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A lily heat-inducible multi-protein bridging factor, *LlMBF1c*, plays a crucial role in plant thermotolerance

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Abstract

Lily (*Lilium* spp.) is popular for its colorful flowers and exquisite scents. Nonetheless, high temperatures often severely reduce its yield production and quality. The implementation of biotechnological approaches to manipulate the expression of key heat-resistant genes is an effective way to improve the thermotolerance of plants. Here, we isolated a gene encoding for a multi-protein bridging factor 1c (MBF1c) from *L. longiflorum* 'White Heaven' (*LlMBF1c*), which was highly similar to MBF1c from *Elaeis guineensis* (*EgMBF1c*). LlMBF1c harbors conserved MBF1 and helix-turn-helix (HTH) domains. Moreover, the expression of *LlMBF1c* and its promoter activity were enhanced under high-temperature conditions. Further analysis indicated that *LlMBF1c* is a transcriptional repressor in both yeast and *Nicotiana benthamiana*. Its protein was located in the nucleus and cytoplasm of *N. benthamiana* leaf cells. Overexpression of *LlMBF1c* in lily and *Arabidopsis* resulted in enhanced thermotolerance in these plants. By contrast, silencing *LlMBF1c* reduced the thermotolerance of lily. Our results identified an important candidate gene that can be utilized to develop thermotolerant lily germplasm.

Keywords Heat stress, LlMBF1c, *Lilium longiflorum*, Thermotolerance

Introduction

High temperatures significantly impact plant growth and result in substantial economic losses worldwide (Lesk et al. 2016). Unlike animals, plants are immobile and thus have developed intricate regulatory mechanisms to cope with challenging conditions, such as regulation of the heat stress transcription factor-heat stress protein module (Hsf-Hsp) (Liu et al. 2023; Li et al. 2023), activation of the calcium-calmodulin (Ca^{2+}-CaM) and plant hormone pathways (Castroverde et al. 2021; Kothari et al. 2021), mobilization of reactive oxygen species (ROS), nucleosome assembly, and protein folding/unfolding (Li et al. 2018; Mittler et al. 2012). Elucidation of the regulatory networks related to heat tolerance in plants is significant for generating heat-resistant varieties.

The multi-protein bridging factor (MBF) proteins are highly conserved transcriptional co-activators in eukaryotic organisms that have been shown to participate in adaptation to heat, drought, salt, and fugus in various plant species. Li et al. (1994) isolated the first MBF1 from *Bombyx mori* (silkworm) and discovered that it had transcription factor properties without DNA binding ability. In yeast (Takemaru et al. 1998) and humans (Kabe et al. 1999), the gene encoding MBF1 is present as a single copy. However, plants possess multiple *MBF1* genes. For example, three *MBF1* genes have been identified in the *Arabidopsis* genome (Suzuki et al. 2011). A total of 23 candidate *MBF1* genes have been identified

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in five Solanaceae species, including five in *Solanum Lycopersicum* (*SlER24*, *SlMBF1a*, *SlMBF1b1*, *SlMBF1b2*, and *SlMBF1b3*), five in *Capsicum annuum* (*CaMBF1a*, *CaMBF1b1*, *CaMBF1b2*, *CaMBF1c1*, and *CaMBF1c2*), four in *Solanum tuberosum* (*StMBF1a*, *StMBF1b1*, *StMBF1b2*, and *StMBF1c*), four in *Lycium barbarum* (*LbaMBF1a1*, *LbaMBF1a2*, *LbaMBF1b*, and *LbaMBF1c*); and three in *Solanum melongena* (*SmeMBF1a*, *SmeMBF1b*, and *SmeMBF1c*) (Xia et al. 2023). In addition, a total of 9 *MBF1* genes have been identified from *Triticum aestivum* (Zhao et al. 2019). The classification of plant MBF1 family members are determined by their protein sequences. MBF1a and MBF1b are classified as members of Group I, while MBF1c is classified in Group II. The members comprising Group I often possess three introns and four exons. Group II genes can have none, single, or two introns. Despite the differences in structures between group I and II proteins, the primary structure of MBF1 proteins remains conserved. The N-terminal domain of MBF1 is referred to as the MBF1 domain, while the C-terminal domain is known as the helix-turn-helix (HTH) domain.

MBF1 proteins are primarily involved in plant stress responses, especially in plant tolerance to heat, salt, drought, and pathogens. According to Guo et al. (2014), the constitutively overexpressed Group I MBF1, isolated from *Capsicum annuum*, resulted in increased leaf damage and elevated electrolyte leakage in *Arabidopsis* seedlings when subjected to cold stress. The transgenic plants exhibited reduced expression of cold-stress-related genes in comparison to the wild-type plants. Kim et al. (2007) identified an *MBF1* gene from maize named *salt tolerance 41* (*SAT41*), whose protein sequence exhibited 82.4% identity to the *Arabidopsis* MBF1a. The constitutive expression of *SAT41* in *Arabidopsis* resulted in an enhanced capacity to tolerant salt stress. The responses of *MBF1* genes to high temperatures have been extensively analyzed across various plant species. Several studies have demonstrated that MBF1c plays a crucial role in regulating the basal thermotolerance of plants (Zou et al. 2019; Tian et al. 2022). In a study by Suzuki et al. (2008), it was observed that MBF1c protein accumulated rapidly in response to heat stress, playing a regulatory role in the activation of several stress-related mechanisms. These included salicylic acid (SA) signaling, trehalose synthesis, ethylene signaling, and the induction of pathogenesis-related protein 1. Zou et al. (2019) isolated an *MBF1c* gene from Chinese kale (*BocMBF1c*), which exhibited high similarity to an *MBF1c* gene from *Arabidopsis* (*AtMBF1c*), overexpression of this gene in *Arabidopsis* enhanced thermotolerance compared to the wild-type plants. *Arabidopsis* lines overexpressing *TaMBF1c* exhibited greater heat stress resistance during the seedling stage compared to the wild type, whereas lines with *TaMBF1c* knockdown displayed heightened sensitivity to heat stress (Tian et al. 2022). Consequently, MBF1c can positively regulate plants' thermotolerance, as it has also been observed in *Retama raetam* (Pnueli et al. 2002), *Pyropia yezoensis* (Uji et al. 2013), *Polytrichastrum alpinum* (Alavilli et al. 2017), and *Triticum aestivum* (Qin et al. 2015).

Lilies exhibit superior quality when grown in a cool climate. Most lily species lack the ability to withstand high temperatures, and their growth, development, quality, and production are severely inhibited under high temperatures (Zhou et al. 2005). As global temperatures continue to rise, there is a need to breed new heat-resistant lily varieties. Traditional breeding through hybridization is time-consuming, while genetic engineering techniques provide a convenient and faster way to breed new varieties. MBF1c has been demonstrated to be a crucial regulator of thermotolerance in plants; however, the functions of MBF1c in lilies have not been elucidated. To provide a theoretical basis for breeding new heat-tolerant varieties, we investigated the involvement and functions of MBF1c, isolated from 'White Heaven', in heat tolerance.

Materials and methods

Plant materials

'White Heaven' seedlings were grown on a Murashige and Skoog medium (MS). 'Sorbonne' and 'White Heaven' plants were cultivated in a greenhouse. *Nicotiana benthamiana* seeds were soaked in water at 4°C for 3 d and then sown and grown on MS for 10 d. The seedlings were transplanted to plastic pots, with 2 seedlings placed in each pot. *Arabidopsis thaliana* seeds were soaked in water at 4°C for 3 d and then sown and grown on MS for 7 d. All plant materials were grown in a controlled environment at 22°C during the day and 16°C at night, under a 16 h light/8 h darkness photoperiod.

DNA and RNA extraction

The DNA was isolated using the CTAB reagent, as described previously, with some modifications (Aboul-Maaty et al. 2019). Briefly, 1 g of leaves were ground into powder, 1 mL of preheated CTAB was added to the powder, and the samples were incubated at 65°C for 1 h. An equal volume of chloroform: isoamyl alcohol (24:1 *v*/*v*) was then added, followed by centrifugation at 13,000 rpm for 15 min. The upper aqueous solution was transferred to a new Eppendorf tube. Ice-cold 100% isopropyl alcohol was added at approximately two-thirds the volume of the samples, which were then incubated at − 20°C and subsequently centrifuged at 13,000 rpm for 5 min. The supernatant was discarded, and the DNA was washed with 70% ethanol and centrifuged at 13,000 rpm for 5 min.

Then, the DNA pellet was resuspended in TE buffer and stored at − 20°C before use.

Total RNA was extracted using TRIzol Reagent (Life Technologies, USA). The quality of RNA was assessed using the Nano-300. The cDNA synthesis was conducted in accordance with the manufacturer's guidelines using a kit (R323-01) manufactured by Vazyme.

Cloning of *LlMBF1c* open reading frame (ORF)

By referring to the transcriptome data we obtained by sequencing, *LlMBF1c* was cloned from the 'White Heaven' cDNA using the High-Fidelity DNA Polymerase Kit. The primers used for gene amplification are listed in Table S1.

Sequence analysis of *LlMBF1c*

The ORF of *LlMBF1c* was determined using the ORF finder tool available at http://www.bioinformatics.org/sms2/orf_find.html. The amino acid sequence was subjected to analysis using the tool. The phylogenetic tree was generated utilizing the software MEGA 7.0. based on the amino acid sequence (Kumar et al. 2016). Clustal X was employed to identify the conserved amino acid motifs (Thompson et al. 2003).

Gene expression determination in lily

14-day-old tissue-cultured 'White Heaven' seedlings underwent heat treatment. The seedlings were treated with 37°C for different durations ranging from 0 to 12 h. The aimed target gene expression levels were detected by qPCR. The primer sets for the specific gene targets that are listed in Table S1. Gene expression analysis was performed in three independent experiments. The $2^{-\Delta\Delta Ct}$ method was applied to assess the relative expression levels of the amplified targets. 18S rRNA was used as a reference gene.

Promoter activity and subcellular localization of *LlMBF1c*

An 836-bp upstream fragment from the transcription initiation codon of *LlMBF1c* was amplified using primers carrying restriction sites, and the amplified product was cloned into pCAMBIA1391-GUS (Abcam, USA). This recombinant plasmid, designated proLlMBF1c-GUS, was then introduced into the *Agrobacterium* strain GV3101, which was used for *Arabidopsis* transformation. Transgenic *Arabidopsis* plants were treated for 1 h treatment at 37°C, followed by staining with X-Gluc, while *Arabidopsis* that did not undergo heat stress treatment were used as control.

Similarly, the ORF of *LlMBF1c* was inserted into the pCAMBIA1300-GFP (Abcam, USA) vector by the homologous recombination technique. The *Pst*I and *Kpn*I restriction endonucleases were employed for the digestion of the pCAMBIA1300-GFP vector, leading to the generation of the pCAMBIA1300-LlMBF1c-GFP construct. The pCAMBIA1300-LlMBF1c-GFP construct was transiently expressed in the leaves of *N. benthamiana* seedlings, and the green fluorescent protein (GFP) signal was detected at 48 h after agro-infiltration by a Zeiss LSM800 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany).

Transcriptional activity analysis in vitro

A yeast system was utilized to assess the transcriptional activation activity of LlMBF1c. The ORF of *LlMBF1c* was inserted into the pGBKT7 plasmid, generating BD-LlMBF1c. The GAL4 and pGBKT7 plasmids were employed as positive and negative controls. The plasmids were transferred into the AH109 strain respectively. The transformed yeast strains were incubated for 3 days and then evaluated by the spot assay (Wu et al. 2023a). β-Galactosidase (GUS) activity was quantified in accordance with the instructions provided in the user manual (Clontech). The primers used for vector construction are listed in Table S1.

Transcriptional activity analysis in vivo

LlMBF1c was inserted into the pEAQ and pEAQ-VP16 plasmids, resulting in pEAQ-LlMBF1c and pEAQ-LlMBF1c-VP16, which encode for the fusion protein, respectively. The empty pEAQ and pEAQ-VP16 plasmids were employed as negative controls, respectively. The pGreenII0800-LUC plasmid was used as the reporter. *Agrobacterium* cultures containing pEAQ, pEAQ-LlMBF1c, pEAQ-VP16, and pEAQ-LlMBF1c-VP16 mixed with the reporter in a 1:1 (*v*/*v*) ratio, respectively. Following infiltration of the *Agrobacterium* into the *N. benthamiana*, the firefly luciferase (LUC) signal was observed. The intensity of LUC signal was measured by Image J 7.0, and the primers used are listed in Supplementary Table S1.

Petal disc transformation and phenotypic analysis of transgenic plants

The ORF of *LlMBF1c* was cloned into the SK-II plasmid. The recombinant plasmid SKII-LlMBF1c and SK-II were transformed into GV3101 (pSoup). A petal disc transformation assay was performed as described by Wu et al. (2023a).

Generation of transgenic *Arabidopsis* and thermotolerance analysis

The generation of *LlMBF1c* transgenic *Arabidopsis* lines was performed in accordance with the methods described by Li et al. (2022). Basal thermotolerance assessment was assessed by exposing 1-week-old seedlings to 45°C for 90 min. For the acquired thermotolerance assessment, 1-week-old seedlings were pre-treated

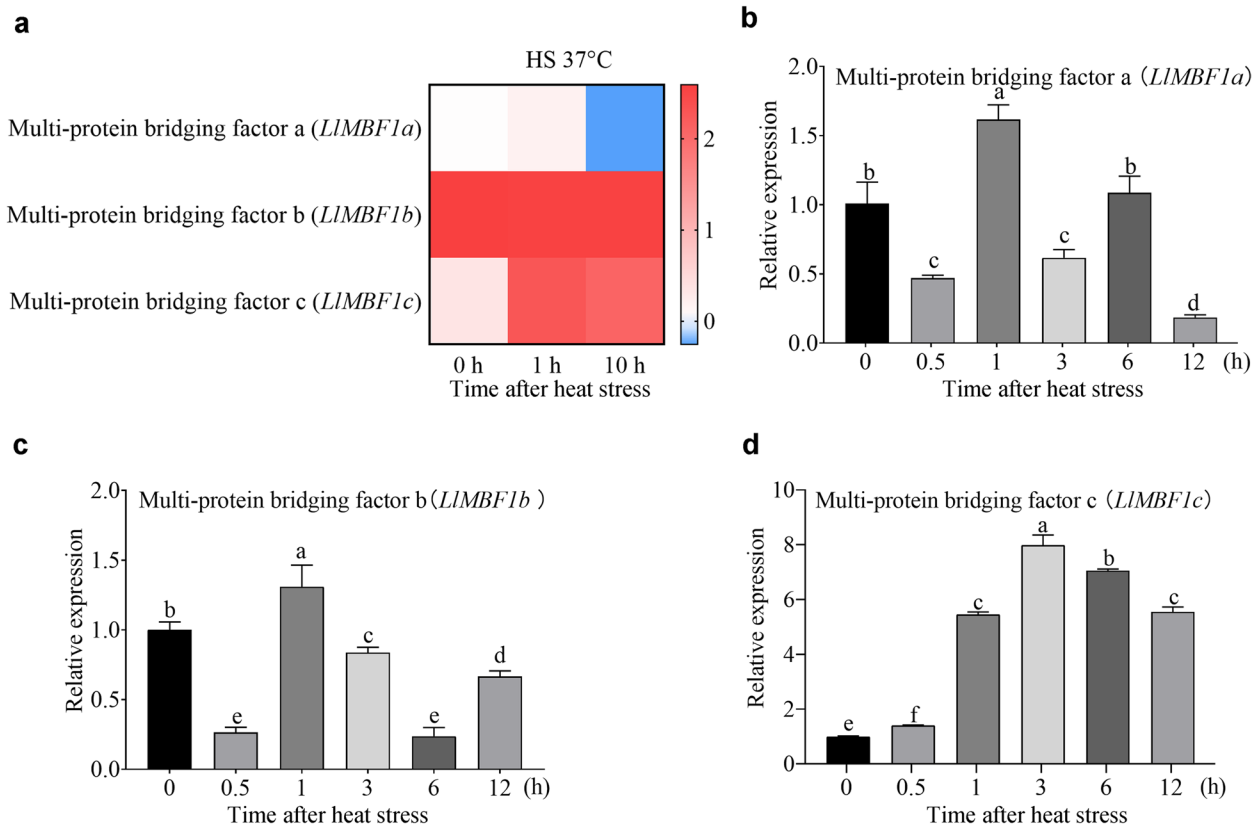


Fig.1 The mRNA levels of multi-protein bridging factor (MBF) genes in *Lilium longiflorum* 'White Heaven'. **a** Relative expression heatmap of *LlMBF1s* in 'White Heaven' after heat treatment. **b-d** Expression of *LlMBF1a* (**b**), *LlMBF1b* (**c**), and *LlMBF1c* (**d**) in lily leaves exposed to 37°C. The 18S rRNA was used as the reference gene. HS, heat stress

at 37°C for 60 min, recovered at 22°C for 120 min, and then placed at 45°C for 240 min. The survival rates of seedlings were recorded after 7 d of recovery at 22°C.

Virus induced gene silencing (VIGS) of *LlMBF1c* in lily and phenotypic analysis of silenced plants

The 3'-end of *LlMBF1c* was amplified and cloned into the pTRV2 vector to specifically silence the target gene. The primer sequences are listed in Supplementary Table S1. *Agrobacteria* carrying the pTRV1 and pTRV2-gene constructs were mixed in equal volumes, and the mixture containing pTRV1 and pTRV2 was used as the control. The *Agrobacterium* mixtures, which contained either pTRV1 and pTRV2 or pTRV1 and pTRV2-target gene constructs were injected into the upper leaves of 'White Heaven'. After injection, the plants were cultivated in the dark for 1 d, after which they were cultivated for an additional 9 d for phenotype analysis.

Data analysis

The ANOVA method was employed for the statistical analysis of *LlMBF1a*, *LlMBF1b,* and *LlMBF1c* expression, GUS activity, and relative LUC intensity. All data, shown as the mean ± SD ($n=3$), were analyzed using Student's *t*-test, * represented $P<0.05$, *** represented $P<0.001$.

Results

LlMBF1c is a heat-inducible gene

Based on their sequences, three MBF1 members were identified in the 'White Heaven' transcriptome under heat treatment: *LlMBF1a*, *LlMBF1b*, and *LlMBF1c*. The expression heatmap of these three *MBF1*s revealed that only *LlMBF1c's* expression increased steadily under 37°C (Fig. 1a). qPCR analysis of the expression of *LlMBF1a*, *LlMBF1b,* and *LlMBF1c* in leaves showed that the expression of *LlMBF1c* increased under 37°C for 0.5 h, with a peak in its expression observed at 3 h after heat treatment (Fig. 1d). However, the expression of *LlMBF1a* and *LlMBF1b* did not exhibit a consistent increase with the heat treatment (Fig. 1b and c). This suggested that

LlMBF1c might be a heat-responsive gene in lily during the heat stress exposure stage.

LlMBF1c encodes for a MBF1 protein

The ORF of *LlMBF1c* was 438 bp and encodes a protein comprising 145 amino acids. A multiple alignment analysis of the amino acid sequences indicated that LlMBF1c contained typical MBF1 and HTH domains (Fig. 2a).A phylogenetic analysis revealed that LlMBF1c formed a cluster with the MBF1c of *Elaeis guineensis* (Fig. 2b).

LlMBF1c promoter activity is enhanced by high temperature, and its protein is located in the cytoplasm and nucleus

The promoter activity of *LlMBF1c* was analyzed under heat stress conditions (37°C, 1 h). X-Gluc staining results indicated that *Arabidopsis* seedlings exhibited low β-glucuronidase (GUS) activity under normal temperatures. In contrast, when exposed to 37°C for 1 h, the GUS activity exhibited a marked increase (Fig. 3a). These results indicated that high temperature can stimulate the activity of the *LlMBF1c* promoter.

Observations of the *N. benthamiana* leaf cells transiently overexpressing the GFP fusion protein revealed

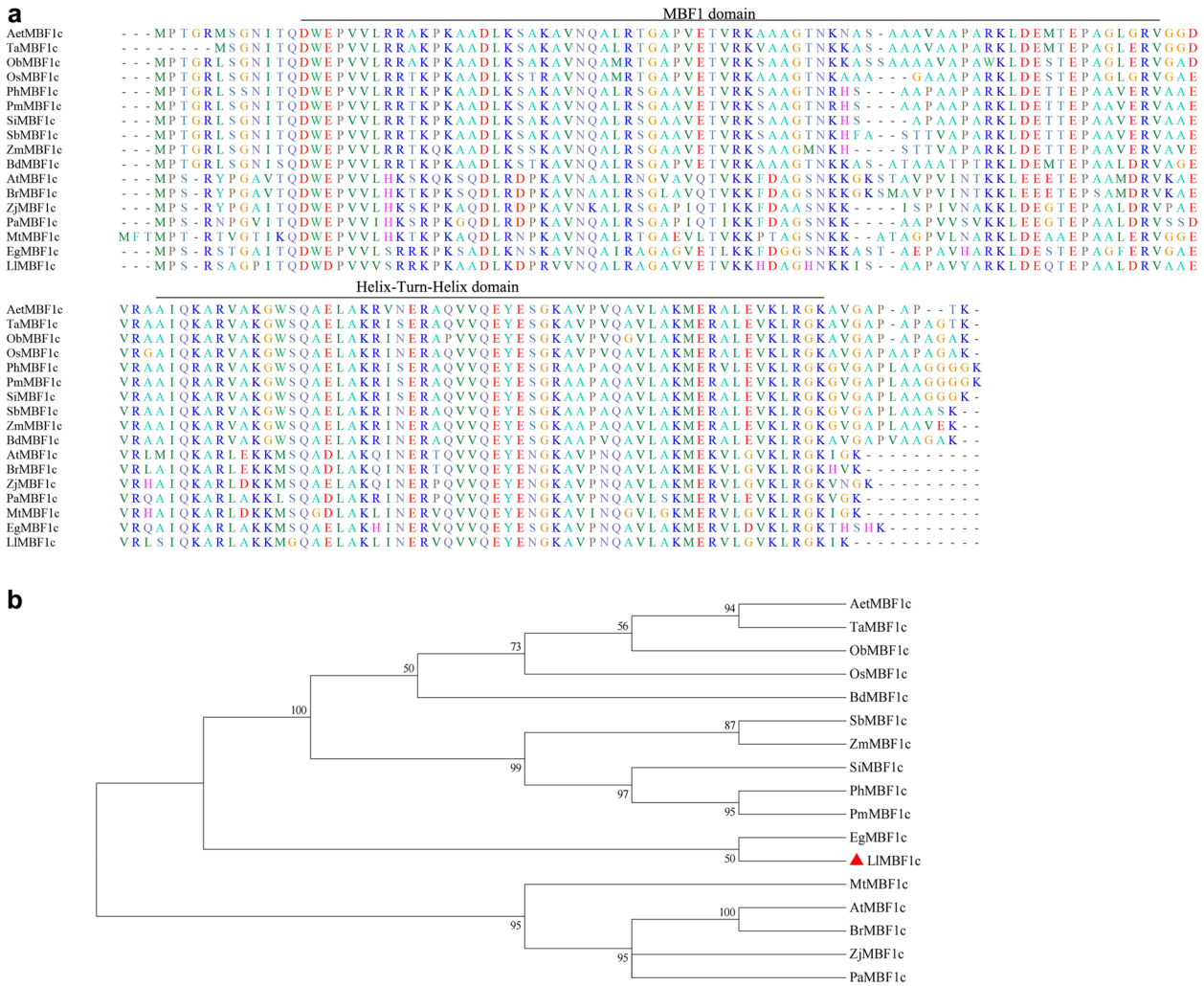


Fig.2 Sequence analysis of multi-protein bridging factor 1c protein in *Lilium longiflorum* (LlMBF1c). **a** Multiple sequence alignment of LlMBF1c amino acid sequences. **b** Phylogenetic analysis of LlMBF1c and MBF1c homologs from other species. The evolutionary tree was constructed using the MEGA 7.0 software. *Aegilops tauschii MBF1c, AetMBF1c; Arabidopsis thaliana MBF1c, AtMBF1c; Brachypodium distachyon MBF1c, BdMBF1c; Brassica rapa MBF1c, BrMBF1c; Elaeis Guineensis MBF1c, EgMBF1c; Medicago truncatula MBF1c, MtMBF1c; Oryza brachyantha MBF1c, ObMBF1c; Oryza sativa MBF1c, OsMBF1c; Prunus avium MBF1c, PaMBF1c; Panicum hallii MBF1c, PhMBF1c; Panicum miliaceum MBF1c, PmMBF1c; Sorghum bicolor MBF1c, SbMBF1c; Setaria italica MBF1c, SiMBF1c; Triticum aestivum MBF1c, TaMBF1c; Ziziphus jujuba MBF1c, ZjMBF1c; Zea mays MBF1c, ZmMBF1c*

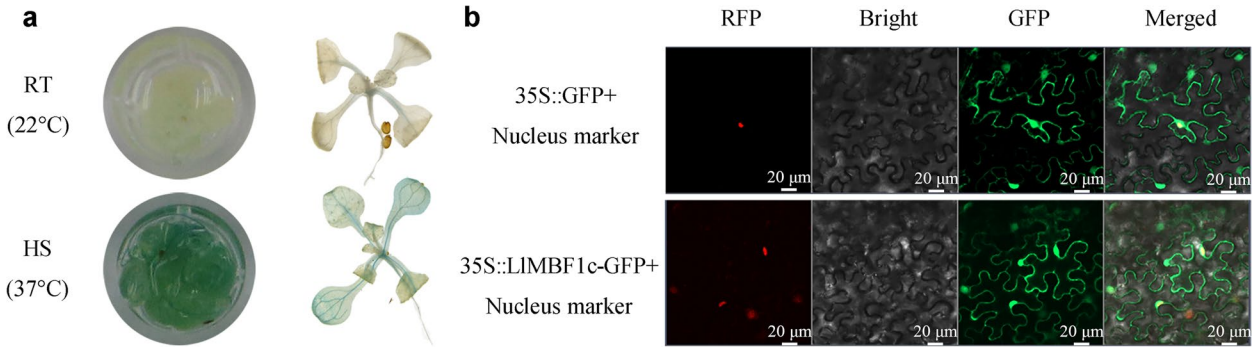


Fig.3 Promoter activity analysis and subcellular localization of multi-protein bridging factor 1c in *Lilium longiflorum* (LlMBF1c). **a** LlMBF1c promoter activity analysis in *Arabidopsis* expressing proLlMBF1c-GUS that was placed at 22°C and 37°C for 1 h. RT, room temperature, 22°C; HS, heat stress, 37°C. **b** Green fluorescence protein (GFP) signals in tobacco co-transfected with LlMBF1c-GFP and the nuclear marker (NLS). Scale bar = 20 μm

that the GFP signal of LlMBF1c-GFP was not only limited to the nucleus but also present in the cytoplasm, similar to the distribution of the signal of the GFP vector in the cytoplasm and nucleus (Fig. 3b). These findings indicate that LlMBF1c is a protein that localized in both the nucleus and cytoplasm.

LlMBF1c is a transcriptional inhibitor

To determine the transactivation capacity of LlMBF1c, vectors carrying the binding domain (BD) were successfully reintroduced into AH109. GAL4-yeast cells carrying the positive control plasmid displayed robust growth on the SD/-Trp-His (-WH) medium and effectively degraded X-α-Gal. However, yeast cells containing BD-LlMBF1c and BD exhibited no growth on the -WH medium and failed to initiate the degradation of X-α-gal. These findings clearly suggested that LlMBF1c does not possess transactivation activity (Fig. 4a). Additionally, we contemplated whether LlMBF1c possessed transcriptional repression activity. A plasmid, designated BD-LlMBF1c-VP16, was constructed and transformed into yeast AH109. BD-VP16 was used as a control. Yeast cells containing BD-VP16 exhibited robust growth on the -WH medium, while BD-LlMBF1c-VP16 could not grow on the medium. This indicated that LlMBF1c possesses transcriptional repressor activity (Fig. 4a). Yeast cells carrying the GAL4 and BD-VP16 plasmids exhibited higher β-galactosidase activity than yeast cells containing only the BD plasmid, BD-LlMBF1c plasmid, and BD-VP16 plasmid (Fig. 4b). The GUS activity assay in yeast cells further confirmed the inhibitory activity of LlMBF1c on transcription.

In addition, the transactivation capacity of LlMBF1c was also determined in *N. benthamiana* leaves using a 5×*UAS* GAL4 transient expression system (Fig. S1). In comparison to the expression of pEAQ alone, pEAQ-*LlMBF1c* exhibited a reduced LUC signal. The result suggested that LlMBF1c had no transcriptional activation capacity in vivo (Fig. 4c). A comparison of the pEAQ-VP16 and pEAQ-LlMBF1c-VP16 expression results revealed a weaker LUC signal, suggesting that LlMBF1c had inhibitory activity on transcription in vivo (Fig. 4d). To summarize, LlMBF1c exhibited inhibitory activity on transcription both in vitro and vivo.

LlMBF1c positively regulates thermotolerance in lilies

To determine the function of LlMBF1c in lily, SK-LlMBF1c was introduced into lily petal discs. The mRNA levels of *LlMBF1c* transiently overexpressed in the petal discs were found to be higher than those of the respective control under both room temperature and heat stress conditions (Fig. 5a). Under heat stress conditions, petal discs overexpressing *LlMBF1c* exhibited less discoloration than the control (Fig. 5b), which suggested that *LlMBF1c* overexpressing petal discs were more resistant to heat stress. Given the usefulness and reliability of relative ion leakage (RIL) as an evaluation index for lily heat damage, we assessed the RIL levels in petal discs transiently overexpressing *LlMBF1c* (Wu et al. 2018a). The overexpression of *LlMBF1c* had no impact on the RIL of petal discs under 22℃. However, it increased less compared to the control after heat stress (HS) (Fig. 5c). Moreover, overexpression of *LlMBF1c* led to a reduction in the accumulation of H_2O_2 after heat stress (Fig. 5d). These findings indicated that the overexpression of *LlMBF1c* enhanced lily thermotolerance.

A VIGS system was employed to suppress the expression of *LlMBF1c* in 'White Heaven'. The expression level of *LlMBF1c* was found to be decreased in the VIGS-LlMBF1c inoculated plants in comparison to the control VIGS inoculated plants (Fig. 5e). Under heat stress conditions, LlMBF1c-knockdown plants displayed more discoloration than the control (Fig. 5f). Under normal conditions, silencing *LlMBF1c* had no impact on their

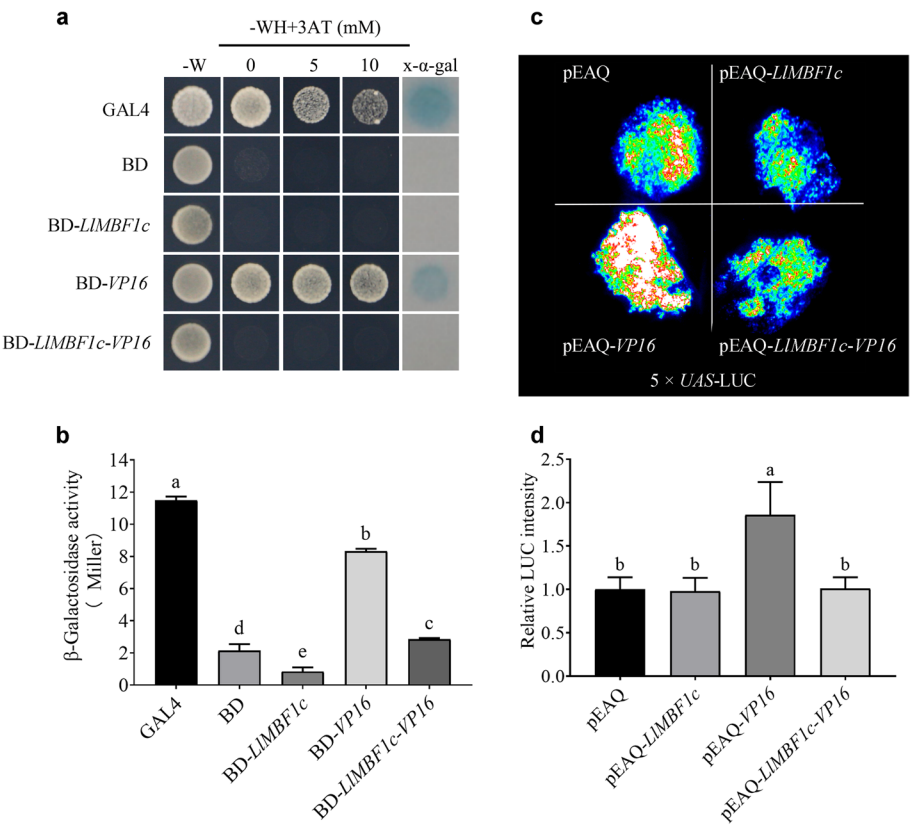


Fig. 4 Transcriptional activation assay of multi-protein bridging factor 1c in *Lilium longiflorum* (LlMBF1c). **a** Transcriptional activation activity determination in the yeast strain AH109. The transformants were screened on an SD/-Trp (-W) medium and an SD/-Trp-His (-WH) medium containing 3-amino-1,2,4-triazole (3-AT) and 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (x-α-gal). **b** β-galactosidase (GUS) activity assay. **c** Detection of the firefly luciferase (LUC) activity in tobacco leaves. **d** Measurement of LUC intensity in tobacco leaves

RIL. However, RIL was observed to be lower in VIGS-LlMBF1c plats than in VIGS-CK plants after HS (Fig. 5g). These findings indicate that the downregulation of *LlMBF1c* reduced lily's thermotolerance.

LlMBF1c overexpression enhances *Arabidopsis* thermotolerance

For the basal thermotolerance assay, *Arabidopsis* seedlings overexpressing *LlMBF1c* were exposed to 45°C for 90 min and subsequently grown at 22°C for 7 d (Fig. 6a). When compared to the wild type, three lines overexpressing *LlMBF1c* had higher survival rates, indicating that *LlMBF1c* expression enhanced the basal thermotolerance of the transgenic seedlings (Fig. 6b). However, when *Arabidopsis* was initially exposed to 37 °C for 1 h, then to 22 °C to recover for 2 h, and subsequently exposed to 45 °C for 240 min (Fig. 6c), no difference was observed in survival rates between the overexpression lines and the wild type (WT), indicating that *LlMBF1c* overexpression was unable to enhance the acquired thermotolerance of *Arabidopsis* seedlings (Fig. 6d). Moreover, the expression of a series of heat-stress-related genes, including *heat shock protein 101* (*AtHSP101*), *heat shock factor A3* (*AtHSFA3*), *dehydration-response element binding protein* (*AtDREB2B*), *galactinol synthase* (*AtGolS*) and *heat shock factor B2A* (*AtHSFB2A*) was higher in

(See figure on next page.)

Fig. 5 Thermotolerance assessment of lily tissues with overexpression or silencing of *multi-protein bridging factor 1c* in *Lilium longiflorum* (*LlMBF1c*). **a** Detection of *LlMBF1c* mRNA levels in petal discs before and after heat stress. **b** Phenotypes of lily petal discs under 22°C (room temperature, RT) and 40°C (heat stress, HS) for 12 h. Scale bar = 1 cm. **c** Relative ion leakage of petal discs at 22°C and 40°C for 12 h. **d** DAB staining of petal discs. **e** Detection of *LlMBF1c* expression in the *LlMBF1c*-silenced leaves before and after heat stress, virus induced gene silencing empty control (VIGS-CK) served as the control. **f**–**g** Phenotypes (**f**) and relative ion leakage (**g**) of VIGS-CK and VIGS-LlMBF1c plants exposed to 22°C and 40°C for 24 h. Data are presented as means ± SD of three replicates (Student's *t*-test, * $P < 0.05$, *** $P < 0.001$)

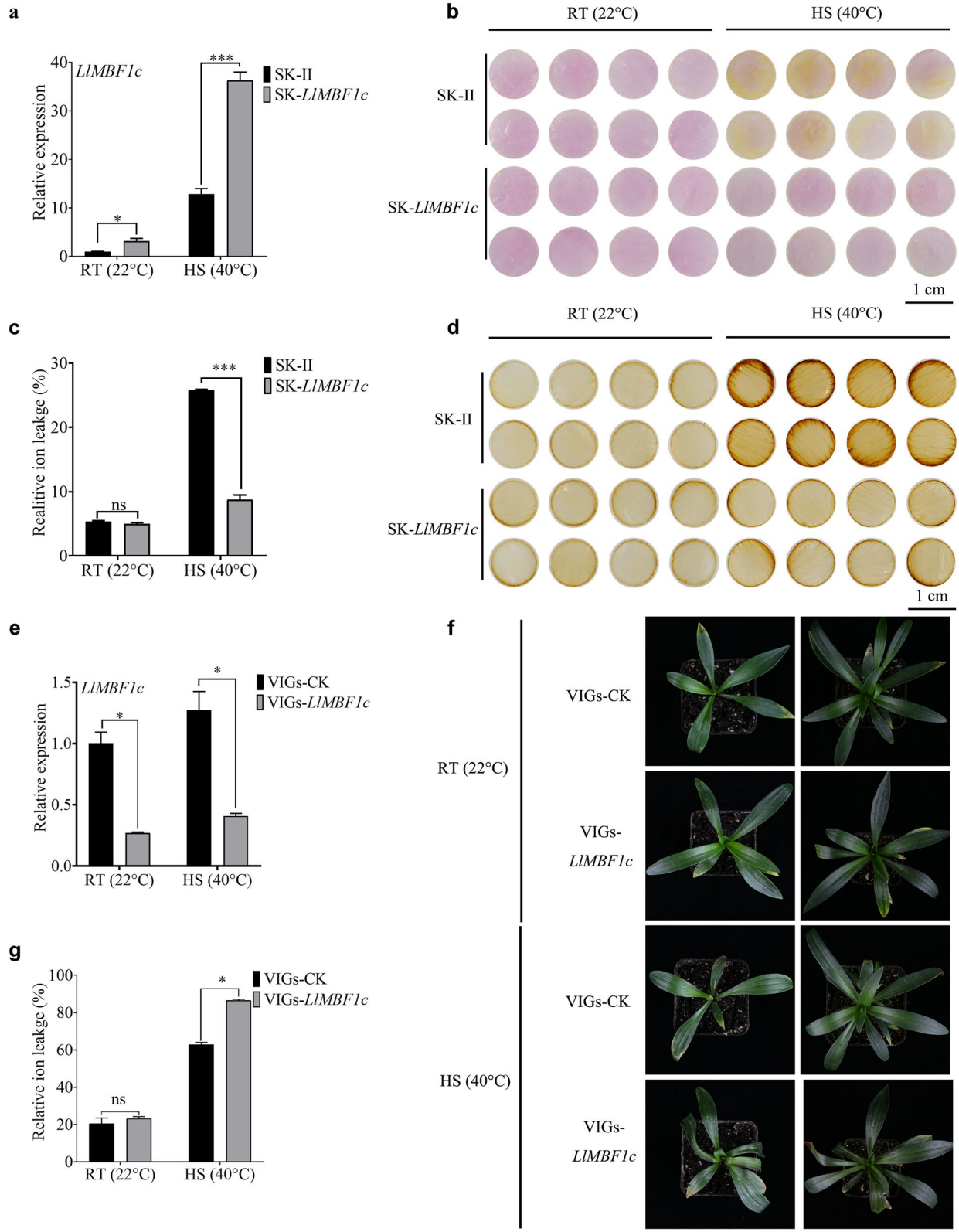


Fig. 5 (See legend on previous page.)

LlMBF1c-overexpressing lines than WT under both normal and heat-stress conditions (Fig. 6). These results suggested that the elevated expression of these heat-stress-related genes might contribute to the enhanced thermotolerance observed in *LlMBF1c*-overexpressing plants.

Discussion

High temperatures severely hamper the development of lilies and the quality of lilies flowers (Zhou et al. 2005). Many heat responsive genes and regulatory modules have been identified in lilies, providing a valuable genetic resource for enhancing the thermotolerance of lilies through genetic engineering approaches. Those genes include members of the HSF family such as *LlHSFA1* (Gong et al. 2014), *LlHSFA2* (Xin et al. 2017), *LlHSFA3* (Wu et al. 2019), and *LlHSFA4* (Wang et al. 2022). Other gene family members include *dehydration-response element-binging protein 2B* from *Lilium longiflorum* (*LlDREB2B*), *WRKY transcription factor 39* from *Lilium longiflorum* (*LlWRKY39*), *ethylene response factor 110* from *Lilium longiflorum* (*LlERF110*), *NAC with transmembrane motif 1-like transcription factor 014* from *Lilium longiflorum* (*LlNAC014*), and *HD-Zip I transcription factor 16* from *Lilium longiflorum* (*LlHB16*) (Wu et al. 2018b; Ding et al. 2021; Wu et al. 2023a; Wu et al. 2022). In this study, we identified a heat-inducible gene in lily, *LlMBF1c*, which enhances plant thermotolerance.

According to their gene structural characteristics, MBF1 genes can be classified into two groups. MBF1a and MBF1b belong to Group I, while MBF1c is assigned to Group II (Jaimes-Miranda and Montes 2020). Group I members are commonly involved in plant developmental processes, whereas MBF1c is a pivotal regulator of thermotolerance (Suzuki et al. 2008). We identified three members of MBF1 family members using the 'White Heaven' transcriptome database. Among them, *LlMBF1c* was steadily upregulated by high temperature (Fig. 1). The rapid induction of *MBF1c* by high temperature has been observed in *Arabidopsis* (Suzuki et al. 2005), wheat (Qin et al. 2015) and Chinese kale (Zou et al. 2019). The expression of *AtMBF1c* was observed to increase after a 5 min exposure at 37 °C, reaching a peak at 1 h (Suzuki et al. 2005). In wheat, the *MBF1c* mRNA levels began to increase after 15 min of exposure to at 40 °C and with a peaked at 1 h (Qin et al. 2015). In response to heat stress,, *BocMBF1c* was rapidly upregulated within 30 min and remained highly expressed for at least 8 h. A similar expression pattern was observed in lily, where *LlMBF1c* was rapidly upregulated under 37 °C for 0.5 h and maintained high expression levels for 12 h (Fig. 1d). The pro*LlMBF1c* GUS activity was found to be enhanced after heat stress treatment for 1 h (Fig. 3a). Those results indicate that *LlMBF1c* is involved in the responses to high-temperatures.

As a transcriptional regulator, MBF1c was located in both the nucleus and cytoplasm (Fig. 3b), similar to its cellular localization in *Arabidopsis* (Suzuki et al. 2008) and wheat (Tian et al. 2022). Given that *LlMBF1c* is located in the nucleus, it is involved in the transcription regulation. A comparison of the transcriptome of mutants and wild-type plants under heat stress, Suzuki (2011) found that *AtMBF1c* functioned as a transcription factor that binds to CTAGA or GGAGG elements and controlled 36 downstream genes during heat stress, including *DREB2A*, *HSFB2A*, and *HSFB2B*. Subsequently, the transcriptional activity of LlMBF1c was evaluated, and found that LlMBF1c has no transcriptional activation capacity, similar to the results reported in *Chrysanthemum morifolium* (Zhao et al. 2022). In addition, our findings indicate that LlMBF1c is a repressor of transcription in both yeast and *N. benthamiana* (Wu et al. 2024). These findings suggest that LlMBF1c may function as a DNA-binding protein, as previously described by Suzuki et al. (2011) and Liu et al. (2023).

It is possible that MBF1c performs analogous functions in disparate plant species due to its high degree of sequence conservation.. *MBF1c*-overexpressing *Arabidopsis* plants were more tolerant to heat stress, whereas the *mbf1c* mutant seedlings exhibited increased sensitivity to high temperatures (Suzuki et al. 2008). The overexpression of the MBF1c gene has been demonstrated to enhance thermotolerance in a number of plant species. For example, *AtMBF1c* overexpression in *Arabidopsis*,

(See figure on next page.)

Fig. 6 Thermotolerance assessment of *Arabidopsis* plants overexpressing *multi-protein bridging factor 1c* in *Lilium longiflorum* (*LlMBF1c*). **a** Phenotypes of wild type (WT) and three transgenic *Arabidopsis* plants (OE1, OE2, and OE3) under the basal thermotolerance assessment. **b** The survival rate of wild type and three transgenic lines under the basal thermotolerance assessment. **c** Phenotypes under the acquired thermotolerance assessment of the WT and three *LlMBF1c*- overexpressing lines. **d** The survival rates of the WT and three transgenic lines under the acquired thermotolerance assessment. **e**-**j** The expression of *Arabidopsis thaliana* genes, including *multi-protein bridging factor 1c* in *Arabidopsis thaliana* (*AtMBF1c*) (**e**), *heat shock protein 101* in *Arabidopsis thaliana* (*AtHSP101*) (**f**), *heat shock factor A3* in *Arabidopsis thaliana* (*AtHSFA3*) (**g**), *dehydration-response element-binging protein* in *Arabidopsis thaliana* (*AtDREB2B*) (**h**), *Galactinol synthase* in *Arabidopsis thaliana* (*AtGolS*) (**i**), *heat shock factor B2A* in *Arabidopsis thaliana* (*AtHSFB2A*) (**j**), in the WT and transgenic plants grown under 22 °C and 37 °C. RT, room temperature; HS, heat stress

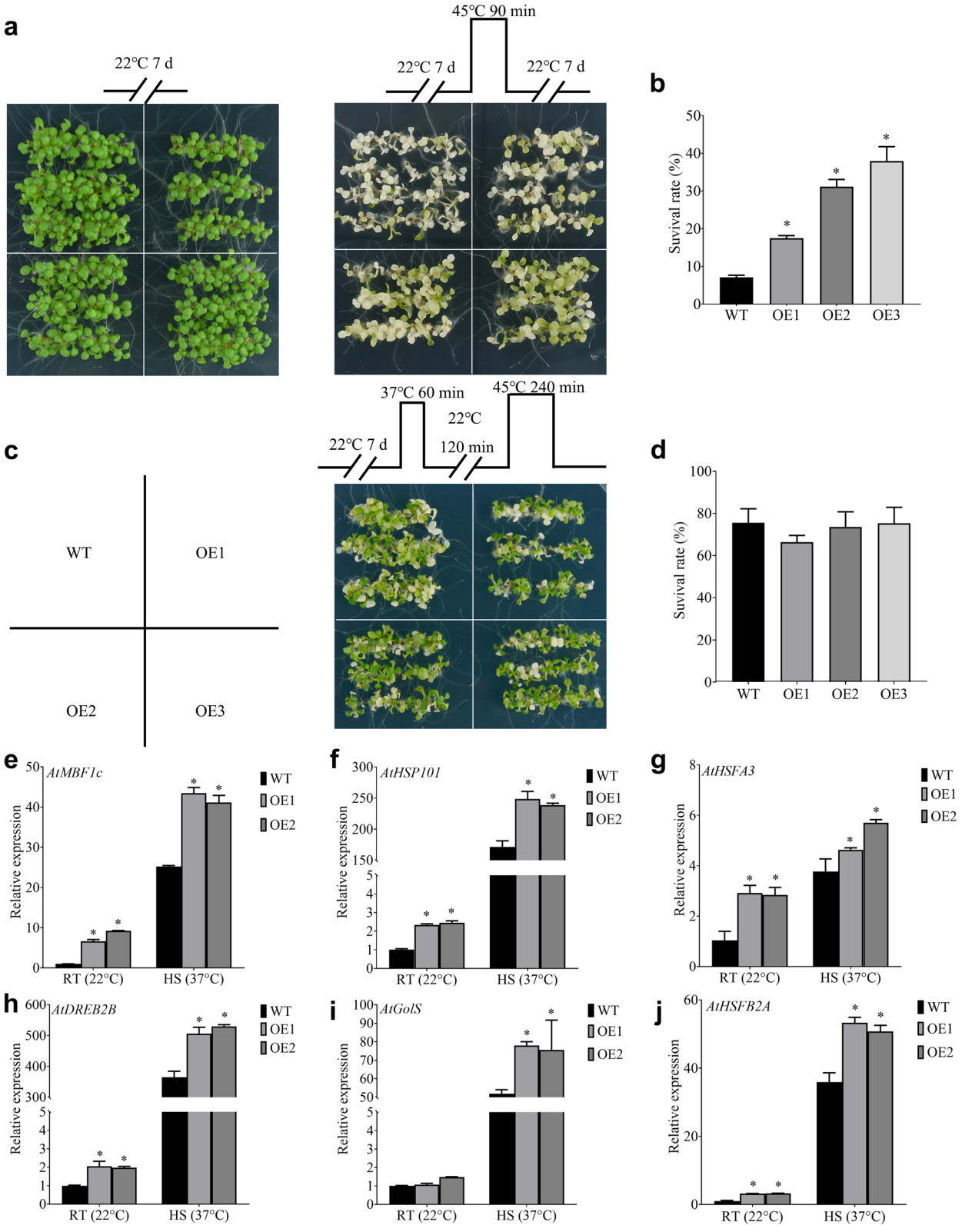


Fig. 6 (See legend on previous page.)

as well as the heterologous expression of *TaMBF1c* in rice and of *BoMBF1c* in *Arabidopsis*, has been shown to confer enhanced thermotolerance (Zou et al. 2019; Jaimes-Miranda and Montes 2020). We found that overexpression of *LlMBF1c* resulted in increased resistance to heat stress, while silencing of *LlMBF1c* decreased the thermotolerance of lily (Figs. 5 and 6), suggesting that LlMBF1c positively regulated thermotolerance of lily plants. Given that *LlMBF1c* is persistently induced by heat and positively regulates heat tolerance, constitutive overexpression of *LlMBF1c* in lily plants may contribute to improving their thermotolerance. Overall, the functions of LlMBF1c were found to be similar to those previously reported genes in other species.

Conclusions

It has been demonstrated that elevated temperatures have a destructive effect on the growth and quality of lilies. In this article, a pivotal heat stress-responsive gene *LlMBF1c* was isolated from a lily cultivar 'White Heaven'. *LlMBF1c* overexpression enhanced thermotolerance of lilies, whereas its silencing had the opposite effect, reducing thermotolerance. It can therefore be concluded that LlMBF1c has significant potential and utility for the development of heat-resistant lily cultivars through molecular breeding techniques in the near future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44281-024-00042-7.

Supplementary Material 1: Fig. S1. The constructs for the transcriptional activity assay in vivo. P_{35S}, Cauliflower Mosaic virus *35S* promoter; LUC, firefly luciferase; REN, *Renilla* luciferase; *LlMBF1c*, *multi-protein bridging factor 1c protein* in *Lilium longiflorum*.

Supplementary Material 2: Table S1. Gene specific primers used in this study

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Authors' contributions
N.T. and Z.W. conceived and designed the experiments; J.X. performed the experiments and the data processing, and wrote the first draft of the manuscript under the supervision of N.T.; L.D. and Y.Z. provided the technical supports; all authors consented to the final version of the manuscript.

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Declarations

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The authors declare that they have no competing interests.

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