The diversity in interaction between HsfA2 and ACTIN leads to differences in heat stress responses among different lily varieties

Yue Wang#, Cunxu Song#, Shi Tong#, Yulei Guo, Xi Yang, Chunyan Li, Yanan Shao, Mingfang Yi and Junna He*

Beijing Key Laboratory of Development and Quality Control of Ornamental Crops, College of Horticulture, China Agricultural University, Beijing 100193, China
# Authors contributed equally: Yue Wang, Cunxu Song, Shi Tong
* Corresponding author, E-mail: hejunna@cau.edu.cn

Abstract

Lily is an important cut flower worldwide and prefers cool environments. The high summer temperature in China affects the quality and quantity of cut lily–flower production. In this study, the heat stress transcription factor HsfA2 was cloned from two cultivated commercial varieties, *Lilium longiflorum* 'White Heaven' and *Lilium* oriental 'Siberia', which have different responses to heat stress. An actin-interacting protein 3 (AIP3) domain was found in LlHsfA2 of *Lilium longiflorum* 'White Heaven' but did not appear in LoHsfA2 of *Lilium* oriental 'Siberia' using prediction from the simple modular architecture research tool (SMART) website. The genes *LlACTIN* and *LoACTIN* were cloned, and their amino acid sequences were found to be the same, so they were named *LACTIN*. There was an interaction between LlHsfA2 and LACTIN, whereas this interaction did not occur between LoHsfA2 and LACTIN based upon the bimolecular fluorescence complementary (BiFC) experiments. The silenced plants of *LlHsfA2* and *LACTIN* were sensitive to heat stress treatment using barley stripe mosaic virus (BSMV) induced gene silencing in 'White Heaven', and *LoHsfA2* silenced plants were also sensitive in 'Siberia'. In contrast, *LACTIN* silenced plants in 'Siberia' were normal. The expressions of *LlHsfA2* or *LACTIN* were reduced in the *LACTIN* or *LlHsfA2* silenced 'White heaven' plants, whereas the expressions of *LoHsfA2* or *LACTIN* were normal in the *LACTIN* or *LoHsfA2* silenced 'Siberia' plants. In conclusion, the diversity of protein interactions between HsfA2 and ACTIN may affect the distinctness of the heat stress response in different lily varieties, which provides new knowledge for further study on the heat stress response in lily.

Introduction

Global warming can directly change the environmental factors required for the growth and development of crops, resulting in changes in their maturity, growth cycle extension, and declines in yield[1–3]. The response of plants to climate change, especially high temperatures, requires more attention and research as plants grow in soil that cannot rid itself of heat. Plants have formed a relatively complex regulatory network to cope with heat stress based on recent research in model plants such as Arabidopsis and tomatoes[4,5]. The heat stress transcription factor–heat shock protein (Hsf–Hsp) pathway is important among the networks, and the Hsf transcription factor is critical in plant responses to heat stress[3,6].

During the heat stress response, Hsf directly regulates the expression of downstream response genes such as transcription factors, chaperone proteins, enzymes, etc.[3,5]. The Hsfs in plants have conserved domains and can be divided into three categories, including HsfA, HsfB, and HsfC, among which HsfA has the largest number and plays major roles in the heat stress response[6]. HsfA2 is only expressed in stress conditions and is induced most strongly by heat stress compared with other HsfAs[7–9]. Overexpression of *HsfA2* in Arabidopsis can improve the heat and anoxia tolerance of transgenic lines[8,10]. At the protein level, HsfA2 interacts with HsfA1 in the nucleus to activate heat stress response genes[11,12]. Recently, HsfA2 could activate the H3K27me3 demethylase relative of early flowering 6 (REF6), which represses HsfA2 and drives early flowering

transgenerational thermomemory in *Arabidopsis*[13]. In addition, HsfA3 could interact with HsfA2 to form heteromeric complexes and drive transcriptional memory after heat stress in *Arabidopsis*[14].

Actin is an important protein that widely exists in eukaryotic cells and forms microfilaments to maintain the stability of the cell's cytoskeleton structure to ensure that a cell can complete the normal physiological metabolism processes[15]. ACTIN is often used as a control gene to compare the different expressions of target genes in various conditions or tissues, as its stable expression makes it a housekeeping gene[16]. ACTIN genes have been cloned, and their function has been studied in many plants, such as Arabidopsis, rice, maize, soybean, etc.[17,18]. At the cellular level, actin can coordinate the establishment of cell polarity, participate in cell wall synthesis and plasma membrane stability, regulate plasmodesmata transport, and direct dynamic cytoplasmic streaming[19,20]. Actin also plays an important role in plant cell morphogenesis, including cell division, cell elongation and expansion, and stomatal movement[21–23]. There are many actin-interacting proteins, such as actin-binding protein (ABP), LIM domain-containing protein (LIM1), and villins (VLNs), that have been found in the research of pollen tube tip growth[24–26]. Although few reports involve actin in stress responses, several research studies showed that actin-interacting proteins could respond to different stresses. As an ABP, actin depolymerizing factors (ADFs) ADF7 and ADF4 were reported to be involved in osmotic and pathogen stress responses in Arabidopsis[27,28]. In addition,

members of the ADF family participate in different stress responses such as aluminum stress, osmotic, heat, cold, salinity, and drought in pigeon pea or Zea maize using genome-wide identification and qRT-PCR analysis[29,30]. These ABPs can regulate actin dynamics to respond to different stresses, which are also associated with the reactive oxygen species (ROS) and calcium pathways[31,32]. However, the molecular mechanism of actin in the plant heat stress response is rarely reported.

Lily is an important cut and potted flower worldwide and occupies an important part of the flower market[33,34]. Most lily varieties prefer cool environments based on their native habitat. However, most areas in China are becoming hotter in the summer overtime, which seriously affects the quality and annual production of cut lily flowers[35]. Therefore, improving the heat resistance of lily is a flower industry and scientific problem that needs to be solved urgently. Previously, we focused on the molecular mechanism of heat stress transcription factors regulating the lily heat stress response and cloned several *HsfAs* from *Lilium longiflorum* 'White Heaven', such as *LlHsfA1*, *LlHsfA2*, *LlHsfA3A*, *LlHsfA3B*, and *LlHsfA4*[12,36–38]. During this research process, we found that *Lilium longiflorum* 'White Heaven' showed more tolerance than *Lilium* oriental hybrids 'Siberia' with the same heat stress treatment; however, the regulatory mechanism of heat resistance differences between these two varieties has not been explored[37]. In this study, *HsfA2* was cloned from these two cultivars, and it was found that LlHsfA2 has an AIP3 domain from *Lilium longiflorum* 'White Heaven', but LoHsfA2 did not contain an AIP3 domain from the *Lilium* oriental hybrids 'Siberia'. Further, LlHsfA2 could interact with LACTIN, whereas LoHsfA2 could not interact. In addition, *LlHsfA2* or *LACTIN* silenced plants were sensitive to heat stress treatment using barley stripe mosaic virus (BSMV) induced gene silencing in 'White Heaven' and silenced LoHsfA2 were sensitive to heat stress in 'Siberia'. We found a new pathway by which HsfA2 regulates the plant heat stress response at the protein level and provided new theoretical support for solving the molecular mechanism of lily heat resistance.

Materials and methods

Plant materials and growth conditions

Longiflorum hybrid 'White heaven' (*Lilium longiflorum*) and Oriental hybrid 'Siberia' (*Lilium* oriental) commercial bulbous plants were cultured in pots containing peat and vermiculite (1:1) in a greenhouse with conditions of 25/20 °C with a 16 h light/8 h dark photoperiod. *Nicotiana benthamiana* plants were cultured in a greenhouse with the conditions of a 16 h light/8 h dark photoperiod at 22 °C.

Gene cloning and sequence analysis

Total RNA was extracted from 'White heaven' and 'Siberia' leaves using the RNAprep Pure Plant Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Then, reverse transcription of 1 μg of RNA using HiScript Q RT Super-Mix Kit (Vazyme, Nanjing, China). The full–length sequences of *LoHsfA2*, *LlACTIN*, and *LoACTIN* were cloned using reverse transcription production by designing primers (Supplemental Table S1) according to transcriptome sequence. A homologous comparison of amino acid sequences was performed using the DNAMAN (Version 7) software, and the conserved domain prediction was carried out using the DNAMAN (Version 7) software and the SMART website (http://smart.embl.de/).

Subcellular localization assay

The coding sequence of *LACTIN* were amplified by PCR using primers and then cloned into a pNC–Cam1304–subC vector to generate LACTIN–GFP, which was used for subcellular localization. LACTIN–GFP and 35S::GFP (as negative control) were transformed into *Agrobacterium tumefaciens* strain GV3101 and cultured in Luria–Bertani medium containing Kanamycin B selective antibiotics. The cultivated *Agrobacterium* was harvested using centrifugation at 3,000 g for 10 min and resuspended with the infection buffer (10 mM 2–(N–morpholino)–ethanesulfonic acid, 10 mM $MgCl_2$, 0.2 μM acetosyringone, pH 5.6) to a final concentration at OD_{600} = 1.0. Then mixed along with the silencing suppressor P19, placed in the dark for about 3 h, and infected into *Nicotiana benthamiana* plants with 4–5 young leaves. After 72 h, a Zeiss LSM710 META confocal microscope observed the fluorescence signal and green fluorescent protein (GFP) images were acquired at an excitation of 488 nm and emission of 525 nm[39]. The primer sequences used for subcellular localization are listed in Supplemental Table S2.

Bimolecular fluorescence complementation (BiFC) assay

The coding sequence of *LACTIN, LlHsfA2,* and *LoHsfA2* were amplified by PCR using primers containing *Spe*I and *Kpn*I sites and then cloned into pSPYCE (YCE) or pSPYNE (YNE) to generate YCE–*LACTIN,* YNE–*LlHsfA2,* and YNE–*LoHsfA2*. All vectors and their corresponding empty vectors (as negative controls) were transformed into *Agrobacterium tumefaciens* strain GV3101 and cultured in Luria–Bertani medium containing Kanamycin B selective antibiotics. The cultivated Agrobacterium was harvested using centrifugation at 3,000 g for 10 min and resuspended with the infection buffer (10 mM 2–(N–morpholino)–ethanesulfonic acid, 10 mM $MgCl_2$, 0.2 μM acetosyringone, pH 5.6) to a final concentration at OD_{600} = 1.0. Different suspension combinations were mixed along with the nuclear marker NF–YA4–mcherry and silencing suppressor P19, placed in the dark for about 3 h, and infected into *Nicotiana benthamiana* plants with 4–5 young leaves. After 72 h, a Zeiss LSM710 META confocal microscope observed the fluorescence signal and green fluorescent protein (GFP) images were acquired at an excitation of 488 nm and emission of 525 nm, and the mcherry images were acquired at an excitation of 543 nm and emission of 615 nm[39]. The primer sequences used for BiFC are listed in Supplemental Table S2.

Functional verification of HsfA2 and LACTIN in different lily varieties using the BSMV silencing system

The BSMV system was used to examine the function of HsfA2 and ACTIN in Longiflorum hybrid 'White heaven' and Oriental hybrid 'Siberia', which contains three vectors: pCaBS–α, pCaBS–β, and pCaBS–γ, and the targeted fragments were ligated to the pCaBS–γ[35,40]. The target fragments with a 262 bp length of the *LoHsfA2* gene and 236 bp length of the *LACTIN* gene were cloned into the pCaBS–γ ligation independent cloning (LIC) vector and transformed into *Agrobacterium* spp. strain EHA105. Agrobacterium cells containing pCaBS–α, pCaBS–β, pCaBS–γLIC, *LoHsfA2*–pCaBS–γLIC, or *LACTIN*–pCaBS–γLIC vectors were cultured in liquid Luria–Bertani medium containing Kanamycin B. The cultivated agrobacterium cells were collected using centrifugation at 3,000 g for 10 min and resuspended in the infecting buffer (10 mM 2–(N–morpholino)

ethanesulfonic acid, 10 mM MgCl$_2$, 0.2 μM acetosyringone, pH 5.6) to a final concentration at OD$_{600}$ = 1.0. Suspensions of pCaBS−α, pCaBS−β, with LoHsfA2−pCaBS−γLIC, LACTIN−pCaBS−γLIC, or pCaBS−γLIC were mixed according to a ratio of 1:1:1 and placed in dark conditions at room temperature for 3 h. The mixed suspension was injected into different leaves of cultivated varieties using syringes without needles; then, the upper leaves were collected to verify whether the gene was silenced after injecting 10 d. All silenced plants were used to examine the heat stress phenotype by treating them at 42 °C for 24 h in a light incubator and then allowing them to recover at 22−23 °C for 1 d. Photographs were taken. The primers used are listed in Supplemental Table S2.

The degree of wilting of the silent strain and the control group was counted according to the criterion of drooping or not drooping of the upper stem leaf bases, and a plant with more than 80% of the upper stem leaf bases drooping was considered to be wilted, and the formula for calculating the percentage of wilting was: Percentage of wilting = (no. of wilted plants/total no. of plants) × 100%.

Gene expression

qRT−PCR was used to examine the expressions of HsfA2 and ACTIN in the silenced plants and analyze the expression levels of HsfA2 and ACTIN in ACTIN−silenced or HsfA2−silenced plants. Leaves of different commercial bulbous plants were collected 10 d after injecting, and RNA was extracted and reverse transcripted for real−time quantitative RT-PCR. The 18S rRNA was used as an internal control, which has been validated in our previous studies. The analysis of qRT−PCR results was performed using the 2$^{-\Delta\Delta Ct}$ method. Each experiment included three technical replicates, and two biological tests were performed; at least one result is shown in the study. All relevant primers are listed in Supplemental Table S3.

Results

Molecular cloning and protein domain analysis of HsfA2 in 'White Heaven' and 'Siberia'

HsfA2 plays an important role in the heat−stress response of plants as an important heat stress transcription factor whose expression is induced rapidly and abundantly by heat[7,9,36]. In order to perform functional analysis and comparison of HsfA2 in different lily varieties, Lilium longiflorum 'White Heaven' and Lilium oriental hybrids 'Siberia' cultivars were used according to their different heat response phenotypes. LlHsfA2 has already been cloned from 'White Heaven' in previous studies[36]. The coding sequence of LoHsfA2 was cloned from the cDNA of 'Siberia' by designing primers according to the transcriptome sequence. The full length of LoHsfA2 was 1,077 bp, encoding a 358 amino acid protein.

Amino acid sequence alignment between LlHsfA2 and LoHsfA2 showed that there were only some differing amino acids between the LlHsfA2 and LoHsfA2 protein sequences (Fig. 1a). The overall homology of LlHsfA2 and LoHsfA2 was high, and the conserved domains in Hsfs, such as the DNA binding domain (DBD), oligomeric domain (HR−A/B), nuclear localization signal (NLS), and activation domain (AHA) and nuclear export signal (NES) are close to each other (Fig. 1a). The SMART website was used to analyze the specific domains in LlHsfA2 and LoHsfA2. The results showed that the AIP3 domain existed
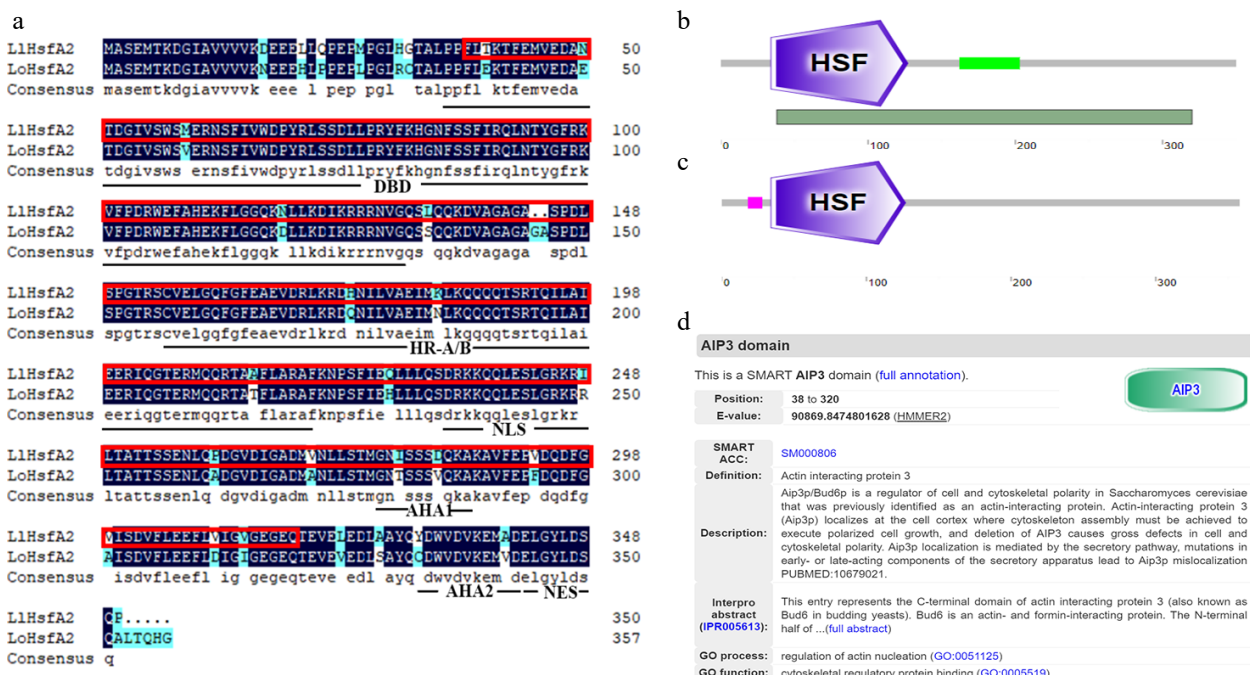

Fig. 1 Amino acid sequence analysis of HsfA2 in 'White Heaven' and 'Siberia'. (a) Amino acid sequence alignment between LlHsfA2 and LoHsfA2. The red boxes indicate the AIP3 domain. DBD, DNA binding domain; HR-A/B: Oligomerization domain; NLS: Nuclear localization sequence; AHA1/AHA2: Activation domains; NES: Nuclear export sequence. (b) The SMART website predicted that LlHsfA2 of 'White Heaven' would contain the HSF and AIP3 domain, and the position of the AIP3 domain was from 38 to 320 amino acids. The dark green bar below represents the location of the AIP3 domain. (c) The SMART website predicted that LoHsfA2 of 'Siberia' only contains the HSF domain. (d) The SMART website comments on the AIP3 domain.

Wang et al. Ornamental Plant Research 2024, 4: e011

Page 3 of 10

in LlHsfA2 from 'White Heaven' (Fig. 1b, d), whereas the same domain was not found in LoHsfA2 from 'Siberia' (Fig. 1c). These results indicate that differences in several amino acids might result in domain diversity between homologous genes from different cultivars.

Molecular cloning and sequence analysis of ACTIN in 'White Heaven' and 'Siberia'

The AIP3 domain in the protein can bind to ACTIN to affect the interaction between proteins in the relevant studies of *Saccharomyces cerevisiae*[41]. Therefore, we speculate that LlHsfA2 may interact with LlACTIN to regulate the heat stress response in lily. *LlACTIN* and *LoACTIN* were cloned by designing primers according to the transcriptome sequence[35,42]. *LlACTIN* was cloned from the cDNA of 'White Heaven', which contains a full length of 1,134 bp and encodes a 377 amino acid protein. *LoACTIN* was cloned from the cDNA of 'Siberia', which contains a full length of 1,134 bp and encodes a 377 amino acid protein. The amino acid sequences of LlACTIN and LoACTIN were homogenously compared. The results showed that the amino acid sequences of LlACTIN and LoACTIN are highly consistent and similar, indicating that the ACTINs are highly conserved in different lily cultivars (Fig. 2a). The phylogenetic tree analysis showed that LACTIN was closest to ACTIN in *Tulipa gesneriana*, *Diospyros lotus*, *Triadica sebifera*, and *Vaccinium dunalianum* (Fig. 2b).

HsfA2 interacts with ACTIN in 'White Heaven' but not in 'Siberia'

In a previous study we found that LlHsfA2 was localized in the nucleus[12]. Here, we explored the subcellular localization of LACTIN. LACTIN was inserted into a binary vector containing an eGFP, transformed into *Agrobacterium*, and injected into *N. benthamiana* to observe the fluorescent signal. The green fluorescence of LACTIN–GFP was observed in the nucleus and cytoplasm (Fig. 3a). These results indicated that LACTIN was a nucleoplasmic co-localized protein. BiFC was used to verify whether different HsfA2s and ACTINs from 'White Heaven' and 'Siberia' interacted with each other, respectively. HsfA2 and ACTIN of two varieties were inserted into YCE and YNE vectors, transformed into *Agrobacterium*, and injected into *N. benthamiana* to observe the fluorescent signal. The results showed that in the combination of LlHsfA2 and LACTIN, the green fluorescence was found in the nucleus and overlapped with the red fluorescence of the nuclear localization marker NF–YA4–mCherry. However, GFP was not observed in the combined LoHsfA2 and LACTIN infection or the control cells (Fig. 3b). These results suggested that LlHsfA2 interacted with LACTIN in the cell nucleus, but LoHsfA2 and LACTIN could not interact with each other.

Virus-induced gene silencing of *HsfA2* and *ACTIN* in two varieties

In order to verify whether the interaction between LlHsfA2 and LACTIN is involved in the heat stress response in lily, virus-induced gene silencing was used to silence *HsfA2* and *ACTIN* in 'White Heaven' and 'Siberia', respectively, and the heat stress phenotypes of the silenced plants were observed. In a previous study, we silenced *LlHsfA2* in 'White Heaven' and found that the silenced strains were sensitive to heat stress treatment[35]. Therefore, *LoHsfA2* and *LACTIN* were silenced in 'White Heaven' or 'Siberia' using BSMV in this study. *LoHsfA2* and *LACTIN* fragments were inserted into BSMV vectors to silence these

genes in lily. Using the silencing system, each gene obtained five silenced strains from 20 infected plants and showed the result of two strains. The expressions of *LoHsfA2* in 'Siberia' (Fig. 4b), *LACTIN* in 'White Heaven' (Fig. 4d), and *LACTIN* in 'Siberia' (Fig. 4f) were decreased compared with their expression in the control plants based on qRT–PCR analysis. All silenced strains were treated at 42 °C for 24 h and allowed to recover for 1 d at room temperature. Compared with the control plants, there was obvious wilting in the leaves of *LoHsfA2* silenced 'Siberia' and *LACTIN* silenced 'White Heaven' (Fig. 4a, c & g), whereas the *LACTIN* silenced 'Siberia' did not have a different phenotype (Fig. 4e, g). These results indicated that silencing of *HsfA2* decreased the thermal tolerance of lilies, and *LACTIN* silencing decreased the thermal tolerance of 'White Heaven', whereas silencing of *LACTIN* did not affect the thermal tolerance of 'Siberia'.

Silencing of *ACTIN* or *HsfA2* affected the expression of each other in 'White Heaven'

In order to examine whether *HsfA2* and *ACTIN* affect each other's expression at the gene level, qRT–PCR was used to detect the expression levels of *ACTIN* and *HsfA2* in the *HsfA2* or *ACTIN*–silenced lines of 'White Heaven' and 'Siberia'. The results showed that the expression of *HsfA2* and *ACTIN* showed a significant change in the *ACTIN* and *HsfA2* silenced 'White Heaven' lines (Fig. 5a, b) but not in the 'Siberia' lines (Fig. 5c, d). Therefore, *HsfA2* and *ACTIN* also affected each other's expression in 'White Heaven' at the gene level. This regulatory relationship may form a positive feedback regulation to mediate heat tolerance and play an important role in the heat stress response of 'White Heaven'.

Discussion

When plants are subjected to heat stress in the environment, many transcription factors are induced to regulate the heat stress response and induce the expression of corresponding stress proteins, thus enhancing the heat tolerance of plants in all aspects[6]. Heat stress transcription factors play an important role in all levels of the heat stress response network and complete strict regulatory processes through the Hsf–Hsp pathway[5,43,44]. In this study, we found that LlHsfA2 from 'White Heaven' could interact with ACTIN, which contains the AIP3 domain (Figs 1 & 3b), indicating that HsfA2 regulates the heat stress response through other pathways more than the Hsf–Hsp pathway.

The protein HsfA2 and ACTIN are conserved in different lily varieties, 'White Heaven' and 'Siberia'

HsfA2 plays an important role in plant response to heat stress; in the early stage of the heat stress response, HsfA1 regulates the expression of *HsfA2*, then HsfA2 interacts with HsfA1 at the protein level to regulate other heat stress responses[7,8,36]. In a long-term heat stress environment, *HsfA2* was induced highly and accumulated continually, which is an enhancer of acquired thermal tolerance[9]. In this study, we cloned LoHsfA2 from *Lilium* oriental 'Siberia' and found it was highly homologous with the LlHsfA2 from *Lilium longiflorum* 'White Heaven'[36] (Fig. 1a), indicating that HsfA2 was conserved in these two varieties. There were only a few amino acid sequences that differed, which caused the presence or absence of the AIP3 domain (Fig. 1). This means that LlHsfA2 contains the AIP3 domain, and LoHsfA2 does not. LlHsfA2 could interact

a

L1ACTIN	MADAEDIQPLVCDNGTGMVKAGFAGDDAPRAVFPSIVGRPRHTGVMVGMG	50
LoACTIN	MADAEDIQPLVCDNGTGMVKAGFAGDDAPRAVFPSIVGRPRHTGVMVGMG	50
Consensus	madaediqplvcdngtgmvkagfagddapravfpsivgrprhtgvmvgmg	

L1ACTIN	QKDAYVGDEAQSKRGILTLKYPIEHGIVSNWDDMEKIWHHTFYNELRVAP	100
LoACTIN	QKDAYVGDEAQSKRGILTLKYPIEHGIVSNWDDMEKIWHHTFYNELRVAP	100
Consensus	qkdayvgdeaqskrgiltlkypiehgivsnwddmekiwhhtfynelrvap	

L1ACTIN	EEHPVLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRT	150
LoACTIN	EEHPVLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRT	150
Consensus	eehpvllteaplnpkanrekmtqimfetfnvpamyvaiqavlslyasgrt	

L1ACTIN	TGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTDSLMKILTERGY	200
LoACTIN	TGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTDSLMKILTERGY	200
Consensus	tgivldsgdgvshtvpiyegyalphailrldlagrdltdslmkiltergy	

L1ACTIN	MFTTTAEREIVRDIKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVI	250
LoACTIN	MFTTTAEREIVRDIKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVI	250
Consensus	mftttaereivrdikeklayvaldyeqeletaksssssveknyelpdgqvi	

L1ACTIN	TIGAERFRCPEVLFQPSLIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIV	300
LoACTIN	TIGAERFRCPEVLFQPSLIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIV	300
Consensus	tigaerfrcpevlfqpsligmeaagihettynsimkcdvdirkdlygniv	

L1ACTIN	LSGGSTMFPGIADRMSKEITALAPSSMKIKVVAPPERKYSVWIGGSILAS	350
LoACTIN	LSGGSTMFPGIADRMSKEITALAPSSMKIKVVAPPERKYSVWIGGSILAS	350
Consensus	lsggstmfpgiadrmskeitalapssmkikvvapperkysvwiggsilas	

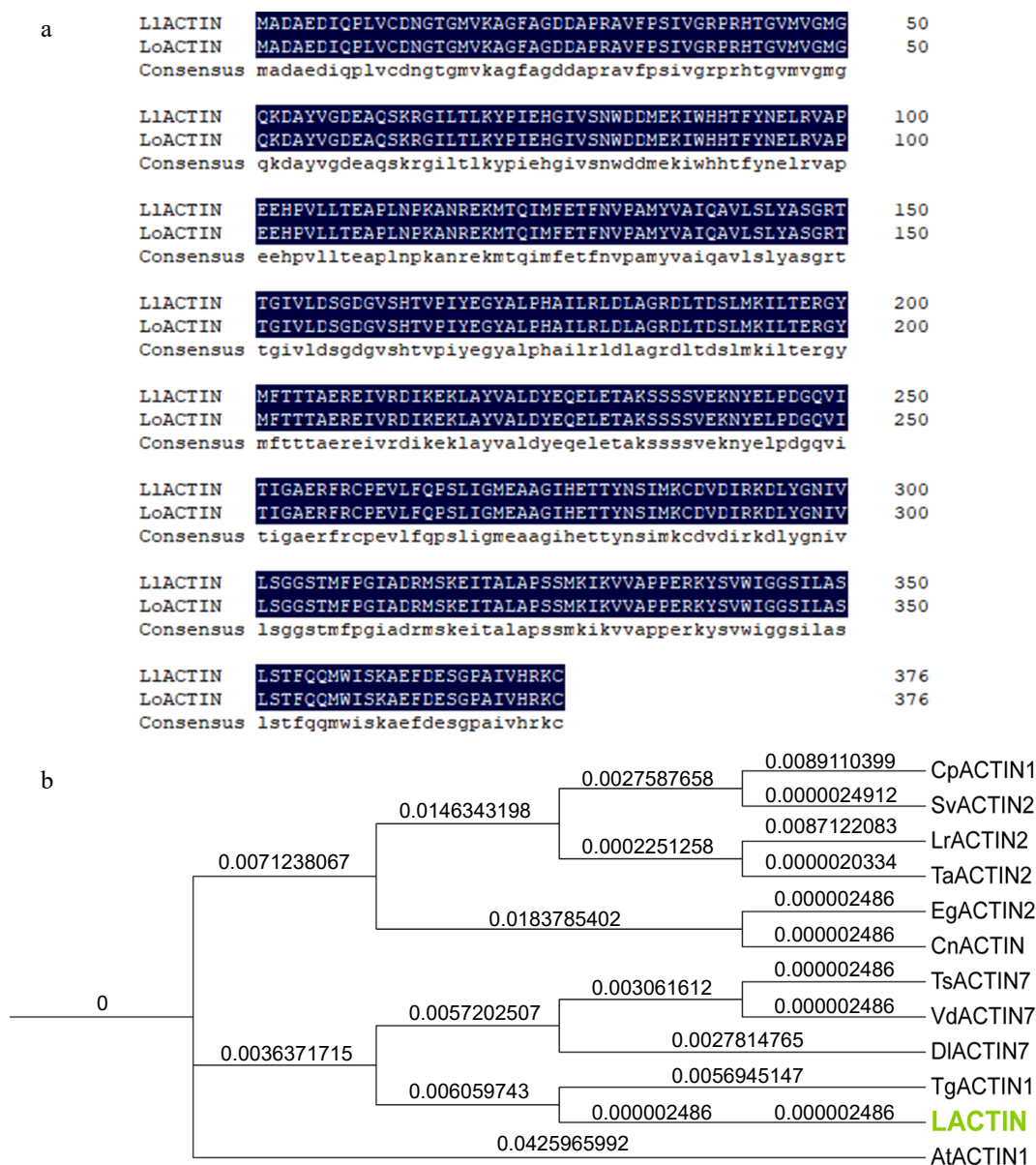|		
L1ACTIN	LSTFQQMWISKAEFDESGPAIVHRKC	376
LoACTIN	LSTFQQMWISKAEFDESGPAIVHRKC	376
Consensus	lstfqqmwiskaefdesgpaivhrkc	

b

Fig. 2 Amino acid sequence alignment and phylogenetic tree analysis of ACTIN in 'White Heaven' and 'Siberia'. (a) Amino acid sequence alignment between L1ACTIN and LoACTIN. (b) Phylogenetic tree of L1ACTIN, LoACTIN, and ACTIN in other species. The ACTIN proteins were from: *Elaeis guineensis* EgACTIN2 (LOC105059495), *Cocos nucifera* CnACTIN (MH017421.1), *Tulipa gesneriana* TgActin1 (AB456684.1), *Lolium rigidum* LrACTIN2(LOC124705928), *Triadica sebifera* TsACTIN7 (KY656700.1), *Diospyros lotus* DlACTIN7 (LOC127809754), *Cenchrus purpureus* CpACTIN1 (MT784734.1), *Triticum aestivum* TaACTIN2 (LOC123048645), *Setaria viridis* SvACTIN2 (LOC117839433), and *Vaccinium dunalianum* VdACTIN7 (OM033722.1). The phylogenetic tree was analyzed by TBtools v1.09876 and drawn by iTOL.

with ACTIN through the AIP3 domain, whereas LoHsfA2 could not function because it lacked this domain (Fig. 3), which may cause differences in heat resistance. This result indicated that although the protein sequence was highly conserved in these two varieties, differences in several amino acids could also lead to differences in the protein domain.

As an important component of the cytoskeleton, actin is required for cell differences, cell growth, and signal transduction, and *ACTIN* is constantly expressed in various tissues and has a high degree of conservation and homology at the RNA and amino acid levels[27,32,45]. The dynamic conversion of the actin cytoskeleton is essential for a variety of plant physiological processes, which are regulated by ABPs and ADFs[32,46]. Several ADFs, such as ADF4 and ADF7, were reported to regulate the biotic or abiotic stress response through actin dynamics and ROS pathways[28]. In this study, we cloned the *ACTIN* from 'White Heaven' and 'Siberia', respectively, and found they encode the same amino acid sequence named LACTIN (Fig. 2a), indicating that the evolution of ACTIN in these two varieties was highly conserved, which was consistent with previous research results. In addition, the phylogenetic tree analysis showed that lily's LACTIN was closest to the actin in monocotyledonous plants (Fig. 2b), which also conforms to the conservation in the evolution of actin.
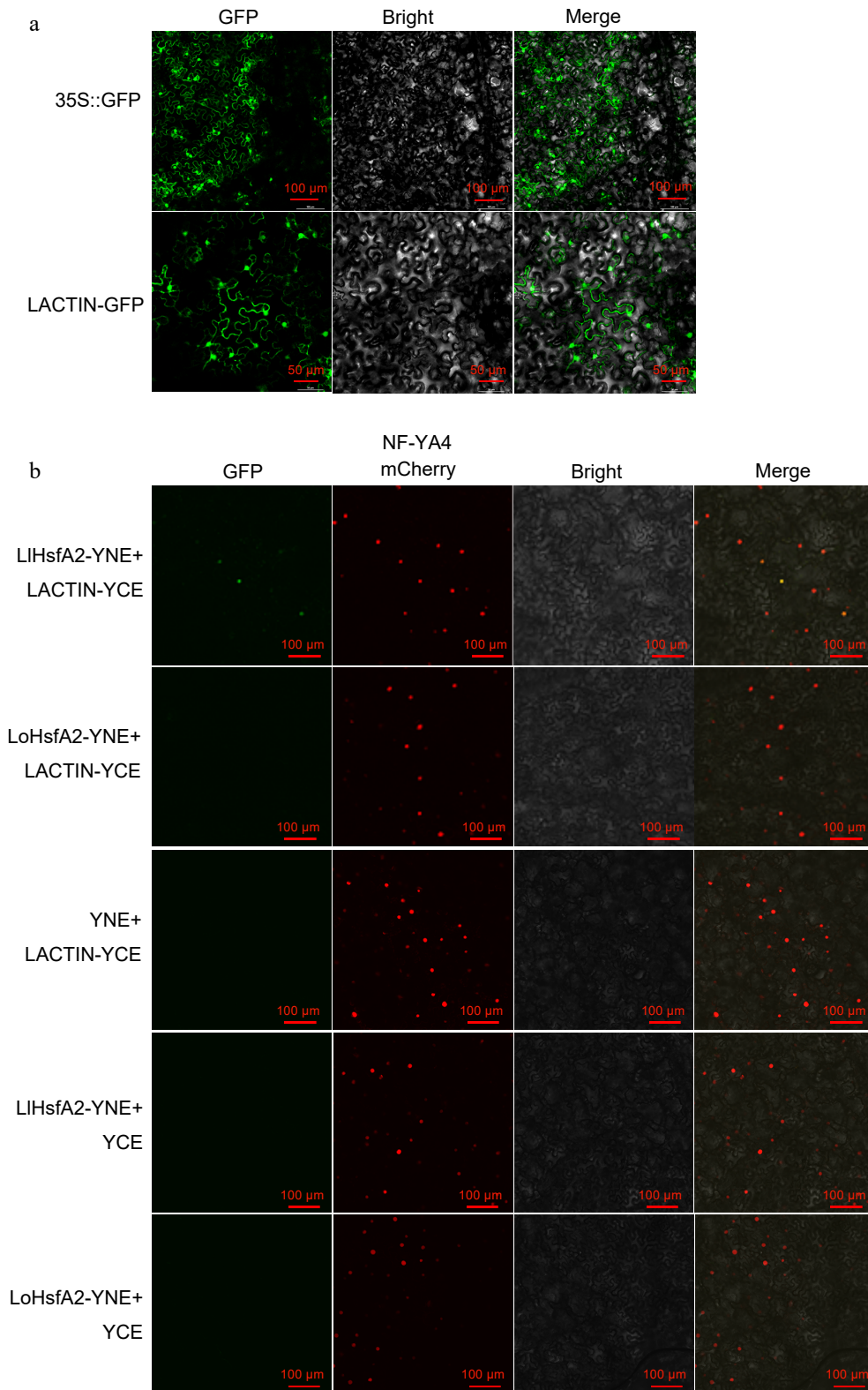
Fig. 3 The interaction relationships of different HsfA2s and ACTINs from 'White Heaven' and 'Siberia'. (a) The subcellular localization of the LACTIN−GFP fusion protein. 35S::GFP was used as the negative control. Green fluorescence were visualized using confocal microscopy 72 h after infiltration. Bars = 100 or 50 μm. (b) BiFC assay. LACTIN−YCE and the empty vector YNE, LlHsfA2-YNE, LoHsfA2-YNE and the empty vector YCE were used as negative controls. NF−YA4−mCherry was used as the nuclear marker. Green and red fluorescence were visualized using confocal microscopy 72 h after infiltration. Bars = 100 μm.
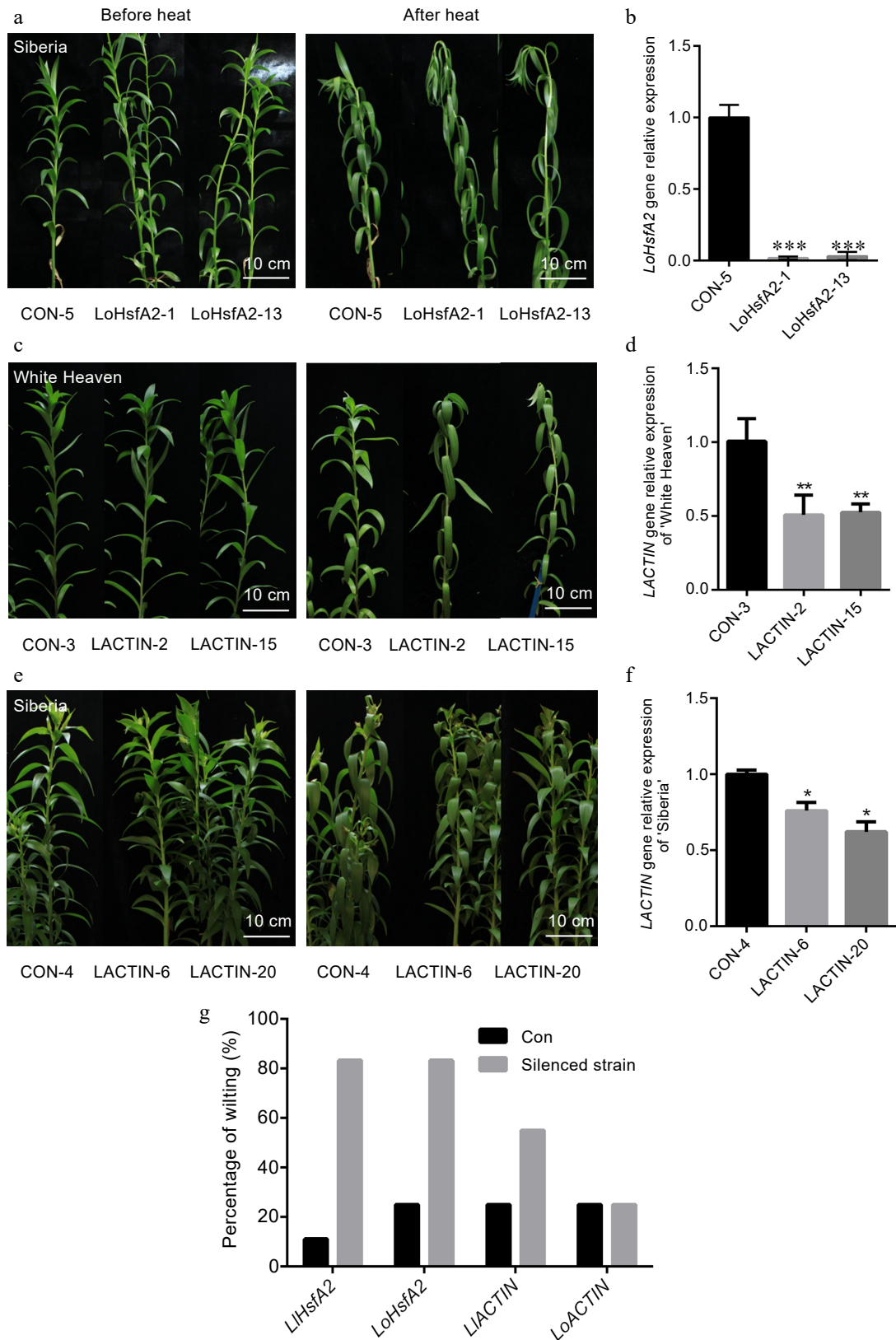
Fig. 4 Heat stress phenotype and gene expression of silenced *HsfA2* and *ACTIN* in 'White Heaven' and 'Siberia'. (a), (c), (e) The phenotypes of the *LoHsfA2*, *LACTIN* silenced 'White Heaven' or 'Siberia' lines before heat and recovered at room temperature for 1 d after treatment with heat stress at 42 °C for 24 h. Bar: 10 cm. (b), (d), (f) Expression of *LoHsfA2*, *LIACTIN* silenced lines based on qRT-PCR. A T-test analysis of variance was employed to identify treatment means that differed statistically. Samples with different stars are significantly different: * *p* <0.05, ** *p* <0.01, and *** *p* <0.001. (g) Percentage wilting of the *LlHsfA2*, *LoHsfA2*, *LACTIN* silenced 'White Heaven' or 'Siberia' lines and negative control lines recovered at room temperature for 1 d after treatment with heat stress at 42 °C for 24 h.
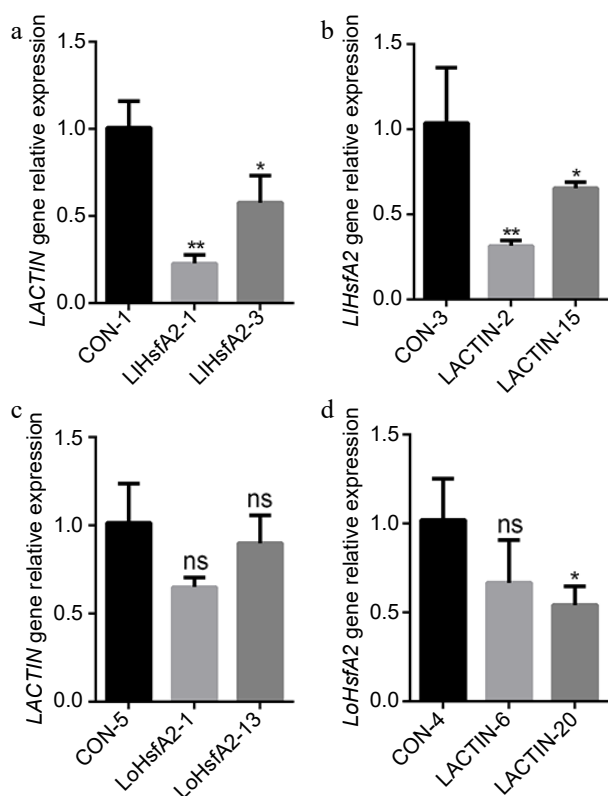
Fig. 5 The expressions of *ACTIN* and *HsfA2* in the *HsfA2* or *ACTIN*-silenced lines of 'White Heaven' and 'Siberia'. (a) The expression of *LACTIN* in the *LlHsfA2* silenced 'White Heaven' line. (b) The expression of *LlHsfA2* in the *LACTIN* silenced 'White Heaven' line. (c) The expression of *LACTIN* in the *LoHsfA2* silenced 'Siberia' line. (d) The expression of *LoHsfA2* in the *LACTIN* silenced 'Siberia' line. A T-test analysis of variance was employed to identify treatment means that differed statistically. Samples with different stars are significantly different: * $p <0.05$, ** $p <0.01$; ns: non-significance.

The different interactions between LlHsfA2 and LACTIN lead to diverse heat stress responses in distinct varieties

Actin-interacting protein (AIP) was first found to interact with actin in yeast[41]. In this study, we found that LlHsfA2 of *Lilium longiflorum* 'White Heaven' contains the AIP3 domain and could interact with actin using the BiFC assay. Meanwhile, the LoHsfA2 of *Lilium* oriental 'Siberia' lacks the AIP3 domain and cannot interact with actin (Figs 1 & 3). Silencing *LlHsfA2* or *ACTIN* induced heat sensitivity in *Lilium longiflorum*, which means that LlHsfA2 and ACTIN can regulate the heat stress response by interacting with each other (Fig. 4c & g). While in *Lilium* oriental 'Siberia' that was more sensitive than 'White Heaven' in heat stress, silenced *LoHsfA2* plants showed sensitivity to heat stress (Fig. 4a & g), but silenced *ACTIN* plants have no obvious phenotype compared with the control plants (Fig. 4e). These results indicated that the different interactions between LlHsfA2 and ACTIN affect the diversity of the heat stress response in cultivated varieties. Similar to the ABPs or ADFs, LlHsfA2 could affect actin filament dynamics or Ca^{2+} or ROS signals to regulate the heat stress response by interacting with actin[27,28]. This regulation mechanism needs further research on whether the HsfA2 could affect actin dynamics.

During the cytoskeleton accumulation and actin aggregation induced by heat stress, small HSPs bind to denatured actin monomers or short oligomers and protect them from aggregation by forming relatively small and highly soluble complexes, which protect the cytoskeleton and the whole cell from damage caused by the accumulation of large insoluble aggregates[47,48]. While studying the effect of heat stress on the actin cytoskeleton of tobacco using two cultured cells, it was found that heat stress induced the depolymerization of actin microfilaments accompanied by the accumulation of Hsp70 binding protein (BiP)[49]. In studies on plants such as tomatoes, it was found that HsfA2 can regulate the transcription of *Hsps* and interact with Hsps to regulate their activity upon exposure to heat stress, which protects the proteins from being misfolded and losing their functional orientation during heat stress[50–52]. In our study, it was found that in 'White Heaven', *LACTIN* and *LlHsfA2* regulated each other's transcription (Fig. 5a, b). However, no such regulation occurred in 'Siberia' (Fig. 5c, d). Therefore, we speculate that LACTIN may regulate the transcription of *LlHsfA2* or interact with LlHsfA2 to affect its protein activity under heat stress, and then LlHsfA2 regulates the transcription of *Hsps* or interacts with Hsps to regulate the activity of Hsps to prevent LACTIN aggregation and cell damage, forming a positive feedback regulation mode of Hsf–Hsp–ACTIN. In addition, actin also plays an important role in guarding cell turgor regulation and shape changes[20,53,54], influencing the process of programmed cell death[55], altering the cytosolic calcium level[56,57], altering ROS signaling[58], changing organelle morphology[59], and controlling stomatal movement[60]. The interaction of LlHsfA2 and LACTIN may also activate LACTIN and protect cells from heat stress damage in these aspects. However, the specific molecular mechanisms of these assumptions need to be further explored.

Conclusions

LlHsfA2 and LACTIN can interact at the protein level and mediate the gene expression of each other in *Lilium longiflorum* 'White Heaven', which protects cells in 'White Heaven' from heat stress damage. In contrast, this interaction and regulatory relationship was not found in 'Siberia', which may be a reason for the difference in heat resistance between these two varieties. This conclusion provides a new area for further research on the response of lily to heat stress and the cultivation of heat-resistant lily varieties.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: He J, Yi M; data collection: Wang Y, Song C, Tong S, Guo Y, Yang X, Li C, Shao Y; analysis and interpretation of results: Wang Y, Song C, Tong S; helpful discussion provided: Yi M; manuscript review and editing: He J. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (https://www.maxapress.com/article/doi/10.48130/opr-0024-0009)

Dates

References

1. Lobell DB, Schlenker W, Costa-Roberts J. 2011. Climate trends and global crop production since 1980. *Science* 333:616−20

2. Zhang J, Li X, Lin H, Chong K. 2019. Crop improvement through temperature resilience. *Annual Review of Plant Biology* 70:753−80

3. Zhou Y, Xu F, Shao Y, He J. 2022. Regulatory mechanisms of heat stress response and thermomorphogenesis in plants. *Plants* 11:3410

4. Mittler R, Finka A, Goloubinoff P. 2012. How do plants feel the heat? *Trends in Biochemical Sciences* 37:118−25

5. Gong Z, Xiong L, Shi H, Yang S, Herrera-Estrella LR, et al. 2020. Plant abiotic stress response and nutrient use efficiency. *Science China Life Sciences* 63:635−74

6. Scharf KD, Berberich T, Ebersberger I, Nover L. 2012. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1819:104−19

7. Scharf KD, Heider H, Höhfeld I, Lyck R, Schmidt E, et al. 1998. The tomato Hsf system: HsfA2 needs interaction with HsfA1 for efficient nuclear import and may be localized in cytoplasmic heat stress granules. *Molecular and Cellular Biology* 18:2240−51

8. Li C, Chen Q, Gao X, Qi B, Chen N, et al. 2005. AtHsfA2 modulates expression of stress responsive genes and enhances tolerance to heat and oxidative stress in *Arabidopsis*. *Science in China Series C: Life Sciences* 48:540−50

9. Charng Y, Liu H, Liu N, Chi W, Wang C, et al. 2007. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiology* 143:251−62

10. Banti V, Mafessoni F, Loreti E, Alpi A, Perata P. 2010. The heat-inducible transcription factor *HsfA2* enhances anoxia tolerance in Arabidopsis. *Plant Physiology* 152:1471−83

11. Chan-Schaminet KY, Baniwal SK, Bublak D, Nover L, Scharf KD. 2009. Specific interaction between tomato HsfA1 and HsfA2 creates hetero-oligomeric superactivator complexes for synergistic activation of heat stress gene expression. *Journal of Biological Chemistry* 284:20848−57

12. Gong B, Yi J, Wu J, Sui J, Khan MA, et al. 2014. LlHSFA1, a novel heat stress transcription factor in lily (*Lilium longiflorum*), can interact with LlHSFA2 and enhance the thermotolerance of transgenic *Arabidopsis thaliana*. *Plant Cell Reports* 33:1519−33

13. Liu J, Feng L, Gu X, Deng X, Qiu Q, et al. 2019. An H3K27me3 demethylase-HSFA2 regulatory loop orchestrates transgenerational thermomemory in *Arabidopsis*. *Cell Research* 29:379−90

14. Friedrich T, Oberkofler V, Trindade I, Altmann S, Brzezinka K, et al. 2021. Heteromeric HSFA2/HSFA3 complexes drive transcriptional memory after heat stress in *Arabidopsis*. *Nature Communications* 12:3426

15. Lian N, Wang X, Jing Y, Lin J. 2021. Regulation of cytoskeleton-associated protein activities: linking cellular signals to plant cytoskeletal function. *Journal of Integrative Plant Biology* 63:241−50

16. Kong Q, Yuan J, Gao L, Zhao S, Jiang W, et al. 2014. Identification of suitable reference genes for gene expression normalization in qRT-PCR analysis in watermelon. *PLoS One* 9:e90612

17. McDowell JM, Huang S, McKinney EC, An Y, Meagher RB. 1996. Structure and evolution of the actin gene family in *Arabidopsis thaliana*. *Genetics* 142:587−602

18. Meagher RB, McKinney EC, Kandasamy MK. 1999. Isovariant dynamics expand and buffer the responses of complex systems: the diverse plant actin gene family. *The Plant Cell* 11:995−1005

19. Kropf DL, Bisgrove SR, Hable WE. 1998. Cytoskeletal control of polar growth in plant cells. *Current Opinion in Cell Biology* 10:117−22

20. McCurdy DW, Kovar DR, Staiger CJ. 2001. Actin and actin-binding proteins in higher plants. *Protoplasma* 215:89−104

21. Galatis B, Apostolakos P. 2004. The role of the cytoskeleton in the morphogenesis and function of stomatal complexes. *New Phytologist* 161:613−39

22. Maisch J, Nick P. 2007. Actin is involved in auxin-dependent patterning. *Plant Physiology* 143:1695−704

23. Rahman A, Bannigan A, Sulaman W, Pechter P, Blancaflor EB, et al. 2007. Auxin, actin and growth of the *Arabidopsis thaliana* primary root. *The Plant Journal* 50:514−28

24. Yokota E, Shimmen T. 1999. The 135-kDa actin-bundling protein from lily pollen tubes arranges F-actin into bundles with uniform polarity. *Planta* 209:264−66

25. Wang HJ, Wan AR, Jauh GY. 2008. An actin-binding protein, LlLIM1, mediates calcium and hydrogen regulation of actin dynamics in pollen tubes. *Plant Physiology* 147:1619−36

26. Qu X, Zhang H, Xie Y, Wang J, Chen N, et al. 2013. *Arabidopsis* villins promote actin turnover at pollen tube tips and facilitate the construction of actin collars. *The Plant Cell* 25:1803−17

27. Tian M, Chaudhry F, Ruzicka DR, Meagher RB, Staiger CJ, et al. 2009. Arabidopsis Actin-Depolymerizing Factor AtADF4 mediates defense signal transduction triggered by the pseudomonas syringae effector AvrPphB. *Plant Physiology* 150:815−24

28. Bi S, Li M, Liu C, Liu X, Cheng J, et al. 2022. Actin depolymerizing factor ADF7 inhibits actin bundling protein VILLIN1 to regulate root hair formation in response to osmotic stress in *Arabidopsis*. *PLoS Genetics* 18:e1010338

29. Huang J, Sun W, Ren J, Yang R, Fan J, et al. 2020. Genome-wide identification and characterization of actin-depolymerizing factor (*ADF*) family genes and expression analysis of responses to various stresses in *Zea Mays* L. *International Journal of Molecular Sciences* 21:1751

30. Cao H, Amin R, Niu L, Song Z, Dong B, et al. 2021. Multidimensional analysis of actin depolymerising factor family in pigeon pea under different environmental stress revealed specific response genes in each subgroup. *Functional Plant Biology* 48:180−94

31. Porter K, Day B. 2016. From filaments to function: the role of the plant actin cytoskeleton in pathogen perception, signaling and immunity. *Journal of Integrative Plant Biology* 58:299−311

32. Qian D, Xiang Y. 2019. Actin cytoskeleton as actor in upstream and downstream of calcium signaling in plant cells. *International Journal of Molecular Sciences* 20:1403

33. Grassotti A, Gimelli F. 2011. Bulb and cut flower production in the genus *Lilium*: current status and the future. *Acta Horticulturae* 900:21−35

34. Xu L, Yang P, Yuan S, Feng Y, Xu H, et al. 2016. Transcriptome analysis identifies key candidate genes mediating purple ovary coloration in asiatic hybrid lilies. *International Journal of Molecular Sciences* 17:1881

35. Zhou Y, Wang Y, Xu F, Song C, Yang X, et al. 2022. Small HSPs play an important role in crosstalk between HSF-HSP and ROS

pathways in heat stress response through transcriptomic analysis in lilies (*Lilium longiflorum*). *BMC Plant Biology* 22:202

36. Xin H, Zhang H, Chen L, Li X, Lian Q, et al. 2010. Cloning and characterization of HsfA2 from Lily (*Lilium longiflorum*). *Plant Cell Reports* 29:875−85

37. Wu Z, Liang J, Wang C, Zhao X, Zhong X, et al. 2018. Overexpression of lily *HsfA3s* in Arabidopsis confers increased thermotolerance and salt sensitivity via alterations in proline catabolism. *Journal of Experimental Botany* 69:2005−21

38. Wang C, Zhou Y, Yang X, Zhang B, Xu F, et al. 2022. The heat stress transcription factor LlHsfA4 enhanced basic thermotolerance through regulating ROS metabolism in lilies (*Lilium Longiflorum*). *International Journal of Molecular Sciences* 23:572

39. Wang K, He J, Zhao Y, Wu T, Zhou X, et al. 2018. EAR1 negatively regulates ABA signaling by enhancing 2C protein phosphatase activity. *The Plant Cell* 30:815−34

40. Yuan C, Li C, Yan L, Jackson AO, Liu Z, et al. 2011. A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. *PLoS One* 6:e26468

41. Amberg DC, Zahner JE, Mulholland JW, Pringle JR, Botstein D. 1997. Aip3p/Bud6p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites. *Molecular Biology of the Cell* 8:729−53

42. Dong T, Wang L, Wang R, Yang X, Jia W, et al. 2023. Transcriptomic analysis reveals candidate genes associated with anther development in *Lilium* Oriental Hybrid 'Siberia'. *Frontiers in Plant Science* 14:1128911

43. von Koskull-Döring P, Scharf KD, Nover L. 2007. The diversity of plant heat stress transcription factors. *Trends in Plant Science* 12:452−57

44. Ding Y, Shi Y, Yang S. 2020. Molecular regulation of plant responses to environmental temperatures. *Molecular Plant* 13:544−64

45. Barrero RA, Umeda M, Yamamura S, Uchimiya H. 2002. Arabidopsis CAP regulates the actin cytoskeleton necessary for plant cell elongation and division. *The Plant Cell* 14:149−63

46. Xiang Y, Huang X, Wang T, Zhang Y, Liu Q, et al. 2007. ACTIN BINDING PROTEIN 29 from *Lilium* pollen plays an important role in dynamic actin remodeling. *The Plant Cell* 19:1930−46

47. Pivovarova AV, Chebotareva NA, Chernik IS, Gusev NB, Levitsky DI. 2007. Small heat shock protein Hsp27 prevents heat-induced aggregation of F-actin by forming soluble complexes with denatured actin. *The FEBS Journal* 274:5937−48

48. Levitsky DI, Pivovarova AV, Mikhailova VV, Nikolaeva OP. 2008. Thermal unfolding and aggregation of actin - stabilization and destabilization of actin filaments. *The FEBS Journal* 275:4280−95

49. Malerba M, Crosti P, Cerana R. 2010. Effect of heat stress on actin cytoskeleton and endoplasmic reticulum of tobacco BY-2 cultured cells and its inhibition by Co^{2+}. *Protoplasma* 239:23−30

50. Ali A, Bharadwaj S, O'Carroll R, Ovsenek N. 1998. HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Molecular and Cellular Biology* 18:4949−60

51. Hahn A, Bublak D, Schleiff E, Scharf KD. 2011. Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. *The Plant Cell* 23:741−55

52. Port M, Tripp J, Zielinski D, Weber C, Heerklotz D, et al. 2004. Role of Hsp17.4-CII as coregulator and cytoplasmic retention factor of tomato heat stress transcription factor HsfA2. *Plant Physiology* 135:1457−70

53. Eun SO, Lee Y. 1997. Actin filaments of guard cells are reorganized in response to light and abscisic acid. *Plant Physiology* 115:1491−98

54. Hwang JU, Suh S, Yi H, Kim J, Lee Y. 1997. Actin filaments modulate both stomatal opening and inward K^+-channel activities in guard cells of Vicia faba L. *Plant Physiology* 115:335−42

55. Franklin-Tong VE, Gourlay CW. 2008. A role for actin in regulating apoptosis/programmed cell death: evidence spanning yeast, plants and animals. *Biochemical Journal* 413:389−404

56. Wang C, Li J, Yuan M. 2007. Salt tolerance requires cortical microtubule reorganization in Arabidopsis. *Plant and Cell Physiology* 48:1534−47

57. Zhao Y, Pan Z, Zhang Y, Qu X, Zhang Y, et al. 2013. The actin-related Protein2/3 complex regulates mitochondrial-associated calcium signaling during salt stress in *Arabidopsis*. *The Plant Cell* 25:4544−59

58. Sun H, Qiao Z, Chua KP, Tursic A, Liu X, et al. 2018. Profilin negatively regulates formin-mediated actin assembly to modulate PAMP-triggered plant immunity. *Current Biology* 28:1882−1895.e7

59. Wang X, Mao T. 2019. Understanding the functions and mechanisms of plant cytoskeleton in response to environmental signals. *Current Opinion in Plant Biology* 52:86−96

60. Li Y, Zhang X, Zhang Y, Ren H. 2022. Controlling the gate: the functions of the cytoskeleton in stomatal movement. *Frontiers in Plant Science* 13:849729