Ectopic over-expression of *BhHsf1*, a heat shock factor from the resurrection plant *Boea hygrometrica*, leads to increased thermotolerance and retarded growth in transgenic *Arabidopsis* and tobacco

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Abstract Plant heat shock transcription factors (Hsfs) are commonly found to be involved in various stress responses. Several Hsfs displayed dwarf phenotype while conferred stress tolerance when over-expressed. However, the underlying mechanisms were not fully understood. Here we report the cloning and characterization of an Hsf (*BhHsf1*) from the resurrection plant *Boea hygrometrica*.

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Drought, heat and wound can induce *BhHsf1* expression. The over-expression of *BhHsf1* conferred growth retardation and stress tolerance in both *Arabidopsis* and tobacco. Evidence was presented to show that the growth retardation of aerial organs in the transgenic plants was resulted from the reduction of cell proliferation. Gene expression profiling using microarray hybridization and pathway analysis showed that Hsps and stress-associated genes were induced whereas the genes related to DNA replication and mitotic cell cycle were down-regulated in *BhHsf1* over-expression *Arabidopsis*, which was in consistence with the observation of the impaired nuclear endoreduplication. Taking together, our results suggest that *BhHsf1* may play dual roles in mediating the processes in heat stress tolerance and growth retardation via regulation of target genes related to stress protection and mitotic cell cycle.

Introduction

Heat shock transcription factors (Hsfs) are a group of proteins conserved in all eukaryotic organisms which mediate the activation of genes responsive to heat stress as the terminal components of the signal transduction chain. Hsfs recognize the heat shock elements (HSEs) presented in promoters of heat shock inducible genes of all eukaryotes (von Koskull-Doring et al. 2007). Compared with other eukaryotes such as yeast, *Drosophila* and vertebrates, which contain one to three Hsfs, plants possess more than 20 Hsf members (Baniwal et al. 2004). Hsfs have a modular structure with an N-terminal DNA binding domain

(DBD) that specifically binds to HSE, a heptad hydrophobic repeat (HR A/B) required for oligomerization, a nuclear localization signal (NLS) enriched in basic amino acids and in most cases, a C-terminal transcriptional activation domain (CTAD) (Nover et al. 1996, 2001). Based on details of their oligomerization domains, plant Hsps are grouped into class A, B and C (Baniwal et al. 2004; Nover et al. 2001; von Koskull-Doring et al. 2007).

Plant Hsfs function in multiple processes of stress response. Several members of Hsfs have been reported to play central roles in the heat stress response (HSR). Among those widely studied, *LpHsfA1a* is a master regulator for induced thermotolerance in tomato and *AtHsfA2* is the dominant Hsf in acquired thermotolerance in *Arabidopsis*. Both genes can enhance thermotolerance when over-expressed and abolished the thermotolerance when knocked-out or interfered (Charng et al. 2007; Mishra et al. 2002; Nishizawa et al. 2006; Ogawa et al. 2007). Besides, plant Hsfs are also involved in other environmental stress tolerance. For example, over-expression of *OsHsfA2e* and *AtHsfA3* elevated tolerance to salt stress in transgenic *Arabidopsis* (Yokotani et al. 2008; Yoshida et al. 2008); over-expression of *AtHsfA2* improved heat and osmotic stress tolerance of plants simultaneously (Ogawa et al. 2007). In addition, HsfA9 was reported to be essential for embryogenesis and seed maturation in sunflower and *Arabidopsis*, which increased our understanding of the diversity of Hsf function (Kotak et al. 2007; Prieto-Dapena et al. 2008).

Growth inhibition is generally observed when plants are stressed. This is a mechanism for plants to prolong energy supply to cope with indisposed environment (Osorio et al. 1998). Several Hsfs including AtHsfA3, AtHsfA2 and OsHsfA2e, among many other types of stress-inducible transcription factors, also confer dwarf phenotype (Ogawa et al. 2007; Yokotani et al. 2008; Yoshida et al. 2008). Final size of plant organ depends on cell number and cell size, which are controlled by two successive processes referred to as cell proliferation and cell expansion, respectively (Anastasiou and Lenhard 2007; Mizukami 2001; Tsukaya 2006). Many genes affecting organ growth by cell division and/or cell expansion are well studied (Breuer et al. 2007; Deprost et al. 2007; Fleury et al. 2007; Jing et al. 2009; West et al. 2004), but the underlying mechanism of Hsf-induced growth retardation is still far from understanding.

A group of so-called resurrection plants is capable of withstanding severe water loss in vegetative tissues (Bartels and Sunkar 2005; Gaff 1971; Oliver et al. 2000). It has been observed that the growth is arrested and metabolic reactions are largely ceased in leaves of resurrection plants soon after desiccation, and resumed rapidly upon rehydration (Bartels and Sunkar 2005; Oliver et al. 2000). Therefore, resurrection plants can be used to identify the possible link between the stress tolerance and the rapid

regulation of growth, which will deepen our understanding on the molecular basis of stress tolerance. Previously, Hsf fragments had been isolated from the resurrection plant *Craterostigma plantagineum* and *Boea hygrometrica* (Bockel 2001; Wang et al. 2009). In this study, we describe the cloning of the full length cDNA of the Hsf from *B. hygrometrica* and functional characterization based on phenotypic analysis and putative target genes using transgenic approach. The aim of our research is to estimate the possibility that BhHsf1 may function in integration of the processes of growth retardation and stress tolerance.

Materials and methods

Plant materials and growth conditions

Boea hygrometrica plants were collected from their natural habitat in Beijing and maintained in a greenhouse with regular irrigation as described previously (Jiang et al. 2007). Tobacco (*Nicotiana tabacum* cv. SR-1 and *Nicotiana benthamiana*) and *Arabidopsis thaliana* (Col-0) were used for transformation. Seeds were sterilized in 1% sodium hypochlorite for 15 min, and were then germinated on MS medium. 7-day-old *Arabidopsis* and 14-day-old tobacco seedlings were transferred to soil, and grown in a chamber at $22 \pm 1°C$ in a photoperiod of 16 h light and 8 h darkness.

Molecular cloning and sequence analysis

A cDNA fragment of *BhHsf1* was identified by differential screening of dehydration-inducible genes from a cDNA library prepared from desiccated leaves of *B. hygrometrica* using macroarray hybridization method as described previously (Wang et al. 2009). The full length coding region was subsequently obtained using the system for Rapid Amplification of cDNA Ends Kit (Invitrogen, CA). Reverse transcription was performed using gene specific primer 5′-TATCCGACGGCTGCTT-3′. Primers 5′-GGGATCCTTTGGAGGGATTTGAT-3′ and 5′-CTGGATCCTCCTTTTGATGTGTTTC-3′ were used in the first and second rounds of PCR amplification with anchor primers 5′-GGCCACGCGTCGACTAGTACG$_{14}$-3′ and 5′-GGCCACGCGTCGACTAGTAC-3′, respectively. The conditions were 94°C denaturation for 2 min, 35 cycles of 94°C 30 s; 55°C 30 s; 72°C 1 min, and 72°C elongation for 10 min for both the first and second PCR amplifications.

The 5′-flanking region of *BhHsf1* was obtained by TAIL-PCR (Liu et al. 1995) using a random primer 5′-(AGCT)GTCGA(GC)(AT)GA(AGCT)A(AT)GAA-3′ and two pairs of nested specific primers 5′-CTGCAGCTACCACAGAAACCAT-3′, 5′-CAGCCTCATCAAGAAACAC

CAAG-3′ and 5′-CTGTTACACGCCCACATTTTC-3′, 5′-GTGTTATTCTTACGTATGATGTGCG-3′.

cDNA and protein sequences were analyzed using BLAST program. Amino acid comparison and multiple alignment was performed using ClustalW program. Phylogenetic tree was constructed by Neighbor Joining method using MEGA 4.0. *Cis*-elements were predicted by PLACE software.

Generation of transgenic plants

BhHsf1 coding sequence was fused between 35S promoter and terminator and the cassette was cloned into pBin19 binary vector (Frisch et al. 1995) to create the *35S:BhHsf1* plasmid. Based on this vector, *GFP* coding region was in-frame fused downstream of *BhHsf1* to generate *35S:BhHsf1-GFP* construct. Besides, the 786 bp promoter of *BhHsf1* was amplified from genomic DNA, and ligated upstreame of *GUS* coding region in pBISN1 vector to generate *ProBhHsf1:GUS* fusion. The resulting plasmids were introduced into tobacco and *Arabidopsis* by *Agrobacterium*-mediated transformation (Clough and Bent 1998; Kapila et al. 1997). At least 30 independent lines for each construct were selected on MS agar plates containing 50 and 100 mg l^{-1} kanamycin (for *Arabidopsis* and tobacco, respectively) and homozygous T_3 generations of transgenic plants were used for further analysis.

Phenotypic analyses

To determine the size of the organs, stem height, leaf length and width were measured with a ruler, fully expanded cotyledons and fifth leaves were photographed for area measurement by ImageJ software (http://rsb.info.nih.gov/ij). To examine the growth kinetics of leaves, the fifth leaves were used to measure blade length and width in 4-day intervals after emergence. In order to study cell number and size, fully expanded fifth leaves (5-week-old plants grown in soil) were cleared to observe palisade cells between the midvein and the leaf margin. The number of palisade cells per unit area of a region was determined, and the area of the leaf blade was multiplied by this value to calculate the total number of palisade cells in the subepidermal layer. Eight regions per leaf and five leaves were analyzed (modified from Horiguchi et al. 2005; Jing et al. 2009). Areas of organs and cells were measured with ImageJ software. Flowers and fully expanded siliques from the same positions on WT and transgenic plants were compared.

Thermotolerance assays

Seedlings were grown on MS agar in Petri dishes. The Petri dishes were sealed with transparent plastic film and then immerged into water bath at different temperatures for heat stress tolerance assays. For tobacco, 2-week-old plants were exposed to 48°C for 2.5 h for the basal thermotolerance assays, and to 40°C for 3 h followed by 50°C for 2 h for the acquired thermotolerance assays (modified from Prieto-Dapena et al. 2008). For *Arabidopsis*, 2-week-old plants were exposed to 45°C for 1 h, for the basal thermotolerance assay and to 37°C for 1 h, 22°C for 3 h, and 49°C for 1 h for the acquired thermotolerance assays (modified from Ogawa et al. 2007). These plants were grown at 22°C after HS treatment for 3 days (for basal thermotolerance assays) and 6 days (for acquired thermotolerance assays) before taking photographs and counting the number of surviving plants.

GUS histochemical analysis

Plants from six independent transgenic *Arabidopsis* lines containing *ProBhHsf1:GUS* were used for GUS staining using X-Gluc as a substrate. After staining, the tissues were soaked in several changes of 95% ethanol and examined under a microscope (Bertrand et al. 2003). For heat-shock experiments, the plants were placed at 37°C for 8 h and were allowed to recover at growth condition for 3 h before histochemical staining. *Arabidopsis* plants that harbor *AtCYCB1;1:GUS* (Colon-Carmona et al. 1999) were crossed with *35S:BhHsf1* homozygous plants. Seven-day-old seedlings of crossed progenies were used for histochemical GUS staining.

GFP visualization

Samples were incubated with 0.1 mg/ml DAPI and illuminated with an argon laser (488 nm wavelength for detection of GFP fluorescence and 405 nm for DAPI, respectively) using a confocal laser scanning microscope (Leica TCS SP5, Germany).

RNA blot and RT-PCR

Total RNA was prepared by a guanidine thiocyanate method (Hu et al. 2000). For RNA blot analysis, total RNA (5 μg per lane) was denatured for 1 h at 55°C, and, separated on a 1% (w/v) denaturing agarose gel, transferred onto HybondTM-N$^+$ nylon membrane (Amersham Phamacia Biotech, UK), fixed by incubating for 2 h at 80°C. Probe labeled with fluorescein and fluorescent signal detection were done using Gene Images Random Prime Labelling Module and Gene Images CDP-Star Detection Module (Amersham Phamacia Biotech, UK) according to manufacture's instruction.

Reverse-transcribed PCR (RT-PCR) was performed to monitor the expression of *BhHsf1* in transgenic tobacco

and *Arabidopsis*. *ACTIN2* and 18S rRNA were used as the internal controls for *Arabidopsis* and tobacco, respectively. Quantitative real time PCR analyses were performed using RNA isolated with Trizol reagent (Invitrogen, US) as previously described (Wang et al. 2009). The primers used are listed in Table S1.

Microarray analysis

Total RNA was isolated with Trizol reagent (Invitrogen) from the aerial part of the 3-week-old seedlings of *35S:BhHsf1* and WT plants grown in parallel and then analyzed using an A-MEXP-1038 Agilent *Arabidopsis* 3 Oligo Microarray 4x44K 015059 G2519F (Agilent Technologies Inc. USA). For each biological replicate, materials from 15 plants were pooled for RNA purification. Two independent experiments were carried out using two independent transgenic lines (OE-15 and OE-21). Cy5- and Cy3-labeled cDNA probes were prepared from total RNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies Inc. USA). Microarray experiments including labeling, hybridizations and data analysis were performed according to the manufacturer's manual. Agilent G2566AA Feature Extraction Software was used to locate and delineate every spot in the array and to integrate each spot's intensity, filtering, and normalization by the Lowess method. All changes in gene expression were statistically significant at $P < 0.01$. Microarray data were submitted and can be accessed from the MIAME checklist format (http://www.mged.org/Workgroups/MIAME/miame.html) under the ArrayExpress accession of E-MTAB-106. Pathways analysis was performed with GeneSpring10 software (http://www.silicongenetics.com) using KEGG and Reactome database.

Flow cytometric analysis

The fully expanded fifth leaves (corresponding to 5-week-old plants) of WT and transgenic *Arabidopsis* OE plants were chopped with a razor in parallel, suspended in cold nuclear isolation buffer (Galbraith et al. 1983) and filtered through a 20 μm filter. Then 4′,6-diamidino-2-phenylindole (DAPI; 0.5 mg ml^{-1}) staining buffer was added to the filtered solution. Nuclear ploidy was analyzed with a Cell Lab Quanta^{TM} SC flow cytometer (Beckman coulter, US).

Results

Cloning and sequence analysis of *BhHsf1*

A cDNA fragment encoding C-terminal of an Hsf was identified previously as a dehydration-inducible gene from a cDNA library from dehydrated leaves of *B. hygrometrica* using a macroarray screen approach (Wang et al. 2009). Subsequently, the corresponding full-length cDNA sequence containing 14 bp of 5′ UTR, 287 bp of 3′ UTR and 1,152 bp of open reading frame (ORF) was cloned using 5′RACE and designated as *BhHsf1* (Accession No. FJ947047). The deduced amino acid sequence includes an Hsf specific DNA-binding domain (DBD), an oligomerization domain with the hydrophobic heptad repeat (HR-A/B), and an adjacent putative nuclear localization signal (NLS) enriched in arginine and lysine (Fig. 1a). The plant Hsfs unique stretch (DSDR) between the third and fourth β-strands within the DBD (Nover et al. 1996) also exists in BhHsf1 (Fig. 1b). Besides, several features for class A Hsfs were found in BhHsf1, including the 21-amino acid insertion linking the cores of HR-A and HR-B, and the putative C-terminal transcriptional activation domain (CTAD motif), which is characterized by aromatic, large hydrophobic, and acidic amino acid residues. However, this protein is lack of any nuclear export signal (NES), which is required for keeping the balance of nuclear export and import for some Hsfs. BLASTp search in GenBank database revealed that BhHsf1 was most closely related to HaHsfA9, a seed-specific Hsf from sunflower, with 45% identity over the whole sequence. Phylogenetic relationship of 20 Hsfs from *A. thaliana*, tomato, sunflower and *B. hygrometrica* based on the sequence of the conserved DBD domain and HR-A/B region has indicated that BhHsf1 belongs to the subgroup containing HaHsfA9 and AtHsfA9 (Fig. 1c).

BhHsf1 expression is regulated by stress and development

A RNA blot was performed to investigate the expression of *BhHsf1* gene in response to dehydration. The transcripts of *BhHsf1* was absent in untreated fresh leaves, but appeared in leaves dehydrated for 2 h, and accumulated to high levels in leaves dehydrated for 8–72 h (Fig. 2), and disappeared after rehydration (data not shown).

To further characterize the regulation of *BhHsf1* gene expression, a 786 bp 5′ flanking sequence of *BhHsf1* was amplified from *B. hygrometrica* genomic DNA (Fig. S1), and cloned in-frame upstream to a β-glucuronidase (*GUS*) reporter gene. The *ProBhHsf1:GUS* cassette was stably transformed into *Arabidopsis*. Histochemical staining of transgenic plants revealed GUS activity at almost all developmental stages (Fig. 3). In young seedlings, GUS staining was detected throughout the plant with strong signals in the actively proliferating tissues of the shoot and root, such as leaf primordial, root tip and the lateral root differentiation zones, as well as cotyledons and vascular tissues (Fig. 3a–d). In adult plants, GUS signals were detected very faint (Fig. 3e). Upon heat, GUS signals were

(A) Full-length cDNA sequence and characteristics of the predicted BhHsf1 protein.

(B) Alignment of the conserved DNA binding domain (DBD) sequences.

(C) Phylogenetic relationship of Hsfs.
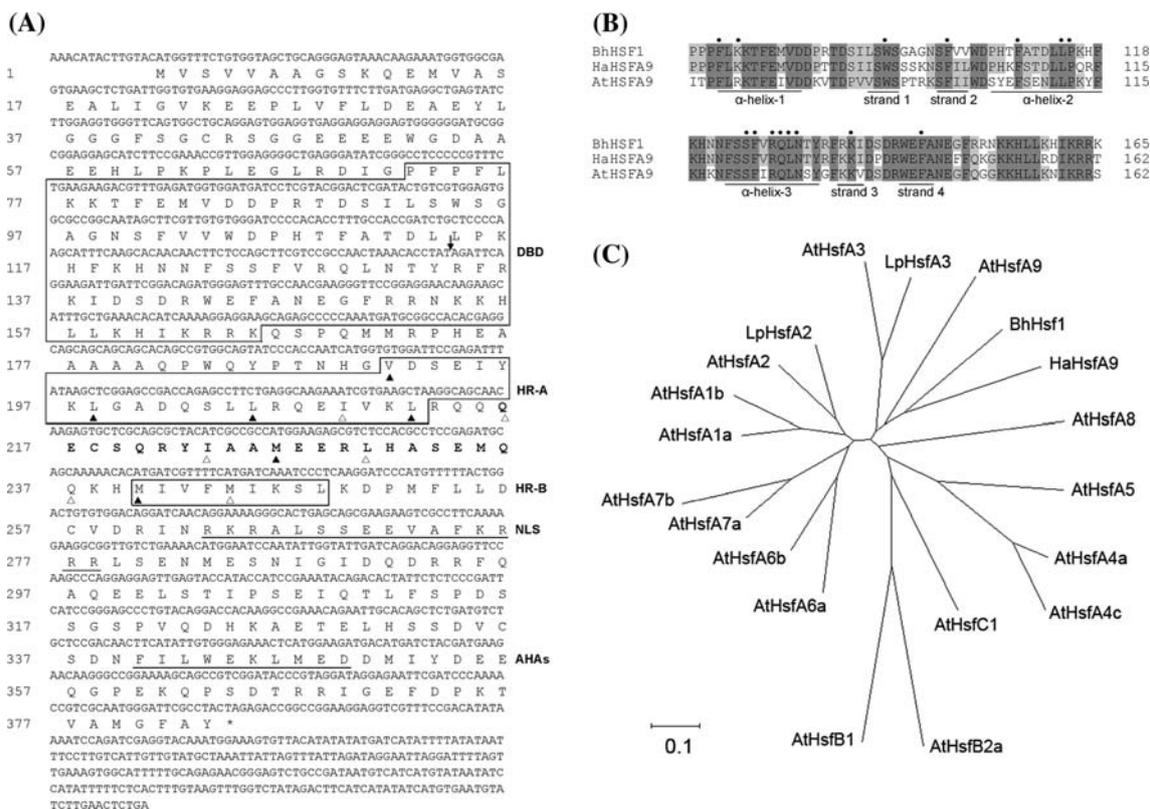
Fig. 1 BhHsf1 sequence characterization and phylogenetic analysis. **a** Full-length cDNA sequence and characteristics of the predicted BhHsf1 protein. *BhHsf1* cDNA contains a short 5′-untranslated region (UTR), a coding region and a poly (A) tail. The *arrow* indicates the position of the conserved intron that is present in corresponding genomic sequence. The deduced translation of the BhHsf1 protein is showed below the coding region sequence (*CDS*). We also indicate putative domains identified by sequence comparison with other Hsfs, the DNA binding domain (*DBD*); the oligomerization domain, with overlapping heptad repeats (HR-A/B) indicated by *filled triangle* and *hollow triangle*; a putative nuclear localization sequence (*NLS*) (*underlined*); the 21-amino acid insertion between HR-A and HR-B (*boldface*); and finally the putative AHA motifs (*underlined*). **b** Alignment of the conserved DNA binding domain (*DBD*) sequences of BhHsf1, HaHsfA9 and AtHsfA9. Amino acids highlighted in *black* are conserved in all three proteins; those highlighted in *grey* are conserved in two of them. The four *β*-sheets and three *α*-helixs are

indicated. *Dots* above indicated the residues with conserved and important functions. **c** Phylogenetic relationship of Hsfs based on amino acid sequence comparison of the DBDs and HR-A/B regions. Amino acid comparison and multiple alignments were performed using the ClustalW program. Phylogenetic tree was generated by Neighbor Joining method using MEGA 4.0. Plant species are abbreviated as follows: At, *Arabidopsis thaliana*; Lp, *Lycopersicon esculentum* (cotton); Ha, *Helianthus annuus* (sunflower); Bh, *Boea hygrometrica*. Sequence data were taken from the GenBank database for the following accession numbers: BhHsf1, FJ947047; HaHsfA9, AAM43804; AtHsfA1a, CAB10555; AtHsfA1b, CAA74397; AtHsfA2, AAC31222; LpHsfA2, CAA47807; AtHsfA3, CAB82937; LpHsfA3, AAF74563; AtHsfA4a, CAA16745; AtHsfA4c, BAB09213; AtHsfA5, CAB10177; AtHsfA6a, BAB11313; AtHsfA6b, BAB01258; AtHsfA7a, CAB41311; AtHsfA7b, CAB86436; AtHsfA8, AFF16564; AtHsfA9, BAA97129; AtHsfB1, CAB16764; AtHsfB2a, CAB63802; AtHsfC1, BAB02003
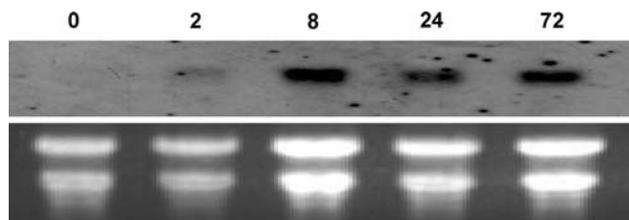
Fig. 2 RNA blot analysis of *BhHsf1* in dehydrated *B. hygrometrica* leaves. The *numbers* above each *lane* indicate the hours after leaves were dehydrated for 0, 2, 8, 24 and 72 h. Each *lane* was loaded with 5 μg mRNA

increased in leaves (Fig. 3f). Wound can also induce stronger GUS signal in leaves (Fig. 3g). In old plants, GUS signals were found in anther and pistil but not in mature siliques (Fig. 3h, i). However, the intensity of GUS signal is not significantly altered after drought stress in *Arabidopsis* (data not shown). One possible reason for this is that the promoter GUS construct may miss regulatory sequences such as the coding sequences (De Almeida et al. 1989) and the promoter-proximal introns (Rose et al. 2008), which play important roles in the regulation of gene expression in some cases.
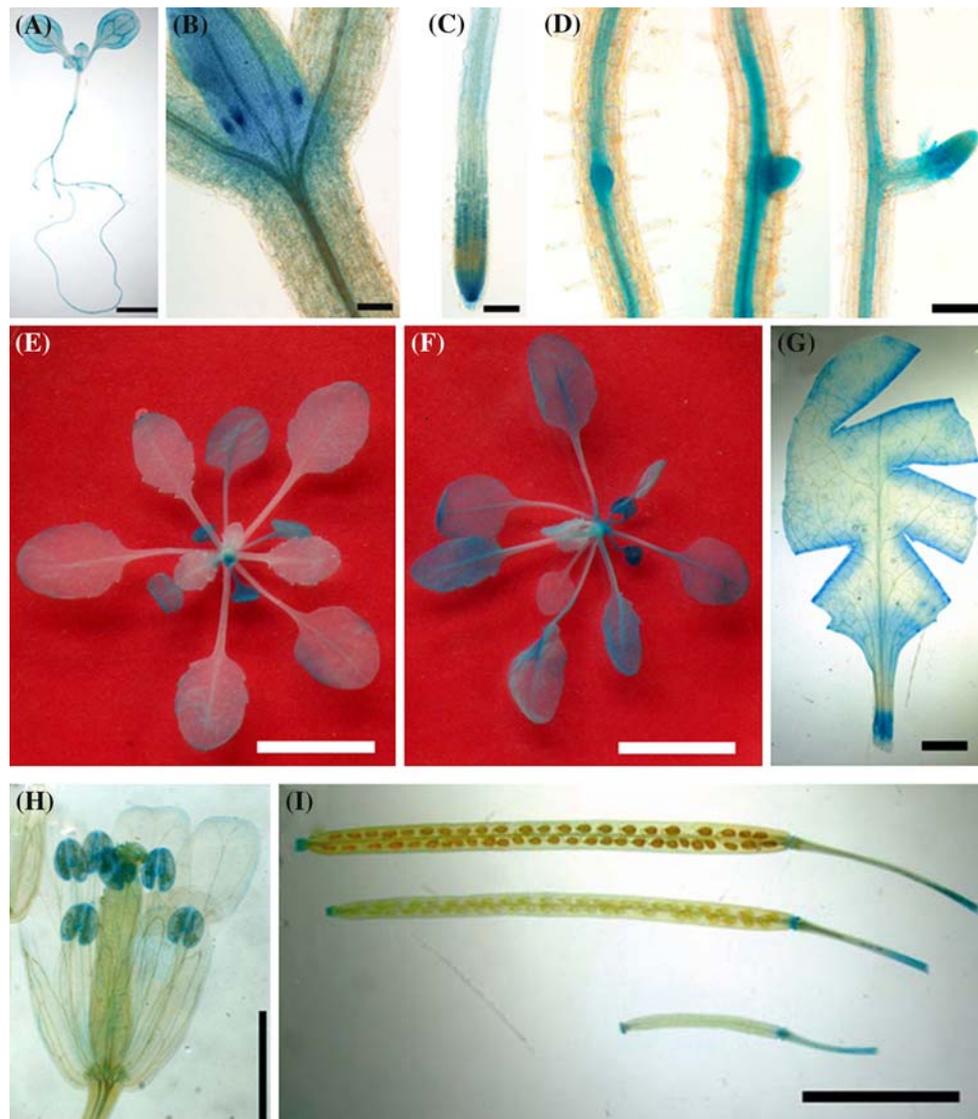
Fig. 3 Histochemical localization of *GUS* expression driven by *BhHsf1* promoter in transgenic *Arabidopsis*. **a** 9-day-old seedling. *Bar* 5 mm. **b** Shoot apex showing the emerging first pair of leaf primordia. *Bar* 20 μm. **c** Meristematic region in root of 7-day-old seedling. *Bar* 20 μm. **d** Lateral root formation of early to late stages (6–12 days after germination, from *left* to *right*). *Bar* 20 μm. Three-week-old seedling untreated (**e**) and heat stressed (**f**). *Bar* 5 mm. **g** Rosette leaves treated with wounding. *Bar* 10 mm. **h** Flower. *Bar* 5 mm. **i** Siliques at different developmental stages. *Bar* 5 mm. GUS staining was observed for at least four different transgenic lines for each experiment

BhHsf1 is localized in the nucleus

The subcellular localization of BhHsf1 was examined by expressing the BhHsf1-GFP fusion protein in tobacco epidermal cells. The BhHsf1-GFP signals were detected mainly in the nuclei, while the control GFP was detected in both nuclei and cytosol (Fig. 4). These results indicate that BhHsf1 protein is targeted to the nucleus, which is consistent with the absence of NES in BhHsf1.

Over-expression of *BhHsf1* in *Arabidopsis* and tobacco results in reduced size of aerial organs

Several Hsfs have been transformed to *Arabidopsis* for functional assessment. In order to gain an insight on the basic function of BhHsf1 in comparison with other plant Hsfs, transgenic *Arabidopsis* ectopically expressing *BhHsf1* under the 35S promoter were constructed. Among a collection of 36 T1 transgenic lines, nearly all showed dwarf

Fig. 4 Nuclear localization of BhHsf1-GFP fusion protein. Tobacco epidermal cells transiently expressing GFP alone (*upper*) and BhHsf1-GFP (*bottom*) proteins were stained with DAPI and observed with a confocal microscope. Note that BhHsf1 is localized within nucleus, whereas that of control GFP is evenly dispersed in the whole epidermal cells. DAPI is stained pseudocolored blue. *Bars* 10 μm

Bright light   **GFP**   **DAPI**   **Merge**
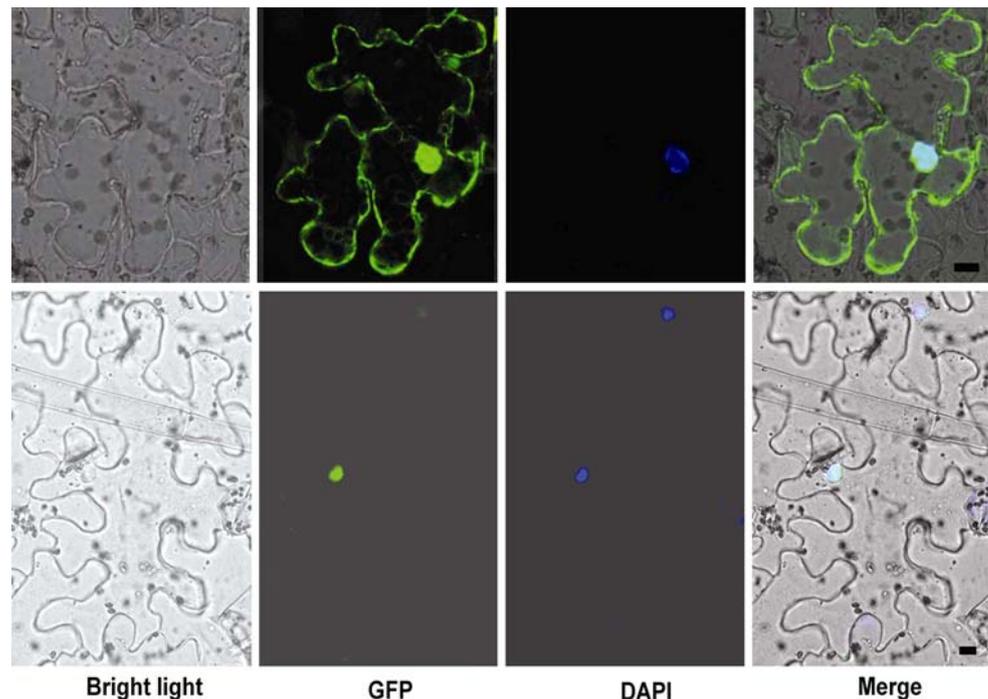
phenotype to various extents: 8 lines were severe, 19 lines moderate and 7 lines weak, as represented by OE-7, OE-15 and OE-21, respectively (Fig. 5b, c), which is in consistence with the expression levels of *BhHsf1* transcripts (Fig. 5a). Plants of these lines were selfed, and kanamycin-resistant T3 seeds were collected for further analyzed.

When grown in parallel, the OE lines exhibited obvious smaller aerial organs compared with wild type (WT) (Fig. 5). For example, cotyledons appeared at approximately the same time after germination in both WT and OE lines, but the final sizes were smaller and the shape was rounder in OE lines (Fig. 5b). The areas of fully expanded cotyledons in 9-day-old seedlings of OE-7, OE-15 and OE-21 were about 40, 55 and 70% of that in the WT, respectively (Fig. 5f). Further measurement revealed that cotyledon length in OE lines was significantly smaller than that in WT, while the cotyledon width was not (Fig. 5g). Consistently, the area of the fully expanded fifth leaves of 5-week-old plants of the OE lines were less than 45% of that in WT of the same age (Fig. 5h); and the reduction in leaf length was more significant than that in the width (Fig. 5i). The sizes of other aerial organs, including stems (Fig. 5n), flowers (Fig. 5j) and siliques (Fig. 5l), were also decreased to some extent, resulting in dwarfish phenotype. No difference between OE plants and WT were seen on the processes of development. In addition, it was observed that the stamens failed to elongate over the pistils in a portion of flowers of OE lines, which resulted in sterile to 46% in OE-7, 25% in OE-15, and 13% in OE-21, respectively (Fig. 5k, m). Overall, compared with WT, *BhHsf1* OE plants exhibited pleiotropic phenotypes. However, the

lengths of the primary roots of OE lines were not significantly different from that of the WT (Fig. 5d), which is consistent with our quantification of the primary root length (data not shown). Thus, no defects were found in the length of the primary root in the OE plants.

To confirm the effects of *BhHsf1* on plant growth, transgenic tobacco plants over-expressing *BhHsf1* were also generated. Three homologous lines, 2-10, 4-9 and 8-2 were obtained (Fig. 6a). In agreement with the *Arabidopsis* OE lines, these transgenic tobacco lines also displayed small-sized leaves (Fig. 6b and c), flowers (Fig. 6d) and stems (Fig. 6e). The length of stamens were also reduced (Fig. 6d), which resulted in the failure of self-pollination.

Reduced organ size in *BhHsf1* OE transgenic plants is attributed mainly to decreased cell proliferation

The most noticeable phenotype of *BhHsf1* OE transgenic plants is the size reduction in aerial organs. Since leaf has been used as a model to investigate the genetic and environmental control of organ size and shape in the plants (Tsukaya 2006), we performed a growth kinetic analysis of the fifth leaf to determine the nature of the defect in leaf growth of the *BhHsf1* OE lines (Fig. 7a, b). The data revealed that the size difference in both leaf blade length and width between OE lines and WT occurred at comparatively early growing stages, but the time that leaves to stop growing in the longitudinal and transversal direction are similar between OE lines and WT (Fig. 7a, b), suggesting that the OE plants may have altered the cell division and thus the leaf expansion rates during the

Fig. 5 Phenotypes of *35S:BhHsf1* transgenic *Arabidopsis* lines. **a** Expression of *BhHsf1* in WT and three OE lines by RT-PCR, *ACTIN2* was used as an internal control. **b** Seedlings of 9-day-old. *Bar* 5 mm. **c** Seedlings of 4-week-old. *Bar* 5 mm. **d** Roots of 9-day-old WT and OE lines. *Bar* 10 mm. **e** Rosette leaves from WT and OE lines arranged in order (*left* to *right*) from the oldest to the youngest. *Bar* 10 mm. WT, OE-7, OE-15 and OE-21 are shown from *top* to *bottom*. Dimensions (**f**, **h**) and areas (**g**, **i**) of cotyledons (**f**, **g**) and the fifth leaves (**h**, **i**) in WT and *BhHsf1* OE lines. Cotyledons and leaves from eight plants of each genotype were measured: average values ± SD are shown; Students' *t*-test, *$P < 0.05$, **$P < 0.01$. Flowers (**j**) and siliques (**l**) of 7-week-old WT and *35S:BhHsf1* plants. *Bars* 5 mm. **k** A normal flower of WT (*left*) and a sterile flower of OE-7 line (*right*) with reduced stamens that is defect in self-pollination. *Bar* 2 mm. **m** Normal ovules in silique of WT (*left*) and aborted ovules in silique of OE-7 (*right*), respectively. *Bar* 2 mm. **n** Seven-week-old plants of WT and OE lines. *Bar* 2 cm. WT, OE-7, OE-15 and OE-21 are shown from *left* to *right* unless indicated
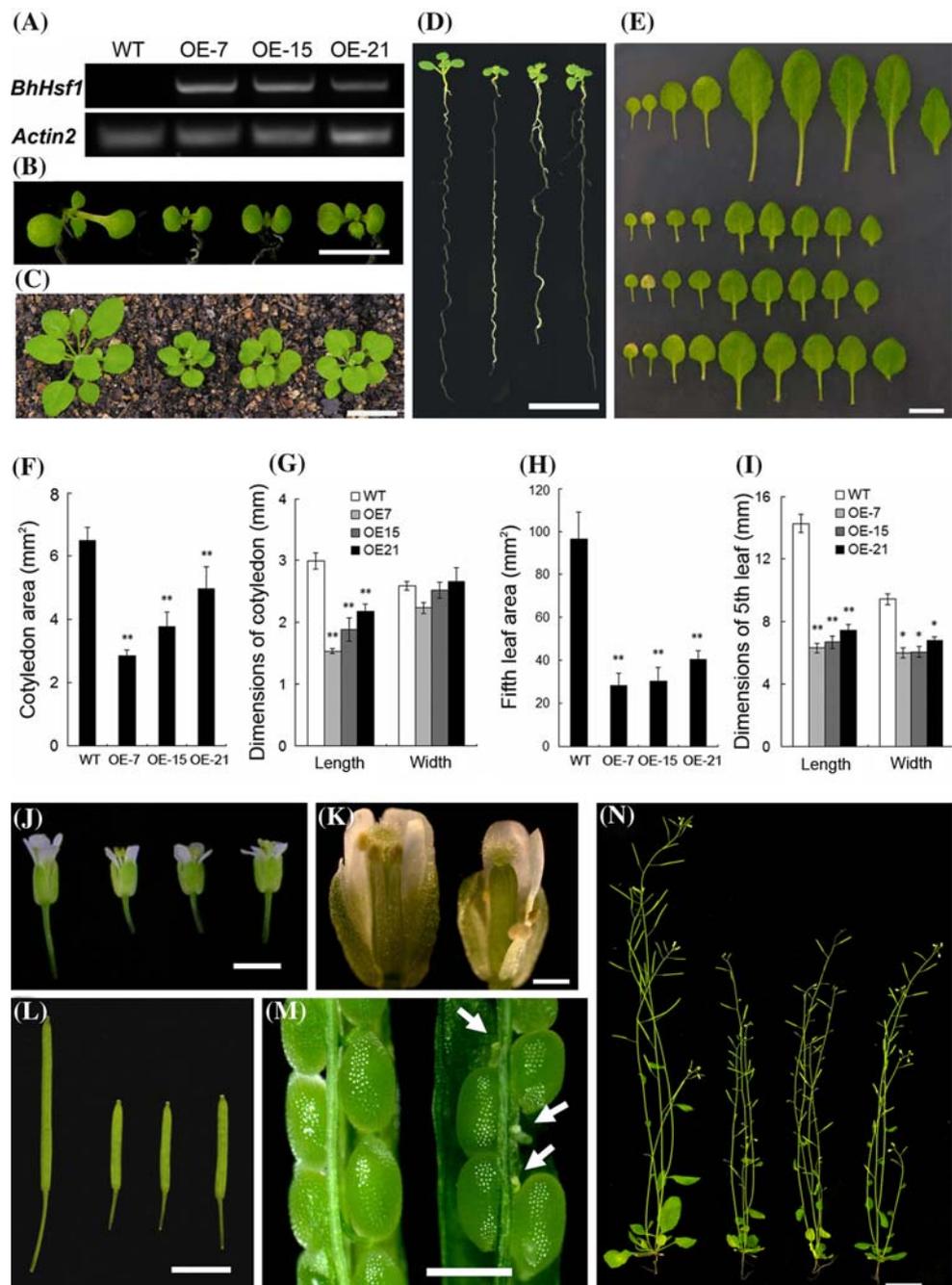
proliferation phase of leaf development but did not affect the duration of proliferation or leaf expansion. To assess the contribution of cell division to the small-sized leaves of OE lines, the palisade cell area and cell number of the fully expanded fifth leaves in WT and OE lines was compared. It was observed that the average area of palisade cells in OE lines was approximately the same as that in WT (Fig. 7c–g). However, the total palisade cell numbers in OE lines were only one-third of that in WT (Fig. 7h). These findings suggest that the over-expression of *BhHsf1* mainly impedes the process of cell proliferation, rather

than cell expansion, during leaf growth. And the effect is correlated with the dosage of *BhHsf1* transcripts in each OE line (Fig. 7). A B-type cyclin *CYCB1;1* promoter fused *GUS* reporter gene, which has been used as a tool to monitor mitotic activity in actively proliferating tissues (Himanen et al. 2002), was introduced into OE-15 and WT by crossing to evaluate the meristem size and activity. The range and intense of the GUS staining in young developing leaves had been obviously reduced in OE plants (Fig. 8), suggesting the cell mitotic division decreased in the site of vigorous division and growth in OE plants.

Fig. 6 Phenotype of
35S:BhHsf1 transgenic tobacco
plants. **a** Relative expression of
BhHsf1 in WT and three
35S:BhHsf1 lines by RT-PCR.
18S rRNA was used as an
internal control. **b** WT and
35S:BhHsf1 plants of 18-day-
old. *Bar* 5 mm. **c** The tenth
leaves of 3-month-old plants.
Bar 2 cm. **d** Flowers of
3-month-old WT and
35S:BhHsf1 plants. Reduced
stamens of various extents are
observed in all OE lines. *Bar*
1 cm. **e** WT and *35S:BhHsf1*
plants of 3-month-old. *Bar*
10 cm. WT, 8-2, 2-10 and 4-9
are shown from *left* to *right*
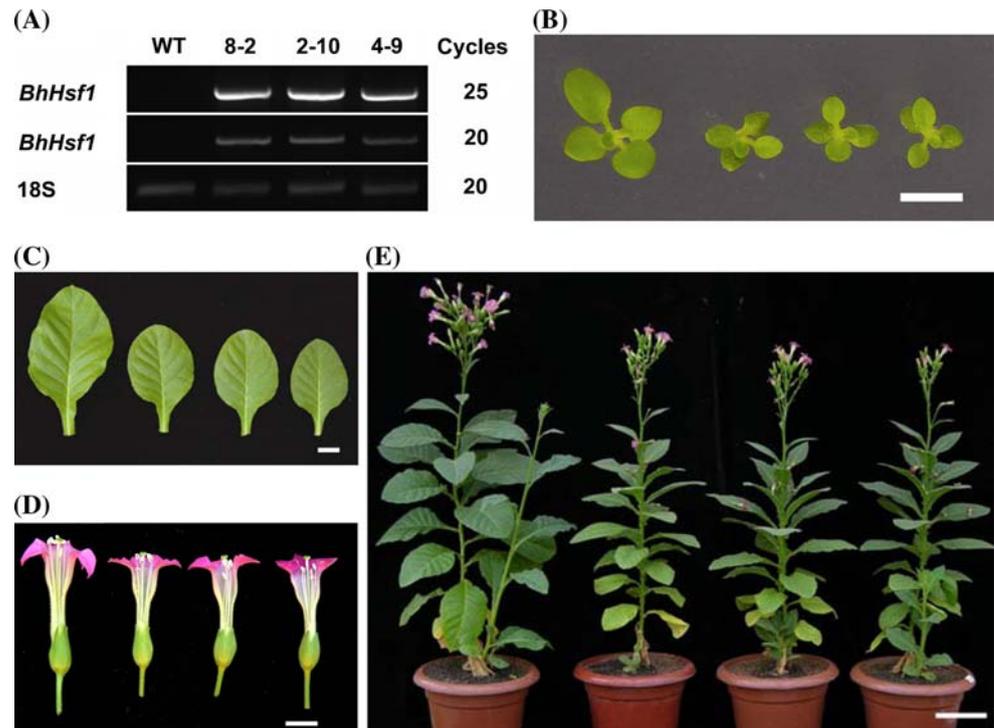
35S:BhHsf1 Arabidopsis and tobacco exhibit increased
thermotolerance

The previously described experiments demonstrated that
Hsfs from various plants were required for basal thermo-
tolerance and/or acquired thermotolerance (Ogawa et al.
2007; Prieto-Dapena et al. 2008; Yokotani et al. 2008). To
determine the effect of *BhHsf1* on plant heat stress
responses, we compared the acquired and basal thermo-
tolerance of transgenic *Arabidopsis* with those of WT:
100% seedlings of OE lines but none of WT display the
acquired thermotolerance (Fig. 9a); meanwhile 50% seed-
lings of OE line but none of WT survived when exposed to
high temperature without a conditioning pretreatment
(Fig. 9b, c). Similarly, we found that over-expression of
BhHsfA1 conferred transgenic tobacco increased both
acquired (Fig. 9d) and basal (Fig. 9e) thermotolerance. The
observation implicated that BhHsf1, like other Hsfs, is
involved in tolerance to heat stress.

Transcriptome analysis of aerial organs in *BhHsf1* OE
Arabidopsis

To investigate the possible molecular mechanisms of
BhHsf1 function in plant growth and stress responses, a
genome-wide expression analysis on the aerial organs of
young plants (3-week-old) was carried out to identify the
genes with altered expression levels in the OE lines.
Approximately 27,718 expressed genes were detected. The
statistical analysis identified a total of 687 differentially

expressed genes between *35S:BhHsf1* and WT at $P < 0.01$,
among which 240 (34.9%) were down-regulated and 447
(65.1%) were up-regulated with fold changes higher than
1.5.

Table 1 listed 59 genes that were up-regulated in OE
lines for at least sevenfold of that in WT. These genes are
involved in diverse aspects of stress response, metabolism,
protein fate, transcription regulation and signal transduc-
tion, implying that *BhHsf1* is involved in multiple pro-
cesses of expression regulation. Many of them that have
been reported to be also induced by other Hsfs include
AtAPX2, *AtGolS1*, *MKP6.25*, and Hsps (Nishizawa et al.
2006; Ogawa et al. 2007). There were 11 Hsp genes that
were up-regulated in OE lines to up to 189-folds, including
genes encoding two Hsp70s and seven sHsp with molecular
weight around 16–30 kDa. sHsps and Hsp70 can function
as molecular champions preventing aggregation of dena-
tured proteins and refold stress-denatured proteins (Gait-
anaris et al. 1990; Goloubinoff et al. 1999; Sheffield et al.
1990). *AtAPX2* encodes cytosolic ascorbate peroxidase that
scavenges reactive oxygen species (Mittler et al. 2004).
AtGolS1 encodes galactinol synthase, the rate-limiting
enzyme of raffinose oligosaccharide synthesis, plays an
important role in compatible solute accumulation in
response to stress (Taji et al. 2002). *AtMKP6.25* encodes a
member of late embryogenesis abundant (LEA) proteins,
which accumulate in response to drought, freezing and salt
stress as protective molecules (Shao et al. 2005; Tunnac-
liffe and Wise 2007). *AtAPX2* and *AtGolS1* were both
induced by stresses such as HS, dehydration, and salt

Fig. 7 Growth rate and anatomical analysis of the fifth leaf of WT and *BhHsf1* OE *Arabidopsis*. a, b Kinetics of leaf growth. The length and width of the fifth leaves of WT and OE lines were examined. For each genotype, more than 12 leaves were continuously observed in 4-day intervals after emergence. Average values ± SE are shown. c–f Palisade cells of the fully expanded fifth leaves in WT and OE lines. The fifth leaves from 5-week-old plants were cleared and visualized under a microscope. *Bar* 50 μm. g Area of palisade cells of leaves in WT and OE lines. Five blades of the fifth leaf from each genotype were cleared, and at least 50 palisade cells were measured in each leaf under a microscope. Average values ± SD are shown. h Mesophyll cell number in WT and OE lines. The area of the leaf blade was multiplied by the number of palisade cells per unit area to calculate the total number of palisade cells in the subepidermal layer. Average values ± SD are shown
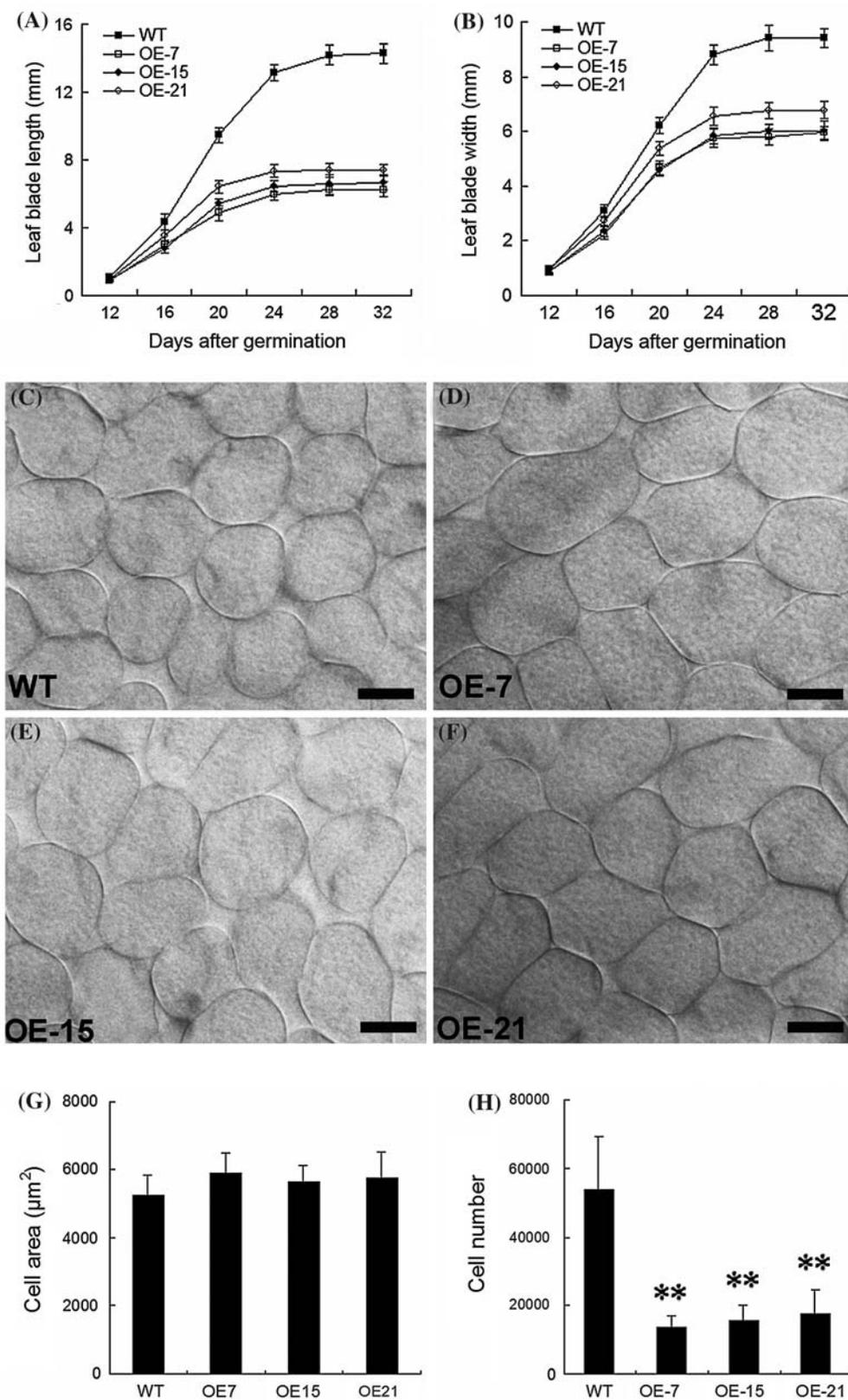
(Chang et al. 2004; Panikulangara et al. 2004). Beside, some other stress-inducible genes were also found to be up-regulated by *BhHsf1*. For example, genes encoding C2 domain-containing protein and glycine-rich protein have been reported to be induced by dehydration (Collett et al. 2004; Neale et al. 2000; Wang et al. 2009). These results
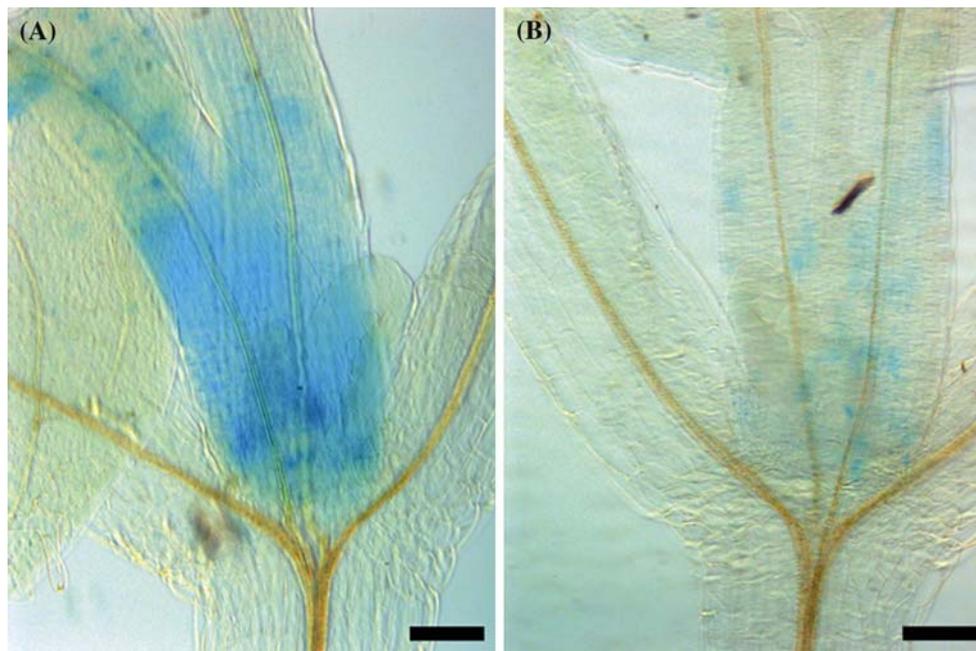
Fig. 8 *CYCB1;1:GUS* expression in young developing leaves of WT (**a**) and *35S:BhHsf1 Arabidopsis* lines (**b**), respectively. *Bars* 20 µm

suggested that *BhHsf1* may regulate a large group of stress related genes, which is in agreement with our observation that the *BhHsf1* OE lines conferred tolerance to heat stress. Five genes with different expression level were further analyzed by real time PCR and the results confirmed the microarray data (Fig. 10).

We also focused on the genes that were down-regulated in the *BhHsf1* over-expression lines (Table S2). Pathway analysis revealed that mitotic cell cycle and DNA replication are the pathways containing the largest number of down-regulated genes. For example, seven and five down-regulated genes were identified from OE-15 falling into pathways of mitotic cell cycle and DNA replication, respectively (Table 2). Although the induction levels of these genes were low, down-regulation of both pathways was statistically significant. Among the genes listed in Table 2, six were present in G1/S Transition and S phase and one was in G2/M Transition. The six genes in G1/S Transition and S phase also involved in DNA replication. Similar sets of genes were also down-regulated in OE-21 with fewer folds, which was consistent with the fact that dwarf phenotype and the expression level of *BhHsf1* in OE-15 is more significant than that in OE-21. The repression of *CDC45* and *MCM10* by BhHsf1 were further confirmed by real time PCR, which were down-regulated to 1.35 ± 0.11 and 1.44 ± 0.06 fold in OE15 when compared to that in WT, respectively. These results indicate that mitotic cell cycle might be blocked in *BhHsf1* OE plants.

BhHsf1 over-expression affects endoreduplication

During *Arabidopsis* leaf development, a certain degree of endoreduplication is generously observed. Molecular evidences have indicated that cell cycle was disturbed in *BhHsf1* OE lines due to deficiency in DNA replication, so we examined whether nuclear endoreduplication was affected in cells of 35S:*BhHsf1* transgenic *Arabidopsis*. Flow cytometric analyses with nuclei of fully expanded fifth leaves revealed differences in the polyploidy among cells of WT and OE lines. It was observed that the ploidy of WT and OE plant cells ranged from 2C to 16C. OE and WT possessed similar frequency of cells at 2C and 4C, however, OE lines showed declined frequency of cell numbers at the higher ploidy levels 8C and 16C (Fig. 11). These observations suggested that over-expression of *BhHsf1* in *Arabidopsis* impaired nuclear endoreduplication.

Discussion

BhHsf1 improves stress resistance

Hsfs play a critical role in response to heat stress in all organisms, especially in sessile plants (von Koskull-Doring et al. 2007). BhHsf1 comprise motifs such as DBD, HR-A/B, NLS and CTAD, which are the modular structures in Hsfs. The existence of 21 amino acids between HR-A/B and the putative AHA motifs enable us to group BhHsf1 into class
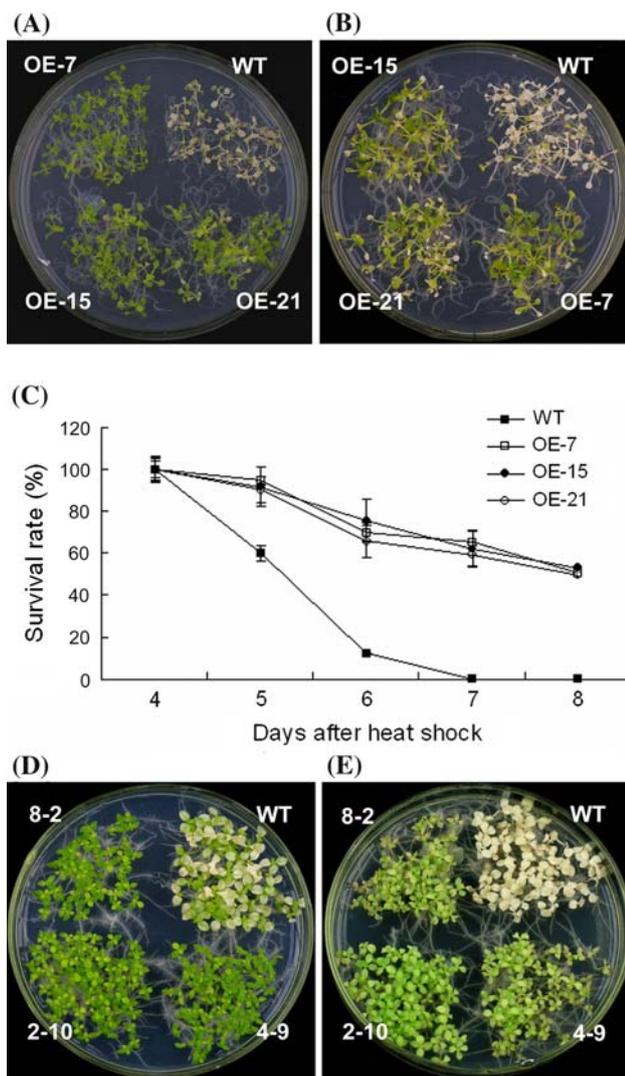
Fig. 9 Thermotolerance of WT and *35S:BhHsf1* transgenic *Arabidopsis* and tobacco plants. Comparison of acquired (**a**) and basal (**b**) thermotolerance among WT and transgenic *Arabidopsis* OE lines. **c** Percentages of the surviving transgenic *Arabidopsis* plants after HS stress at 45°C for 1 h. For each experiment, more than 60 plants were used; values are means ± SD from three independent measurements. Acquired (**d**) and basal (**e**) thermotolerance of WT and *35S:BhHsf1* transgenic tobacco lines

A. BhHsf1 possesses highest sequence similarity with HaHsfA9 and AtHsfA9, which expressed during seed development. Recent studies have suggested that DT in seeds may be adopted by resurrection plants to develop vegetative DT (Moore et al. 2009; Oliver et al. 2000). The relevant anagenesis of BhHsf1 with seed-specific HsfA9 and AtHsfA9 fits this hypothesis.

Previous studies have demonstrated a link between Hsfs and different environment stresses (Li et al. 2005; Nishizawa et al. 2006; Ogawa et al. 2007). In this study, evidence from Northern blot and GUS staining supports the link between *BhHsf1* and abiotic stress. Although no

significant drought-triggered changes of *BhHsf1* promoter activity was detected in *Arabidopsis*, which might be due to the potential flaw of the promoter-*GUS* expression system. The fact that *BhHsf1* transcript was induced by drought treatment in *B. hygrometrica* and the *proBhHsf1:GUS* activity was up-regulated by heat shock and wound suggests a role for this putative transcription factor in regulation of plant responses to these abiotic stresses. Besides, like its homologue *HaHsfA9*, *BhHsf1* is able to confer heat tolerance when over-expressed. Gene expression comparison between Hsf over-expression lines and WT had revealed many common genes that were up-regulated by BhHsf1 and other plant Hsfs that had been investigated. For example, *AtAPX2*, *AtGolS1*, *MKP6.25* and almost the same set of Hsps including *Hsp22.0-ER*, *Hsp18.1-CI*, *Hsp17.6B-CI*, *Hsp17.6C-CI*, *Hsp25.3-P*, *Hsp26.5-P*, *Hsp70* and *Hsp70T-2* were up-regulated by *AtHsfA2*, *AtHsfA3*, *OsHsfA2e* and *BhHsf1* (Nishizawa et al. 2006; Ogawa et al. 2007; Yokotani et al. 2008; Yoshida et al. 2008). HSEs are located in 5′ upstream region of all these genes, suggesting that they could be direct target genes of these Hsfs. This indicates the evolutionarily conserved mechanism of plant stress adaptation *via* Hsf-mediating activation of genes that function in ROS scavenging, osmo-adjust and macromolecular protection.

BhHsf1 leads to retarded organ growth

The growth of plant aerial organs is always arrested during stress conditions (Bartels and Sunkar 2005). It is considered that plants may preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief by inhibition of shoot growth (Osorio et al. 1998).

Over-expression of many stress-induced transcription factors resulted in enhanced stress tolerance and also growth retardation to some extent. For example, over-expression of *AtDREB2A* (Liu et al. 1998), *AtAREB1* (Furihata et al. 2006), *OsHsfA2e* (Yokotani et al. 2008) exhibited slightly retarded growth; over-expression of *AtDREB1A* (Kasuga et al. 1999), *AtHsfA3* (Yoshida et al. 2008) and *AtHsfA2* (Ogawa et al. 2007) led to severe dwarf phenotype. The function of these factors in stress resistance has been investigated intensively; however, the molecular and cellular mechanism underlying the growth inhibition by these factors was less studied.

When ectopically expressed *BhHsf1* in *Arabidopsis*, cell numbers decreased significantly in the leaves. In addition, other organs of *Arabidopsis* and tobacco, such as stems, siliques and flowers, also showed smaller sizes, suggesting that *BhHsf1* over-expression may generally decrease cell proliferation in aerial organs. GUS expression driven by the *BhHsf1* promoter was detected in the actively

Table 1 Up-regulated genes in *BhHsf1* OE plants compared with WT

AGI locus	Description	Fold change	*P* value
HSP			
AT4G10250*	Hsp22.0-ER	189.2	2.97E-06
AT4G27670*	Hsp25.3-P	92.8	2.19E-06
AT1G52560*	Hsp26.5-P	24.8	3.37E-04
AT5G59720*	Hsp18.1-CI	19.6	1.22E-04
AT3G12580*	Heat shock protein 70, putative/Hsp70	18.4	7.66E-04
AT1G53540*	Hsp17.6C-CI	14.7	1.80E-06
AT4G25200*	Hsp23.6-M	14.6	5.46E-06
AT2G20560	DNAJ heat shock family protein (Hsp40)	12.9	1.33E-04
AT2G32120*	Heat shock protein 70 family protein/Hsp70T-2	9.2	6.48E-05
AT2G03020*	Heat shock protein-related	9.1	1.41E-04
AT2G29500*	Hsp17.6B-CI	7.4	3.96E-05
Transcription factor			
AT5G65080	MADS-box family protein	13.0	5.59E-04
AT4G08590*	C3HC4-type RING finger	12.0	1.20E-05
AT5G58010	bHLH transcription factor GBOF-1	10.7	7.64E-05
AT1G01355	CCCH-type/C3HC4-type RING finger	9.9	3.73E-04
AT4G31615	Transcriptional factor B3 family protein	7.3	1.23E-03
Stress response			
AT3G28740*	Cytochrome P450 family protein	60.1	2.47E-05
AT3G09640*	APX2	18.7	1.52E-05
AT2G36750	UDP-glucosyl transferase family protein	18.4	1.79E-03
AT3G17520*	MKP6.25, an LEA domain-containing protein	16.9	3.46E-06
AT5G45080	Disease resistance protein-related	15.5	2.11E-04
AT4G12400*	Stress-inducible protein	8.6	6.91E-04
AT4G23680	Major latex protein-related	8.1	6.22E-04
Metabolism			
AT5G43300*	Glycerophosphoryl diester Phosphodiesterase family protein	36.9	2.40E-05
AT5G25450*	Ubiquinol-cytochrome C reductase complex 14 kDa protein	12.6	2.23E-04
AT2G29370	Tropinone reductase	10.4	7.22E-05
AT2G47180*	Galactinol synthase (GolS1)	7.9	8.96E-04
AT5G14860	UDP-glucoronosyl/UDP-glucosyl transferase	7.4	8.91E-04
Protein fate			
AT1G62290*	Aspartyl protease family protein	14.8	1.20E-04
AT5G12110*	Elongation factor 1B alpha-subunit 1 (eEF1B alpha1)	13.1	7.48E-05
AT5G48570*	Peptidyl-prolyl cis–trans isomerase	9.9	3.62E-04
AT2G23030	Protein kinase	9.9	1.92E-04
AT3G05780	Lon protease	9.4	1.36E-03
AT4G21230	Protein kinase family protein	9.0	1.83E-03
AT4G21323*	Subtilase family protein	7.9	2.75E-04
Cellular transport			
AT1G60970	Clathrin adaptor complex small chain family protein	41.7	5.52E-04
Signal transduction			
AT1G72660*	Developmentally regulated GTP-binding protein	13.6	5.98E-04
Unclassified protein			
AT1G60110	Jacalin lectin family protein	19.5	2.84E-04
AT1G60095	Jacalin lectin family protein	18.0	8.86E-05
AT5G50790	Nodulin MtN3 family protein	13.4	1.40E-03

Table 1 continued

AGI locus	Description	Fold change	P value
AT5G11100*	C2 domain-containing protein	13.3	1.41E-03
AT2G43660	Glycosyl hydrolase family protein 17	11.2	8.41E-04
AT5G13170	Nodulin MtN3 family protein	9.5	8.73E-07
AT2G05510	Glycine-rich protein	9.5	1.17E-03
AT1G72980	LOB domain family protein	9.3	1.47E-04
AT1G42110	Transposase	8.8	1.65E-03
AT1G12740	Cytochrome P450 family protein	8.2	2.29E-03
AT4G04260	Bromo-adjacent homology (BAH) Domain-containing protein	7.8	2.04E-03
AT5G03010	Kelch repeat-containing protein	7.5	3.38E-05
AT2G36540	NLI interacting factor (NIF) family protein	7.1	1.33E-04
Unknown protein			
AT5G07330*	Expressed protein	34.1	1.89E-05
AT4G23493*	Expressed protein	28.5	5.36E-06
AT1G66060	Hypothetical protein	19.5	2.31E-05
AT1G03070*	Expressed protein	11.1	7.58E-04
AT3G07150	Hypothetical protein	10.0	2.34E-04
AT1G17285	Expressed protein	8.8	3.75E-04
AT3G28270*	Expressed protein	7.0	1.04E-04
AT5G61080	Expressed protein	7.0	8.26E-04

Fold change indicates the average of up-regulation in both lines compared to WT. The genes marked with asterisks are those also up-regulated in plants over-expressing *AtHsfA2*, *AtHsfA3* and *OsHsfA2e* (Ogawa et al. 2007; Yokotani et al. 2008; Yoshida et al. 2008)
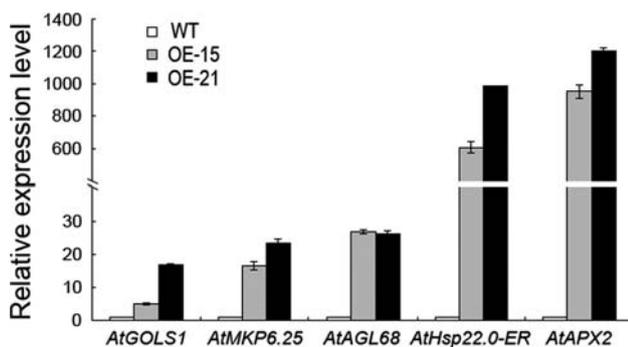
Fig. 10 Quantitative real time PCR analysis of *AtGOLS1*, *At-MKP6.25*, *AtAGL68*, *AtHsp22.0-ER* and *AtAPX2* in WT, OE-15 and OE-21 seedlings

proliferating tissues such as root tips, root hair differentiation zones, and leaf primordial, but was less in mature rosette leaves. The association of GUS expression with proliferating regions suggested a role of BhHsf1 in regulating cell proliferation. Considering its role as a transcription factor, BhHsf1 likely regulate cell cycle related genes to restrict cell proliferation. Genome-wide genes expression profile uncovered that the over-expression of *BhHsf1* repressed the genes involved in the pathways of the meiotic cell cycle and DNA replication, thus providing a strong evidence to support the role of BhHsf1 in regulation of cell proliferation via transcriptional regulation of target gene expression. We found the genes in these pathways were only slightly down-regulated, which could be explained that this type of genes are mainly limited in apical meristems and their meristem expression may be largely diluted in the sample of aerial part of the plants used for the microarray experiment. This is testified by the fact that *AtCYCB1;1:GUS* expression was decreased significantly in the actively proliferating zone of *35S:BhHsf1* lines, but *AtCYCB1;1* transcripts were not detected differently expressed in OE lines and WT in microarray analysis.

Previously, it was suggested that endoreduplication was coupled with cell size determination (Jing et al. 2009; Tsukaya 2008); however, controversial results were also reported (De Veylder et al. 2001; Jasinski et al. 2002; Schnittger et al. 2003). For example, over-expression of the cyclin-dependent kinase (CDK) inhibitor genes NtKIS1a and AtKRPs in *Arabidopsis* leaves resulted in inhibited endoreduplication but the cells were relatively larger (De Veylder et al. 2001; Jasinski et al. 2002). In this study, we found that the DNA replication was deficient and the cell division and endoreduplication were affected in BhHsf1 OE *Arabidopsis* lines; however, the cell size was not different from that in WT. Thus our data suggested a partial uncoupling of cell size and nuclear DNA content.

Table 2 Down-regulated cell cycle related genes in *BhHsf1* OE plants compared with WT

Genbank Accession	Description	Fold change		Cell cycle, mitotic	DNA replication
		OE-15	OE-21		
AT1G07270	Cell division control protein CDC6b	1.8	1.4	G1-S, S	+
AT1G44900	Similar to minichromosome maintenance protein 2 (MCM2)	1.6	1.0	G1-S, S	+
AT4G12620	Similar to origin recognition complex subunit 1 (ORC1B)	1.5	1.0	G1-S, S	+
AT3G25100	CDC45	1.7	1.5	G1-S, S	+
AT2G29570	Proliferating cell nuclear antigen 2 (PCNA2)	1.7	1.5	G1-S, S	+
AT2G20980	Similar to MCM10	1.5	1.4	G1-S, S	+
AT4G35620	CYCB2;2	1.6	1.0	G2-M	

The P values of the genes were less than 0.05
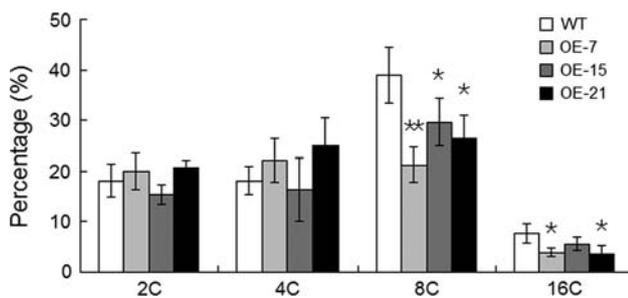
Fig. 11 Percentage of cells with different nuclear ploidy. Nuclear DNA Content was analyzed using flow cytometry in fully expanded fifth leaves of 32-day-old WT and BhHsf1 OE seedlings. Average values ± SDs are shown ($n = 3$)

The decline of cell proliferation is often observed when plants are exposed to drought, high salinity and freezing (Xiong and Zhu 2002). For example, mitotic and DNA-synthetic activities had declined when the moss *Polytrichum formosum* was subjected to dehydration (Mansour and Hallet 2001); soybean developed smaller leaves with less cell numbers under drought condition (Randall and Sinclair 1988). In plants, many components of the cell cycle machinery have been demonstrated to be conserved throughout the eukaryotes; however, it is not clear whether or how these proteins are regulated by stress response. Cyclin-dependent kinase (CDK) is a key factor in control of cell division (Cockcroft et al. 2000; West et al. 2004) and has been reported to be repressed during water stress (Setter and Flannigan 2001). CDK inhibitor ICK1 can block cells at G2/M but allow S-phase. The over-expression of ICK1 in *Arabidopsis* resulted in dwarf plants and enhanced drought tolerance (Kang et al. 2002; Wang et al. 1998, 2000).

The fact that several Hsfs from different plant species display dwarf phenotype suggests that the regulation of cell cycle may be another conserved mechanism of Hsfs to perform fundamental roles in the control of plant growth. According to our data on the stress-inducible expression and dual roles on enhancement of stress tolerance and growth retardation, it is possible that BhHsf1 may conduct signal transduction in both processes of stress response and growth inhibition, serving as positive regulator of genes related to stress tolerance and negative regulator of genes related to DNA replication and mitotic cell cycle synergistically. Therefore, it is likely that the rapid dehydration induction of *BhHsf1* may attribute to the fast acquisition of desiccation tolerance and quick cease of growth in *B. hygrometrica* upon water loss. However, whether this is the case in resurrection plants is still a misery due to the lack of efficient genetic transformation methods.

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