


ARTICLE

Open Access

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

Liping Ding^{1,2}, Ze Wu^{1,2,3}, Renda Teng^{1,2}, Sujuan Xu^{1,2}, Xing Cao^{4,5}, Guozhen Yuan^{1,2}, Dehua Zhang^{1,2} and Nianjun Teng^{1,2} 

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*, *AtGolS1*, *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 physiochemical changes in plant cells and even lead to severe economic losses in crops and other economically important plants^{2,3}. 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 warming^{8,9}. Therefore, an understanding of the HSR mechanisms of lily under HS is essential to improve the thermotolerance of lily.

Correspondence: Nianjun Teng (njteng@njau.edu.cn)

¹Key Laboratory of Landscaping Agriculture, Ministry of Agriculture and Rural Affairs, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

²Baguazhou Science and Technology Innovation Center of Modern Horticulture Industry, Nanjing 210043, China

Full list of author information is available at the end of the article

These authors contributed equally: Liping Ding, Ze Wu

© The Author(s) 2021



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

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^{10,11}. 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^{12,13}. Currently, most studies on thermotolerance in lily focus on HSFs. The overexpression of *LIHSFA1* and *LIHSFA2* from lily in Arabidopsis can enhance the thermotolerance of transgenic lines^{6,7,14}. Two *HSA3* homologs of lily, *LIHSFA3A* and *LIHSFA3B*, increase the thermotolerance of transgenic Arabidopsis plants, possibly through a proline-mediated pathway¹⁵. In addition, *LIHSFA3A* and *LIHSFA3B* from lily can form a regulatory mechanism involving heat-inducible alternative splicing to sustain balance in the HSR¹⁶. Furthermore, lily *LIDREB2B*, a member of the DREB subfamily of the ERF/AP2 TF family, can increase the basal thermotolerance (BT) and acquired thermotolerance (AT) of transgenic Arabidopsis¹⁷.

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^{18–20}. In Arabidopsis, MBF1 cofactors are encoded by three genes: *AtMBF1a* (*AT2G42680*), *AtMBF1b* (*AT3G58680*), and *AtMBF1c* (*AT3G24500*)²¹. Among these genes, *AtMBF1c* is related to thermotolerance and functions upstream of salicylic acid (SA), ethylene, and trehalose signaling^{22,23}. A previous study reported that *AtMBF1c* is regulated by AtHsfA1 cofactors because an *hsf1* quadruple mutant shows suppressed expression of *AtMBF1c* during HS²⁴. 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^{25–27}. The typical feature of WRKY TFs is the WRKY domain, which contains an invariant WRKYGQK sequence and a zinc finger motif (CX_{4–5}CX_{22–23}HXH or CX₇CX₂₃HXC)^{28,29}. 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_{4–5}CX_{22–23}HXH and the zinc finger motif of group III is CX₇CX₂₃HXC²². Group II is further classified into five subgroups (IIa to IIe) based on different conserved short motifs^{22,28}. 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^{30,31}. This type of binding can regulate the

expression of target stress genes and thus increase plant stress tolerance^{29,32}.

In recent years, increasing evidence has shown that WRKYs are related to thermotolerance^{33–36}. 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 thermotolerance by regulating the cooperation between the SA- and JA-activated signaling pathways³⁴. Here, we identify a WRKY-IId factor in lily, LIWRKY39, which was induced by HS and can interact with LiCaM3 in a Ca²⁺-dependent manner. The thermotolerance 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 LiCaM3 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'³⁷. 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 WRKYGQK sequence, one zinc-binding motif C-X₅-C-X₂₃-H-X₁-H, one HARF (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

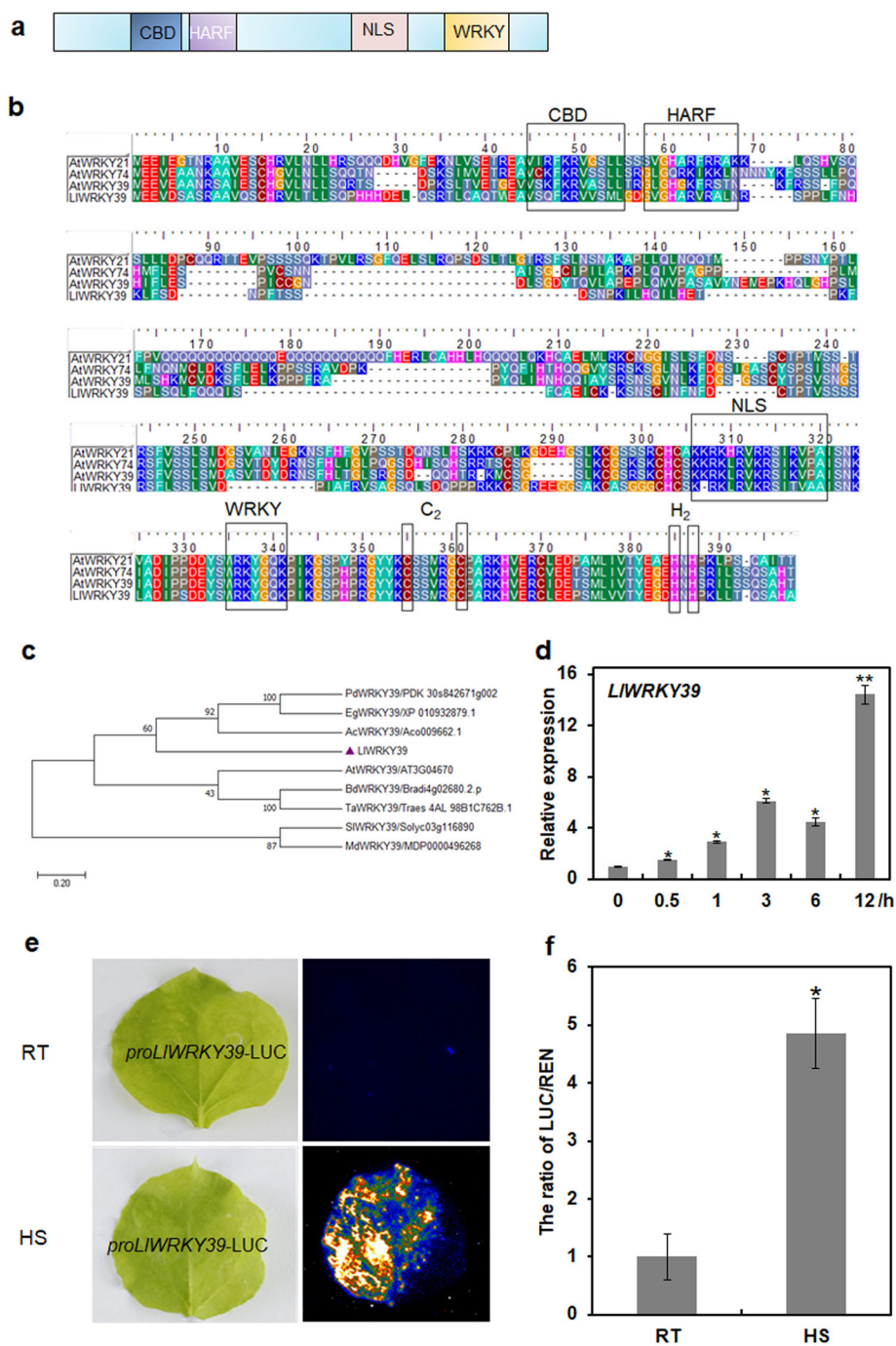


Fig. 1 *LIWRKY39* is a heat-inducible member of the WRKY group IId of transcription factors. **a** Structural diagram of the WRKY IId transcription factor. **b** Multiple alignments of *LIWRKY39* with *AtWRKY39* (AT3G04670), *AtWRKY74* (AT5G28650), and *AtWRKY21* (AT2G30590) using the Clustal W algorithm 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 *LIWRKY39* with other plant WRKY proteins was determined by the neighbor-joining method with 500 bootstrap replicates. *LIWRKY39* 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 domestica, SI solanum lycopersicum. **d** Relative expression of *LIWRKY39* in lily leaves under HS for different lengths of time. Each bar indicates the mean \pm 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 \pm SD of three repeated experiments (* $P < 0.05$, Student's *t* test)

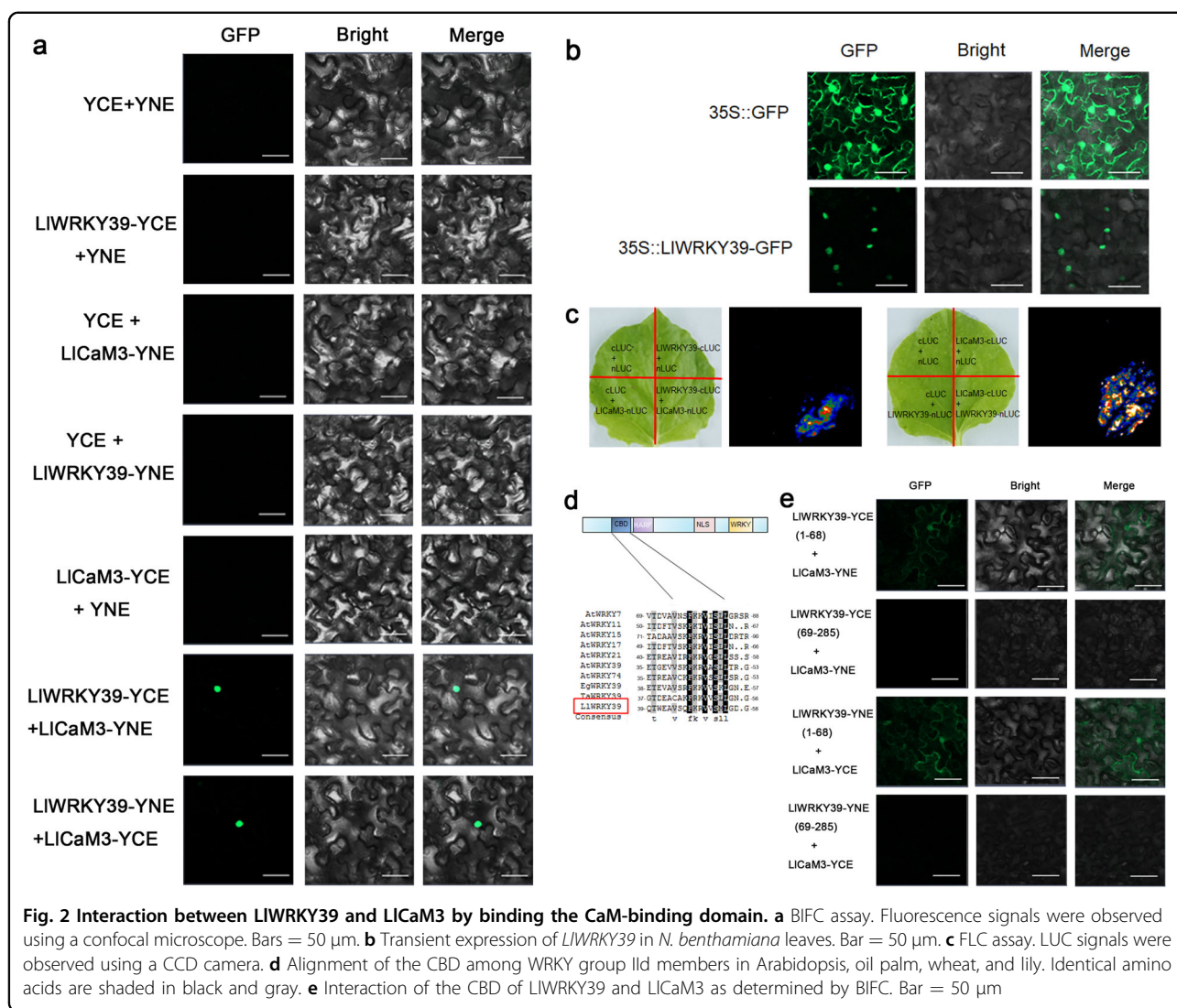
between LIWRKY39 and pineapple AcWRKY39 (Fig. 1c), which are both noncereal monocot species.

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 subjection 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 protein³⁹. Here, we found that the fluorescence of the LIWRKY39-GFP fusion protein appeared only in the nucleus (Fig. 2b), which might explain why the fluorescence emitted by LIWRKY39 interacting with LICaM3 appeared only in the nucleus. Similarly, firefly luciferase complementation imaging (FLC) assays also showed that LIWRKY39 interacted with LICaM3 (Fig. 2c). However, compared with that of homologous genes, the CBD of LIWRKY39 is not highly conserved (Fig. 2d). Therefore, we sought to determine whether the interaction between LIWRKY39 and LICaM3 depends on this domain. We truncated LIWRKY39 into two parts, one segment with the CBD and one without the CBD. In the BIFC assay, LIWRKY39 with the CBD interacted with LICaM3, but LIWRKY39 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 LIWRKY39 and LICaM3 in the cytoplasm and nucleus³⁰. In a split-ubiquitin assay with yeast cells, similar results were observed (Supplementary Fig. S3), which implies that the interaction between LICaM3 and LIWRKY39 depends on the CBD region.

LICaM3–LIWRKY39 interaction depends on Ca²⁺

To determine whether the LICaM3–LIWRKY39 interaction depended on Ca²⁺, we conducted a transient FLC assay with *N. benthamiana* leaves and CaCl₂ and calcium

ion chelator ethylene glycol tetraacetic acid (EGTA) treatments. The interaction signal in the leaves treated with CaCl₂ 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 LICaM3–LIWRKY39 interaction might depend on Ca²⁺ (Fig. 3).

Overexpression of *LIWRKY39* increases the thermotolerance of transgenic plants

In a transient expression assay, the overexpression of *LIWRKY39* 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 *LIWRKY39* under HS, indicating that the transient expression of *LIWRKY39* 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 *LIWRKY39* 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

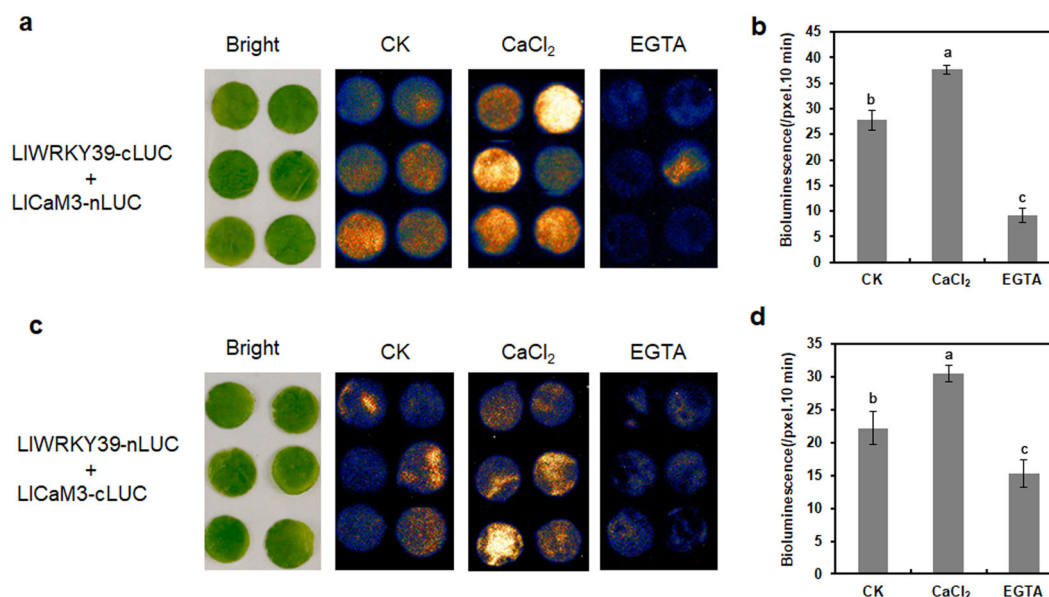


Fig. 3 Effects of CaCl₂ and EGTA treatment on the interaction between LIWRKY39 and LICaM3 in *N. benthamiana* leaves. **a** LUC bioluminescence in *N. benthamiana* leaves coinfiltrated with mixed bacterial solutions of LIWRKY39-cLUC and LICaM3-nLUC by different treatments. **b, d** LUC bioluminescence intensity was quantified using Andor Solis v15 software. **c** LUC bioluminescence in *N. benthamiana* leaves coinfiltrated with mixed bacterial solutions of LIWRKY39-nLUC and LICaM3-cLUC by different treatments. Values represent the mean ± SD of three independent experiments. Significant differences are indicated by Student–Newman–Keuls test

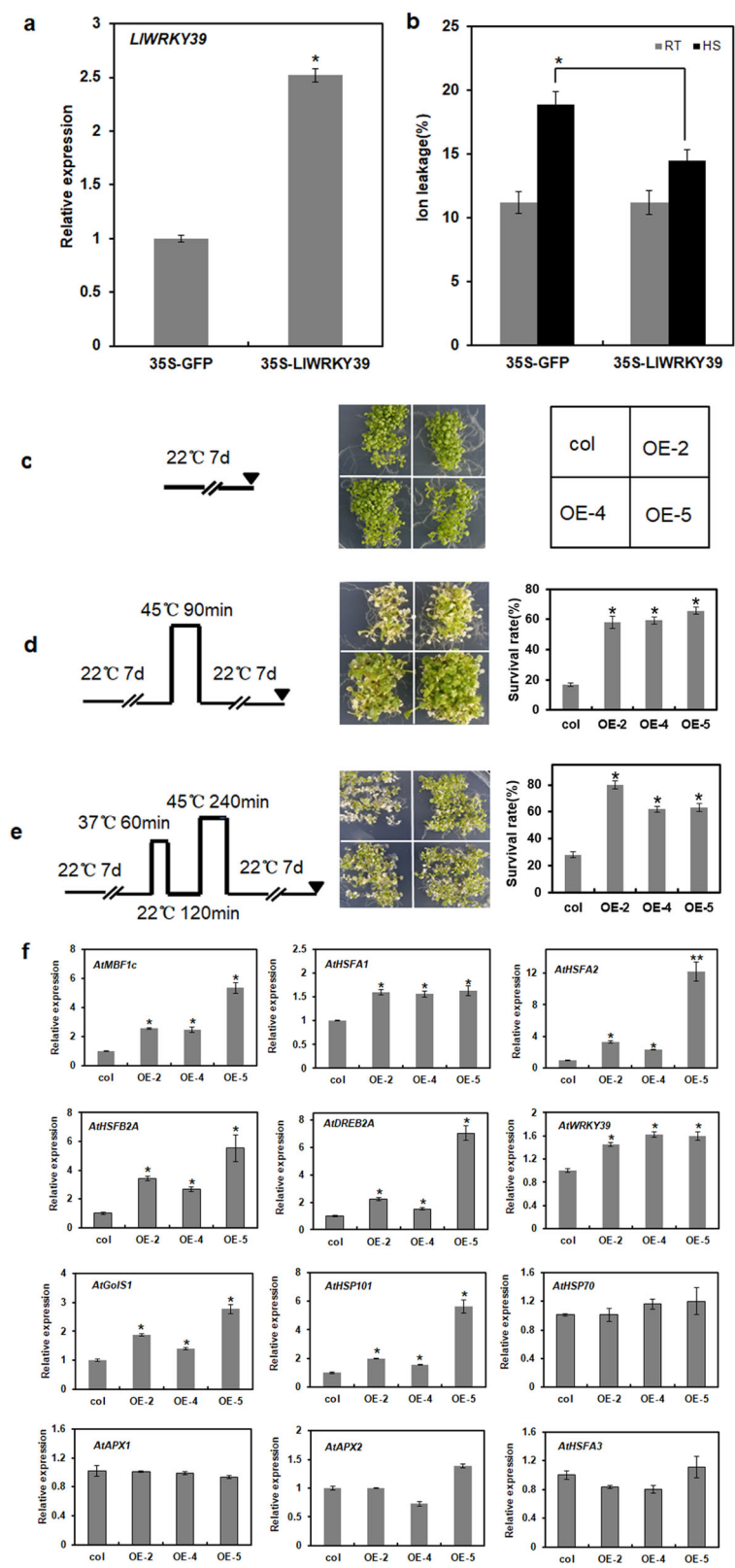


Fig. 4 (See legend on next page.)

(see figure on previous page)

Fig. 4 Thermotolerance assay in lily and transgenic Arabidopsis lines. **a** The expression of *LIWRKY39* in transient-overexpressing lily. **b** Relative ion leakage (%) of lily leaves under RT and HS (42 °C, 2 h). Each bar indicates the mean \pm SD of three repeated experiments (*t* test, * $P < 0.05$). **c** Phenotypes of one-week-old seedlings before exposure to HS. **d** Phenotypes of one-week-old seedlings after the BT test. Left: schematic representations of BT; right: survival rate of BT. **e** Phenotypes of one-week-old seedlings after the AT test. Left: schematic representations of AT; right: survival rate of AT. A bar shows the mean of three independent experiments (* $P < 0.05$, *t* test). **f** Relative expression levels of HS-inducible genes in the transgenic plants. Each bar indicates the mean \pm SD of three repeated experiments. Significant differences are indicated by *t* test (* $P < 0.05$ and ** $P < 0.01$)

AtWRKY39 increased significantly in transgenic Arabidopsis (Fig. 4f), which might facilitate the increase in thermotolerance of transgenic lines. Unexpectedly, the expression of *AtHSFB2A*, a transcriptional repressor⁴⁰, was also significantly induced. In addition, the expression of *AtHSP70*, *AtAPX1*, *AtAPX2*, and *AtHSFA3*, which are the downstream genes of *AtHSFA1*, *AtHSFA2*, and *AtDREB2A*^{41–43}, 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 the *LIHSFA1*, *LIHSFA2*, and *LIDREB2B* genes were enhanced in lily leaves overexpressing *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^{22,23}. 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-amino-1,2,4-triazole (3-AT), which implies that *LIWRKY39* has a high affinity for the W-box

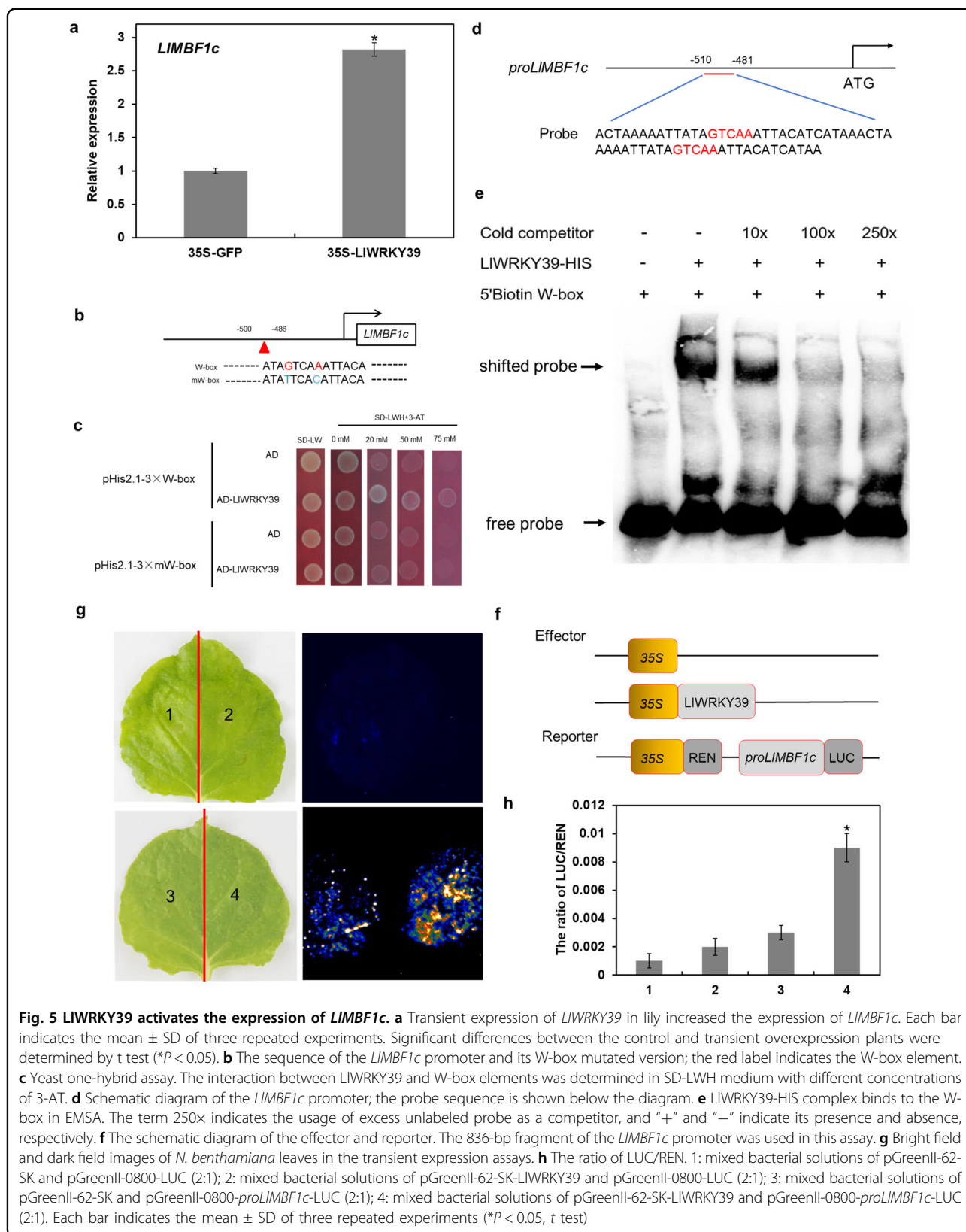
element (GTCAA). However, when the GTCAA sequence was mutated to TTAC, *LIWRKY39* failed to bind to it (Fig. 5c), implying that the G and A residues in GTCAA are essential for the recognition and combination of *LIWRKY39*. To further verify these results, we performed an electrophoretic mobility shift 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.

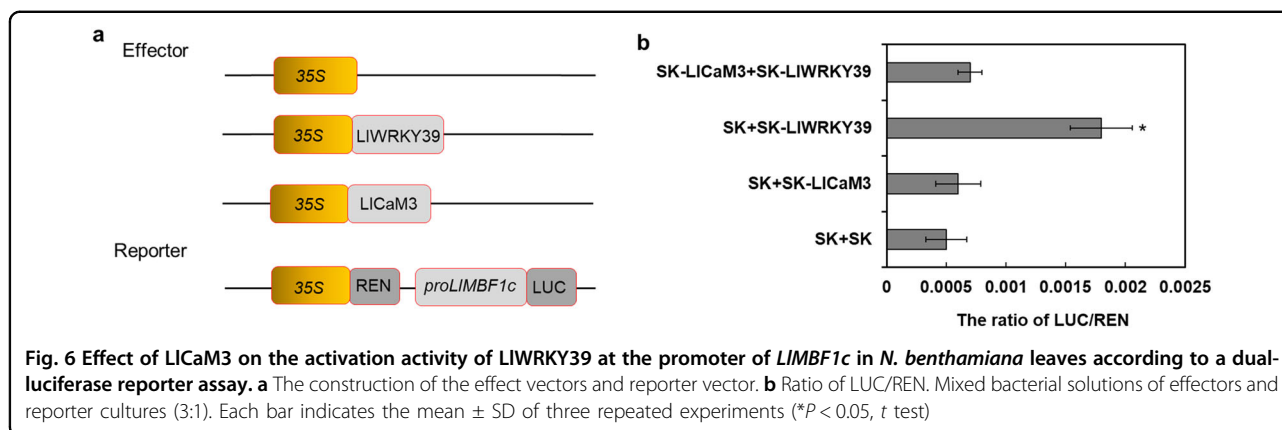
LiCaM3 negatively affects LIWRKY39 in the transactivation of LIMBF1c

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^{44–47}. 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 expression⁴⁸. Most Ca^{2+} sensors are proteins with one or more EF-hands that have Ca^{2+} -binding helix-turn-helix structures⁴⁹. Ca^{2+} sensors with EF-hands are roughly classified into two groups: sensor responders and sensor relays^{50,51}. CaMs are a group of well-characterized Ca^{2+} sensor relays that do not have any functional





domains; however, when they bind to Ca^{2+} , they can activate or inactivate interacting proteins by relaying the signal⁵². 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 (VSSF[K/R]VISLL in the C-region), which is also called a CBD domain⁵³. AtWRKY7 belongs to the WRKY group IId subfamily, and all members of this subfamily interact with Ca^{2+} /CaM in Arabidopsis⁵³. Here, we identified a new WRKY IId protein, LIWRKY39, from lily, which has a similar primary motif (HARE:RTGHARFRR[A/G]P) that is the distinctive characteristic of WRKY group IId TFs²⁸. Additionally, LICaM3, which is reportedly associated with HS in lily³⁹, interacts with LIWRKY39 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 residues⁵⁴. The CBD of AtWRKY7 does not belong to either of the two classical CBD motifs; however, AtWRKY7 is a Ca^{2+} -dependent CBD⁵³. LIWRKY39 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 LIWRKY39 and LICaM3 depends on the CBD in the N-region of LIWRKY39, 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 HS³⁴. 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^{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^{58–60}. Nevertheless, HSF2A, a transcriptional repressor⁴⁰, 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^{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^{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^{22,23}. The homolog of *MBF1c* in lily, *LIMBF1c*, can be activated by LIWRKY39 by directly binding the W-box element in the promoter 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

proteins, play negative roles in the plant basal defense response^{64,65}. *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) signaling⁶⁴. 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 genes⁶⁵. 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 response⁶⁶. 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 extent¹⁹. ABA signaling can also be involved in the HSR^{67,68}. 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, which directly activates the expression of *LIMBF1c* and participates in the establishment of the HSR. Simultaneously, the feedback regulation mechanism also begins to respond, and Ca²⁺ enters the cell to activate LICaM3, which upon interaction with LIWRKY39, can 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,

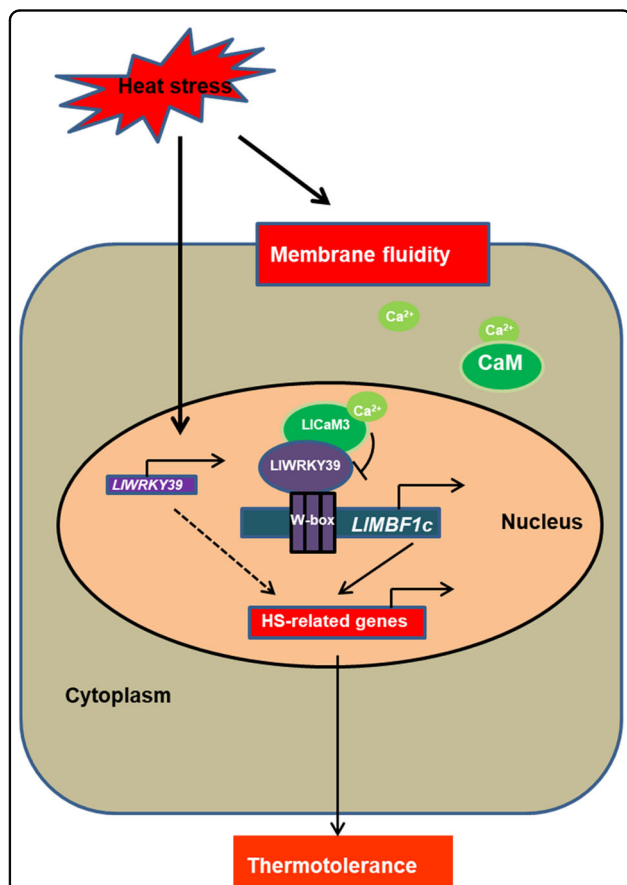


Fig. 7 A proposed working model of LIWRKY39-mediated HSR under HS. When lily suffers from high temperature, LIWRKY39 is rapidly induced, and its protein directly binds to the W-box element on the promoter of *LIMBF1c* to activate its expression. The feedback regulation mechanism also begins to respond, 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

Japan). Primers for gene cloning are listed in Supplementary Table S3. The physicochemical properties of LIWRKY39 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 *LIWRKY39* 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 (YCE:YNE:P19 = 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 *LIWRKY39* and *LlCaM3* were cloned into pCAMBIA1300-nLUC vectors, and the ORFs with stop codons of *LIWRKY39* 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 *LIWRKY39* 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 NMY51. The interaction was identified by spot assay on Leu-, Trp-, His-, and Ade-deficient SD medium with 3-AT (20 mM). All the primers used for vector construction are shown in Supplementary Table S3.

CaCl₂ and EGTA treatment

The bacterial solutions (*LIWRKY39-cLUC* with *LlCaM3-nLUC* and *LIWRKY39-nLUC* with *LlCaM3-cLUC*) were collected by centrifugation and resuspended in buffer (10 mM MgCl₂, 200 mM acetosyringone (AS), 10 mM 2-morpholino ethanesulfonic acid (MES), pH 5.6) to a final OD₆₀₀ of 1.0. Then, the resuspended bacterial solutions were placed in the dark for 3 h. To induce transient expression, 1-cm diameter samples were cut from *N. benthamiana* leaves with a puncher. Then, the samples were placed into the bacterial suspension and infiltrated under vacuum for 10–15 min until the samples became transparent. After the vacuum was released, the samples were placed on medium (0.4% agar) in the dark. After 24 h, the samples were transferred to medium (0.4% agar) with CaCl₂ (20 mM) or EGTA (20 mM) and incubated for 24 h. The samples on medium (0.4% agar) were

used as controls. The fluorescence signal was observed with a luminometer (PIXIS1024B, China). The LUC activity measurement was performed using Andor Solis v15 software as described in a previous study⁶⁹.

Subcellular localization of LIWRKY39

The ORF without the stop codon of *LIWRKY39* was inserted into a pCAMBIA1300-GFP vector to generate a fusion protein (LIWRKY39-GFP). The primers used for vector construction are shown in Supplementary Table S3. The empty vector pCAMBIA1300-GFP was used as the control. The plasmids pCAMBIA1300-GFP and pCAMBIA1300-LIWRKY39-GFP were transformed into *A. tumefaciens* strain GV3101, and then, the bacterial solution was infiltrated into *N. benthamiana*. A confocal laser-scanning microscope (LSM800, Zeiss, Germany) was used to observe the fluorescence.

Transactivation activity assay of LIWRKY39 in yeast

The ORF of *LIWRKY39* was inserted into the pGBKT7 vector using specific primers (Supplementary Table S3). The pGBKT7 vector, pGBKT7-GAL4, and pGBKT7-LIWRKY39 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-LIWRKY39 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 PCR⁷². PLACE databases (<http://www.dna.affrc.go.jp/PLACE/>) were used to analyze the *cis*-elements in the promoters.

Promoter activity analysis of *LIWRKY39*

The isolated 737-bp fragment of the *LIWRKY39* 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 *LIWRKY39* under HS. The bacterial solution of pCAMBIA1300-LIWRKY39 was collected by centrifugation and resuspended in a sucrose solution (5%). Arabidopsis plant genetic transformation was performed according to the floral dip method⁷³. 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 identification of the transgenic lines.

Phenotypic analysis and heat stress responsive gene expression in transgenic plants

Seeds of three transgenic lines (OE-2, OE-4, OE-5) and Col-0 were sown on the same MS medium, and after incubation, they were placed into the culture room at 4 °C for 3 days. To compare the thermotolerance of the transgenic line and wild-type (WT) plants, 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), *AtGolS1* (AT2G47180), *AtMBF1c* (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 *LIMBF1c* 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. *LIWRKY39* 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 *LIMBF1c* promoter was cloned into pGreenII-0800-LUC to obtain the reporter vector. The ORF of *LIWRKY39* was cloned into a pGreenII-62-SK vector to generate the effector vector. These vectors were transformed into *A. tumefaciens* strain GV3101 (pSoup). 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 study⁷⁴. 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 study⁷⁵. The samples were loaded onto a prerun native 4% polyacrylamide gel with TBE buffer as the electrolyte. After electroblotting 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 *LIWRKY39* and *LICaM3* were cloned into pGreenII-62-SK⁷⁶ for use as effector vectors. A fragment of the *LIMBF1c* 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.

Acknowledgements

This work was supported by the National Key R&D Program of China (Grant No. 2019YFD1000400), the National Natural Science Foundation of China (31902055), the High Level Talent Project of the Top Six Talents in Jiangsu, China (NY-077), the Natural Science Foundation of Jiangsu Province, China (BK20190532), and the Fundamental Research Funds for the Central Universities (KJQN202032). We thank the Central Laboratory of the College of Horticulture of Nanjing Agricultural University, which is a large-scale instrument sharing platform.

Author details

¹Key Laboratory of Landscaping Agriculture, Ministry of Agriculture and Rural Affairs, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China. ²Baguazhou Science and Technology Innovation Center of Modern Horticulture Industry, Nanjing 210043, China. ³College of Agriculture, Nanjing Agricultural University, Nanjing 210095, China. ⁴State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China. ⁵College of Life Science, Zaozhuang University, Zaozhuang 277160, China

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 assistances. X.C. provided the *LIMBF1c* 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 The online version contains supplementary material available at <https://doi.org/10.1038/s41438-021-00473-7>.

Received: 5 October 2020 Revised: 24 November 2020 Accepted: 3 December 2020

Published online: 04 February 2021

References

- Hasanuzzaman, M., Nahar, K., Alam, M. M., Roychowdhury, R. & Fujita, M. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *Int. J. Mol. Sci.* **14**, 9643–9684 (2013).
- Wahid, A., Gelani, S., Ashraf, M. & Foolad, M. R. Heat tolerance in plants: An overview. *Environ. Exp.* **61**, 199–223 (2007).
- Long, S. P. & Ort, D. R. More than taking the heat: Crops and global change. *Curr. Opin. Plant Biol.* **13**, 241–248 (2010).
- Fu, W. et al. Molecular cloning and analysis of a cytosolic HSP70 gene from *Enteromorpha prolifera* (Ulvoophyceae, Chlorophyta). *Plant Mol. Biol. Rep.* **28**, 430–437 (2010).
- Ohama, N., Sato, H., Shinozaki, K. & Yamaguchi-Shinozaki, K. Transcriptional regulatory network of plant heat stress response. *Trends Plant Sci.* **22**, 53–65 (2017).
- Xin, H. et al. Cloning and characterization of HSFA2 from lily (*Lilium longiflorum*). *Plant Cell Rep.* **29**, 875–885 (2010).
- Gong, B. et al. LHSA1, a novel heat stress transcription factor in lily (*Lilium longiflorum*), can interact with LHSA2 and enhance the thermotolerance of transgenic *Arabidopsis thaliana*. *Plant Cell Rep.* **33**, 1519–1533 (2014).
- Grover, A., Mittal, D., Negi, M. & Lavania, D. Generating high temperature tolerant transgenic plants: achievements and challenges. *Plant Sci.* **205**, 38–47 (2013).
- Teixeira, E. I., Fischer, G., Van Velthuisen, H., Walter, C. & Ewert, F. Global hot-spots of heat stress on agricultural crops due to climate change. *Agric. For. Meteorol.* **170**, 206–215 (2013).
- Mitsuda, N. & Ohme-Takagi, M. Functional analysis of transcription factors in *Arabidopsis*. *Plant Cell Physiol.* **50**, 1232–1248 (2009).
- Golldack, D., Lueking, I. & Yang, O. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep.* **30**, 1383–1391 (2011).
- Nover, L. et al. *Arabidopsis* and the heat stress transcription factor world: how many heat stress transcription factors do we need? *Cell Stress Chaperones* **6**, 177–189 (2001).
- Von Koskull-Doering, P., Scharf, K. D. & Nover, L. The diversity of plant heat stress transcription factors. *Trends Plant Sci.* **12**, 452–457 (2007).
- Xin, H. et al. Over-expression of LHSA2b, a lily heat shock transcription factor lacking trans-activation activity in yeast, can enhance tolerance to heat and oxidative stress in transgenic *Arabidopsis* seedlings. *Plant Cell Tissue Organ Cult.* **130**, 617–629 (2017).
- Wu, Z. et al. Overexpression of lily HSFA3s in *Arabidopsis* confers increased thermotolerance and salt sensitivity via alterations in proline catabolism. *J. Exp. Bot.* **69**, 2005–2021 (2018).
- Wu, Z. et al. Alternative splicing provides a mechanism to regulate LHSA3 function in response to heat stress in lily. *Plant Physiol.* **181**, 1651–1667 (2019).
- Wu, Z. et al. A canonical DREB2-type transcription factor in lily is post-translationally regulated and mediates heat stress response. *Front. Plant Sci.* **9**, 243 (2018).
- Pamela Arce, D. et al. The analysis of an *Arabidopsis* triple knock-down mutant reveals functions for MBF1 genes under oxidative stress conditions. *J. Plant Physiol.* **167**, 194–200 (2010).
- Florenza Di Mauro, M. et al. MBF1s regulate ABA-dependent germination of *Arabidopsis* seeds. *Plant Signal. Behav.* **7**, 188–192 (2012).
- Blombach, F. et al. Archaeal MBF1 binds to 30s and 70s ribosomes via its helix-turn-helix domain. *Biochem. J.* **462**, 373–384 (2014).
- Tsuda, K. & Yamazaki, K. Structure and expression analysis of three subtypes of *Arabidopsis* MBF1 genes. *Biochim. Biophys. Acta* **1680**, 1–10 (2004).
- Suzuki, N., Bajad, S., Shuman, J., Shulaev, V. & Mittler, R. The transcriptional co-activator MBF1c is a key regulator of thermotolerance in *Arabidopsis thaliana*. *J. Biol. Chem.* **283**, 9269–9275 (2008).
- Suzuki, N., Sejima, H., Tam, R., Schlauch, K. & Mittler, R. Identification of the MBF1 heat-response regulon of *Arabidopsis thaliana*. *Plant J.* **66**, 844–851 (2011).
- Yoshida, T. et al. *Arabidopsis* HSFA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol. Genet. Genomics* **286**, 321–332 (2011).
- Eulgem, T. & Somssich, I. E. Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* **10**, 366–371 (2007).
- Pandey, S. P. & Somssich, I. E. The role of WRKY transcription factors in plant immunity. *Plant Physiol.* **150**, 1648–1655 (2009).

27. Zhao, X. Y. et al. The MdWRKY31 transcription factor binds to the MdRAV1 promoter to mediate ABA sensitivity. *Horti. Res.* <https://doi.org/10.1038/s41438-019-0147-1> (2019).
28. Eulgem, T., Rushton, P. J., Robatzek, S. & Somssich, I. E. The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* **5**, 199–206 (2000).
29. Rushton, P. J., Somssich, I. E., Ringler, P. & Shen, Q. J. WRKY transcription factors. *Trends Plant Sci.* **15**, 247–258 (2010).
30. Ulker, B. & Somssich, I. E. WRKY transcription factors: From DNA binding towards biological function. *Curr. Opin. Plant Biol.* **7**, 491–498 (2004).
31. Giolkowski, I., Wanke, D., Birkenbihl, R. P. & Somssich, I. E. Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. *Plant Mol. Biol.* **68**, 81–92 (2008).
32. Ishihama, N. & Yoshioka, H. Post-translational regulation of WRKY transcription factors in plant immunity. *Curr. Opin. Plant Biol.* **15**, 431–437 (2012).
33. Wu, X., Shiroto, Y., Kishitani, S., Ito, Y. & Toriyama, K. Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing OsWRKY11 under the control of HSP101 promoter. *Plant Cell Rep.* **28**, 21–30 (2009).
34. Li, S., Zhou, X., Chen, L., Huang, W. & Yu, D. Functional characterization of Arabidopsis thaliana WRKY39 in heat stress. *Mol. Cells* **29**, 475–483 (2010).
35. Li, S., Fu, Q., Chen, L., Huang, W. & Yu, D. Arabidopsis thaliana WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance. *Planta* **233**, 1237–1252 (2011).
36. He, G. H. et al. Drought-responsive WRKY transcription factor genes TaWRKY1 and TaWRKY33 from wheat confer drought and/or heat resistance in Arabidopsis. *BMC Plant Biol.* **16**, 116 (2016).
37. Wang, X. et al. Cytological and molecular characteristics of pollen abortion in lily with dysplastic tapetum. *Hortic. Plant J.* **5**, 281–294 (2019).
38. Rieping, M. & Schoffl, F. Synergistic effect of upstream sequences, CCAAT box elements, and HSE sequences for enhanced expression of chimeric heat-shock genes in transgenic tobacco. *Mol. Gen. Genet.* **231**, 226–232 (1992).
39. Cao, X. et al. Involvement of Ca²⁺ and CaM3 in regulation of thermotolerance in lily (*Lilium longiflorum*). *Plant Mol. Biol. Rep.* **31**, 1293–1304 (2013).
40. Ayako, N. Y. et al. HSF1d and HSF1e involved in the transcriptional regulation of HSF2 function as key regulators for the HSF signaling network in response to environmental stress. *Plant Cell Physiol.* **52**, 933–945 (2011).
41. Liu, H. C. & Chang, Y. Y. Common and distinct functions of Arabidopsis class A1 and A2 heat shock factors in diverse abiotic stress responses and development. *Plant Physiol.* **163**, 276–290 (2013).
42. Li, C. et al. AtHSFA2 modulates expression of stress responsive genes and enhances tolerance to heat and oxidative stress in Arabidopsis. *Sci China C Life Sci.* **48**, 540–550 (2005).
43. Yoshida, T. et al. Functional analysis of an Arabidopsis heat-shock transcription factor HSF3 in the transcriptional cascade downstream of the DREB2A stress-regulatory system. *Biochem. Biophys. Res. Commun.* **368**, 515–521 (2008).
44. Hepler, P. K. Calcium: a central regulator of plant growth and development. *Plant Cell* **17**, 2142–2155 (2005).
45. Mcainsh, M. R. & Pittman, J. K. Shaping the calcium signature. *New Phytol.* **181**, 275–294 (2009).
46. Defalco, T. A., Bender, K. W. & Snedden, W. A. Breaking the code: Ca²⁺ sensors in plant signalling. *Biochem. J.* **425**, 27–40 (2010).
47. Hashimoto, K. & Kudla, J. Calcium decoding mechanisms in plants. *Biochimie* **93**, 2054–2059 (2011).
48. Luan, S., Lan, W. & Lee, S. C. Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL-CLPK network. *Curr. Opin. Plant Biol.* **12**, 339–346 (2009).
49. Day, I. S., Reddy, V. S., Ali, G. S. & Reddy, A. S. N. Analysis of EF hand-containing proteins in Arabidopsis. *Genome Biol.* **3**, RESEARCH0056 (2002).
50. Sanders, D., Pelloux, J., Brownlee, C. & Harper, J. F. Calcium at the crossroads of signaling. *Plant Cell* **14**, S401–S417 (2002).
51. Kudla, J., Batistic, O. & Hashimoto, K. Calcium signals: the lead currency of plant information processing. *Plant Cell* **22**, 541–563 (2010).
52. Reddy, A. S. N., Ali, G. S., Celesnik, H. & Day, I. S. Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell* **23**, 2010–2032 (2011).
53. Park, C. Y. et al. WRKY group IId transcription factors interact with calmodulin. *FEBS Lett.* **579**, 1545–1550 (2005).
54. Rhoads, A. R. & Friedberg, F. Sequence motifs for calmodulin recognition. *FASEB J.* **11**, 331–340 (1997).
55. Ogawa, D., Yamaguchi, K. & Nishiuchi, T. High-level overexpression of the Arabidopsis HSF2 gene confers not only increased thermotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *J. Exp. Bot.* **58**, 3373–3383 (2007).
56. Chang, Y. Y. et al. A heat-inducible transcription factor, HSF2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiol.* **143**, 251–262 (2007).
57. Liu, H. C., Liao, H. T. & Chang, Y. Y. The role of class A1 heat shock factors (HSF1s) in response to heat and other stresses in Arabidopsis. *Plant Cell Environ.* **34**, 738–751 (2011).
58. Zheng, Z., Abu Qamar, S., Chen, Z. & Mengiste, T. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant J.* **48**, 592–605 (2006).
59. Lippok, B. et al. Expression of AtWRKY33 encoding a pathogen- or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Mol. Plant Microbe Interact.* **20**, 420–429 (2007).
60. Mao, G. et al. Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *Plant Cell* **23**, 1639–1653 (2011).
61. Kim, M. J. et al. Abiotic and biotic stress tolerance in Arabidopsis overexpressing the multiprotein bridging factor 1a (MBF1a) transcriptional coactivator gene. *Biochem. Biophys. Res. Commun.* **354**, 440–446 (2007).
62. Yan, Q. et al. The grape VvMBF1 gene improves drought stress tolerance in transgenic Arabidopsis thaliana. *Plant Cell Tissue Organ Cult.* **118**, 571–582 (2014).
63. Zhao, Q. et al. Overexpression of a multiprotein bridging factor 1 gene DgMBF1 improves the salinity tolerance of chrysanthemum. *Int. J. Mol. Sci.* **20**, 245 (2019).
64. Kim, K. C., Fan, B. & Chen, Z. Pathogen-induced Arabidopsis WRKY7 is a transcriptional repressor and enhances plant susceptibility to *Pseudomonas Syringae*. *Plant Physiol.* **142**, 1180–1192 (2006).
65. Journot-Catalino, N., Somssich, I. E., Roby, D. & Kroj, T. The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana. *Plant Cell* **18**, 3289–3302 (2006).
66. Guo, W. L. et al. Reduced tolerance to abiotic stress in transgenic Arabidopsis overexpressing a *Capsicum annuum* multiprotein bridging factor 1. *BMC Plant Biol.* **14**, 138 (2014).
67. Huang, Y. C., Niu, C. Y., Yang, C. R. & Jinn, T. L. The heat stress factor HSF6b connects ABA signaling and ABA-mediated heat responses. *Plant Physiol.* **172**, 1182–1199 (2016).
68. Zhang, X., Wang, X., Zhuang, L., Gao, Y. & Huang, B. Abscisic acid mediation of drought priming-enhanced heat tolerance in tall fescue (*Festuca arundinacea*) and Arabidopsis. *Physiol. Plant.* **167**, 488–501 (2019).
69. Lu, J., Bai, M., Ren, H., Liu, J. & Wang, C. An efficient transient expression system for gene function analysis in rose. *Plant Methods* **13**, 116 (2017).
70. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* **25**, 402–408 (2001).
71. Willems, E., Leyns, L. & Vandesompele, J. Standardization of real-time PCR gene expression data from independent biological replicates. *Anal. Biochem.* **379**, 127–129 (2008).
72. Liu, Y. G. & Chen, Y. High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques* **43**, 649–654 (2007).
73. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735–743 (1998).
74. Fan, Y. et al. The RhHB1/RhLOX4 module affects the dehydration tolerance of rose flowers (*Rosa hybrida*) by fine-tuning jasmonic acid levels. *Hortic. Res.* **7**, 74 (2020).
75. Sun, W. et al. Hairy leaf 6, an AP2/ERF transcription factor, interacts with OsWOX3B and regulates trichome formation in rice. *Mol. Plant* **10**, 1417–1433 (2017).
76. Hellens, R. P. et al. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods* **1**, 13 (2005).