforts, the biological roles of most plant bHLHs remain poorly understood (Liu et al. 2014).

The genus *Tamarix* (tamarisk, salt cedar) is woody halophyte, growing as shrubs or small trees, and is widely distributed in the saline soils of drought-stricken areas of Central Asia (Kalir and Poljakoff-Mayber 1976, Sher and Marshall 2003). *Tamarix hispida*, a species of genus *Tamarix*, is highly tolerant to salinity and drought (Pan et al. 2011), indicating that it has efficient abiotic stress defense systems and is suitable for abiotic stress tolerance analysis.

Previously, we had constructed eight transcriptomes from *T. hispida* after $NaHCO_3$ treatment for 0, 12, 24 and 48 h, and identified 15 *bHLHs* that are differentially regulated by $NaHCO_3$ treatment (Wang et al. 2014). We further identified a *bHLH* gene, *ThbHLH1*, which is highly induced by salt, and selected it for further study. Our studies showed that ThbHLH1 modulates abiotic stress tolerance by regulating the expression of genes to activate a series of stress-related physiological changes, including osmotic potential, calcium ion in stress signaling cascades and reactive oxygen species (ROS) scavenging. This study might provide a new insight into the functions of the bHLH proteins during stress tolerance.

Materials and methods

Plant materials and growth conditions

Seeds of *T. hispida* were seeded into a pot containing a mixture of turf peat and sand (2 : 1 v/v), in a greenhouse under controlled conditions of 70–75% relative humidity, 14 h light/10 h darkness photocycle and 24 °C. Well-watered 2-month-old seedlings were watered on their roots with a solution of 400 mM NaCl, 100 μM ABA, 20% (w/v) PEG6000 or 50 μM methyl viologen (MV), after watering for 3, 6, 9, 12 and 24 h for studies of *ThbHLH1* gene expression. Seedlings watered with fresh water were harvested at the corresponding time points as controls. *Arabidopsis* plants grew in pots containing a mixture of perlite/soil (2 : 1 v/v) in a greenhouse (70–75% relative humidity; 16 h light/8 h darkness photocycle; 22 °C).

Cloning of the ThbHLH1 gene and plant transformation

The full-length cDNA sequence of *ThbHLH1* (GenBank number: KM101094) was cloned based on the transcriptomes of *T. hispida* (Wang et al. 2014). The coding region (CDS) of *ThbHLH1* was cloned into pROKII under the control of the 35S promoter (35S::bHLH). An inverted repeat cDNA fragment of *ThbHLH1* was inserted into RNAi vector pFGC5941 (pFGC::bHLH) to silence the expression of *ThbHLH1*. All the primers used are shown in Table S1 available as Supplementary Data at *Tree Physiology* Online. Transient transformation of whole *T. hispida* plantlets, as well as the salt and drought treatments, was performed using a method for transient transformation of whole plantlets (Ji et al. 2014). The 6-week-old plantlets of *T. hispida* with similar size were used for transformation. Three kinds of transiently transfected *T. hispida* plantlets were generated, i.e., plants transformed with 35S::bHLH for overexpressing *ThbHLH1* (OE), plants transformed with pFGC::bHLH for RNAi-silenced *ThbHLH1* (IE) and control (transformed with empty pROKII vector). The *ThbHLH1* overexpression construct was transformed into *Arabidopsis* using the *Agrobacterium*-mediated floral dip transformation method to generate transgenic *Arabidopsis* OE lines.

Subcellular location analysis

The CDS of *ThbHLH1*, without the termination codon, was fused to the N-terminus of the green fluorescent (*GFP*) gene (Clontech, Palo Alto, CA, USA) under the control of *ThbHLH1* promoter, to generate the ProThbHLH1::ThbHLH1-GFP construct. Transient transformation of ProThbHLH1::ThbHLH1-GFP and 35S::GFP into whole *T. hispida* plantlets was performed according to Ji et al. (2014). After transformation for 48 h, the transiently transformed plantlets were treated with NaCl (150 mM), ABA (20 μM) or mannitol (250 mM) for 3 h and their roots were visualized using confocal laser scanning microscopy LSM700 microscope (Zeiss, Jena, Germany). The plasmid encoding the 35S::ThbHLH1-GFP and 35S::GFP was introduced into onion epidermal cells by particle bombardment (Bio-Rad, Hercules, CA, USA). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (10 μg ml^{-1}) in phosphate-buffered saline for 3 min. The transformed cells were analyzed using confocal laser scanning microscopy on an LSM700 microscope.

Stress tolerance analysis

Transgenic *Arabidopsis* plants of T_3 generation harboring the *ThbHLH1* gene were used in stress tolerance tests. The *Arabidopsis*

seeds were sown on 1/2 MS medium, or 1/2 MS containing 125 mM NaCl or 150 mM mannitol for 1 week at 25 °C, and the germination rates of each transgenic plant or Col-0 plant were measured. The seeds were sown on 1/2 MS medium for 3 days, and were transferred into 1/2 MS medium or 1/2 MS medium plus 150 mM NaCl or 175 mM mannitol for 2 weeks for root length and fresh weight measurement. The chlorophyll contents of detached leaves were measured as described by Lichtenthaler (1987).

Physiological analysis

The 6-week-old *T. hispida* plantlets grown at 1/2 MS medium or 1/2 MS containing 150 mM NaCl or 250 mM mannitol for 48 h were used for physiological analysis. The 3-week-old *Arabidopsis* plants grown in soil were watered with a solution of 150 mM NaCl or 250 mM mannitol for 48 h, and were harvested for physiological analysis. The superoxide dismutase (SOD) and peroxidase (POD) activities and malondialdehyde (MDA) level measurements were performed as in Wang et al. (2010), electrolyte leakage assay was performed as in Ji et al. (2014) and H_2O_2 level measurement was performed as in Dal Santo et al. (2012). Proline content was measured according to Bates et al. (1973). Betaine content was measured according to Rajashekar et al. (1999).

The seedlings were incubated in solution of NaCl (150 mM) or mannitol (250 mM) for 0.5, 1 and 2 h. Nitroblue tetrazolium (NBT), diaminobenzidine (DAB) and Evans blue staining for *T. hispida* and *Arabidopsis* plants were performed as in Fryer et al. (2002) and Zhang et al. (2011).

Reactive oxygen species levels in root tips and intact guard cells were monitored using 2,7-dichlorofluorescin diacetate (H_2DCF-DA) (Invitrogen, CA, USA), as described by Pei et al. (2000). For propidium iodide (PI) (Beyotime, Shanghai, China) staining, the seedlings were incubated in solution of NaCl (150 mM) or mannitol (250 mM) for 30 min, and then incubated with PI solution (50 μg ml^{-1}) for 30 min, rinsed with sterile water and visualized on a confocal laser microscope with excitation at 488 nm and emission at 516 nm. Transpirational water loss was determined according to Zhang et al. (2011).

Analysis of intracellular calcium ion (Ca^{2+}) levels in root tips was monitored using Fluo-3-acetoxymethyl (AM) ester staining (Beyotime). The *T. hispida* or *Arabidopsis* seedlings were incubated in a solution of NaCl (150 mM) or mannitol (250 mM) for 2 h, then incubated with Fluo-3-AM solution (5 μM) for 40 min at 37 °C in the dark, and were washed with sterile water three times. Fluo-3 was excited by argon laser light at 488 nm and fluorescence was measured at 515 nm.

Real-time reverse transcription-polymerase chain reaction analysis

For expression analyses in *T. hispida*, *Actin* (GenBank number: FJ618517) and *β-tubulin* (GenBank number: FJ618519) were used as internal references (see Table S2 available as Supplementary Data at *Tree Physiology* Online). For expression analyses in *Arabidopsis*, *actin3* (AT3G53750) and *α-tubulin* (AT1G50010) were used as internal references (see Table S2 available as Supplementary Data at *Tree Physiology* Online). cDNA was produced by reverse transcription of total RNA from different tissues and plant species as indicated (Takra, Dalian, China). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with an Opticon 2 System (Bio-Rad). The reaction mixture contained 10 μl of SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 0.5 μM each of forward and reverse primers and 2 μl of cDNA template (equivalent to 50 ng of total RNA) in a volume of 20 μl. The PCR procedure was as follows: 94 °C for 30 s, followed by 45 cycles at 94 °C for 12 s, 60 °C for 30 s, 72 °C for 40 s and 1 s at 82 °C for plate reading. A melting curve was generated to assess the purity of the amplified products. Three biological replicates were performed, and relative expression levels were calculated according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Determination of the binding of G-box to ThbHLH1

Three tandem copies of G-box and its mutants were cloned into pHIS2 as reporter construct, and the CDS of ThbHLH1 was cloned into pGADT7-Rec2 as effector (Clontech). The primers used are shown in Table S3 available as Supplementary Data at *Tree Physiology* Online. The binding of G-box and its mutants to ThbHLH1 was determined by Y1H assay. To study their interaction in plants, three tandem copies of G-box and its mutants were fused to the minimal 35S promoter to drive a *β-glucuronidase (GUS)* gene in a reformed pCAMBIA1301 (in which the region of 35S::Hygromycin was deleted and a 46 bp minimal promoter replaced the 35S promoter in the region between HindIII and *β-glucuronidase (GUS)* as reporters, and the primers used are shown in Table S4 available as Supplementary Data at *Tree Physiology* Online. Each reporter and effector (35S::bHLH) vector were cotransformed into *Arabidopsis* Col-0 plants using the transient transfection method according to Ji et al. (2014); the transformed plants were treated with salt and drought or under normal growth conditions for 3 h, and the fluorometric GUS activity was determined (Jefferson et al. 1987, Jefferson 1989). To normalize the efficiencies of transformation, the *luciferase* (*Luc*) gene under the control of 35S promoter (35S::Luc) was also cotransformed and measured by a luminescence assay. Three independent experiments were performed.

Statistical analyses

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software. Data were compared using Student's *t*-test. Differences were considered to be significant if $P < 0.05$. * $P < 0.05$.

Results

Cloning the ThbHLH1 gene

To select the *bHLH* genes from *T. hispida* that are potentially involved in abiotic stress, 15 *bHLH* genes that were differentially

regulated by $NaHCO_3$ were identified from the transcriptome of *T. hispida* (Wang et al. 2014), and their expression in response to salt stress was studied using real-time PCR. All the primers used are shown in Table S5 available as Supplementary Data at *Tree Physiology* Online. The results showed that the expression of ThbHLH1 is highly induced by salt stress (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), suggesting that it might be involved in abiotic stress tolerance, and was selected for further study. The open reading frame of *ThbHLH1* is 924 bp in length and encodes a protein of 308 amino acids. ThbHLH1 and 149 bHLH proteins from *Arabidopsis* were aligned based on their amino acid sequences for phylogenetic analysis. The phylogenetic tree suggested that ThbHLH1 shares most sequence similarities with two uncharacterized *Arabidopsis* genes, *AtbHLH007* (AT1G03040) and *AtbHLH059* (AT4G02590) (see Figure S2 available as Supplementary Data at *Tree Physiology* Online), indicating that ThbHLH1 should belong to subfamily XI, as do AT1G03040 and AT4G02590.

ThbHLH1 is a nuclear protein that is induced by abiotic stresses and ABA stimuli

The ProThbHLH1::ThbHLH1-GFP construct was transiently transformed into whole 2-month-old *T. hispida* plantlets (Ji et al. 2014). *Tamarix hispida* plantlets transformed with 35S::GFP were used as the control. Green fluorescent signals in *T. hispida* plantlets transformed with ProThbHLH1::ThbHLH1-GFP were observed in the nucleus under normal conditions (Figure 1A), and the accumulation of ThbHLH1-GFP in nucleus increases with the addition of salt, ABA and mannitol in root tips after stress for 3 h (Figure 1A). To further confirm the nucleus localization of ThbHLH1, the plasmid encoding the 35S::ThbHLH1-GFP and 35S::GFP control was introduced into onion epidermal cells. The GFP signals of ThbHLH1-GFP were localized to the nucleus of onion epidermal cells, whereas GFP was found to be uniformly distributed throughout the cells (Figure 1B). These results indicated that ThbHLH1 is a nuclear-localized protein whose expression can be induced by ABA stimuli, salt and osmotic stress.

The expression of ThbHLH1 in T. hispida is responsive to abiotic stress and ABA stimulus

The 2-month-old *T. hispida* plantlets were used for studies of *ThbHLH1* gene expression. *ThbHLH1* was significantly induced by treatments with NaCl, ABA, PEG6000 or MV (Figure 2). In addition, the induced expression of *ThbHLH1* by NaCl, ABA, PEG6000 or MV is also consistent with the subcellular location analysis (Figure 2), indicating that the expression of *ThbHLH1* is induced by abiotic stress, and ThbHLH1 protein is accumulated in the nucleus.

Generation of transgenic Arabidopsis plants overexpressing ThbHLH1

We generated the *Arabidopsis* plants overexpressing *ThbHLH1*, and the T_3 homozygous lines were used for analysis. Ten independent T_3 homozygous *Arabidopsis* lines overexpressing *ThbHLH1* were generated. Reverse transcription-PCR confirmed that *ThbHLH1* was expressed in the transgenic plants (see Figure S3 available as Supplementary Data at *Tree Physiology* Online). Two independent *ThbHLH1* transgenic lines (OE1 and OE3) were randomly selected for further study.

Constitutive expression of ThbHLH1 in Arabidopsis enhances salt tolerance

There was no difference in seed germination between the transformed and Col-0 wild-type plants under normal growth conditions. However, the plants overexpressing ThbHLH1 exhibit an increase in seed germination rate under NaCl (125 mM) or mannitol (150 mM) stress compared with Col-0 plants (Figure 3a and b). Additionally, the chlorophyll contents of transgenic and Col-0 plants were similar before abiotic stress treatments; however, the two transgenic lines had significantly higher chlorophyll levels than the Col-0 under stress conditions (Figure 3c). Under normal growth conditions, there was no difference in growth and phenotype between Col-0 and the transgenic lines (Figure 3d–f). The transgenic lines showed significantly improved root growth and fresh weight gain under NaCl or mannitol stress compared with Col-0 plants (Figure 3d–f). Stress tolerance assay of plants grown in soil also showed that there was no difference in both growth phenotypes and fresh weights between transgenic and Col-0 plants under normal conditions, indicating that overexpression of *ThbHLH1* do not influence growth phenotype. However, both *ThbHLH1*-transformed lines grew better and displayed significantly enhanced fresh weight gain than the Col-0 plants under stress conditions (Figure 3f and g). These results suggested that overexpression of *ThbHLH1* significantly improves the tolerance to salt and drought stress.

Generation of transiently transfected T. hispida plantlets with transient overexpression or knockdown of ThbHLH1

To investigate the expression of *ThbHLH1* in plants overexpressing ThbHLH1 (OE), in RNAi-silenced *ThbHLH1* (IE) plants and in the control *T. hispida* plantlets (transformed with empty pROKII), real-time RT-PCR was performed. The expression level of *ThbHLH1* in the control plants after transformation for 48 h was used as the calibrator (designated as 1) to normalize the expression of *ThbHLH1*. The results showed that compared with the control plants, the transcripts of *ThbHLH1* were significantly increased in the OE plants; meanwhile, the transcripts of *ThbHLH1* were significantly decreased in the IE plants (Figure 4). These results indicated that the transcripts of *ThbHLH1* were significantly increased and decreased in the OE and IE plants, and are therefore suitable for gain- and loss-of-function studies.

ThbHLH1 positively regulates the biosynthesis of proline and glycine betaine

The levels of proline and glycine betaine were compared among the control, OE and IE transiently transfected *T. hispida* plantlets. There was no significant difference in proline or glycine betaine contents among the three kinds of transiently transfected plants under normal growth conditions; however, upon salt or osmotic treatment, both the proline and glycine betaine contents were significantly different among the three kinds of transfected plants. The OE plants had the highest proline and glycine betaine contents, followed by the control plants, and the IE plants showed the lowest proline and betaine contents (Figure 5a and b). We further examined the expression of genes related to the biosynthesis of proline or glycine betaine, including two delta 1-pyrroline-5-carboxylate synthetase (*P5CS*) genes (*ThP5CS1* and *ThP5CS2*, GenBank numbers: KM101096 and KM101097) and two betaine aldehyde dehydrogenase (*BADH*/*ALDH*, belonging to the aldehyde dehydrogenase family) genes (*ThBADH1* and *ThBADH2*, GenBank numbers: KM101098 and KM101099). Under salt or osmotic treatment conditions, the expressions of

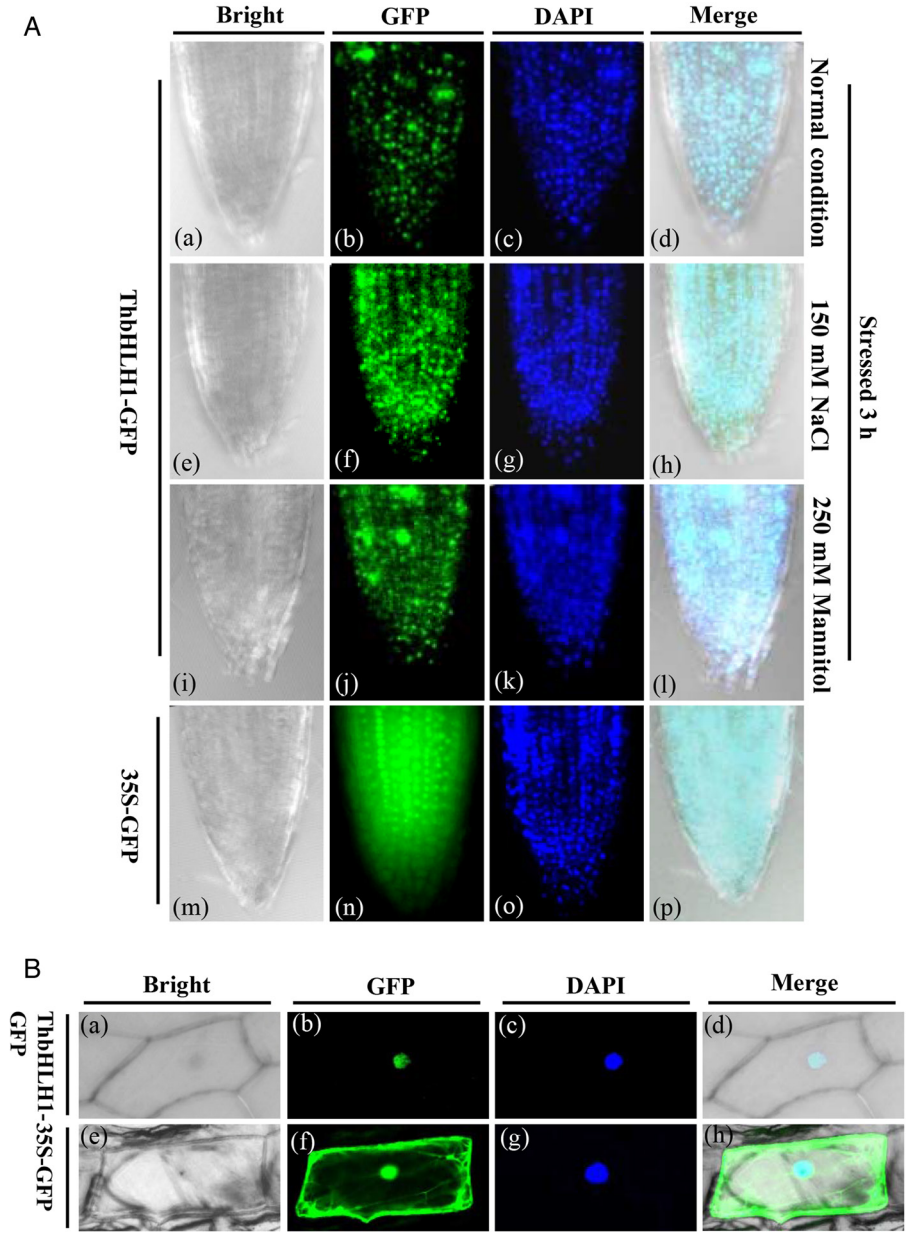

Figure 1. Subcellular localization of the ThbHLH1 protein. (A) Subcellular localization of ThbHLH1 protein in roots of *T. hispida*. The construct ProThbHLH1::ThbHLH1-GFP or 35S::GFP (as a control) was transiently transformed into *T. hispida*. The transformed plants were grown under normal conditions, or were treated with 150 mM NaCl, 20 μM ABA or 250 mM mannitol for 3 h, and their roots were visualized. (B) Subcellular localization of ThbHLH1 in onion epidermal cells. The construct for 35S::ThbHLH1-GFP and 35S::GFP plasmid was introduced into onion epidermal cells by particle bombardment. The nuclei of the onion cells were visualized by DAPI staining.

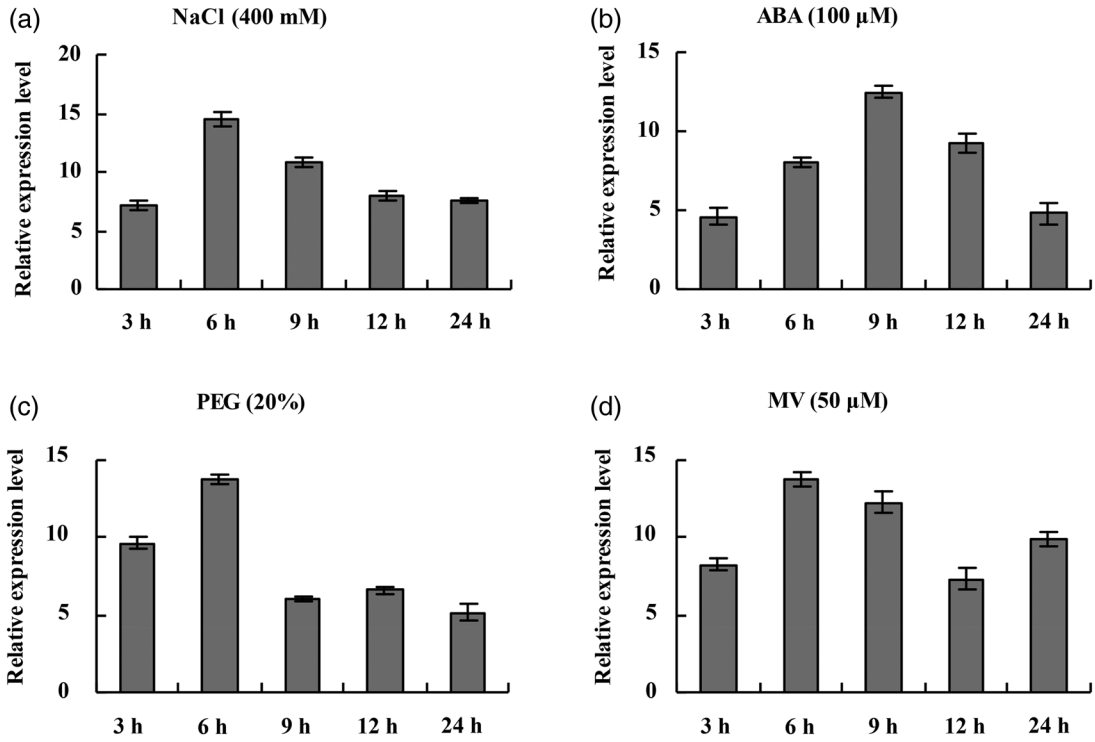

Figure 2. Expression patterns of ThbHLH1 in whole *T. hispida* are responsive to abiotic stress and ABA stimulus. (a–d) The expression patterns of *ThbHLH1* in whole 2-month-old *T. hispida* plantlets are responsive to treatment with 400 mM NaCl (a), 100 μM ABA (b), 20% PEG6000 (c) or 50 μM MV (d). The expression level of *ThbHLH1* under normal growth conditions was designed as 1 to normalize the expression *ThbHLH1* under other conditions.

all the *P5CS* and *BADH* genes were significantly highest in the OE plants, followed by in the control plants, and lastly in the IE plants (Figure 5c–f).

To confirm the physiological roles of *ThbHLH1* studied from *T. hispida* plantlets, we further performed the above physiological analyses on *Arabidopsis* plants stably transformed with *ThbHLH1* and Col-0 plants. There was no significant difference in both proline and glycine betaine contents among the wild-type and OE lines under normal conditions. However, upon salt or osmotic treatment, both proline and glycine betaine contents in two OE lines were significantly higher than those in Col-0 plants (see Figure S4 available as Supplementary Data at *Tree Physiology* Online). We further examined the expression of proline and glycine betaine biosynthesis genes, including *AtP5CS1* (AT2G39800), *AtP5CS2* (AT3G55610), *AtALDH3* (AT4G34240) (Kirch et al. 2001) and *AtALDH10A8* (AT1G74920). Under salt or osmotic conditions, the expressions of *AtP5CS1*, *AtP5CS2* (see Figure S4C and D available as Supplementary Data at *Tree Physiology* Online), *AtALDH3* and *AtALDH10A8* (see Figure S4E and F available as Supplementary Data at *Tree Physiology* Online) in two OE lines were both significantly higher than that in Col-0 plants. These results are consistent with those from *T. hispida*.

Overexpression of ThbHLH1 decreases cell death, water loss rate and MDA accumulation

Evans blue in situ staining on *T. hispida* plantlets indicated that, compared with the control plants, cell death greatly decreased in the OE plants, but greatly increased in the IE plants under salt and osmotic stress conditions (Figure 6a). An electrolyte leakage assay further confirmed these Evans blue staining results (Figure 6b). A water loss assay showed that the OE plants exhibited delayed water loss relative to the control, but IE plants displayed the highest water loss rate under dehydration conditions (Figure 6c). Moreover, under normal growth conditions, there was no difference in MDA levels among these three kinds of transiently transfected *T. hispida* plantlets. However, under salt and osmotic stress conditions, the IE plants displayed the highest MDA content, followed by control plants; the OE plants had the lowest MDA level (Figure 6d). These results indicated that the expression of *ThbHLH1* could reduce cell death, water loss rate and MDA accumulation under abiotic stress conditions.

Cell death in transgenic *Arabidopsis* plants overexpressing *ThbHLH1* was assessed by Evans blue and PI biochemical staining. Cell death was notably reduced after salt and osmotic treatments in OE plants when compared with the control Col-0 plants (see Figure S5a and b available as Supplementary Data at *Tree Physiology* Online). Meanwhile, both OE lines showed significantly decreased electrolyte leakage compared with Col-0 plants under salt and osmotic treatment conditions (see Figure S5C available as Supplementary Data at *Tree Physiology* Online). In addition, MDA content in the OE *Arabidopsis* plants was significantly decreased compared with Col-0 plants when exposed to salt and drought treatments (see Figure S5D available as Supplementary Data at *Tree Physiology* Online). The water loss rates

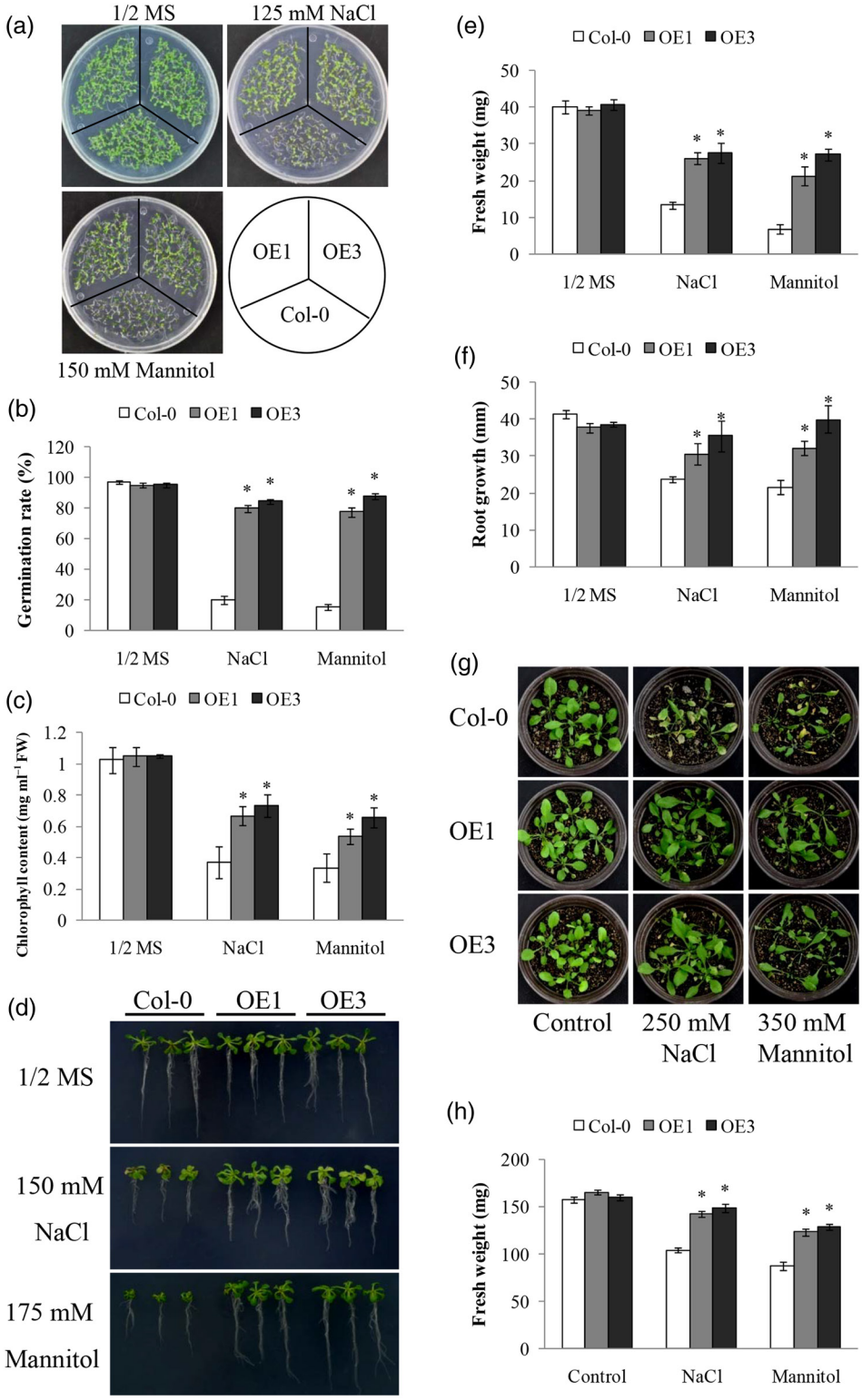

Figure 3. The stress tolerance of *Arabidopsis* plant overexpressing *ThbHLH1*. (a) Seed germination of *ThbHLH1*-transformed lines and Col-0 plants under normal conditions (1/2 MS), salt (NaCl) and drought (mannitol). (b) Seed germination rates assay. (c) Chlorophyll contents assay. (d) The growth phenotype of *ThbHLH1*-transformed and Col-0 plants. Analysis of fresh weight (e) and root length (f). The plants were grown in 1/2 MS medium (control) or 1/2 MS medium plus NaCl (salt) or mannitol (osmotic) for 2 weeks for above analyses. Growth phenotype (g) and fresh weight (h) of *Arabidopsis* plants. The 3-week-old plants in soil were treated with 250 mM NaCl or 350 mM mannitol for 10 days, and then their phenotypes were photographed. Control: under normal conditions. Asterisks ($P < 0.05$) indicate significant difference compared with Col-0 plants.

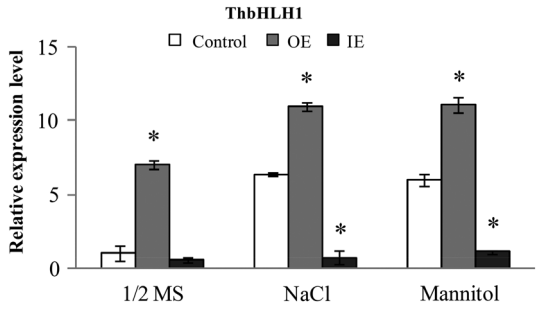

Figure 4. Expression of *ThbHLH1* in control, OE and IE *T. hispida* plantlets. The plants were grown in 1/2 MS medium (normal conditions) or 1/2 MS supplied with 150 mM NaCl or 250 mM mannitol for 48 h, and the expression of *ThbHLH1* was determined. The expression level of *ThbHLH1* in control plants under normal conditions was used as the calibrator (designed as 1) to normalize the expression of *ThbHLH1*. Control: *T. hispida* plantlets transformed with empty pROKII; OE: *T. hispida* overexpressing *ThbHLH1*; IE: *T. hispida* with RNAi-silenced *ThbHLH1*. Asterisks indicate significant ($P < 0.05$) difference from control plants.

in OE lines were significantly decreased compared with those in Col-0 plants (see Figure S5E available as Supplementary Data at *Tree Physiology* Online). These results were fully consistent with those from *T. hispida* plantlets.

ThbHLH1 regulates the Ca^{2+} concentration in response to abiotic stress

The concentration of free intracellular Ca^{2+} ions in transiently transfected *T. hispida* plantlets was assessed by Fluo-3-AM staining. Upon salt and osmotic treatment, the Ca^{2+} concentration in the roots of OE lines was the highest, followed by the control plants; the IE lines showed the lowest Ca^{2+} concentration (Figure 7a). The expressions of the *Calmodulin* (*CaM*) genes (*ThCaM1* and *ThCaM2*, GenBank numbers: KM101100 and KM101101) were further studied in the roots of *T. hispida*. The results showed that under stress conditions, the transcript levels of both *CaM* genes were highest in the OE plants, followed by in the control plants; the IE plants showed the lowest expression levels (Figure 7b and c).

The Ca^{2+} in the roots of *Arabidopsis* was also detected using Fluo-3-AM staining, and the Ca^{2+} concentration in both OE lines was substantially higher than that in Col-0 plants (see Figure S6A available as Supplementary Data at *Tree Physiology* Online). Meanwhile, the expression of the two *CaM* genes (AT2G41110 and AT1G66410) was also significantly increased in OE plants compared with Col-0 plants when exposed to NaCl and mannitol (see Figure S6B and C available as Supplementary Data at *Tree Physiology* Online). These results are consistent with those obtained in *T. hispida* (Figure 7).

Overexpression of ThbHLH1 improves the ROS scavenging ability

To study ROS accumulation, DAB and NBT in situ staining were performed, which can stain two prominent ROS species, H_2O_2 and O^{2-}, respectively. Both DAB and NBT staining on *T. hispida* plantlets showed that the OE plants had the lowest H_2O_2 and O^{2-} levels; however, the IE plants showed highest H_2O_2 and O^{2-} levels (see Figure S7A and B available as Supplementary Data at *Tree Physiology* Online). Consistent with DAB staining, measurement of H_2O_2 showed that H_2O_2 content in the IE plants was highest, followed by in the control plants; the OE plants had the lowest H_2O_2 content (see Figure S7C available as Supplementary Data at *Tree Physiology* Online).

As POD and SOD are the two main ROS scavenging enzymes, we further studied their activities on *T. hispida* plantlets. There were no significant differences in POD and SOD activity among the control, OE and IE plants under normal conditions (see Figure S7D and E available as Supplementary Data at *Tree Physiology* Online). However, under salt and osmotic stress conditions, OE plants showed the significant ($P > 0.05$) highest POD and SOD activities, followed by control plants; the IE plants had the significantly ($P > 0.05$) lowest POD and SOD activities (see Figure S7D and E available as Supplementary Data at *Tree Physiology* Online).

We further studied the expression of *POD* and *SOD* genes to determine whether the improved POD and SOD activities were reflected by enhanced expression of the *POD* and *SOD* genes. In total, three *POD* (*ThPOD1–3*, GenBank numbers: KF756934–KF756936) and three *SOD* (*ThSOD1–3*, GenBank numbers: KF756930–KF756932) genes that were studied previously (Wang et al. 2010) were included in this study. The results revealed that compared with the control plants, the expression levels of all the studied *POD* and *SOD* genes were significantly increased in the OE plants, but significantly decreased in the IE plants under salt and osmotic stress conditions (see Figure S8 available as Supplementary Data at *Tree Physiology* Online).

We further performed the above analyses on *Arabidopsis* plants. Biological staining in situ with DAB and NBT and the measurement of H_2O_2 level all confirmed that ROS levels were significantly reduced in the OE lines compared with Col-0 plants under salt and drought stress conditions (see Figure S9A, B and G available as Supplementary Data at *Tree Physiology* Online). The cellular level of ROS in guard cells and root tips was further examined using H_2DCF-DA fluorescence staining. Reactive oxygen species levels in the guard cells and the root tips of the OE lines were significantly lower than those in Col-0 plants (see Figure S9C and D available as Supplementary Data at *Tree Physiology* Online). These observations further indicated that *ThbHLH1* expression enables plant cells to decrease the amount of ROS to alleviate the damage caused by stress. Peroxidase and SOD activity analysis indicated that the both OE lines displayed significantly improved POD and SOD activities compared with Col-0 plants under salt and drought stress conditions (see Figure S9E and F available as Supplementary Data at *Tree Physiology* Online).

We examined the expression of *POD* and *SOD* genes in *Arabidopsis* plants, including three *Arabidopsis POD* genes (AT1G14550, AT2G18140 and AT5G58400) and three *SOD*

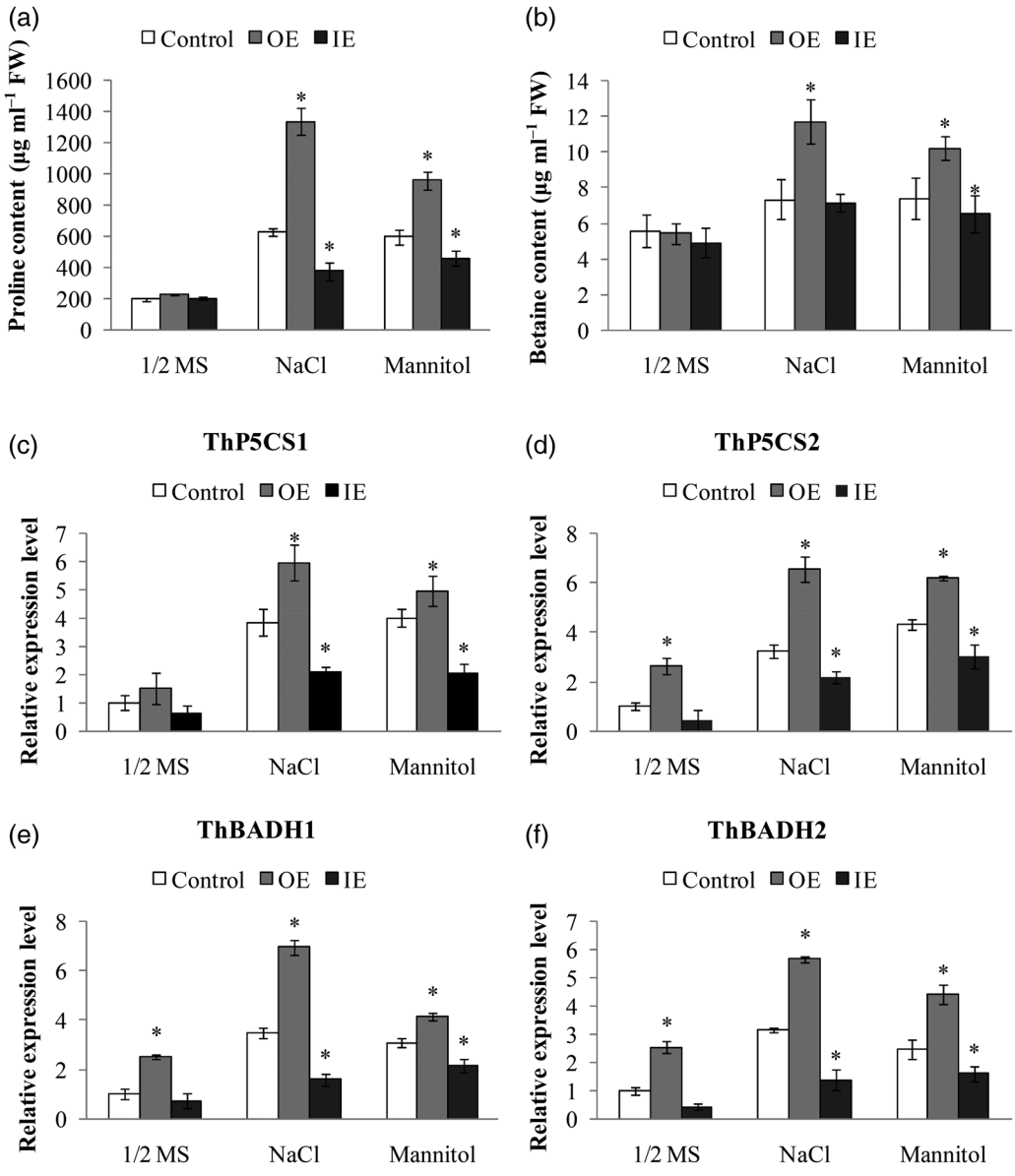

Figure 5. The levels of proline and glycine betaine in transiently transfected *T. hispida* plantlets. Analysis of the contents of proline (a) and glycine betaine (b). The expression of the genes involved in proline (c and d) and glycine betaine (e and f) biosynthesis. The plants were grown in 1/2 MS medium or 1/2 MS medium containing 150 mM NaCl or 250 mM mannitol for 48 h, and were analyzed. Asterisks indicate significant ($P < 0.05$) difference from control plants.

genes (AT1G08830, AT2G28190 and AT3G10920). The results revealed that compared with the Col-0 plants, the expression levels of all the studied *POD* and *SOD* genes were significantly increased in the OE lines under salt and osmotic stress conditions (see Figure S10 available as Supplementary Data at *Tree Physiology* Online). These results are consistent with the results from *T. hispida*, indicating that ThbHLH1 can decrease ROS accumulation induced by abiotic stresses.

The expression of genes involved in abiotic stress

The expressions of the late embryogenesis abundant protein (LEA, *ThLEA1* and *ThLEA2*, GenBank numbers: KM101102 and KM101103) and heat shock protein (HSP, *ThHSP1* and *ThHSP2*, GenBank numbers: KM101104 and KM101105) genes in the OE, control and IE *T. hispida* plantlets were studied (Figure 8). The results revealed that under salt and mannitol treatments, these genes showed significantly higher expression levels in OE *T. hispida* plantlets compared with the control; meanwhile, the expressions of these genes in IE plants were significantly lower than in control plants. These results showed that ThbHLH1 could induce the expressions of these *LEA* and *HSP* genes.

The binding affinity of ThbHLH1 to G-box is in response to NaCl and mannitol

Previous studies showed that bHLH proteins could bind to G-box motif with the sequence of 'CACGTG' (Toledo-Ortiz et al. 2003,

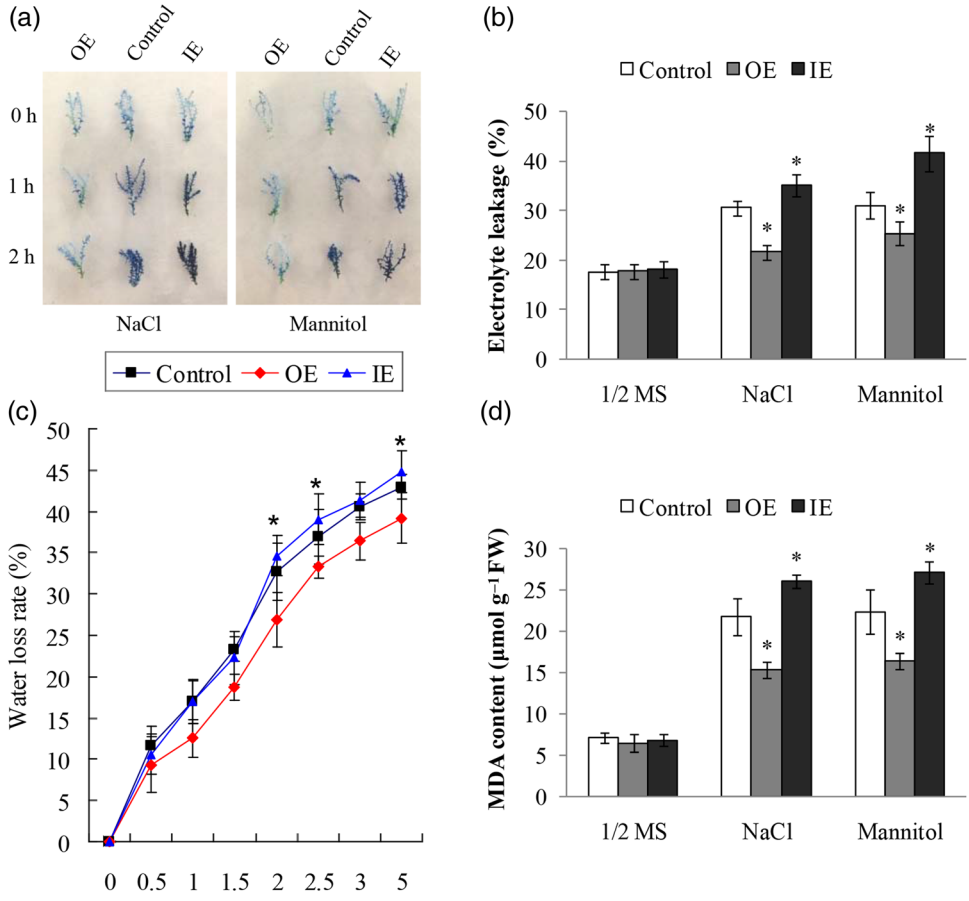

Figure 6. Cell death, MDA content and water loss rate analyses. (a) Evans blue staining of cell death. (b) Electrolyte leakage assay. (c) Water loss rate analysis. (d) MDA level analysis. The plants were grown in 1/2 MS medium or 1/2 MS medium containing 150 mM NaCl or 250 mM mannitol for 48 h, and used for analysis. Asterisks indicate significant ($P < 0.05$) difference from control plants.

Kong et al. 2012, Zhang et al. 2013). To study whether ThbHLH1 also can bind to G-box motif, the binding of ThbHLH1 to G-box and the mutated G-box motifs was studied using Y1H. The results showed that ThbHLH1 binds the G-box, but failed to bind to any of the mutated sequences (Figure 9a), indicating that the binding of ThbHLH1 to G-box is specific.

The effector construct (pROKII–ThbHLH1) was transiently transformed into *Arabidopsis* plants together with two G-box trimer reporter constructs, pCAM-GBOX or pCAM-GM7, where the latter contains a mutated G-box trimer, and GUS activities were determined (Figure 9b). Consistent with the Y1H results, under normal conditions, ThbHLH1 was able to transactivate the G-box reporter, but not the mutated G-box construct (Figure 9c), in line with ThbHLH1 specifically binding to the G-box motif. Additionally, under salt and drought stress condition, the transactivation of the G-box reporter by ThbHLH1 is significantly increased (Figure 9c). We further determined the ratio of the expression of *GUS* gene to that of *ThbHLH1* in coexpression of the effector and reporter system, and this ratio is significantly increased under NaCl or mannitol treatment conditions (Figure 9d). This result indicated that compared with ThbHLH1, the expression of *GUS* gene is significantly elevated when exposed to NaCl or mannitol. Therefore, increased GUS activity in response to salt and osmotic stress is not caused by increased ThbHLH1 transcription. Taken together, these data indicate that transactivation of target genes by ThbHLH1 is mediated by G-box elements, and that this G-box-mediated transactivation by ThbHLH1 increases when plants are exposed to abiotic stress.

Discussion

ThbHLH1 improves stress tolerance by osmotic adjustment

Compatible solutes, including glycine betaine and proline, are the two main solutes used by plants for osmotic adjustment. Glycine betaine can improve the abiotic stress tolerance by stabilizing complex proteins and enzyme quaternary structures, maintaining the highly ordered state of membranes, and reducing the efflux of K^+ ions and H_2O_2 accumulation (Park et al. 2006, Chen and Murata 2008). Proline acts both as a radical scavenger and as an osmotic agent to protect photosynthetic activity and cells from damage under abiotic stress, which can also maintain sustainable growth under long-term stress (Silva-Ortega et al. 2008, Kavi Kishor and Sreenivasulu 2014). Therefore, these two solutes

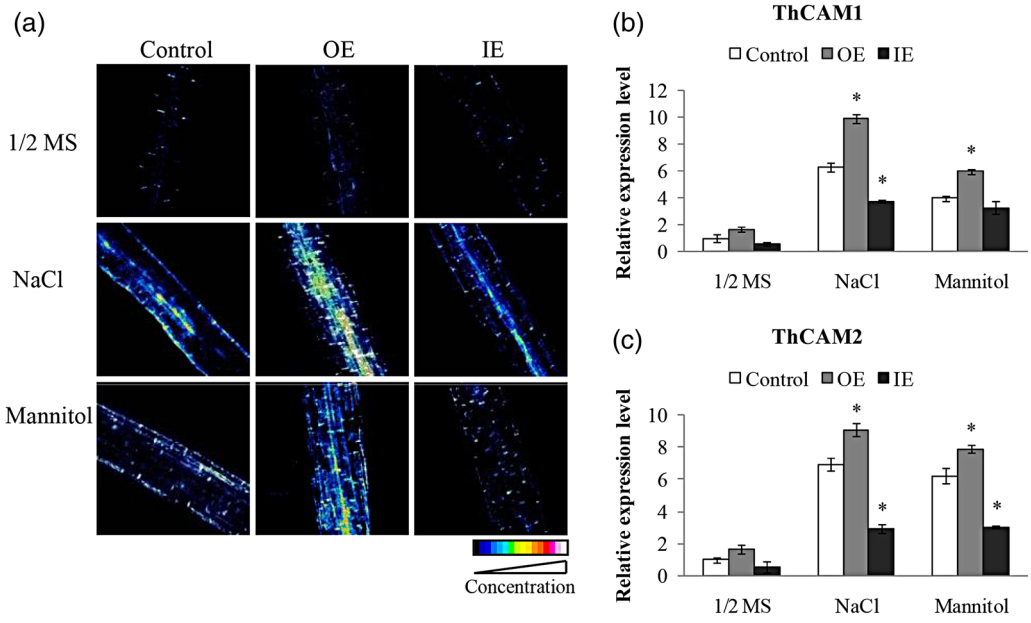

Figure 7. Analysis of Ca^{2+} and the expression of *CaM* genes in transiently transfected *T. hispida* plantlets. (a) Analysis of Ca^{2+} in the roots of *T. hispida* plantlets using Fluo-3-AM ESTER staining. *Tamarix hispida* plantlets were incubated in a solution of 150 mM NaCl or 250 mM mannitol for 2 h, and were then stained with Fluo-3-AM ESTER. Black to blue shows a low Ca^{2+} level, red color indicates a high level and green to yellow color means middle level of Ca^{2+}. (b and c) The expression of *CaM* genes in the roots of *T. hispida* in response to NaCl or mannitol. The GenBank numbers of *ThCaM1* and *ThCaM2* are KM101100 and KM101101. Asterisks indicate significant ($P < 0.05$) difference from control plants.

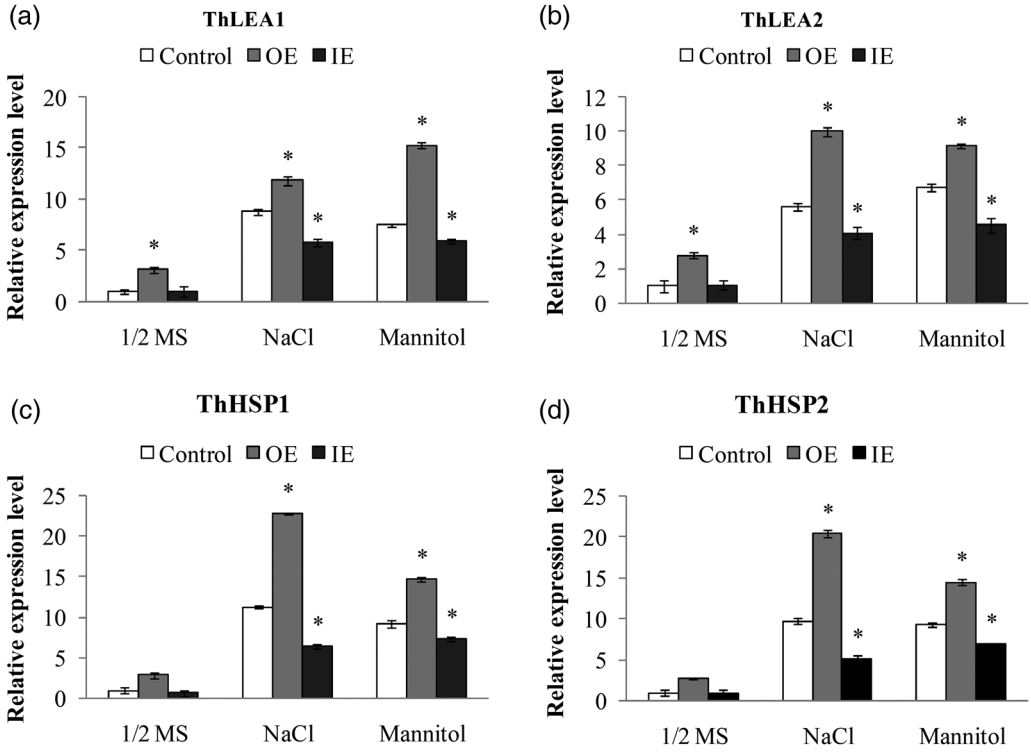

Figure 8. The expression of stress-related genes in transiently transfected *T. hispida* plantlets. For each gene, its expression level in the control plants under normal conditions was used as the calibrator (designed as 1) to normalize the relative expression under other conditions. The plants were grown in 1/2 MS medium or 1/2 MS medium containing 150 mM NaCl or 250 mM mannitol for 48 h, and used for analysis. Asterisks indicate significant ($P < 0.05$) difference from control plants.

play important roles in plant stress tolerance. In plants, P5CS is the key enzyme in proline biosynthesis. Aldehyde dehydrogenases including betaine ALDHs (BADH/ALDH) are the rate-limiting enzymes in biosynthesis of glycine betaine.

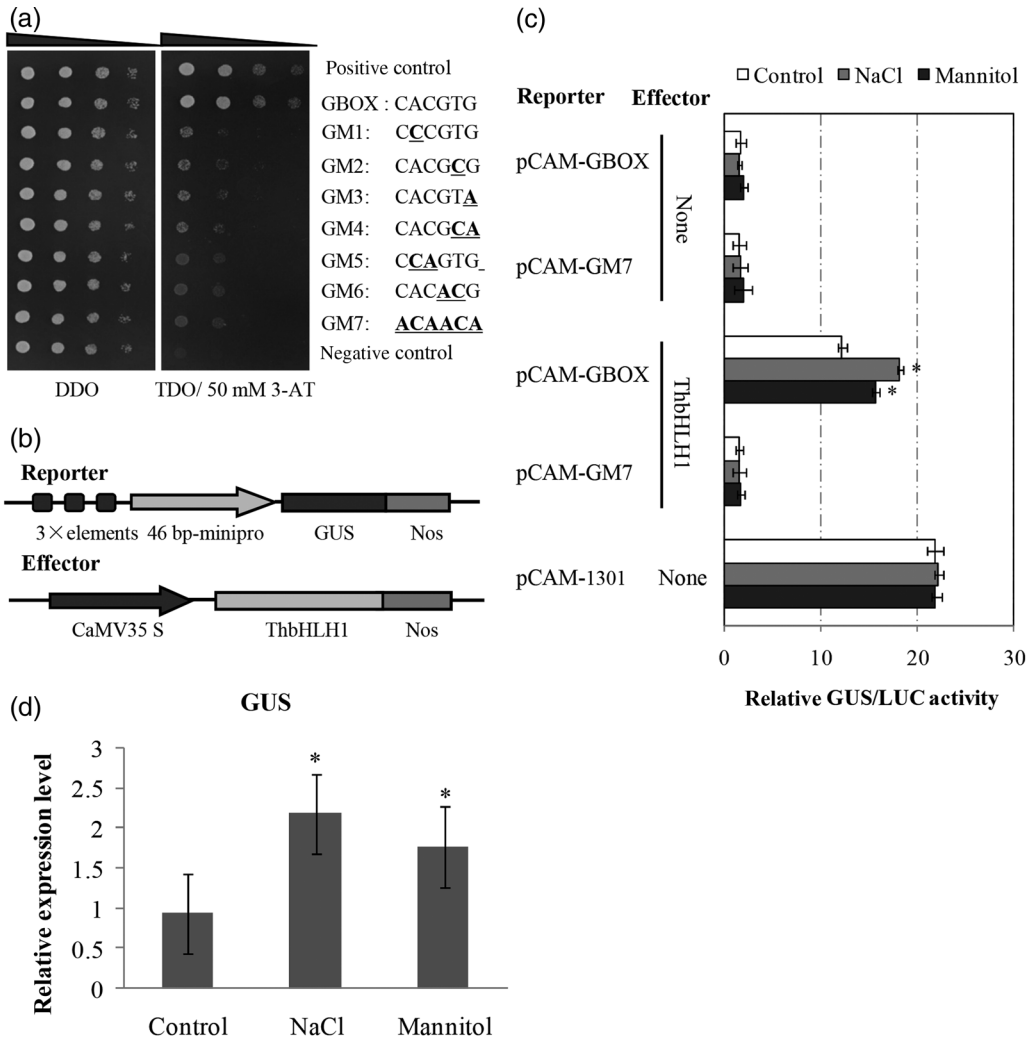

Figure 9. Interaction of ThbHLH1 with the G-box motif. (a) Analysis of the binding of ThbHLH1 to G-box using Y1H. The sequences of the G-box and its mutants are shown on the right panel. The transformants grown on double drop out medium: SD/-Leu/-Trp medium were used as positive controls for transformant growth. (b) Schematic diagram of the effector and reporter constructs used in the coexpression in *T. hispida* plantlets. (c) Transactivation of a G-box reporter by ThbHLH1 in transiently transfected *Arabidopsis* plants in response to salt and osmotic stress. Positive control, involved in transformation with the reformed pCAMBIA1301 containing the 35S promoter upstream of the *GUS* gene; None, cotransformation of empty pROKII and the reporters. The transformation efficiencies were normalized using LUC activity. (d) Comparison of the ratio of the transcript level of *GUS* gene (reporter) with that of ThbHLH1 (effector) in *Arabidopsis* under normal or salt and osmotic stress conditions. Asterisks indicate significant ($P < 0.05$) difference from the controls.

In the present study, *ThbHLH1* is found to induce the expression of *P5CS* and *BADH*/*ALDH* genes in both *T. hispida* and *Arabidopsis* plants (Figure 5; see Figure S4 available as Supplementary Data at *Tree Physiology* Online); meanwhile, the transcript levels of *P5CS* and *BADH*/*ALDH* genes were, respectively, positively correlated with the proline and glycine betaine contents (Figure 5; see Figure S4 available as Supplementary Data at *Tree Physiology* Online). These results suggest that ThbHLH1 activates the biosynthesis of proline and glycine betaine by inducing the expression of *P5CS* and *BADH*/*ALDH* genes, and these elevated proline and glycine betaine levels adjust the osmotic potential, which results in improved abiotic stress tolerance. What deserves to be mentioned is that in *Arabidopsis* plants, the *P5CS* and *ALDH* genes were all highly induced by *ThbHLH1* (see Figure S4 available as Supplementary Data at *Tree Physiology* Online). Meanwhile, there are several G-box motifs present in the promoter regions of all these genes (not shown), suggesting that ThbHLH1 directly regulates the expression of *P5CS* and *ALDH* genes via binding to the G-box motifs in their promoters.

ThbHLH1 mediates singling cascades in response to abiotic stress

In plants, Ca^{2+} is a ubiquitous intracellular second messenger that mediates complex responses to environmental and developmental signal cues. The external and internal signals of plants can strongly and rapidly enhance cytosolic calcium, and ultimately induce specific stress responses to improve plant survival. In this

signaling cascade, the CaMs are the major calcium sensor proteins that can bind calcium and regulate the activity of downstream target proteins (Yang et al. 2013), playing a crucial role in cellular signaling cascades.

Our results showed that intracellular Ca^{2+} levels were significantly increased in the roots of both *T. hispida* and *Arabidopsis* plants overexpressing *ThbHLH1*, while the roots of IE *T. hispida* plantlets had the lowest Ca^{2+} levels in roots (Figure 7a; see Figure S6A available as Supplementary Data at *Tree Physiology* Online), suggesting that increased *ThbHLH1* expression causes increased levels of Ca^{2+} upon exposure to salt and osmotic stress. The Ca^{2+} levels in plants were positively correlated with stress tolerance, suggesting the importance of Ca^{2+} as secondary messengers in stress tolerance. In addition, the expression levels of *CaM* genes were significantly elevated due to ThbHLH1 expression (Figure 7b and c; see Figure S6B and C available as Supplementary Data at *Tree Physiology* Online). Taken together, these results suggest that ThbHLH1 can mediate signaling cascades in response to salt and osmotic stress by enhancing levels of intracellular Ca^{2+}. We propose that the increased Ca^{2+} levels activate the expression of *CaM* genes (Tidow and Nissen 2013), which then regulate the activity of downstream target proteins to improved stress tolerance.

ThbHLH1 improves ROS scavenging capability

Increased ROS scavenging is a common mechanism to induce stress tolerance in plants. In the present study, overexpression of *ThbHLH1* was related to reduced ROS accumulation (see Figures S7 and S9 available as Supplementary Data at *Tree Physiology* Online), suggesting that *ThbHLH1* is involved in ROS scavenging. Meanwhile, *ThbHLH1* induces an increase in the expression level of *POD* and *SOD* genes in both *T. hispida* and *Arabidopsis* plants (see Figures S7 and S9 available as Supplementary Data at *Tree Physiology* Online) to enhance their activities (see Figures S7 and S9 available as Supplementary Data at *Tree Physiology* Online). Additionally, overexpression of *ThbHLH1* was related to significantly decreased MDA content under abiotic stress conditions (Figure 6d; see Figure S5D available as Supplementary Data at *Tree Physiology* Online), indicating that lipid peroxidation in cell membrane was also decreased due to expression of *ThbHLH1*. Therefore, *ThbHLH1* could regulate the expression of ROS scavenging genes to improve stress tolerance.

ThbHLH1 regulates HSP and LEA genes to improve stress tolerance

We further examined whether ThbHLH1 can regulate other genes related to stress tolerance, those encoding HSP and LEA proteins. Increasing synthesis of HSPs is an early and important adaptive strategy in cells when exposed to all types of stress (Chen et al. 2014). HSPs are key factors in maintaining cellular homeostasis under adverse conditions. HSPs assist the correct folding of proteins and the refolding of misfolded proteins accumulated under stress conditions, promote the degradation of misfolded or denatured proteins and also participate in stress signal transduction (Christou et al. 2014). LEA proteins act as molecular chaperones or shields to prevent irreversible protein aggregation, and can also stabilize the membrane by replacing water or inducing preferential hydration during desiccation conditions (Serrano and Montesinos 2003). Therefore, both HSP and LEA proteins are important in plant stress tolerance. Given their importance in stress tolerance, we further studied whether ThbHLH1 regulates their expression. The *LEAs* and *HSPs* from *T. hispida* that are homologous to the *LEAs* and *HSPs* involved in stress tolerance were studied. The results showed that *ThbHLH1* induces the expression of these genes (Figure 8), suggesting that ThbHLH1 can also improve stress tolerance by regulating the expression of *LEAs* and *HSPs*.

The G-box element mediates DNA binding and osmoticum-enhanced transactivation by ThbHLH1

In the transiently transfected plants overexpressing *ThbHLH1*, the expression of genes such as *PODs*, *SODs*, *P5CS*, *BADH*, *HSP*, *LEA* and *CaM* was significantly higher under NaCl or mannitol than under normal conditions (Figures 5, 7 and 8). The 35S promoter is not reported to be induced by osmotic stress, and we confirmed that *ThbHLH1* expression in transgenic *Arabidopsis* OE plants does not increase upon exposure to NaCl or mannitol (see Figure S11 available as Supplementary Data at *Tree Physiology* Online). Further, we established that ThbHLH1 binds the G-box element in yeast (Figure 9a). As shown in Figure 9b–d, ThbHLH1 transactivates a trimeric G-box reporter, and this transactivation is significantly increased by NaCl or mannitol. Meanwhile, the ratio of the expression level of *GUS* to that of *ThbHLH1* under NaCl or mannitol treatment condition is significantly higher than under normal conditions, further supporting the conclusion in Figure S11 available as Supplementary Data at *Tree Physiology* Online, i.e., that enhanced GUS expression upon osmotic stress is not caused by altered *ThbHLH1* expression. Put together, the fact that ThbHLH1 binds the G-box in yeast, and that its transactivation of the G-box reporter in planta is increased by NaCl or mannitol, suggests that ThbHLH1 directly binds the G-box in planta, and that this binding is increased by osmotic stress. Taken together, we propose that the binding affinity of ThbHLH1 to G-box is increased by NaCl and mannitol, and this increased binding elevates the expression of genes regulated by ThbHLH1.

The reliability of the transient transformation system

In the present study, we employed a transient expression system to study the function of *ThbHLH1*, and our results showed that the transiently transformed plants displayed significantly enhanced or decreased expression of *ThbHLH1* (Figure 4), and were therefore suitable for gain- and loss-of-function studies. In transient expression systems, expression of transgenes is not influenced by positional effects (Wroblewski et al. 2005), and

our transient transformed system was found to be reliable and suitable for studies of abiotic stress tolerance (Ji et al. 2014). To ensure that results are reliable, we performed three biologically independent experiments, and each experiment contained at least 20 transformed seedlings. Furthermore, to confirm the results obtained from *T. hispida* plantlets, *Arabidopsis* plants overexpressing *ThbHLH1* were generated. Our analyses showed that *ThbHLH1* positively regulates stress tolerance in transgenic *Arabidopsis* plants. In addition, the biological staining with DAB, NBT and Evans blue, measurements of the content of proline, glycine betaine, Ca^{2+}, H_2O_2 and MDA, determination of the activities of POD and SOD, and analyses of the electrolyte leakage and water loss rate in transgenic *Arabidopsis* plants were all fully consistent with those studied from the transiently transfected *T. hispida* plantlets. These results confirmed that the transient transformation system is reliable for stress tolerance studies.

Conclusion

A *bHLH* gene was cloned from *T. hispida*, and was found to specifically bind to G-box motif. ThbHLH1 positively regulates a series of genes to improve abiotic stress tolerance, including *P5CS*, *BADH*, *CAM*, *POD*, *SOD*, *LEA* and *HSP*. The highly induced expression of these genes leads to increased levels of proline and glycine betaine, improved Ca^{2+} level as a second stress messenger and enhanced ROS scavenging capability. All these physiological changes improve the abiotic stress tolerance in plants. This study improves our understanding of the function of bHLH proteins involved in abiotic stress.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Acknowledgments

We are grateful to our colleagues who have shared their knowledge and reagents, at the State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University).

Conflict of interest

None declared.

Funding

This work was supported by the Hundred Talents Program of the Chinese Academy of Sciences and the Project of the Culture of Young Scientists in Scientific Innovation in Xinjiang Province (2013711001).

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