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ORIGINAL ARTICLE

Over-expression of *LlHsfA2b*, a lily heat shock transcription factor lacking trans-activation activity in yeast, can enhance tolerance to heat and oxidative stress in transgenic *Arabidopsis* seedlings

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Abstract Heat shock transcription factor (Hsf) is a central regulator in the heat signaling transduction pathway in eukaryotes. In this study, we isolated an *HsfA2* gene named *LlHsfA2b* from the leaves of lily (*Lilium longiflorum* 'White Heaven') using the rapid amplification of cDNA ends (RACE) technique. Multiple alignment and phylogenetic analyses showed that LlHsfA2b has critical domains of Hsf class A. Expression analyses revealed that *LlHsfA2b* could be induced by heat and H_2O_2, but not by NaCl, drought, or abscisic acid (ABA) treatments; moreover, the transcript of *LlHsfA2b* were induced by heat earlier than the one of *LlHsfA2* in lily. Following transient expression of *LlHsfA2b* in onion epidermal cells, GFP-LlHsfA2b was observed in the cell nucleus. Different from all known *HsfA2*s, *LlHsfA2b* failed to display trans-activation activity in yeast cell. In transgenic *Arabidopsis* overexpressing *LlHsfA2b*, the putative downstream genes of AtHsfA1d/e and AtHsfA2 were activated slightly under unstressed conditions. The transgenic *Arabidopsis* seedlings displayed enhanced tolerance to heat and oxidative stresses. The transient reporter assay exhibited that LllHsfA2b had a trans-activation activity in tobacco mesophyll protoplasts. At the same time, yeast two-hybrid assay showed that the putative LhHsfA2b interacted with either AtHsfA1d or AtHsfA2. In summary, our data reveal that *LlHsfA2b* is a novel gene associated with tolerance to heat and oxidative stress in lily, and is thought to function probably by directly activating downstream genes or by dimerization or trimerization with other HsfA protein harboring activation activity.

Keywords *Lilium longiflorum* · Heat shock transcription factor · Heat shock protein · Thermotolerance

Abbreviations

H_2O_2	Hydrogen peroxide
Apx	Ascorbate peroxidase
DAB	Diaminobenzidine
UTR	Untranslated region
RT-PCR	Reverse transcription polymerase chain reaction
Hsp	Heat shock protein
Hsf	Heat shock transcription factor

Introduction

Temperatures above their optimum can lead to growth retardation and even death of organisms. Similar to bacteria and animals, plants have evolved a series of defense responses to this adverse condition, in which the accumulation of heat shock proteins (Hsps) plays an important role, usually by sustaining the cellular protein homeostasis

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caused by heat shock (HS) (Wang et al. 2004; Kotak et al. 2007; Steven 2008; Liu and Charng 2012). Hsps are activated by heat shock transcription factors (Hsfs) through the binding of the latter to heat shock elements (HSEs), which are alternating units of the sequence (5′-AGAAnnTTCT-3′), in Hsp promoters (Wu 1995). Many studies have revealed that Hsfs play a central role in the heat signaling transduction pathway (Baniwal et al. 2004; Miller and Mittler 2006; Kotak et al. 2007; Von Koskull-Döring et al. 2007).

Cloning and characterization of *Hsf* were initially carried out in yeast (Sorger and Pelham 1988; Wiederrecht et al. 1988), and subsequently, the corresponding genes were cloned from *Drosophila* (Clos et al. 1990), mammals (Schuetz et al. 1991; Rabindran et al. 1991; Sarge et al. 1991) and tomato (Scharf et al. 1990). The identification of crystal structures of DNA binding domains (DBDs) accelerated the pace at which the Hsf modular structure was elucidated. Close to the N-terminus, DBD is the most conserved region for recognition and binding of HSEs, and consists of three helical bundles and four stranded antiparallel β-sheets (Damberger et al. 1994; Harrison et al. 1994; Vuister et al. 1994; Schultheiss et al. 1996). At the C-terminus of DBD, the oligomerization domain (OD), which is a hydrophobic region A/B (HR-A/B), contains a coiled-coil structure similar to that of the leucine-zipper-type protein interaction domains responsible for trimerization (Peteranderl and Nelson 1992; Peteranderl et al. 1999). In most cases, a nuclear localization signal, which is a cluster of residues rich in lysine and arginine, exists adjacent to HR-A/B (Lyck et al. 1997; Mattaj and Englmeier 1998). The C-terminal activation domain (CTAD) is the least conserved region in both sequence and size, is an acidic domain, and is enriched in aromatic, hydrophobic and acidic acids (AHAs) responsible for transactivation (Nover and Scharf 1997; Döring et al. 2000). At the C-terminus, the leucine-rich nuclear export signal (NES) and nuclear localization signal (NLS) are associated with cytoplasmic distribution of Hsf protein (Scharf et al. 1998; Heerklotz et al. 2001).

Hsf is widely recognized as having an important role in the heat signal transduction pathway in plants. Detailed genetic analysis (Mishra et al. 2002) showed that *HsfA1a* functions as a master regulator of heat shock response (HSR) by activating Hsps and other Hsfs in tomato (*Lycopersicon esculentum*). In *Arabidopsis*, overexpression of *HsfA1*a or *HsfA1b* caused constitutive expression of *Hsps* under normal conditions and led to enhanced basal thermotolerance (Lee et al. 1995; Prändl et al. 1998), whereas an *hsfA1a hsfA1b* double mutant exhibited a loss of thermotolerance (Lohmann et al. 2004). *GmHsfA1* was cloned and characterized from soybeans (*Glycine max*), and its overexpression was found to enhance the thermotolerance of transgenic soybeans (Zhu et al. 2006). *ZmHsfA3*, which is a direct target of dehydration-responsive element binding protein (DREB2A), was shown to play a key role in acquired thermotolerance in *Zea mays* (Qin et al. 2007). Intriguingly, several plant *Hsfs*, such as *HsfA2s* in tomato (Scharf et al. 1998) and *Arabidopsis* (Busch et al. 2005), are HS-inducible genes themselves (Nover et al. 2001). Tomato HsfA2 is seen exclusively after HS, and represents the dominant Hsf of the HS response when plants are subjected to repeated cycles of HS and recovery (Baniwal et al. 2004). Li et al. (2005) reported that overexpression of *AtHsfA2* elevated both heat and oxidative tolerance in transgenic *Arabidopsis*. More detailed analysis has shown that HsfA2 is a heat-inducible transactivator that sustains the expression of *Hsp* genes and extends the duration of acquired thermotolerance in *Arabidopsis* (Charng et al. 2007). Ectopic overexpression of *OsHsfA2e* led to increased thermotolerance in cotyledons, rosette leaves, inflorescence stems, and seeds in transgenic *Arabidopsis* (Yokotani et al. 2008). The lily is an important ornamental flower, which accounts for a large part of the worldwide cut-flower market (Sato and Milloshi 2006). In general, the lily is well adapted to cool conditions at about 18–22 °C. However, high temperatures may cause stagnation of the vegetative growth, diminished cut-flower quality, and even degeneration of the bulb. Most parts of China have high temperatures in summer, which are detrimental to lilies, and thus it would be beneficial for horticulturists if the tolerance of lilies to heat could be improved. Studies on heat tolerance and heat-inducible oxidative stresses in lilies have been restricted mainly to the response of the antioxidant enzyme system to heat (Yin et al. 2008). There has been even less investigation into lily thermotolerance at the molecular level (Xin et al. 2010; Gong et al. 2014).

Previously, *HsfA2* from *Lilium longiflorum* was cloned and characterized, and this gene may play an important role in the heat signaling transduction pathway in lily (Xin et al. 2010). In the current study, we carried out cloning and functional analysis of the *HsfA2b* gene, encoding another HsfA2 protein, from lily (*L. longiflorium* 'White Heaven'). The expression of *LlHsfA2b* responded to heat and H_2O_2 stresses. As a putative transcription factor, LlHsfA2b was observed in the nucleus, but lacked trans-activation activity in yeast. However, LlHsfA2b could activate the reporter of GUS driven by *AtHsp21* promoter, and the transgenic *Arabidopsis* seedlings overexpressing *LlHsfA2b* displayed enhanced tolerance to heat and oxidative stresses.

Materials and methods

Lily growth conditions and stress treatments

The *longiflorum* hybrid 'White Heaven' was cultured on Murashige and Skoog (MS) medium at 22 °C in a culture room with a photoperiod of 18 h light and 8 h dark.

For heat or drought treatment, 30-day-old lily explants were exposed to 37 °C or kept on filter papers on a super-clean bench for 2 h. For H_2O_2, NaCl or abscisic acid (ABA) treatment, 30-day-old lily explants were transferred to MS solution as control or MS solution containing 1 mM of H_2O_2, 250 mM of NaCl or 100 μM of ABA for 2 h.

To detect the expression of the target gene in various organs, leaves, stems and bulbs were sampled from five 30-day-old individual lily plants at 22 or 37 °C for 2 h.

For detection of gene expression levels at different time points during the HS process, triangular flasks carrying 30-day-old lily plants on MS medium were exposed to 37 °C for various times. In these assays, five plants were pooled together as a single independent replicate, and 0.1 g of each sample was used for total RNA extraction and reverse transcription. Each experiment was repeated three times.

Molecular cloning of HsfA2b from lily

Total RNA was extracted by Trizol reagent (Invitrogen, USA) from 0.1 g of leaves of 'White Heaven' explants incubated at 37 °C for 1 h, and 1 μg of total RNA were used to synthesize cDNA by Super Script II (Invitrogen). To obtain the 3′ sequence of the target gene, an adaptor primer AP1 was used for cDNA synthesis. The adaptor primer AP2 was then used to amplify the 3′ region by nested PCR using two degenerate primers, PF1 and PF2, which were designed according to the alignment of nucleotide sequences of well-known HsfA2s from *Arabidopsis*, tomato and rice. For the 5′ terminal sequence, a TaKaRa 5′-full RACE kit was used with three gene-specific primers. PR1 was used for cDNA synthesis as described above. Subsequently, PR2 and PR3 were used to amplify the 5′ terminus of the target gene using nested PCR with the two primers supplied with the kit. The coding region of *LlHsfA2b* was amplified using Primestar (TaKaRa) with two primers (P1 and P2). The PCR products were ligated onto PMD-18T and sequenced, then stored at −80 °C for further use. All primers used for gene cloning were listed in Table 1.

PCR was carried out in 20 μL reactions containing 2 μL of 10 × PCR buffer, 1.6 μL of 2.5 mM dNTPs, 0.4 μL of 20 μM primers, 0.2 μL of ExTaq (5 U/μL) and 1 μL of cDNA. The reaction conditions were as follows: 1 cycle at 94 °C for 5 min, 5 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min 30 s; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s; and 1 cycle of 72 °C for 10 min. The PCR products were ligated into PMD-18T (TaKaRa, Japan) for sequencing.

Table 1 Primers used for gene cloning

Primers	Sequences
AP1	CCGGATCCTCTAGAGCGGCCGCT17
AP2	CCGGATCCTCTAGAGCGGCCGC
PF1	AAGACGTAYGANATGGTGGANGA
PF2	ACTTCAAGCAYARCAAYTTCTC
PR1	GATGCTTCTGGCCTCTCAG
PR2	GCGAATTCCCATCTATCTGTT
PR3	AACCATAGGTATTGAGCTG
P1	CCCGGGATGAATCCACCGCACCAG
P2	GTCGACTCAACTTCCTTCCTCTTTC

Multiple alignment and phylogenetic tree analyses

Conserved DBD and HR-A/B portions (with the linker between them) of the deduced amino acid sequences were used for multiple alignments using Clustal X2.0 with default parameters. The phylogenetic tree was constructed using a neighbor-joining method by MEGA5 according to the alignment result.

Gene expression analysis by real-time RT-PCR in lily

Real-time RT-PCR was used to analyze the expression of *LlHsfA2b*. Total RNA was extracted by Trizol reagent (Invitrogen) from the samples described above. After treatment with DNaseI (Invitrogen), 0.1 μg of total RNA was sampled to synthesize cDNA using Superscript II (Invitrogen). Real-time RT-PCR was performed using TaKaRa SYBR Premix Ex Taq and an ABI 7500 system. The reaction was carried out in 20 μL reactions containing 10 μL of 2 × PCR mix, 0.2 μL each primer (10 μM), 1 μL of 10 × diluted cDNA and 8.6 μL of sterilized saline water under the following conditions: 1 cycle of 30 s at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C. Threshold cycle (Ct) was calculated using *LlActin* as an endogenous control. The primers are listed in Table 2.

Trans-activation activity analysis in yeast

The complete *LlHsfA2b* open reading frame (ORF) was sub-cloned between the *Sma*I and *Sal*I sites of pGBKT7 (Clontech). The recombinant plasmid was transformed into the yeast strain AH109 for HIS activity measurement. In contrast, the yeast strain harboring LlHsfA2 recombinant plasmid was used for a positive control.

Subcellular localization of LlHsfA2b

The open reading frame (ORF) region of *LlHsfA2b* was cloned into PUC18 vector at a *Sma*I site to fuse at the

Table 2 Primers used for qRT-PCR in this study

Gene names	Forward	Reverse
AtHsfA1d	GCACTCGAATGGACAGAACTCATC	CAGTGGACTGTTGCTGCTGTCT
AtHsfA1e	CAACAACCTCAAGTTCAGCACTC	CAAGTTCCTGCATAAGGACGTTC
AtHsfA2	TCTTGGAGTAATGGTCGTAACAGC	GCTATGCTTGAAGTAACGTGGAAG
AtHsfA7a	CATCGTATCTTGGAACAGAGGAG	GTGTTTGAAATGACGAGGAAGGAG
AtHsf70-5	TCCTCAGAACACTGTATTCGATGC	CAGTGGAGTATGTCACTCTGAAC
AtHsp25.3-P	GCTGCATCGGCTCTATGTTCAC	TTCCGTGGGAACGAAACCGAG
AtApx2	TCCGTGGTCTTATTGCCGAGAAG	CTCTTGGGGATGCCTTATCGTC
AtActin	GGTAACATTGTGCTCAGTGGTGG	CTTTCAGGTGGTGCAACGACCT
LlHsfA2	AAGCACGGCAACTTCTCCAG	TCCTTCAAGAGATTCTTCTG
LlHsfA2b	CGGGTAGTCTCGTGGAGCAG	AGGTATTGAGCTGGCGCACG
LlActin	GACAATGGAACTGGAATGGT	GGATTGAGCCTCATCTCCGA

C-terminal region of the GFP. The recombinant plasmids were introduced into onion epidermal cells using the particle bombardment method. In parallel, the empty vector was also transformed into the cells as a control. After incubation overnight, the GFP signal was observed under a TE2000-E (Nikon) confocal microscope.

Generation of transgenic *Arabidopsis*

After its sequence on PMD18-T was confirmed, the target fragment was digested by *Sam*I/*Sal*I and cloned into pCAMBIA 1301 under the control of a cauliflower mosaic virus 35S promoter. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* GV3101 by the freeze–thaw method.

Arabidopsis thaliana (ecotype; Col-0) was grown at 22 °C under long-day conditions (16 h light/8 h dark) for transformation and functional analysis of *LlHsfA2*b. A simplified floral dip protocol (Clough and Bent 1998) was used for *Arabidopsis* transformation. Mature seeds were harvested and sown on MS medium containing 50 μg/mL of hygromycin B. All seeds including wild-type were stratified at 4 °C for 2 days and transferred into a growth room at 22 °C under long-day conditions (16 h light/8 h dark). In total, 25 independent transgenic lines were obtained in this screening, and of these, three typical lines were used for further analysis.

Expression analysis of downstream genes by qRT-PCR in transgenic plants

Seeds of wild-type and transgenic plants were sown onto MS medium and transformed in the growth room after incubation at 4 °C for 2 days. Fifteen-day-old wild type or transgenic *Arabidopsis* plant was used for RNA extraction. Five plants were pooled together for one sample, and three independent replicates were carried out. DNA synthesis was carried out similarly to the description of the molecular cloning above. Realtime PCR was performed as described above. The primers used for reverse transcription PCR (RT-PCR) are shown in Table 2.

Measurement of ascorbate peroxidase activity

For transgenic and wild-type *Arabidopsis*, 50 2-week-old seedlings from each line were used for ascorbate peroxidase (Apx) activity measurement as described previously (Xin et al. 2010). Apx activity was determined by detecting the change in absorbance values at 290 nm (E = 2.8 mM/cm).

Investigation of the tolerance of transgenic plants to heat and oxidative stress

Four-day-old seedlings were incubated in a hot water bath at 45 °C for 60 min, and transferred to 22 °C. The number of surviving plants was recorded every day after heat treatment. After 8 days, these plants were photographed.

For oxidative treatment, 7-day-old seedlings were transformed on MS plates containing 0 and 20 μM Rose Bengal (RB), and photographed after another 7 days. At the same time, the root length of plants was measured.

Transient reporter assay in tobacco mesophyll protoplasts

Genomic DNA of *Arabidopsis* leaves was extracted using the genomic DNA extraction kit (TIANGEN). The promoter of *AtHsp21* was amplified using the genomic DNA (~50 ng) as a template. The primers used for promoter cloning was listed in Table 3. Except for 55 °C as annealing temperature, the PCR system and condition were same as the description above. The 948-bp promoter segment was ligated in front of the GUS gene by a *Bam*HI site in

the pGK-GUS vector. Transient reporter assay was accomplished according to the method described by Döring et al. (2000).

Yeast two-hybrid assay

A yeast two-hybrid kit (Clontech) was used to detect the interaction between LlHsfA2b and AtHsfAs. The complete *AtHsfA1d* and *AtHsfA2* ORFs were amplified (primers listed in Table 3) and sub-cloned at a *SmaI* site of pGADT7, respectively. The different combination of LlHsfA2b-pGBKT7 (as the bait) and AtHsfAs-pGADT7 (as the prey) were co-transformed into the yeast strain AH109 for activity analysis. The operation was carried out as described by the kit instruction.

Results

Molecular cloning and sequence analysis of *LlHsfA2b*

The cDNA of *LlHsfA2b* was 1,298 bp in length, containing 55 bp of 5′-UTR and 286 bp of 3′-UTR (Fig. 1a). This gene had a 945 bp ORF, which encodes a deduced protein of 315 amino acids with a predicted molecular weight (MW) of 36.39 kDa and an isoelectric point (PI) of 6.29.

Using the full-length amino acid sequence as a query, the NCBI BLAST search revealed that the deduced LlHsfA2b possessed a conserved DBD with a high degree of similarity to those of OsHsfA2d, OsHsfA2e, AtHsfA2 and LpHsfA2 (78.5, 76.3, 74.2 and 73.1%, respectively). Multiple alignment showed that LlHsfA2b contained all the conserved domains of HsfA (Fig. 1b). Of the HsfA2 members, LlHsfA2b was the nearest to OsHsfA2d, with a similarity of just 55.1%, implying that *LlHsfA2b* is a novel gene encoding HsfA2. Besides the conserved DBD, the deduced HsfA2b had other functional domains including OD, CTAB and NLS. In contrast to LlHsfA2, AtHsfA2 and LpHsfA2, which have two CTAB regions (highlighted in yellow and green in Fig. 1a, respectively), LlHsfA2b, similar to OsHsfA2e, had just one. In addition, unlike other Hsf A2 members, LlHsfA2b had no NES in its C-terminus (Fig. 1b). The protein structure of the deduced LlHsfA2b was depicted in comparison with LlHsfA2 in Fig. 2a.

To confirm the relationship between LlHsfA2b and other members of Hsf class A from tomato, *Arabidopsis*, and rice, a phylogenetic tree was constructed using DBD, HR-A, HR-B and the linker between HR-A and -B using Clustal (×1.83). As shown in Fig. 2b, a neighbor-joining tree showed that LlHsfA2b belonged to the Hsf A2 group and was nearer to OsHsfA2s.

LlHsfA2b transcript was induced by heat and H_2O_2, but not by salt, drought, or ABA

When exposed to 37 °C for 2 h, *LlHsfA2b* transcripts could be detected in roots, bulbs and leaves, and no obvious difference was observed in levels (Fig. 3a). As shown in Fig. 3b, the transcript of *LlHsfA2b* could be induced by heat and H_2O_2 stresses, but not by NaCl, drought or ABA treatments. Subsequently, the expression profile of *LlHsfA2b* in lily under heat treatment was investigated. In contrast to *LlHsfA2*, the time points of both the rise and peak of *LlHsfA2b* transcripts were earlier during the 37 °C treatment in lily (Fig. 3c).

LlHsfA2b lacked transactivation activity

Yeast one-hybrid system was used to detect whether LlHsfA2b had transactivation activity. On medium lacking histidine, the yeast transformed with the construct containing complete ORF of *LlHsfA2* but not *LlHsfA2b*, was able to survive (Fig. 4a).

LlHsfA2b was localized to the nucleus

The subcellular localization of LlHsfA2b was investigated by transient expression in onion epidermal cells. For GFP-LlHsfA2b, fluorescence was observed on cell membrane, in punctate structures and slightly in cytosol, however the highest was found in the nucleus; by contrast, the fluorescent signal of GFP alone was observed throughout the cells (Fig. 4b).

Table 3 Primers used for amplification of *AtHsfA1d* and *AtHsfA2* ORFs and *AtHsp21* promoter

Gene name	Forward	Reverse
AtHsfA1d	CATGGATGTGAGCAAAGTAAC	TCAAGGATTTTGCCTTGAGAGA
AtHsfA2	CATGGAAGAACTGAAAGTGG	TTAAGGTTCCGAACCAAGAAAAC
AtHsp21 Promoter	GGATTCCTCATATGTTTCTGCAA	GGATTCTGGTGACAAAGGATCCAAC

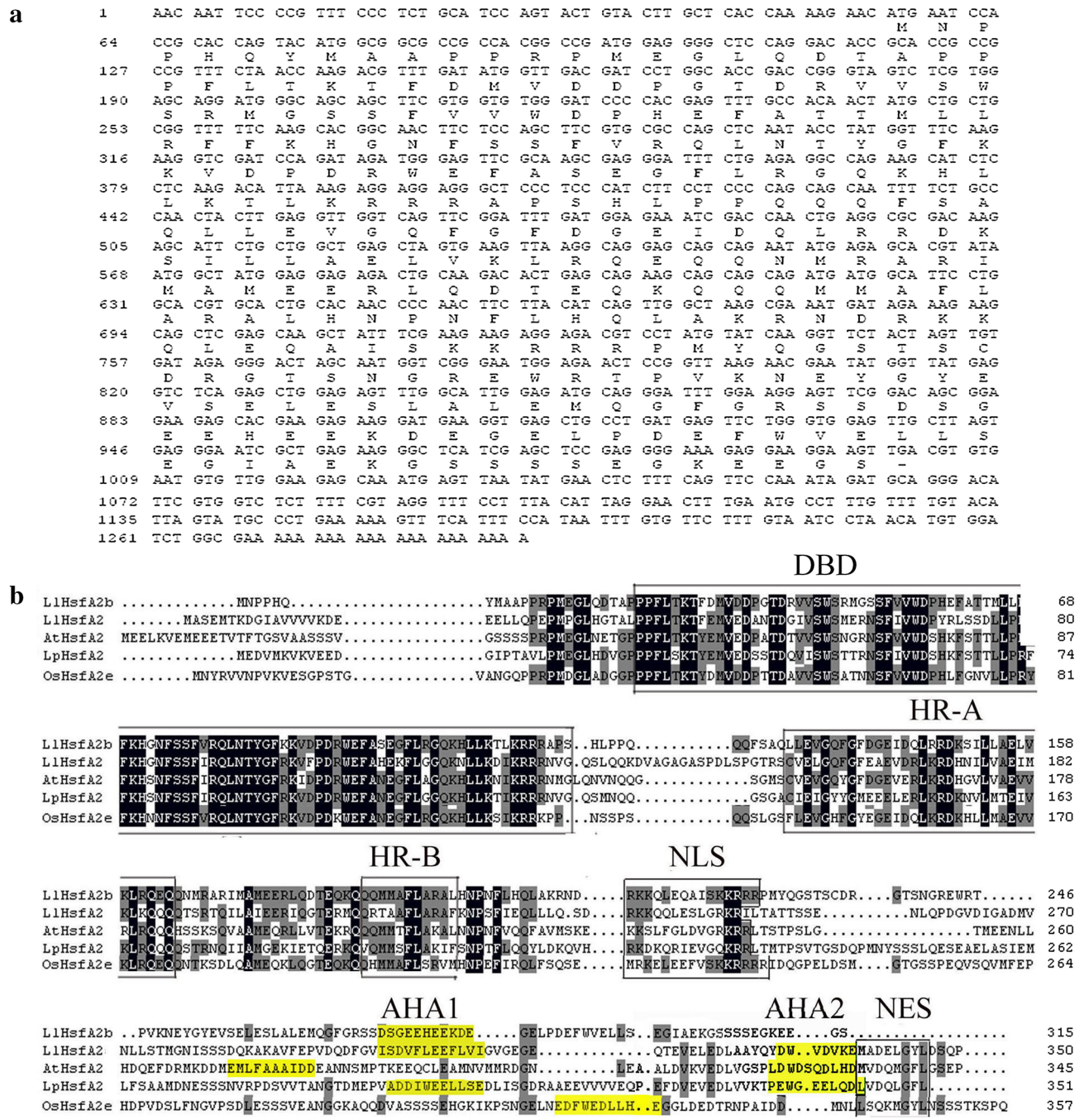


Fig. 1 Sequences analysis on *LlHsfA2b* and the predicted protein. **a** Sequences of *LlHsfA2b* and predicted protein encoded by *LlHsfA2b*; **b** multi-alignment of LlHsfA2b, LlHsfA2, LpHsfA2, AtHsfA2, and OsHsfA2e. Main domains and motifs are indicated by *square frames* with the captions above them

Overexpression of *LlHsfA2b* led to activation of downstrream genes of HsfA2 and increased Apx activity in transgenic *Arabidopsis*

Three independent transgenic *Arabidopsis* lines were used for further analyses. The expression levels of *AtHsfA2, AtHsfA7a* and the putative downstream genes of AtHsfA2, including *AtHsp70-5, AtHsp25.3-P* and *AtApx2*, in transgenic plants were found to be increased compared with the wild-type under unstressed conditions (Fig. 5a). Apx activity in transgenic plants was significantly higher than the one of wild-type (P-values <0.05; Fig. 6a).

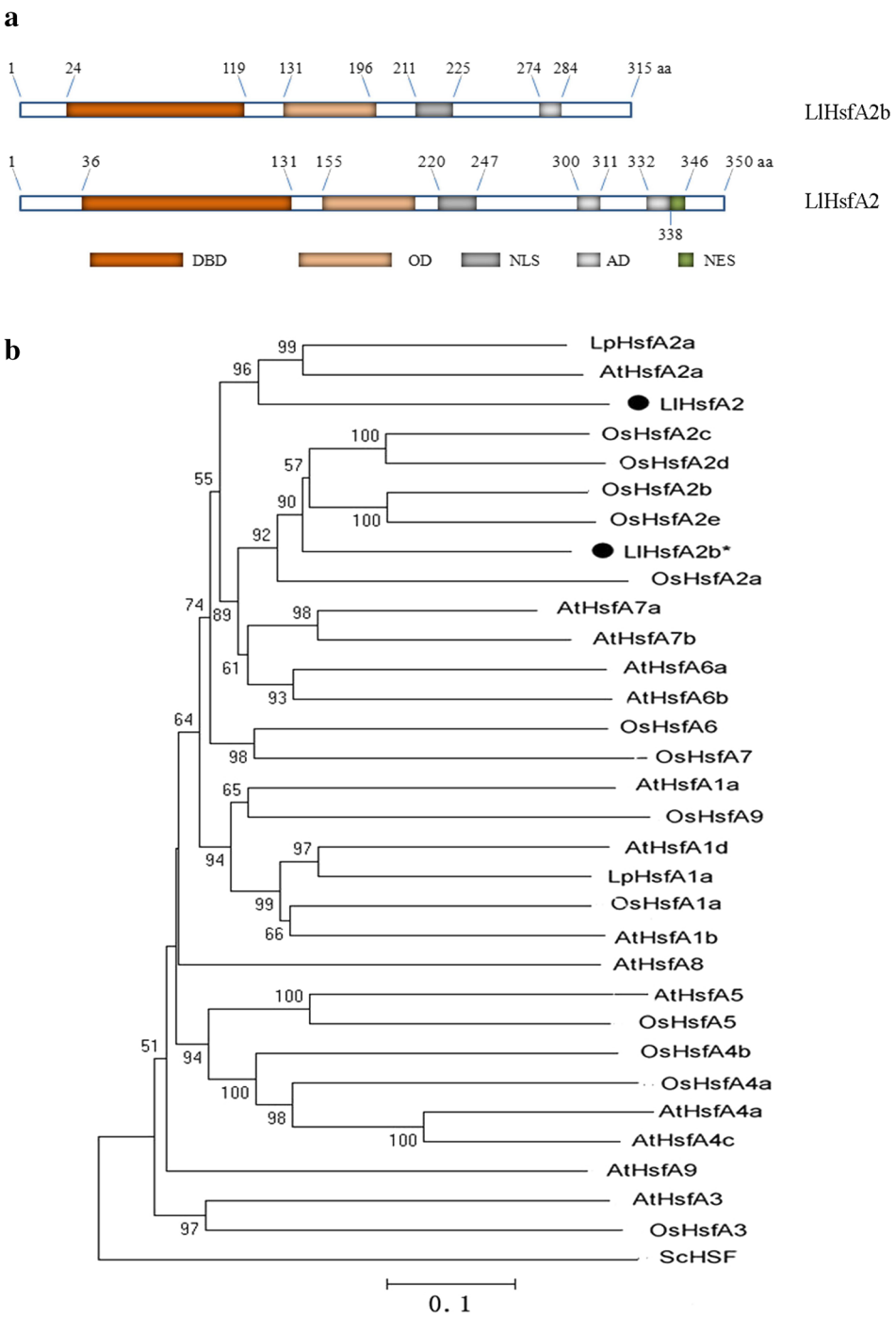


Fig. 2 Structure comparison of LlHsfA2b and LlHsfA2 and phylogenetic tree of HsfA2s. **a** The diagram of protein structures of LlHsfA2b and LlHsfA2. **b** Phylogenetic tree of LlHsfA2b, LlHsfA2, and other Hsf class A members: LlHsfA2 (ADM47610.1), AtHSFA1a (At4g17750); AtHSFA1b (At5g16820); AtHSFA1d (At1g32330); AtHSFA2a (At2g26150); AtHSFA3 (AT5g03720); AtHSFA4a (AT4g18880); AtHSFA4c (AT5g45710); AtHSFA5 (AT4g13980); AtHSFA6a (AT5g43840); AtHSFA6b (AT3g22830); AtHSFA7a (AT3g51910); AtHSFA7b (AT3g63350); AtHSFA8 (AT1g67970); AtHSFA9 (AT5g54070); LpHSFA1a (CAA47869); LpHSFA2a (CAA47870); OsHSFA1a (NP_001051938); OsHSFA2a (AAP13005); OsHSFA2b(NP_001059028); OsHSFA2c (NP_001064617); OsHSFA2d (NP_001049047); OsHSFA2e (NP_001051552);OsHSFA3 (NP_001047003); OsHSFA4a (NP_001044247); OsHSFA4b (NP_001056127);OsHSFA5 (NP_001046889); OsHSFA6 (NP_001057889); OsHSFA7 (NP_001043378); and OsHSFA9 (NP_001049429)

Overexpression of LlHsfA2b enhanced tolerance of transgenic *Arabidopsis* to heat and oxidative stress

Subsequently, the growth of transgenic plants was investigated under heat and oxidative stresses compared with normal conditions. All three lines overexpressing *LlHsfA2b* survived after treatment at 45 °C for 1 h, whereas wild-type plants did not (Fig. 5c). On day 8 after heat treatment, the survival rates of three independent transgenic lines were more than 80%, whereas those of wild-type plants were less than 20% (Fig. 5b).

In addition, transgenic plants could grow healthily on MS medium with 20 μM of RB, even though plant growth was found to be inhibited compared with plants on MS alone (Fig. 6b). The root lengths of transgenic

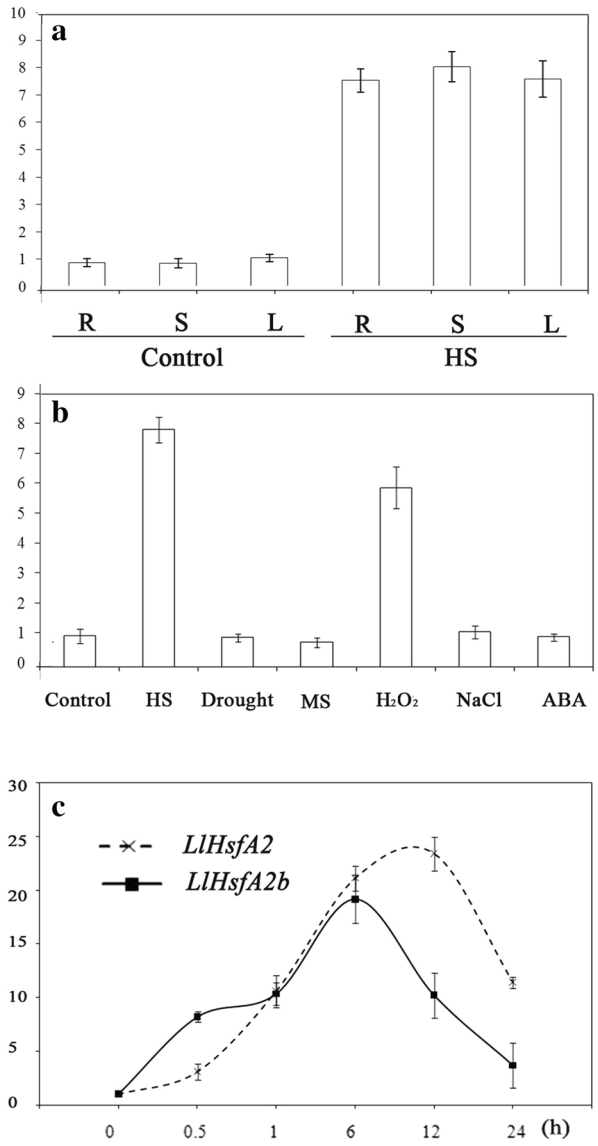


Fig. 3 Expression analyses of *LlHsfA2b* in lily. **a** Expression of *LlHsfA2b* in different organs under heat stress (*B* bulbs; *R* roots; *L* leaves; *C* control; *H* heat stress). **b** Expression pattern of *LlHsfA2b* induced by different stresses in leaves. **c** Relative expression levels of *LlHsfA2* and *LlHsfA2b* by real-time RT-PCR. *LlActin* was used as a loading control

plants were significantly longer than those of wild type when exposed to 20 μM RB (Fig. 6c).

Activator potential of LlHsfA2 and LlHsfA2b in GUS assays

As shown in Fig. 7a, in contrast to the control, both LlHsfA2 and LlHsfA2b could activate the GUS gene driven by *AtHsp21* promoter (Fig. 7a).

Interaction between LlHsfA2b and AtHsfA1d or AtHsfA2

The all transformed yeast cells could grow on the medium without Trp and Leu; however, just two groups of yeast cells carrying the combinations of either LlHsfA2b/AtHsfA1d or LlHsfA2b/AtHsfA2 could grow on the medium without Trp, Leu, His and Ade (Fig. 7b).

Discussion

In this study, a novel *HsfA2* was isolated from *L. longiflorum*. The deduced LlHsfA2b possessed all functional motifs except an obvious NES (Fig. 1b). An insertion of 21 amino acids was found between HR-A and HR-B, which is characteristic of HsfA, implying that this gene should be an HsfA member (Fig. 1b). Searching the protein database using the DBD, OD and their linker regions as a query showed that the putative HsfA2 matched best to HsfA2b-like protein from *Phoenix dactylifera*, and was thus designated LlHsfA2b. Phylogenetic tree analysis revealed that LlHsfA2b is most closely related to OsHsfA2a among the well-known HsfAs from *Arabidopsis*, rice and tomato; moreover, LlHsfA2 and LlHsfA2b were, respectively, divided into dicot and monocot groups, implying that these two genes diverged before occurrence of dicots and monocots (Fig. 2b).

A heat-inducible expression pattern is characteristic of known *HsfA2* members (Scharf et al. 1998; Busch et al. 2005; Li et al. 2005; Charng et al. 2007; Xin et al. 2010). As expected, in roots, bulbs and leaves, *LlHsfA2b* was found to be induced by heat treatments (Fig. 3a). It was also reported previously (Li et al. 2005) that *AtHsfA2* was induced by oxidative stress. As shown in Fig. 3b, *LlHsfA2b* was induced by 1 mM H_2O_2, which is consistent with previous results for *AtHsfA2* and *LlHsfA2* (Li et al. 2005; Xin et al. 2010). It was also observed that *AtHsfA2* was induced by osmotic stress (Ogawa et al. 2007), which imply that AtHsfA2 may be involved not only in heat but also in osmotic signaling pathways. As shown in Fig. 2b, however, transcripts of *LlHsfA2b* could not be induced when exposed to 250 mM NaCl for 2 h (Fig. 3a), which was unexpected. As described in a previous study (Qin et al. 2007), genes induced by salt are usually induced by drought and ABA also. However, transcript levels of *LlHsfA2b* in lily were not enhanced by drought and ABA treatments in the current study (Fig. 3b). These data suggest that *LlHsfA2b* may respond to heat signals and heat downstream signals such as reactive oxygen species rather than to salt or drought stressors. There is a possibility that, unlike *AtHsfA2, LlHsfA2b* is involved exclusively in the heat signaling pathway. In addition, the *LlHsfA2b* peaks were found to occur earlier

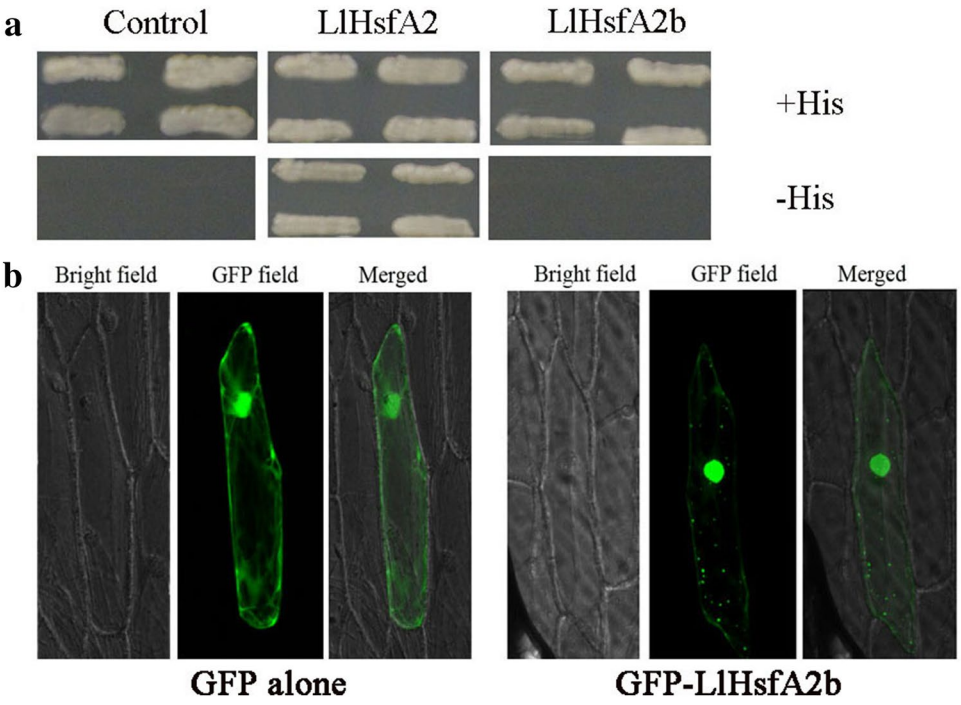


Fig. 4 Trans-activation activity and sub-cellular localization of the deduced LlHsfA2b. **a** Trans-activation activity of LlHsfA2s in yeast. **b** Sub-cellular localization of LlHsfA2b in onion epidermal cells

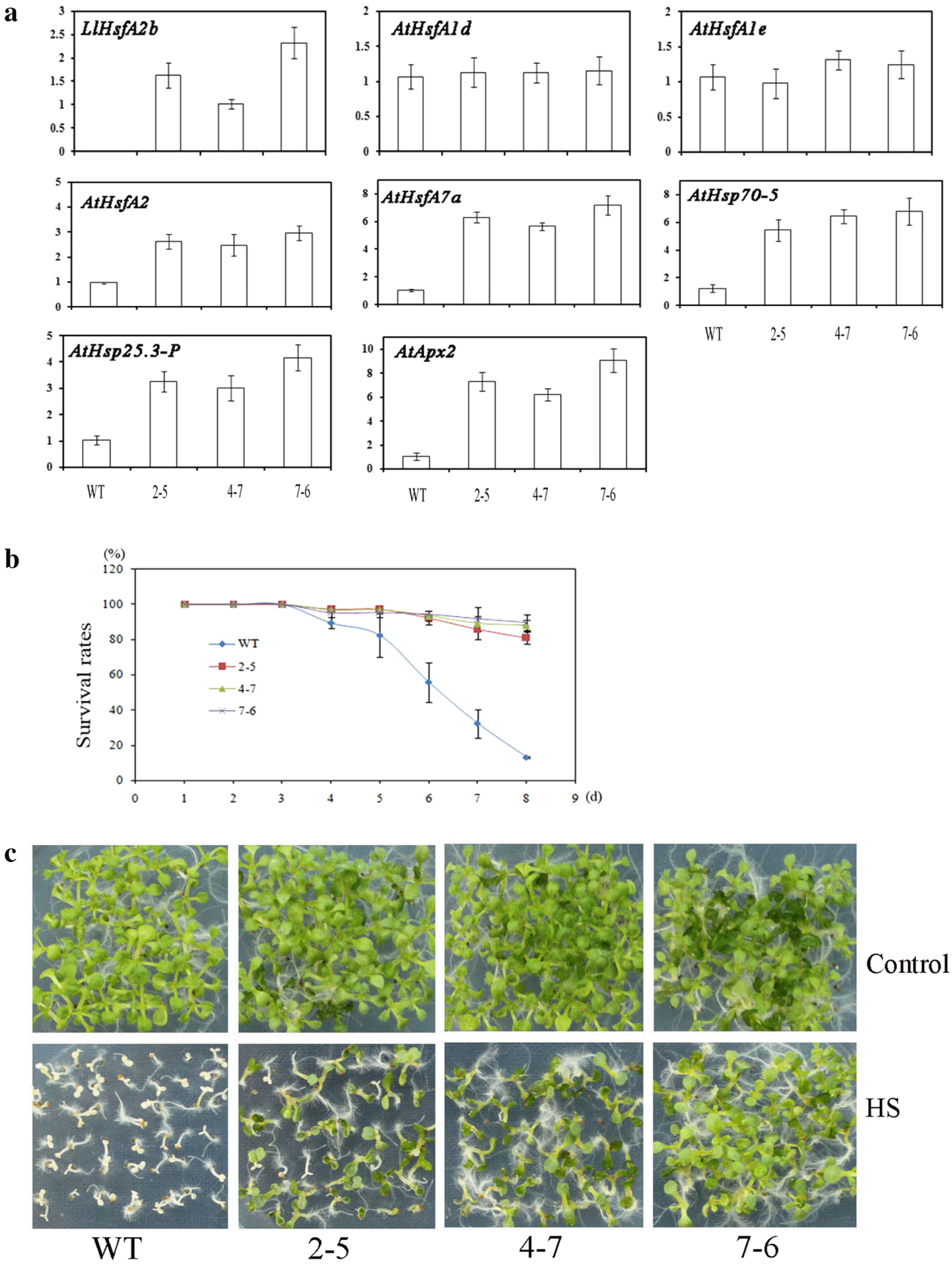
than the *LlHsfA2* peaks under heat treatment (Fig. 3c), which indicates that, in contrast to HsfA2, HsfA2b may be responsive to heat signals at an earlier stage of HS in lily 'White Heaven'.

In eukaryotes, it is necessary in the regulation of their downstream genes for transcription factors to contain nuclear localization signals that direct them to the nucleus (Whiteside and Goodbourn 1993). In this study, the stronger GFP-LlHsfA2b signals were detected in the nucleus compared with the empty vector (Fig. 4b), which is in agreement with the results for AtHsfA2 and OsHsfA2e (Li et al. 2005; Yokotani et al. 2008). The prolonged nuclear localization of LlHsfA2b might be caused by the deletion of NES, which is in agreement with the localization of AtHsfA2 with a mutational NES (Kotak et al. 2004). The slightly GFP-LlHsfA2b signals were observed on cell membrane and in the cytosol, which indicates that LlHsfA2b might play other roles except transcription regulation. In details functional analysis of LlHsfA2b would be carried out in the next step.

The function of class A Hsfs as transcription activator is mediated by the AHA motifs located their C-terminus (Scharf et al. 2012). AtHsfA2 (Kotak et al. 2004), OsHsfA2e (Yokotani et al. 2008) and LlHsfA2 (Xin et al. 2010) were active in yeast monohybrid assay. Kotak et al. (2004) found that the function of AtHsfA2 as a transcription activator was determined dominantly by W (Trp) and L (Leu) located in AHA2 motif. This result implies that W and L in AHA might play critical role on the activator function of AtHsfA. At an aspect of *trans*-activation domain, LlHsfA2b was like OsHsfA2e, and both of them had just one AHA. In this study, LlHsfA2b lacked the function of transcription activator in yeast monohybrid assay (Fig. 4a), and we speculated the phenomenon might be owing to an absence of W and L in its alone AHA motif (Fig. 1b).

To elucidate the detail function of LlHsfA2b, we conducted an ectopic expression of *LlHsfA2b* in *Arabidopsis*. After that, we found that ectopic expression of *LlHsfA2b* enhanced slightly the expression of *AtHsp70-5, AtHsp25.3-P* and *AtApx2* in the AtHsfA2 regulon under unstressed conditions (Fig. 5a). In *Arabidopsis*, the double knockout of AtHsfA1d/A1e led to the reduced induction of Hsfs A2 and A7a (Nishizawa-Yokoi et al. 2011), whereas the knockout of AtHsfA2 caused the reduced levels for *Hsp70-5, Hsp25.3-P* and *Apx2* (Charng et al. 2007). These results revealed the specific downstream genes of AtHsfA1d/A1e and AtHsfA2, respectively. In this study, the expression levels of *AtHsfA7a* and *AtHsfA2* belonging to the target genes of AtHsfA1d and AtHsfA1e were found to be increased in the transgenic plants. Moreover, the expression levels of *AtHsp70-5, AtHsp25.3-P and AtApx2* which located to the AtHsfA2 downstream were also enhanced. However, there were no significant differences at the transcription levels of *AtHsfA1d* and *AtHsfA1e* between transgenic and wild type plants (Fig. 5a).

To further analyze the function of *LlHsfA2b* in transformed plant, a transient assay was carried out in tobacco mesophyll protoplasts. LlHsfA2b as well as LlHsfA2 activated the GUS activity driven by *AtHsp21* promoter (Fig. 7a). These data showed that LlHsfA2b could


a
LlHsfA2b
AtHsfA1d
AtHsfA1e
AtHsfA2
AtHsfA7a
AtHsp70-5
AtHsp25.3-P
AtApx2
WT
2-5
4-7
7-6
b
(%)
Survival rates
WT
2-5
4-7
7-6
(d)
c
Control
HS
WT
2-5
4-7
7-6

◂**Fig. 5** Investigation on the expression of the putative HsfA2-related genes and thermotolerance in transgenic *Arabidopsis* plants overexpressing *LlHsfA2b*. **a** Expression levels of AtHsfAs and their putative downstream genes. **b** Survival rates of wild type or transgenic plants. Seeds of wild-type and transgenic *Arabidopsis* were sown onto MS plates. The plates containing 4-day-old seedlings were transferred to a water bath at 45 °C for 60 min, then were kept at 22 °C. *Error bars* represent SD, based on data from three independent replicates. **c** Enhanced thermotolerance of transgenic *Arabidopsis* overexpressing *LlHsfA2b*. These plants were photographed on 10 days after HS

activate directly the downstream genes. On the other hand, a yeast two-hybrid assay showed that the predicted LlHsfA2b could interact with either AtHsfA1d or AtHsfA2 (Fig. 7b). We concluded that LlHsfA2b could combine AtHsfA1s including AtHsfA1d to elevate the expression of *AtHsfA2* and *AtHsfA7a*, and then the increased AtHsfA2 led to the enhanced expression of its target genes. At the same time, LlHsfA2b could also activate the target genes of AtHsfA2 by the combination with AtHsfA2. The phenomenon that transcriptional factors (TFs) do not have activator activity but function by interacting with other TFs carrying activator activity has been found in *Arabidopsis*. The class B proteins of *Arabidopsis*, PISTILLATA (PI) and APETALA3 (AP3), do not have activator activity but activate downstream through the formation of complex with a class A protein AP1 (Honma and Goto 2001). Based on the analysis above, we speculated that LlHsfA2b may function as a TF to activate directly the downstream gene or through the interaction with other LlHsfAs harboring transactivation activity. *Lilium longiflorum*, however, has a bigger genome with estimated C-value of 35.2 (http://data.kew.org/cvalues) probably harboring more HsfA members. Which A members may interact LlHsfA2b to function in lily will be answered in the next step.

In summary, we report the isolation and characterization of a novel lily *HsfA2* gene from the lily cultivar 'White Heaven', designated *LlHsfA2b*. The divergence of LlHsfA2b and LlHsfA2 occurred before the one of dicots and monocots. Expression analysis confirmed that *LlHsfA2b* responded to heat and oxidative stresses and this action was earlier than the one of *LlHsfA2* in lily 'White Heaven'. *LlHsfA2b* could activate slightly the expression of downstream genes in lily heat signal pathway at early stage of HS and help lily to adopt the following change. These results elucidate the biological function of *LlHsfA2b*, which is at least partly different from the known *HsfA*2s. This finding should deepen our understanding of the transcriptional regulation of Hsfs, and provide a new gene to modify plant thermotolerance trait by transformation techniques.

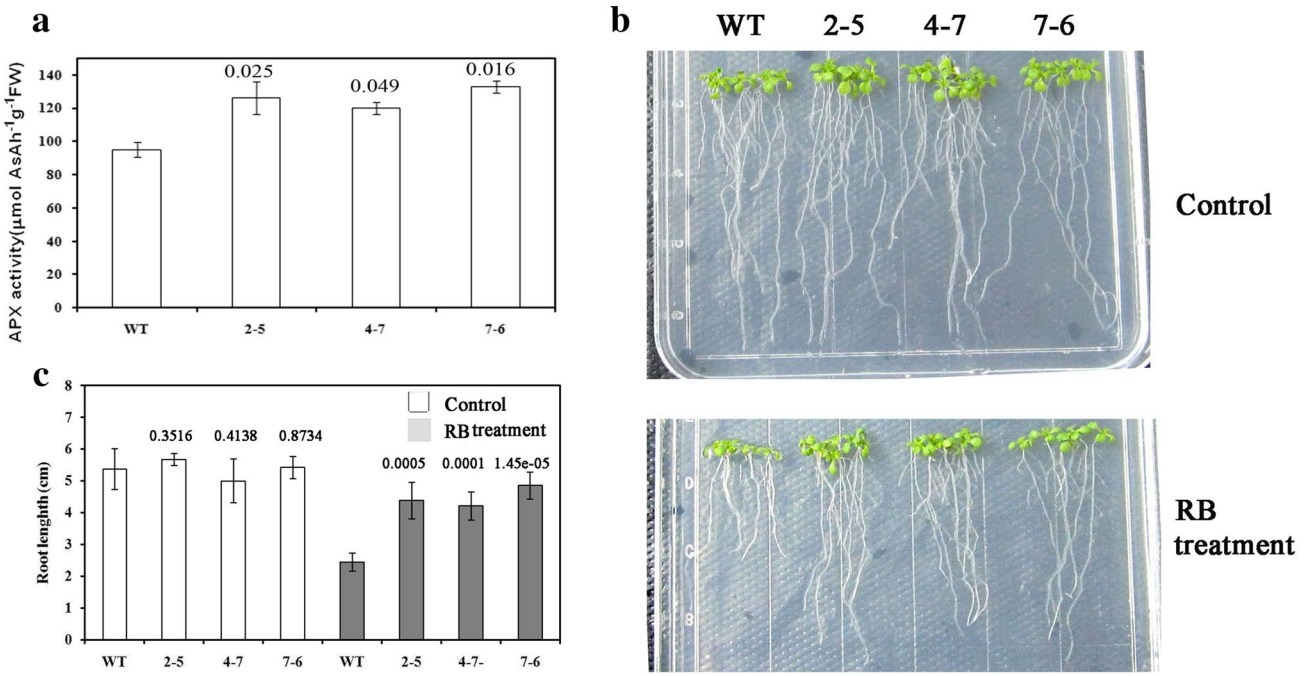


Fig. 6 Increased tolerance of transgenic *Arabidopsis* overexpressing *LlHsfA2b* to oxidative stress. **a** Apx activity of wild type and transgenic plants. **b** Tolerance of transgenic plant seedlings to oxidative stress. 7-day-old seedlings were transferred to MS with 20 μM RB and photographed on 7 days after treatment. **c** Root lengths of wild type and transgenic plants under RB treatment. The root lengths of 20 plants each sample were measured. The *numbers above the columns* are *P* values for comparisons between wild-type and corresponding transgenic plants by Student's *t* test

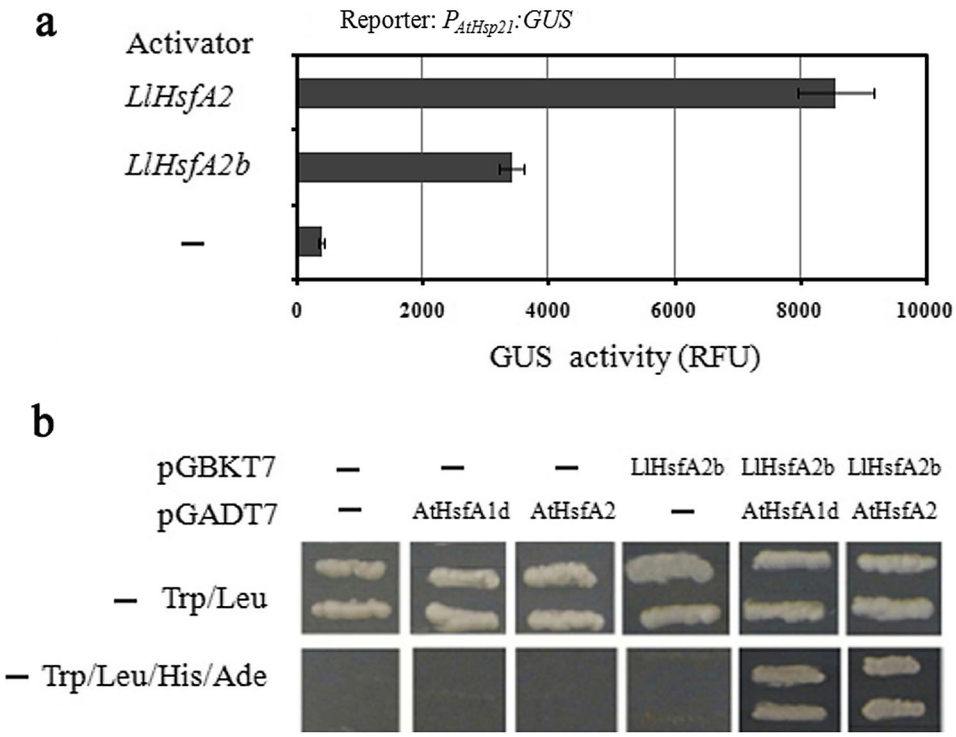


Fig. 7 Transient report assay and the interaction of LlHsfA2b with AtHsfA1d or AtHsfA2. **a** The activator potential of LlHsfA2b and LlHsfA2 was tested in tobacco protoplasts using *AtHsp21GUS* reporter. The GUS activities (rel. fluorescence units, RFU) were represented with three independent replicates. The *error bars* represented standard deviation. **b** The interaction of LlHsfA2b with either AtHsfA1d or AtHsfA2 was exhibited by growth on selection medium without Trp, Leu, His and Ade

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Author Contributions HBX and HZ performed the main experiments such as cloning and expression analysis of *LlHsfA2b, Arabidopsis* transformation, and phenotype analyses of transgenic *Arabidopsis* plants. QLL performed analysis on tranactivation activity and sub-cellular localization of the deduced protein. QLL and XHZ performed real-time PCR. XHZ and helped to analyze the data. AXD and LC helped to prepare the *Arabidopsis* for transformation. HBX wrote this manuscript. RCC and MFY designed the experiment and revised this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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