LIWRKY39 is involved in thermotolerance by activating LIMBF1c and interacting with LICaM3 in lily (Lilium longiflorum)

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Abstract

WRKY transcription factors (TFs) are of great importance in plant responses to different abiotic stresses. However, research on their roles in the regulation of thermotolerance remains limited. Here, we investigated the function of LIWRKY39 in the thermotolerance of lily (Lilium longiflorum ‘white heaven’). According to multiple alignment analyses, LIWRKY39 is in the WRKY IId subclass and contains a potential calmodulin (CaM)-binding domain. Further analysis has shown that LICaM3 interacts with LIWRKY39 by binding to its CaM-binding domain, and this interaction depends on Ca2+. LIWRKY39 was induced by heat stress (HS), and the LIWRKY39-GFP fusion protein was detected in the nucleus. The thermotolerance of lily and Arabidopsis was increased with the ectopic overexpression of LIWRKY39. The expression of heat-related genes AtHSFA1, AtHSFA2, AtMBF1c, AtGoS1, AtDREB2A, AtWRKY39, and AtHSP101 was significantly elevated in transgenic Arabidopsis lines, which might have promoted an increase in thermotolerance. Then, the promoter of LIMBF1c was isolated from lily, and LIWRKY39 was found to bind to the conserved W-box element in its promoter to activate its activity, suggesting that LIWRKY39 is an upstream regulator of LIMBF1c. In addition, a dual-luciferase reporter assay showed that via protein interaction, LICaM3 negatively affected LIWRKY39 in the transcriptional activation of LIMBF1c, which might be an important feedback regulation pathway to balance the LIWRKY39-mediated heat stress response (HSR). Collectively, these results imply that LIWRKY39 might participate in the HSR as an important regulator through Ca2+CaM and multiprotein bridging factor pathways.

Introduction

High temperature is one of the unfavorable factors affecting the growth of plants, generally impairing photosynthetic activity and negatively affecting cell division and growth1. Extreme high temperatures may result in a series of morphoanatomical and physiophysical changes in plant cells and even lead to severe economic losses in crops and other economically important plants2,3. Plants must produce various defense mechanisms against high temperature, including the accumulation of heat shock proteins (HSPs) and complex regulatory networks as established by transcription factors (TFs)4,5.

Lily (Lilium spp.) is one of the most popular cut flower products worldwide because of its attractive shape and color6. Lily adapts well to cool conditions but is sensitive to high temperatures (>30 °C), which not only reduces the quality of cut flowers but also leads to the degeneration of the bulb7. However, high temperatures will become an unavoidable environmental stress factor in the future because of the irreversible trend in global warming8,9. Therefore, an understanding of the HSR mechanisms of lily under HS is essential to improve the thermotolerance of lily.
TFs play major roles in increasing the stress tolerance of plants since they can regulate critical downstream genes by binding to cis-elements in gene promoters. In the HSR, HS transcription factors (HSFs) can directly regulate the expression of downstream genes by binding to HS elements (HSEs; nGAAnnTTCn) in the promoters of downstream genes in response to HS. Currently, most studies on thermostolerance in lily focus on HSFs. The overexpression of LISHSAF1 and LISHSAF2 from lily in Arabidopsis can enhance the thermostolerance of transgenic lines. Two HSE3 homologs of lily, LISHSA3A and LISHSA3B, increase the thermostolerance of transgenic Arabidopsis plants, possibly through a proline-mediated pathway. In addition, LISHSA3A and LISHSA3B from lily can form a regulatory mechanism involving heat-inducible alternative splicing to sustain balance in the HSR. Furthermore, lily LLDREB2B, a member of the DREB subfamily of the ERF/AP2 TF family, can increase the basal thermostolerance (BT) and acquired thermostolerance (AT) of transgenic Arabidopsis.

Multiprotein bridging factor 1 (MBF1) is a highly conserved transcriptional coactivator with various forms involved in the regulation of diverse processes, such as oxidative stress, hormone-regulated seed germination, and translation. In Arabidopsis, MBF1 cofactors are encoded by three genes: AtMBF1a (AT2G42680), AtMBF1b (AT3G58680), and AtMBF1c (AT3G24500). Among these genes, AtMBF1c is related to thermostolerance and functions upstream of salicylic acid (SA), ethylene, and trehalose signaling. A previous study reported that AtMBF1c is regulated by AtHsfA1 cofactors because an hsfA1 quadruple mutant shows suppressed expression of AtMBF1c during HS. Despite much information available indicating how plant MBF1c genes respond to HS, many questions about the relationship between MBF1c and other important TFs, e.g., WRKY TFs, remain.

WRKY TFs can participate in multiple adverse responses during plant growth and development. The typical feature of WRKY TFs is the WRKY domain, which contains an invariant WRKYQK sequence and a zinc finger motif (CX_4−5CX_22−23HXH or CX_3CX_23HXC). The WRKY superfamily is classified into three groups based on the number of conserved WRKY signatures (two WRKY sequences in group I and one WRKY sequence in groups II and III) and the composition of the zinc finger motif in which the zinc finger motif of groups I and II is CX_4−5CX_22−23HXH and the zinc finger motif of group III is CX_2CX_23HXC. Group II is further classified into five subgroups (Ia to Ie) based on different conserved short motifs. WRKY TFs can recognize and bind to W-box elements (TTGACC/T) in the promoters of resistance-related genes. The core sequence TGAC is essential for WRKY recognition, as indicated by different binding experiments. This type of binding can regulate the expression of target stress genes and thus increase plant stress tolerance.

In recent years, increasing evidence has shown that WRKYs are related to thermostolerance. However, studies on WRKY TFs are primarily focused on crops or model plants such as rice, wheat, and Arabidopsis, and little research has been carried out on lily. In A. thaliana, the group II WRKY protein AtWRKY39 may play a positive role in thermostolerance by regulating the cooperation between the SA- and JA-activated signaling pathways. Here, we identify a WRKY IId factor in lily, LIWRKY39, which was induced by HS and can interact with LCaM3 in a Ca^2+−dependent manner. The thermostolerance of transgenic plants increased with the overexpression of LIWRKY39. Further analysis indicated that LIWRKY39 can bind to the promoter of LIMBF1c and activate its expression. However, the interaction between LCaM3 and LIWRKY39 negatively affected the transactivation of LIMBF1c induced by LIWRKY39, which may imply feedback regulation of LIWRKY39 to maintain a balance during the HSR. The results of this study may help to reveal the biological function and mechanism of LIWRKY39 under HS and provide an important theoretical foundation for further perfecting the HS signal transduction network regulated by lily TFs.

Results
LIWRKY39 is a heat-inducible member of the WRKY group IId family of transcription factors

We searched AtWRKY39 from the Arabidopsis database with the online TAIR tool (https://www.arabidopsis.org/) and selected putative WRKY39 from the pollen transcriptome database of "little kiss". Then, we cloned putative WRKY39 from 'white heaven'. Thus, the candidate was designated LIWRKY39. The full-length cDNA sequence of LIWRKY39 contains an 858-bp open reading frame (ORF) that encodes a 285-amino acid protein. The phylogenetic tree including all WRKY proteins in Arabidopsis showed that LIWRKY39 is closely related to AtWRKY39, AtWRKY74, and AtWRKY21 (Supplementary Fig. S1), which suggests that LIWRKY39 is a member of the WRKY group IId family. To identify the basic characteristics of LIWRKY39, amino acid sequence alignment of LIWRKY39, AtWRKY39, AtWRKY74, and AtWRKY21 was performed. The sequences exhibited a similar signature, which included a WRKYQK sequence, one zinc-binding motif C−X_5−6−X_5−6−H−X_1−H, one HARP (RTGHARFRR[A/G]P) motif, a nuclear localization site (NLS), and a potential calmodulin (CaM)-binding domain (CBD) (Fig. 1b). Then, we investigated the evolutionary relationship between LIWRKY39 and WRKY39 factors in different plants, including Arabidopsis, tomato, apple, wheat, brachypodium, date, oil palm, and pineapple. The phylogenetic results indicated that the closest relationship was established.
Fig. 1 LlWRKY39 is a heat-inducible member of the WRKY group IId of transcription factors. a Structural diagram of the WRKY IId transcription factor. b Multiple alignments of LlWRKY39 with AtWRKY39 (AT3G04670), AtWRKY74 (AT5G28650), and AtWRKY21 (AT2G30590) using the Clustal W algorithm with default parameters. The approximate 60-amino acid WRKY domain, CaM-binding domain (CBD), HARF motif, and nuclear localization site (NLS) are indicated by black boxes. c Phylogenetic relationship of LlWRKY39 with other plant WRKY proteins was determined by the neighbor-joining method with 500 bootstrap replicates. LlWRKY39 is highlighted with a purple triangle. The following abbreviations are used to indicate the scientific names of plants with WRKY: AC ananas comosus, EG elaeis guineensis, PD phoenix dactylifera, BD brachypodium distachyon, TA triticum aestivum, AT arabidopsis thaliana, MD malus domestics, SI solanum lycopersicum. d Relative expression of LlWRKY39 in lily leaves under HS for different lengths of time. Each bar indicates the mean ± SD of three repeated experiments (*P < 0.05 and **P < 0.01, Student’s t test). e Analysis of the promoter activity in N. benthamiana leaves under room temperature (RT) and HS (37 °C 2 h). f The relative value of LUC/REN. The ratio of LUC/REN at RT was set to 1 for normalization. All the values represent the mean ± SD of three repeated experiments (*P < 0.05, Student’s t test)
between LIWRKY39 and pineapple AcWRKY39 (Fig. 1c), which are both noncereal monocot species.

LIWRKY39 can be rapidly induced under HS at 37 °C for 0.5 h, and following prolonged HS for 12 h, the expression of LIWRKY39 is enhanced significantly (Fig. 1d). The expression results suggested that LIWRKY39 is induced by high temperature over a long period. To further examine the underlying mechanism of LIWRKY39 under HS, we isolated and analyzed the promoter of LIWRKY39. Different kinds of cis-elements were found in the promoter of LIWRKY39, such as light-responsive elements (I-BOX and GATA-BOX), an element for pollen-specific expression (POLLEN1), a binding site for WRKY TFs (W-box), CCAAT-BOX, and others, indicating that LIWRKY39 may be regulated by different types of TFs (Supplementary Table S1). Among these motifs, CCAAT-BOX is related to HS, and it can act cooperatively with HSEs to increase HS promoter activity38. To identify the promoter activity of LIWRKY39 under HS, we conducted a transient luciferase reporter assay with Nicotiana benthamiana leaves. The LUC signal increased profoundly after subject to HS, although under normal conditions, the LUC signal was very low, which implied that HS activated the promoter activity of LIWRKY39 (Fig. 1e, f). Thus, these results indicate that LIWRKY39 is a heat-inducible WRKY group IId factor.

**LICaM3 interacts with LIWRKY39 by binding the CBD**

In the protein sequence assay, a potential CBD was found in LIWRKY39 (Fig. 1b), which suggests that LIWRKY39 is a CaM-binding protein (CBP). Therefore, we evaluated whether LIWRKY39 interacted with a heat-inducible CaM, LICaM3, from lily (Supplementary Fig. S2). Bimolecular fluorescence complementation (BIFC) showed that the fluorescence generated upon LIWRKY39 interacting with LICaM3 was emitted only in the nucleus (Fig. 2a). A previous study showed that LICaM3 is a
cytoplasmic and nuclear protein\textsuperscript{39}. Here, we found that the fluorescence of the LlWRKY39-GFP fusion protein appeared only in the nucleus (Fig. 2b), which might explain why the fluorescence emitted by LlWRKY39 interacting with LlCaM3 appeared only in the nucleus. Similarly, firefly luciferase complementation imaging (FLC) assays also showed that LlWRKY39 interacted with LlCaM3 (Fig. 2c). However, compared with that of homologous genes, the CBD of LlWRKY39 is not highly conserved (Fig. 2d). Therefore, we sought to determine whether the interaction between LlWRKY39 and LlCaM3 depends on this domain. We truncated LlWRKY39 into two parts, one segment with the CBD and one without the CBD. In the BIFC assay, LlWRKY39 with the CBD interacted with LlCaM3, but LlWRKY39 lacking the CBD did not (Fig. 2e). Notably, the interaction signal was observed throughout the cytoplasm and nucleus, which may be explained by the deletion of the NLS of LlCaM3 and LlWRKY39 in the cytoplasm and nucleus\textsuperscript{30}. In a split-ubiquitin assay with yeast cells, similar results were observed (Supplementary Fig. S3), which implies that the interaction between LlCaM3 and LlWRKY39 depends on the CBD region.

**LlCaM3–LlWRKY39 interaction depends on Ca\textsuperscript{2+}**

To determine whether the LlCaM3–LlWRKY39 interaction depended on Ca\textsuperscript{2+}, we conducted a transient FLC assay with *N. benthamiana* leaves and CaCl\textsubscript{2} and calcium ion chelator ethylene glycol tetraacetic acid (EGTA) treatments. The interaction signal in the leaves treated with CaCl\textsubscript{2} was stronger than that in the leaves treated with water, but the interaction signal was significantly repressed in the leaves treated with EGTA, which implies that the LlCaM3–LlWRKY39 interaction might depend on Ca\textsuperscript{2+} (Fig. 3).

**Overexpression of LlWRKY39 increases the thermotolerance of transgenic plants**

In a transient expression assay, the overexpression of *LlWRKY39* in lily leaves did not affect their relative ion leakage under normal growth conditions. However, the relative ion leakage of 'white heaven' leaves in the control was significantly higher than that of leaves transformed with *LlWRKY39* under HS, indicating that the transient expression of *LlWRKY39* can protect the cells of lily from high temperature (Fig. 4a, b). Four homozygous T3 transgenic Arabidopsis lines were identified (Supplementary Fig. S4), and three transgenic Arabidopsis lines (OE-2, OE-4, and OE-5) were selected for BT and AT analyses. The survival rate of the WT plants was significantly lower than that of the three transgenic lines under both HS conditions (Fig. 4d, e), which showed that the overexpression of *LlWRKY39* can increase the BT and AT of transgenic Arabidopsis. In addition, the expression levels of HS-inducible genes *AtHSFA1*, *AtHSFA2*, *AtHSP101*, *AtDREB2A*, *AtMBF1c*, *AtGolS1*, and

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**Fig. 3 Effects of CaCl\textsubscript{2} and EGTA treatment on the interaction between LlWRKY39 and LlCaM3 in *N. benthamiana* leaves.**

**a** LUC bioluminescence in *N. benthamiana* leaves coinfiltred with mixed bacterial solutions of LlWRKY39-cLUC and LlCaM3-nLUC by different treatments. **b, d** LUC bioluminescence intensity was quantified using Andor Solis v15 software. **c** LUC bioluminescence in *N. benthamiana* leaves coinfiltred with mixed bacterial solutions of LlWRKY39-nLUC and LlCaM3-cLUC by different treatments. Values represent the mean ± SD of three independent experiments. Significant differences are indicated by Student–Newman–Keuls test.
Fig. 4 (See legend on next page.)
AtWRKY39 increased significantly in transgenic Arabidopsis (Fig. 4f), which might facilitate the increase in thermostolerance of transgenic lines. Unexpectedly, the expression of AtHSFB2A, a transcriptional repressor, was also significantly induced. In addition, the expression of AtHSP70, AtAPX1, AtAPX2, and AtHSFA3, which are the downstream genes of AtHSFA1, AtHSFA2, and AtDREB2A, did not change significantly, suggesting that LIWRKY39 also participated in other pathways to control the expression of these genes. We also measured the expression level of genes such as LIHSFA1, LIHSFA2, and LIDREB2B in transiently overexpressed LIWRKY39 lily leaves. The qRT-PCR results showed that the expression levels of LIHSFA1, LIHSFA2, and LIDREB2B genes were enhanced in lily leaves over-expressing LIWRKY39 (Supplementary Fig. S5).

LIWRKY39 activates the expression of LIMBF1c

MBF1c is an extremely conserved transcriptional coactivator that plays an important role in the HSR. Given that the overexpression of LIWRKY39 can increase the expression of AtMBF1c in Arabidopsis (Fig. 4f), Agrobacterium-mediated transient transformation was performed with lily leaves to verify whether the same regulation mode exists in lily. The overexpression of LIWRKY39 in lily activated the expression of LIMBF1c (Fig. 5a), although LIWRKY39 showed no transactivation activity in yeast cells (Supplementary Fig. S6). In addition, LIMBF1c and LIWRKY39 shared similar expression patterns under HS (Supplementary Fig. S7). To further identify the regulatory mechanism between LIWRKY39 and LIMBF1c, we isolated and analyzed the promoter of LIMBF1c. The LIMBF1c promoter contained various cis-elements, such as drought responsiveness elements (MYB2AT); light responsiveness elements (I-BOX and GATA-BOX); CCAAT-BOX, the binding site of the WRKY TF family (W-box); and others (Supplementary Table S2). Then, we conducted a yeast one-hybrid assay to confirm that LIWRKY39 binds to the W-box element in the promoter region (−500 to −486 bp) of LIMBF1c. The yeast cells cotransformed with LIWRKY39 and the W-box element of the LIMBF1c promoter survived on SD medium without Leu, Trp, and His (SD-LWH), even in the presence of 75 mM 3-aminouridine (3-AT), which implies that LIWRKY39 has a high affinity for the W-box element (GTCAA). However, when the GTCAA sequence was mutated to TTCAC, LIWRKY39 failed to bind to it (Fig. 5c), implying that the G and A residues in GTCAA are essential for the recognition and combination of LIWRKY39. To further verify these results, we performed an electrophoretic mobility shift assay (EMSA). As shown in Fig. 5e, the LIWRKY39-HIS complex bound to the W-box element in the LIMBF1c promoter and produced a mobility shift, which implies that LIWRKY39 binds to the LIMBF1c promoter via the W-box to regulate its expression. The effector-reporter assay showed that the N. benthamiana leaves cotransformed with control combinations emitted a very low LUC signal, but the signal increased significantly after cotransformation with LIWRKY39 and proLIMBF1c-LUC, and the ratio of LUC/REN was also significantly higher than that of the control (Fig. 5g, h). Therefore, these results suggest that LIWRKY39 can activate the expression of LIMBF1c and that this activation may be achieved by directly binding the LIMBF1c promoter.

LiCaM3 negatively affects LIWRKY39 in the transactivation of LIMBF1c

To understand how the LIWRKY39–LiCaM3 interaction affects the function of LIWRKY39, we performed a dual-luciferase reporter assay. The ratio of LUC/REN when coexpressing LIWRKY39 together with LiCaM3 was significantly lower than that when only LIWRKY39 was expressed (Fig. 6b), which suggests that the LIWRKY39–LiCaM3 interaction represses the activation ability of LIWRKY39 for its target genes.

Discussion

Calcium, a universal secondary messenger, is one of the main signal transducers and regulators in plant cells. The transient changes in cytosolic Ca\(^{2+}\) concentration are called Ca\(^{2+}\) signatures, and Ca\(^{2+}\) sensors respond to these changes by activating or inactivating target proteins to participate in specific biochemical processes and regulate gene expression. Most Ca\(^{2+}\) sensors are proteins with one or more EF-hands that have Ca\(^{2+}\)-binding helix-turn-helix structures. Ca\(^{2+}\) sensors with EF-hands are roughly classified into two groups: sensor responders and sensor relays. CaMs are a group of well-characterized Ca\(^{2+}\) sensor relays that do not have any functional
Fig. 5 LIWRKY39 activates the expression of LIMBF1c. a Transient expression of LIWRKY39 in lily increased the expression of LIMBF1c. Each bar indicates the mean ± SD of three repeated experiments. Significant differences between the control and transient overexpression plants were determined by t test (*P < 0.05). b The sequence of the LIMBF1c promoter and its W-box mutated version; the red label indicates the W-box element. c Yeast one-hybrid assay. The interaction between LIWRKY39 and W-box elements was determined in SD-LWH medium with different concentrations of 3-AT. d Schematic diagram of the LIMBF1c promoter; the probe sequence is shown below the diagram. e LIWRKY39-HIS complex binds to the W-box in EMSA. The term 250× indicates the usage of excess unlabeled probe as a competitor, and “+” and “−” indicate its presence and absence, respectively. f The schematic diagram of the effector and reporter. The 836-bp fragment of the LIMBF1c promoter was used in this assay. g Bright field and dark field images of N. benthamiana leaves in the transient expression assays. h The ratio of LUC/REN. 1: mixed bacterial solutions of pGreenII-62-SK and pGreenII-0800-LUC (2:1); 2: mixed bacterial solutions of pGreenII-62-SK-LIWRKY39 and pGreenII-0800-LUC (2:1); 3: mixed bacterial solutions of pGreenII-62-SK and pGreenII-0800-proLIMBF1c-LUC (2:1); 4: mixed bacterial solutions of pGreenII-62-SK-LIWRKY39 and pGreenII-0800-proLIMBF1c-LUC (2:1). Each bar indicates the mean ± SD of three repeated experiments (*P < 0.05, t test)
domains; however, when they bind to Ca$^{2+}$, they can activate or inactivate interacting proteins by relaying the signal\cite{52}. AtWRKY7 was the first WRKY TF to be reported to interact with CaM in a Ca$^{2+}$-dependent manner by binding a short conserved motif (VSSFK[K/R]VISLL in the C-region), which is also called a CBD domain\cite{53}. AtWRKY7 belongs to the WRKY group IId subfamily, and all members of this subfamily interact with Ca$^{2+}$/CaM in Arabidopsis\cite{53}. Here, we identified a new WRKY IId protein, LlWRKY39, from lily, which has a similar primary protein, LlCaM3, which is reportedly associated with HS in lily\cite{39}, interacts with LlWRKY39 by binding the CBD in a Ca$^{2+}$-dependent manner (Figs. 2 and 3). Ca$^{2+}$-dependent CBD motifs are normally grouped into two categories, namely, motifs 1-5-10 and 1-8-14, the numbers of which imply the positions of conserved hydrophobic residues\cite{54}. The CBD of AtWRKY7 does not belong to either of the two classical CBD motifs; however, AtWRKY7 is a Ca$^{2+}$-dependent CBP\cite{53}. LlWRKY39 may be similar to AtWRKY7, with a CBD that is not consistent with either of the two major classes (Fig. 2d). In this study, the interaction between LlWRKY39 and LlCaM3 depends on the CBD in the N-region of LlWRKY39, although it is not completely consistent with the CBD of the WRKY IId TFs in Arabidopsis (Fig. 2c, d), which suggests that the basic CBD can interact with the CaM.

In the present study, the induction of LIWRKY39 expression by HS implies that LIWRKY39 might participate in the regulation of HSR (Fig. 1d). Therefore, we identified the function of LIWRKY39 in the HSR process. The overexpression of LIWRKY39 increased the thermotolerance of lily and Arabidopsis (Fig. 4b, d, e). These results were consistent with those of previous studies on AtWRKY39 in Arabidopsis, in which the overexpressing plants showed greater thermotolerance during both seed germination and seedling growth than wrky39 mutants or wild-type plants under HS\cite{34}. In the current study, some of the HS-responsive genes, namely, AtHSFA1, AtHSFA2, AtDREB2A, AtMBF1c, AtHSP101, and AtGolS1, were significantly upregulated in the transgenic lines (Fig. 4f). Their elevated expression may contribute to an increase in thermotolerance since all of these genes play positive roles in thermotolerance\cite{22,34,55–57}. Notably, the basal expression of AtWRKY39 was also upregulated in the transgenic lines, possibly because of the self-activation of endogenous AtWRKY39. W-box elements in the promoters of stress-induced genes and WRKY genes indicate that WRKY TFs can also be self-regulated, e.g., WRKY33. The expression level of AtWRKY33 is usually low in healthy plants, but when plants are exposed to environmental stimuli, activated AtWRKY33 induces its own expression to generate a feedback mechanism for the rapid and strong induction of AtWRKY33 target genes that respond to biotic or abiotic stresses\cite{58–60}. Nevertheless, HSFB2A, a transcriptional repressor\cite{40}, is also induced, which is speculated to be a mechanism that maintains the balance in the HSR. In addition, although AtHSFA1, AtHSFA2, and AtDREB2A were induced in the transgenic plants, the expression of their target genes\cite{41,43}, AtHSP70, AtAPX1, AtAPX2, and AtHSFA3, was not affected (Fig. 4f), which also suggests that another negative pathway exists in the LIWRKY39-mediated HSR to sustain the balance in the HSR. Among the detected genes, an important MBF1, AtMBF1c, was significantly induced in transgenic Arabidopsis. MBF1 proteins are highly conserved transcriptional coactivators that can build bridges between TFs and transcriptional regulation machinery\cite{22,61–63}. MBF1c is a critical heat-response regulator in the HSR and functions upstream of SA, trehalose, and ethylene signaling to participate in the establishment of thermotolerance\cite{22,63}. The homolog of MBF1c in lily, LIMBF1c, can be activated by LIWRKY39 by directly binding the W-box element in the promter of LIMBF1c (Fig. 5), which suggested that LIWRKY39 might be an upstream regulator of LIMBF1c.

The proteins of the CaM-binding subgroup, AtWRKY7, AtWRKY11, and AtWRKY17, of the WRKY group IId

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**Fig. 6 Effect of LlCaM3 on the activation activity of LIWRKY39 at the promoter of LIMBF1c in N. benthamiana leaves according to a dual-luciferase reporter assay. a** The construction of the effect vectors and reporter vector. **b** Ratio of LUC/REN. Mixed bacterial solutions of effectors and reporter cultures (3:1). Each bar indicates the mean ± SD of three repeated experiments (*P < 0.05, t test)**.
proteins, play negative roles in the plant basal defense response. Similarly, wrky7 mutants exhibited increased resistance to *Pseudomonas syringae* 1, whereas plants overexpressing AtWRKY7 displayed a higher sensitivity to the pathogen by repressing the expression of SA-regulated defense genes or repressing weakly activated jasmonic acid (JA) signaling. Similarly, wrky11 mutants showed increased resistance to *P. syringae* 1, and wrky11 and wrky17 double mutants exhibited a higher increase in resistance, which was due to the decreased JA levels and the downregulation of JA-responsive genes. In contrast to previous studies, LIWRKY39 activated the expression of the downstream gene *LIMBF1c*. Additionally, the interaction between LIWRKY39 and LICaM3 repressed the expression of LIMBF1c activated by LIWRKY39 (Fig. 6), suggesting that Ca²⁺/CaM played an important role in inhibiting excessive activation of LIWRKY39-mediated thermotolerance to sustain balance in the HSR. Additional examples show that MBF1 proteins can reduce tolerance to stresses, although MBF1 transcription cofactors often play positive roles against many stresses. For example, in pepper, CaMBF1 transcript levels are dramatically decreased in response to salt or cold stress, which suggests that MBF1 family factors have a negative effect on the stress response. In addition, AtMBF1 genes can relieve abscisic acid (ABA)-dependent inhibition of germination, which implies that MBF1 proteins negatively regulate the ABA-dependent response to some extent. ABA signaling can also be involved in the HSR. In this study, the interaction between LICaM3 and LIWRKY39 weakened the transcriptional activity of LIMBF1c activated by LIWRKY39 to a certain extent, suggesting that this feedback regulation balances the HSR to prevent the damage caused by excessive activation responses. There may be a similar regulatory mechanism in the downstream genes of LIMBF1c because some genes that negatively regulate thermotolerance in transgenic lines were upregulated, e.g., HSFB2A, and the expression of downstream HSP genes was not changed, e.g., HSP70, implying the existence of negative feedback regulation in the LIWRKY39-mediated HSR.

In conclusion, LIWRKY39 may act as a downstream component of the CaM-mediated calcium signaling pathway that lies upstream of LIMBF1c in the HSR. Considering these findings, we propose a simplified working model that may shed light on the mechanisms of the LIWRKY39-mediated HSR (Fig. 7). When lily is exposed to high temperature, LIWRKY39 is rapidly induced, and its protein directly binds to the W-box element on the promoter of LIMBF1c to activate its expression. The feedback regulation mechanism also begins to respond, and Ca²⁺ flows into the cell to bind and activate LICaM3; then, LICaM3 interacts with LIWRKY39 to inhibit its activation-inducing function promoting the expression of downstream genes, e.g., LIMBF1c, which might contribute to the prevention of the side effects caused by excessive activation to sustain balance in the HSR.

**Materials and methods**

**Plant materials and growth conditions**

The lily hybrid *Lilium longiflorum* ‘white heaven’ used in this experiment was cultured on Murashige and Skoog (MS) medium. *Arabidopsis thaliana* (Col-0) and *N. benthamiana* were grown in potting medium. All plant materials were cultured in a growth room at 22 °C with a 16-h photoperiod.

**Molecular cloning and sequence analysis of LIWRKY39**

Total RNA was extracted from the leaves of ‘white heaven’ with TRIzol reagent according to the manufacturer’s instructions (Invitrogen, USA). cDNA was biosynthesized by a reverse transcription system (TaKaRa,
Japan). Primers for gene cloning are listed in Supplementary Table S3. The physicochemical properties of LlWRKY39 were estimated by EXPASY (http://web.expasy.org/compute_pi/, default setting).

**Protein interaction assays**
In the BIFC assay, the ORFs without stop codons of LlWRKY39 and LlCaM3 were amplified and inserted into pSPYCE (M) and pSPYNE173 vectors using a recombinant ligase (Vazyme, Nanjing, China). The reconstructed vectors and empty vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. The combination of bacterial solutions (YCEYNEP19 = 3:3:1) was coinfiltrated into the leaves of *N. benthamiana*. After 48 h, a confocal laser-scanning microscope (LSM800, Zeiss, Germany) was used to observe the fluorescence signal. In the FLC assay, the ORFs without stop codons of LlWRKY39 and LlCaM3 were cloned into pCAMBIA1300-nLUC vectors, and the ORFs with stop codons of LlWRKY39 and LlCaM3 were cloned into pCAMBIA1300-cLUC vectors using recombinant ligase (Vazyme, Nanjing, China). The reconstructed vectors were transformed into *A. tumefaciens* strain GV3101. The corresponding combination of bacterial solutions (cLUC:nLUC = 1:1) was infiltrated into the leaves of *N. benthamiana*. After 48 h, the fluorescence signal was observed with a luminometer (PIXIS1024B, China). For a yeast two-hybrid assay, the split-ubiquitin vectors of pPR3-N and pBT3-STE were used. The ORF of LlCaM3 was inserted into pPR3-N, and the fragments (1 to 60; 1 to 45) of LlWRKY39 were cloned into pBT3-STE. The empty vectors pPR3-N and pBT3-STE were used as negative controls. The corresponding plasmids were cotransformed into yeast strain AH109. The ORF without the stop codon of LlWRKY39 was inserted into the pGBKT7 vector using specific primers (Supplementary Table S3). The pGBK7 vector, pGBK7-GAL4, and pGBK7-LlWRKY39 were transformed into yeast strain AH109. The transformed yeast cells were incubated on SD medium lacking Trp at 30°C for 3 days. Positive clones were selected on SD medium (lacking Trp and His) containing 3-AT at 30°C for 3 days.

**Subcellular localization of LlWRKY39**
The ORF of LlWRKY39 was inserted into the pGGBKT7 vector using specific primers (Supplementary Table S3). The pGGBKT7 vector, pGGBK7-GAL4, and pGGBK7-LlWRKY39 were transformed into yeast strain AH109. The transformed yeast cells were incubated on SD medium lacking Trp at 30°C for 3 days. Positive clones were selected on SD medium (lacking Trp and His) containing 3-AT at 30°C for 3 days.

**Heat stress treatment of lily**
To detect gene expression levels under HS, one-month-old ‘white heaven’ plants were exposed to 37°C for 0, 0.5, 1, 3, 6, and 12 h. Heat treatment was applied in a temperature incubator (GZL-P80-A, Nanjing, China) without light. Samples of leaves were harvested for qRT-PCR analysis after heat treatment.

**Transient transformation in lily leaves**
The bacterial solutions of pCAMBIA1300-LlWRKY39 and pCAMBIA1300 (control) were collected by centrifugation and resuspended in the same buffer as described for the CaCl2 and EGTA treatment. The resuspended bacterial solutions were placed in the dark for 5 h and infiltrated into the leaves of ‘white heaven’. After 72 h, the infiltrated leaves were harvested for qRT-PCR analysis.

**Gene expression analysis in lily**
Total RNA was extracted using TRIzol as described above. A HiScript II Kit with gDNA Eraser (Vazyme, Nanjing, China) was used for cDNA biosynthesis with an oligo dT primer. qRT-PCR was performed using a 20-μL reaction system. Lily 18S rRNA was used as the endogenous gene. Three independent technical replicates were performed for each of three biological replicates. The
relative expression level was calculated with the $2^{-\Delta\Delta Ct}$ method$^{70,71}$. The primers for qRT-PCR are shown in Supplementary Table S3.

**Isolation and analysis of promoter sequences**

The genomic DNA of ‘white heaven’ was extracted using a plant DNA extraction kit (Zomanbio, Beijing, China) following the manufacturer’s instructions. The promoter was isolated using hi-TAIL PCR$^{72}$. PLACE databases (http://www.dna.affrc.go.jp/PLACE/) were used to analyze the cis-elements in the promoters.

**Promoter activity analysis of L1WRKY39**

The isolated 737-bp fragment of the L1WRKY39 promoter was fused to a pGreenII-0800-LUC vector using a recombinant ligase (Vazyme, Nanjing, China). The constructed vector and an empty vector (control) were transformed into A. tumefaciens strain GV3101 (pSoup), which was used to transform N. benthamiana leaves. After 48 h, one-half of the leaves with infiltrated material were treated at 37 °C for 2 h, and allowed to recover from HS at 22 °C for 12 h; then, the fluorescence signal was observed with a luminometer (PIXIS1024B, China). A luciferase reporter assay system (Promega, USA) was used to measure the activity of firefly luciferase (LUC) and Renilla luciferase (REN). The primers for vector construction are shown in Supplementary Table S3.

**Plant transformation and generation of transgenic lines**

It is difficult to obtain lily transgenic plants since a stable genetic transformation system of lily has not yet been established. Therefore, the model plant A. thaliana (Col-0) was used to verify the biological function of L1WRKY39 under HS. The bacterial solution of pCAMBIA1300-L1WRKY39 was collected by centrifugation and resuspended in a sucrose solution (5%). Arabidopsis plant genetic transformation was performed according to the floral dip method$^{73}$. The harvested seeds were selected on MS medium containing 30 mg/L hygromycin until the T3 generation. One-week-old seedlings were collected to extract RNA for RT-PCR hybridization until the T3 generation. One-week-old seedlings were treated with two HS tests. One was a BT-test in which seedlings were directly treated at 45 °C (shown in Fig. 4d), whereas the other was an AT-test in which seedlings were first treated at 37 °C for 1 h, recovered for 2 h at 22 °C, and then treated again at 45 °C (shown in Fig. 4e). After these treatments, the plants were placed in the culture room for a 7-day recovery, and the phenotypes and survival rates of the seedlings were recorded. One-week-old seedlings of the transgenic and WT plants were collected to determine the expression levels of heat-related genes: AtHSFA1 (AT4G17750), AtHSFA2 (AT2G26150), AtHSFA3 (AT5G03720), AtHSFB2A (AT5G62020), AtDREB2A (AT2G40340), AtWRKY39 (AT3G04670), AtAPX2 (AT3G09640), AtAPX1 (AT1G07890), AtGDS1 (AT2G47180), AMB1F1c (AT3G24500), AtHSP70 (AT3G12580), and AtHSP101 (AT1G74310). AtActin2 (AT3G18780) was used as the endogenous gene. The primers used for qRT-PCR are shown in Supplementary Table S3.

**Yeast one-hybrid assay**

A fragment (-500 to -486) of the L1MBF1c promoter with three repeats was cloned into pHis2.1 to obtain the pHis2.1–3 × W-box; two sites of this fragment (Fig. 5b) were mutated and cloned into pHis2.1 to generate pHis2.1–3 × mW-box. L1WRKY39 was amplified and inserted into the pGADT7 vector. Different combinations of plasmids were cotransformed into yeast strain Y187 to identify positive clones. SD medium lacking Trp, Leu, and His was used to detect possible interactions. The primers used for vector construction are listed in Supplementary Table S3.

**Luciferase reporter assay**

The 836-bp fragment of the L1MBF1c promoter was cloned into pGreenII-0800-LUC to obtain the reporter vector. The ORF of L1WRKY39 was cloned into a pGreenII-62-SK vector to generate the effector vector. These vectors were transformed into A. tumefaciens strains GV3101. The mixed bacterial solution of the TF and promoter cultures (2:1) for the induction analysis was used to infiltrate N. benthamiana leaves. After 48 h, the LUC fluorescence of the infiltrated leaves was detected, and LUC and REN activities were measured using luciferase reporter assay reagents (Promega) as described by a previous study$^{74}$. Three replicate experiments were used for statistical analysis by Student’s t test.

**Electrophoretic mobility shift assay (EMSA)**

An EMSA was performed using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher, New York, USA) according to the manufacturer’s protocol. The biotinylated probes for the EMSAs were synthesized by TSINGKE Biological Technology (Nanjing). The recombinant proteins were purified as described in a previous study$^{75}$. The samples were loaded onto a prerun native 4% polyacrylamide gel with TBE buffer as the electrolyte. After electrophoretically run onto a nylon membrane (Millipore, Darmstadt, Germany) and UV cross-linking for 2 min, the...
membrane was incubated in blocking buffer for 15 min and rinsed in washing buffer for 20 min. Finally, a CCD camera was used to visualize the signals in the membrane.

**Measurement of relative ion leakage**

*Agrobacterium*-mediated transformation was performed as described above, and then ‘white heaven’ plants were treated with HS at 42 °C for 2 h. The leaves were harvested to measure ion leakage (percentage) according to a previously described method6.

**Dual-luciferase reporter assay**

The ORFs of *LlWRKY39* and *LICaM3* were cloned into pGreenII-62-SK76 for use as effector vectors. A fragment of the *LMBF1c* promoter was inserted into pGreenII-0800-LUC76, which was used as the reporter vector. These vectors were transformed into *A. tumefaciens* strain GV3101 (pSoup). Mixed bacterial solutions of effector and reporter cultures (3:1) were used to infiltrate the leaves of *N. benthamiana*. The LUC and REN activity levels were measured as described above. The primers for the vector construction are shown in Supplementary Table S3.

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**Author contributions**

N.T. conceived this project and designed all research with help from Z.W. L.D. and Z.W. conducted the experiments and processed the data. R.T. performed the EMSA. S.X., G.Y., X.C., and D.Z. provided technological assistance. X.C. provided the *LMBF1c* and *LICaM3* sequences. L.D. and Z.W. wrote the first draft of the article, and all the authors read and revised the article.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Supplementary information**

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