

Article

SIFBX38, an F-Box Protein, Enhances Thermotolerance in Tomato

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Abstract

Heat stress, intensified by global warming, poses a great threat to plant growth and crop production. However, the molecular mechanisms underlying heat stress response (HSR) remain largely unclear. In this study, we identified and characterized *SIFBX38*, an F-box gene in tomato. *SIFBX38* was predominantly expressed in leaves and fruits, and its expression levels were induced by heat stress and various phytohormones, including ABA, JA and SA. Subcellular location analysis revealed that *SIFBX38* resides in both the nucleus and cytoplasm in *N. benthamiana* leaf cells, but it displays no transcriptional activity. Overexpression of *SIFBX38* (OE) lines conferred enhanced heat stress tolerance, as evidenced by improved photosynthetic efficiency, elevated accumulation of ascorbic acid (AsA), stronger protective enzyme activities, and upregulation of HSR-related genes in *SIFBX38*-OE lines under heat stress condition. To identify potential interacting proteins, yeast two-hybrid (Y2H) library screening and further Y2H verification indicate that *SIFBX38* may interact with *SlbHLH058*. Collectively, these findings establish *SIFBX38* as a positive regulator of thermotolerance in tomato and provide a basis for further mechanistic studies of its role in HSR.

Keywords: *SIFBX38*; F-box; HSR; ascorbic acid (AsA); tomato

1. Introduction

Global warming-driven temperature rise poses a substantial threat to crop yields and global food security worldwide. It has been revealed that once the temperature exceeds the optimal temperature range of a plant by 5–10 °C, the plant suffers from heat stress (HS) [1]. Constitutive exposure to heat stress can cause irreversible damage to plant growth and development [2]. Studies have shown that high temperature adversely affects the efficiency of photosynthesis, for it causes a disruption of chloroplast membranes, manifests thylakoid disintegration, imbalances the chlorophyll a/b ratio, and even poses the risk of chlorophyll degradation, which finally causes leaf yellowing and dehydration-induced wilting [3,4]. Likewise, sustained high temperatures impair the structure and function of mitochondria, leading to the uncoupling of oxidative phosphorylation, decreased respiratory efficiency, reduced ATP synthesis, and enhanced generation of reactive oxygen species (ROS) [5]. Massive deposition of ROS that has not been scavenged efficiently can inflict damage to cellular macromolecules [6]. Therefore, HS can cause irreversible damage to various organelles and cell structures, and disrupt metabolic processes, which ultimately impairs



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plant development. However, the mechanisms underlying plant thermotolerance are becoming increasingly clear.

The cell membrane serves as the primary site for sensing the temperature fluctuations in the environment. Changes in the membrane fluidity, lipid composition and integrity can be detected by membrane-associated proteins, which in turn mediate a heat-induced influx of Ca^{2+} ions into the cell [7]. Similarly, the endoplasmic reticulum (ER) is particularly vulnerable to heat stress, which disrupts protein homeostasis and leads to the accumulation of unfolded protein, rapidly activating the cytoprotective unfolded protein response (UPR) [2]. Numerous transcription factors, including members from the NAC and bZIP families, have been implicated in UPR regulation. In response to HS-caused UPR, transcription factors NAC062 and NAC089 translocate from the plasma membrane and ER, respectively, to the nucleus, thereby activating downstream HSR-related pathways. It is noteworthy that both NAC062 and NAC089 are regulated by bZIP60, whereas NAC089 is additionally regulated by bZIP28 [8]. These two bZIP factors, bZIP60 and bZIP28, contribute to HS-related UPR through regulations on the mRNA splicing and protein modification levels, respectively [9,10]. Beyond those ER- and membrane-associated transcription factors, nuclear transcription factors such as HSFs (heat stress factors) are essential for the successful activation of HSR as most pathways are *HsfA* dependent.

HsfA1 plays a central role in inducing HSR in plants. In normal temperature conditions, its transcription activity is blocked by HSP proteins, while HS can release it from the protein complex to activate thermotolerance [10]. In addition, *HsfA2* functions downstream of *HsfA1*, and in tomato it can interact with *HsfA1* to form a super-activate heterodimer in HSR [11,12]. In addition, other Hsfs, such as *HsfB* and *HsfC* have been reported in thermotolerance as well [13]. *DREB2A* (*Dehydration-Responsive Element Binding 2*), another downstream gene of *HsfA1*, is a primary regulator of both heat and drought response. In addition, both JUB1 and MBF1c act to increase the transcription of *DREB2A* as well [12]. Although most regulations are dependent on HSFs, there are some that are independent of them. For example, REV4 and REV8 can initiate HS-related gene expression without HSFs [14]. Therefore, uncovering the function of genes and deciphering underlying mechanism are vital to fully understand HSR in plants.

The F-box domain-containing gene family is one of biggest gene families in plants. Though some research has reported its function in HSR in plants, none has been reported in tomato. Therefore, identifying and characterizing the function of F-box genes is vital to understand HSR in tomato. The F-box domain, consisting of around 40-amino acid domains, was first identified in the cyclin F protein [15]. Thus, it was named F-box domain, marking the origin of the F-box protein family. The F-box domain of cyclin F bound with the S-phase kinase-associated protein 1 (SKP1) is essential for the ubiquitin-mediated degradation of these protein complexes [16]. Later, it was found that F-box proteins recognize and bind substrates for the SKP1-cullin 1(CUL1)-F-box protein (SCF) ubiquitin ligase complexes to mediate the degradation of proteins [17]. Thus, the process is stringently regulated for F-box protein to recruit potential substrates to form specific SCF complexes in response to stimuli of development and stresses. Further mechanism analysis found that F-box protein can recognize a particular substrate through both phosphorylation and non-phosphorylation [18–20].

In plants, the F-box protein family is one of the biggest gene families. There are approximately 700 members in *Arabidopsis* and rice [21,22]. However, few studies have identified it in thermotolerance response [23,24]. In tomato, 139 F-box genes have been identified by a genome-wide bioinformatic study [25]. Among them, ten genes including *SIFBX38* have been identified as having a possible function in stress response [25]. In this study, in-depth analysis of gene expression patterns, subcellular location, gene function and

a possible molecular mechanism of *SIFBX38* in thermotolerance response was conducted, which will be of great significance for crop breeding in the future.

2. Materials and Methods

2.1. Plant Materials and Treatment

Solanum lycopersicum Mill. cv. Ailsa Craig (AC) and *SIFBX38* overexpression (OE) tomato plants used for seed collection were grown under natural conditions in the experimental fields of Southwest University in Chongqing, China. Eight-leaf plants used for heat stress (42 °C, 6000 Lx, 60%RH), cold treatment (4 °C, 6000 Lx, 60%RH) and drought treatment were grown in a greenhouse. As for drought stress, wilting plants were recovery watered for one day before sample collection. Leaf samples after treatments at different time points (0 h, 4 h, 8 h and 24 h) were collected for RNA extraction and RT-qPCR analysis. Phytohormones ethylene (Eth), abscisic acid (ABA), jasmonic acid (JA) gibberellin and (GA) and salicylic acid (SA) were sprayed on the leaves of the tomato plants, and mixed leaf samples were collected at different time points (1 h, 3 h, 6 h, 12 h and 24 h) for RNA extraction and RT-qPCR analysis (n = 3).

2.2. Vector Construction and Tomato Transformation

The 1110 bp CDS of *SIFBX38* (*Solyc03g026170*) was amplified and cloned into a linearized (digested by BamHI and SacI) p2024 vector driven by the CaMV35S promoter. Primers used in this study are listed in Table S1. Constructs were introduced into the callus of tomato cotyledons using the *Agrobacterium tumefaciens* strain LB4404 [26]. T₂ transgenic plants overexpressing *SIFBX38* were obtained and used in this study.

2.3. RNA Extraction and RT-qPCR Analysis

Total RNA was extracted using a fast total RNA kit (L5045786T) from BIOGUANG company (Chongqing for China) following the instructions provided. First-strand cDNA was produced by a reverse transcriptase kit (BG0070) from BIOGUANG company and diluted 20 times before being used for a quantitative RT-PCR (qRT-PCR). The expression of the ACTIN gene was used as internal control between different samples or genes. Primers used are listed in Table S1.

2.4. Photosynthetic Characteristics and Chlorophyll Contents Determination

Three healthy plants of AC and three *SIFBX38*-OE lines before and after heat stress treatment were chosen randomly for the measurement of the net photosynthetic rate (Pn), intercellular CO₂ concentration (Ci), stomatal conductance (Cs) and transpiration rate (Tr) with a photosynthetic apparatus (LI-6800, LI-COR, USA) in the greenhouse. For further analysis, the chlorophylls in the leaves of AC and *SIFBX38*-OE lines were measured by a Beckman 22S spectrophotometer. Briefly, approximately 0.1 g fresh leaf samples (n = 3) were cut and immersed in 20 mL ethanol and put in darkness for one day before the measurement of absorbance at OD₆₆₅ and OD₆₄₉. The content of total chlorophyll a and chlorophyll b was calculated as described previously [27].

2.5. Plant Physiological Indices Analysis

The activity of peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) was determined using a POD assay kit (A084-3-1), CAT assay kit (A007-1-1), APX test kit (A123-1-1) and SOD assay kit (A001-3-2) from Nanjing Jiancheng Bioengineering Institute Company (Nanjing, China). The experiments were conducted following the instructions provided by the manufacturer. The malondialdehyde (MDA) and proline were measured with a thiobarbituric acid (TBA) reaction as described

previously [26]. The ascorbic acid (AsA) in leaves was measured ($n = 3$) using a Vitamin C assay kit from Nanjing Jiancheng Bioengineering Institute Company (Nanjing, China).

2.6. Transient Expression Assays in *Arabidopsis* Protoplasts

The CDS of *SIFBX38* was cloned into a linearized (digested by *Xba*I and *Eco*RI) GAL vector with a seamless cloning kit (H0526281) from YEASEN company (Shanghai for China). The empty vector and VP16 were used as negative and positive control, respectively. The *Pro35S: Renilla-LUC* activity ($n = 3 \times 3$) was measured as a control to normalize LUC activity among samples through a luminometer (GloMax; Promega, USA). The *Arabidopsis* protoplasts were isolated following a method described previously [28].

2.7. Transient Expression Assays in *Nicotiana benthamiana* Leaves

The pCA1300 vector harboring GFP under the control of the CaMV35S promoter was used for analyses of *SIFBX38* subcellular localization in the leaves of *N. benthamiana*. Briefly, the *Agrobacterium tumefaciens* strain GV3101 containing *SIFBX38*-GFP plasmid transformed by heat-shock was grown in YEB medium with the antibiotics kanamycin and rifampicin to OD₆₀₀ values of 0.6–0.8. Cells were suspended in MES buffer (10 mM MgCl₂, 10 mM MES; pH 5.6) to an OD₆₀₀ of 0.4 and kept in darkness for about 3 h before inoculation. DAPI solution, the nucleus indicator, was inoculated in leaf cells two hours before observation. Leaves were analyzed at 2 days after transformation using a confocal microscope (SU3500, HITACHI, Japan).

2.8. Phylogenetic Analysis

Homologous protein sequences of *SIFBX38* from five eudicots, three monocots and one ancient vascular species were obtained from NCBI by BLAST (2.14.0) search. The phylogenetic tree was constructed after multiple alignments using the maximum likelihood method using MEGA11 software (11.0.13) [29]. The numbers next to the branches are bootstrap support values.

2.9. Yeast Two-Hybrid Assay

A yeast two-hybrid assay was conducted in Y2H Gold yeast strain cells using the GAL4 two-hybrid system. Briefly, *SIFBX38*-pGADT7 and pGBKT7 vectors were transformed into Y2H Gold yeast strain using a PEG/LiAc-mediated transformation. The positive colony containing *SIFBX38*-pGADT7 was put to grow on a TDO (Trp, Leu and Ade) medium to test autoactivation of *SIFBX38* before carrying out library screening. The library that was constructed using tomato leaves was already available in our laboratory. The single yeast colony containing *SIFBX38*-pGADT7 and 2 mL of the Y2H library yeasts were grown in $2 \times$ YPDA to an OD₆₀₀ of 0.6 before mixing. The mixed yeasts were growing under 30 °C and 80 rpm condition for around 12 h until most “micky-like” yeasts were in a normal round morphology. Thereafter, the yeasts were collected to grow on QDO plates lacking Trp, Leu, Ade and His but containing AbA (100 ng/mL). The PCR was used to amplify the gene in the colonies and was identified by sequencing and BLAST search in the Sol Genomics Network.

3. Results

3.1. *SIFBX38* Is a Typical and Conserved F-Box Protein in Tomato

F-box genes related to stress response have rarely been reported in tomato. A previous study has conducted a genome-wide analysis of FBXs in tomato, and found that *SIFBX38* may function in stress response [25]. In this study, we intend to focus on the function of *SIFBX38* in tomato. It has been identified that *SIFBX38* encodes a protein

with 369 amino acid residues with the N-terminal conserved F-box domain (8–53 aa) (Figure 1a). Phylogenetic analysis determined twenty-five proteins, including SIFBX38 (XP 004234361.1), from tomato, pepper, soybean, Arabidopsis, rape, rice, corn and the ancient species *Brachypodium distachyon* and *Selaginella moellendorffii* (Figure S1). In general, FBXs from eudicots and monocots evolved separately into two branches, while those from the ancient fern *Selaginella moellendorffii* are clustered in one branch independently (Figure 1b). The three other FBXs in tomato were clustered closer with those from *Solanaceae* plants, while SIFBX38 was clustered in another branch with those from pepper and soybean (Figure 1b).

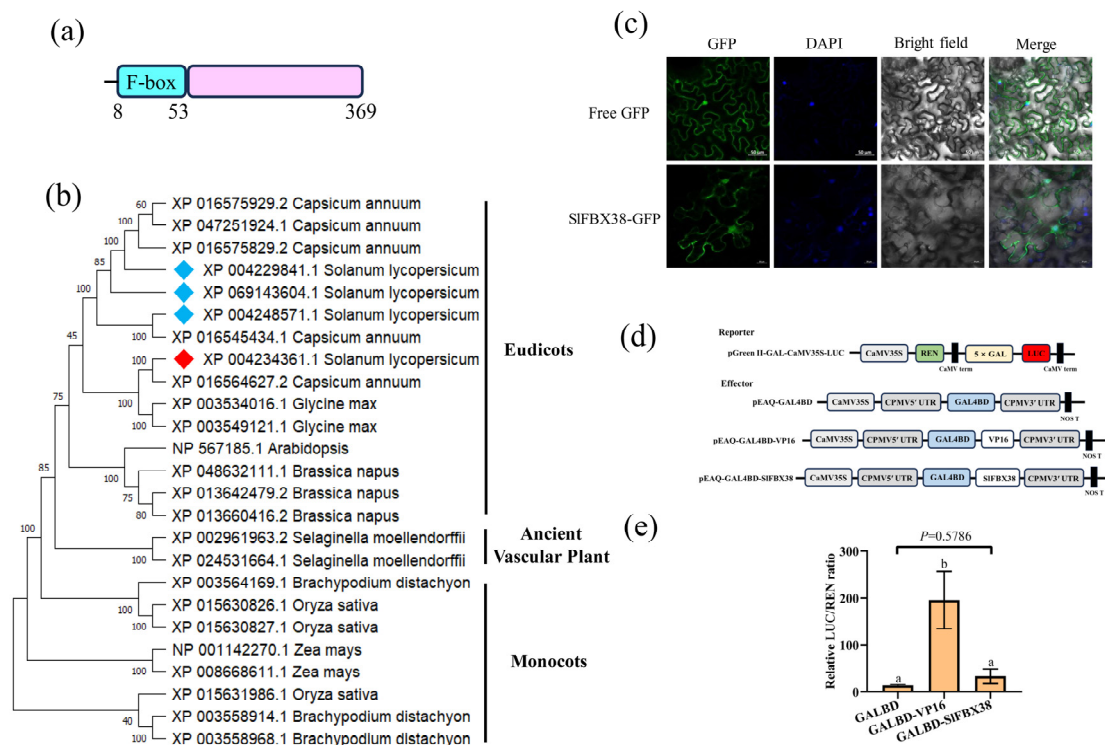


Figure 1. The protein characterization of SIFBX38 in tomato. (a) Schematic diagram of SIFBX38 protein structure. Blue box indicates F-box domain; pink box indicates non-conserved protein sequence. (b) Phylogenetic analysis of SIFBX38 and homologous proteins in plants. (c) Subcellular localization of SIFBX38-GFP and free GFP in *N. benthamiana* leaves. The blue DAPI signal was used as the indicator of the nucleus; the signal from free GFP was used as control. (d) The construction of the transcription factor activity assay. (e) The measurement of transcription factor activity. The LUC activity of empty vector *Pro35S: GAL*, the positive control *Pro35S: GAL-VP16* and *Pro35S:SIFBX38-GAL* were measured 16 h after transformation. Values represent mean $\pm$ SD ($n = 3$). The different letters indicate significant differences (ANOVA with Duncan's multiple comparisons; $p < 0.05$).

SIFBX38 was predicted to be located in the nucleus (<https://www.genscript.com/tools/wolf-psort>, accessed on 23 February 2026). To identify the subcellular location of SIFBX38, SIFBX38-GFP was expressed in *N. benthamiana* leaves. The fluorescence signal of SIFBX38-GFP can be observed in the nucleus as indicated by a blue DAPI signal, and in the cytoplasm and cell membrane as well (Figure 1c). However, the exact part within the cytoplasm remains to be further elucidated. A GAL4-BD-SIFBX38 fusion protein was generated to determine whether SIFBX38 has transcription activation ability. The results showed that no significant difference was observed in LUC activity between SIFBX38 and an empty vector, while LUC activity of VP16 was significantly higher (Figure 1d,e). Thus, the SIFBX38 protein is located in the nucleus and cytoplasm to mediate their function, but it may not function as a transcription factor in its current form.

3.2. The Expression Profile of *SIFBX38* in Tomato

To uncover the tissues that locate *SIFBX38*, the spatiotemporal expression pattern of *SIFBX38* was investigated. The results showed that *SIFBX38* was expressed in almost all tissues, but was highly expressed in leaves in an age-dependent manner (Figure 2a). In addition, *SIFBX38* showed relatively high expression levels in fruit at all development stages as well, but relatively low expression levels in the root, abscission zone and floral organs of the flowers (Figure 2a). Thus, *SIFBX38* may function mainly in leaves and fruits.

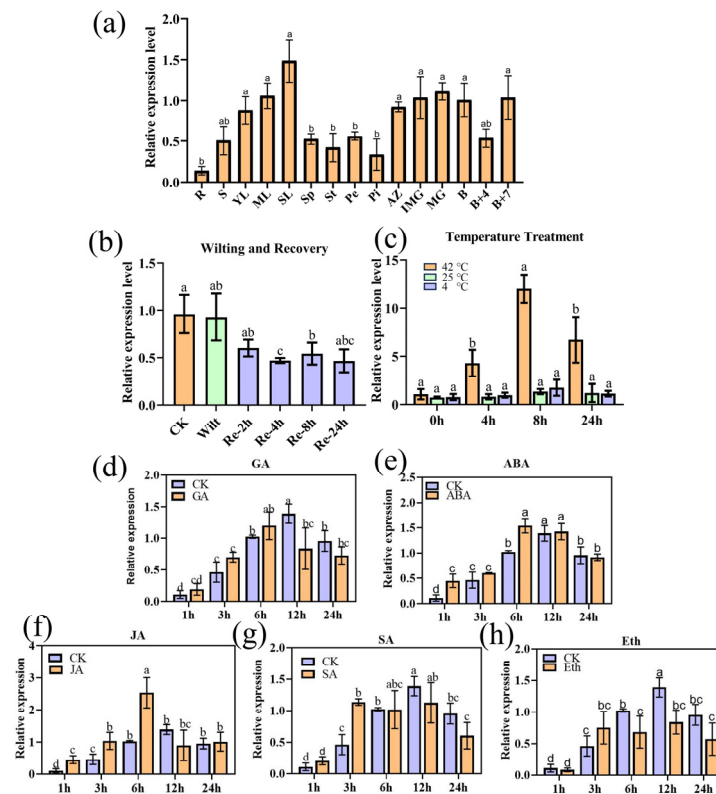


Figure 2. The expression pattern of *SIFBX38* in tomato. (a) The relative expression of *SIFBX38* in different tissues. R: root; S: stem; YL, ML, SL: indicate young leaves, mature leaves and senescent leaves, respectively; Sp: sepal; St: stamen; Pe: petal; Pi: pistil; AZ: abscission zone; IMG-B+7: indicates fruit at immature green (IMG), mature green (MG), breaker (B), 4 days after breaker (B+4) and 7 days after breaker stage (B+7), respectively. (b) Relative expression of *SIFBX38* under drought stress condition; CK: plants with regular watering; Re-4 h, Re-8 h, Re-24 h: indicate different time points after watering of wilting plants. (c) The relative expression of *SIFBX38* under cold (4 °C) or heat stress conditions (42 °C). Plants growing at 25 °C were used as control. (d–h) Phytohormone-induced expression of *SIFBX38* at 1, 3, 6, 12 and 24 h after spraying solutions with GA (d), ABA (e), JA (f), SA (g), and Eth (h), respectively. All data represent mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Duncan's multiple comparisons; $p < 0.05$).

To further elucidate whether *SIFBX38* participates in drought and adverse temperature conditions, the expression levels of *SIFBX38* were analyzed in these unfavorable growth conditions. The results showed that *SIFBX38* cannot respond to drought stress (Figure 2b) for no significant difference was observed in the expression levels of *SIFBX38* at wilting or recovering time points caused by drought stress (Figure 2b). As for adverse temperature treatments, no significant difference was observed after cold treatment (4 °C) at different time points (0 h, 4 h, 8 h and 24 h) (Figure 2b). However, the expression level of *SIFBX38* was significantly induced at the 4 h, 8 h and 24 h time points after heat stress treatment (42 °C). Thus, it is speculated that *SIFBX38* may function in thermotolerance response.

In addition, phytohormones play vital roles in multiple biological processes. To understand whether phytohormones could induce the expression of *SIFBX38*, ethylene (Eth), abscisic acid (ABA), jasmonic acid (JA) and gibberellic acid (GA) were sprayed on the leaves of the AC plants. Leaf samples were collected at different time points to see whether *SIFBX38* could be induced by a certain phytohormone. The results demonstrated that *SIFBX38* cannot respond to GA, but can be induced by ABA, JA or SA at several time points (Figure 2d). However, Eth can slightly repress the expression of *SIFBX38* (Figure 2d).

3.3. Overexpression of *SIFBX38* Exhibits Enhanced Tolerance to HS

Considering that *SIFBX38* was highly expressed in leaves and was heat-inducible, it is speculated that *SIFBX38* in leaves may participate in heat stress responses. To verify our hypothesis, the overexpression lines of *SIFBX38* were generated and those three (OE-2, OE-8 and OE-15) with the highest expression levels were used for heat stress treatment (Figure 3a). The results showed that the AC plants severely wilted, while the *SIFBX38*-OE lines were slightly influenced by heat stress and showed better growth performance (Figure 3b,c). At the same time, the abaxial surface of the leaves before and after heat stress treatment were observed by SEM. No significant differences in structure and stomatal number were observed between AC and the *SIFBX38*-OE lines (Figure S1). Furthermore, no significant differences in plant height and stem diameter were observed between AC and *SIFBX38*-OE lines at either normal or heat stress conditions (Figure 3d,e). Thus, overexpression of *SIFBX38* may not influence plant growth though it may play a positive role in heat stress response.

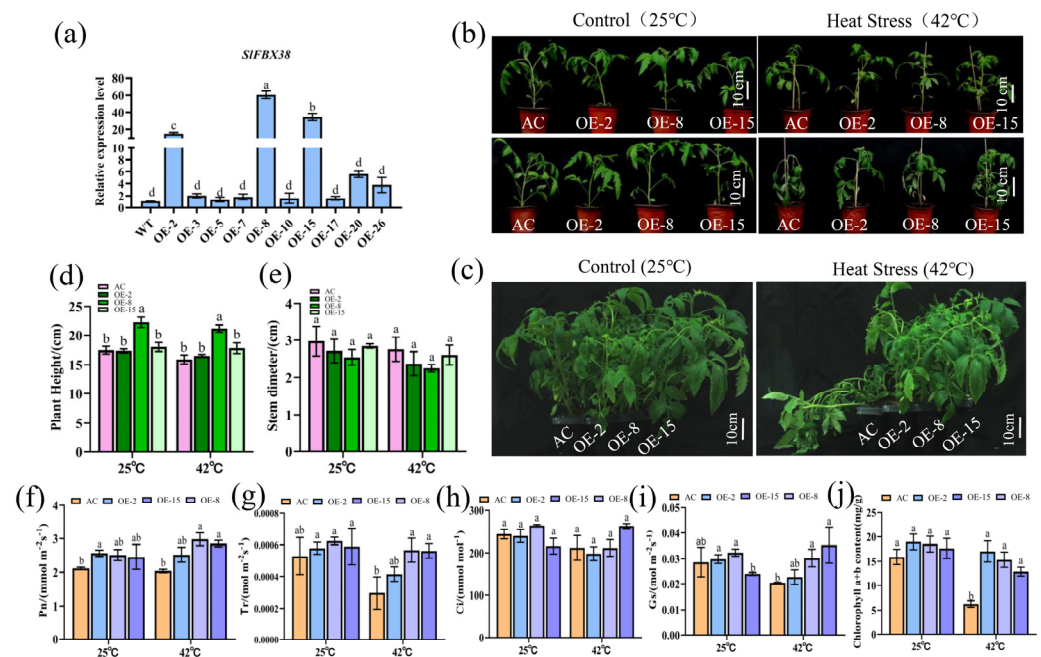


Figure 3. Analysis of *SIFBX38*-OE lines in thermotolerance response. (a) The expression of *SIFBX38* in different lines. Values represent mean $\pm$ SD ($n = 3$). (b) The AC plants and the OE lines with or without heat stress treatment. (c) A group of AC plants and OE lines with or without heat stress treatment. (d,e) The plant height (d) and stem diameter (e) of plants in normal and heat stress condition. Values represent mean $\pm$ SD ($n = 5$). (f–i) The measurement of net photosynthetic rate (f), transpiration rate (Tr) (g) and stomatal conductance (Cs) (h), and intercellular CO_2 concentration (Ci) (i). (j) The content of chlorophyll a $\pm$ b. Values represent mean $\pm$ SD ($n = 3$). Different letters indicate significant differences (ANOVA with Duncan's multiple comparisons; $p < 0.05$).

To elucidate how overexpression of *SIFBX38* could enhance heat stress tolerance, photosynthetic characteristics were measured. The results showed that almost no difference was

observed between AC and *SIFBX38* OE lines in photosynthetic characteristics (Figure 3f–i). However, the net photosynthetic rate (Figure 4f), transpiration rate (Tr) (Figure 3g) and stomatal conductance (Cs) (Figure 3h) were higher in *SIFBX38*-OE lines, while almost no significant difference was observed in intercellular CO₂ concentration (Ci) after heat stress response (Figure 3i). Consistent with this, the content of chlorophylls was higher in *SIFBX38*-OE lines under heat stress, while there is no significant difference in normal growing conditions (Figure 3j).

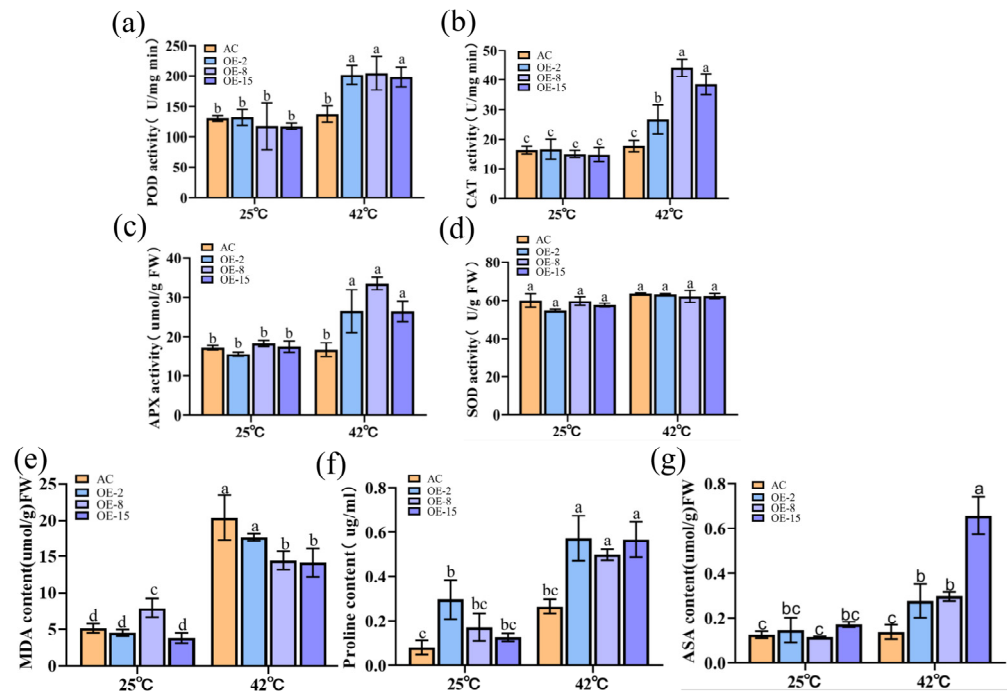


Figure 4. Physiological indices analysis of *SIFBX38*-OE lines after heat stress. (a–d) The POD (a), CAT (b), APX (c) and SOD (d) activity in AC and three *SIFBX38*-OE lines at normal or heat stress growing conditions. (e–g) The content of MDA (e), proline (f) and AsA (g) in AC and *SIFBX38*-OE plants at normal or heat stress growing conditions. Values represent mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Duncan's multiple comparisons; $p < 0.05$).

To ensure the better performance of plants of *SIFBX38*-OE lines under heat stress, the activities of antioxidant protective enzymes and metabolites were determined. The activities of POD, CAT and APX were all significantly higher in *SIFBX38*-OE lines under heat stress treatment, while no difference was observed in normal growing conditions (Figure 4a–c). However, SOD activities were of no significance in either normal or heat stress conditions (Figure 4d). Consistent with the results of enzyme activities, the content of MDA, proline and AsA were elevated in *SIFBX38* OE lines under 42 °C, while there is no difference in normal growing conditions (Figure 4e–g). Thus, overexpression of *SIFBX38* enhanced the tolerance of tomato plants to heat stress.

3.4. The Expression Dynamics of HS-Related Genes

Transcription factors *Hsfs*, especially *HsfA1s*, play vital roles in thermotolerance signaling. To identify whether *SIFBX38*-mediated heat tolerance response was dependent on *HsfA1s*, the expression levels of *Hsfs* and heat tolerance-related genes were determined after heat stress treatment. The results showed that no significant differences were observed in the transcription levels of *SIHsfA1a*, *SIHsfA1b*, *SIHsfA2* and *SIHsfB1* in normal growing conditions (Figure 5a–d). However, under a 42 °C heat stress condition, enhanced expressions of *SIHsfA1a*, *SIHsfA1b*, *SIHsfA2* and *SIHsfB1* were all observed at the 8, 12, 24 h time points

of the heat stress treatment (Figure 5a–d). At the 36 h time point, most signal transductions were over, hence no significance was observed in the expression of *SlHsfA1a*, *SlHsfA1b*, *SlHsfA2* and *SlHsfB1* genes (Figure 5a–d). This was consistent with the phenotype that AC plants were wilting, while those of OE lines stood still. At the same time, enhanced expression levels of *SIDREB2A2* and *SIDREB2A1* were observed in OE lines at the 8, 12, 24 h and 12, 24, 36 h time points, respectively (Figure 5e,f).

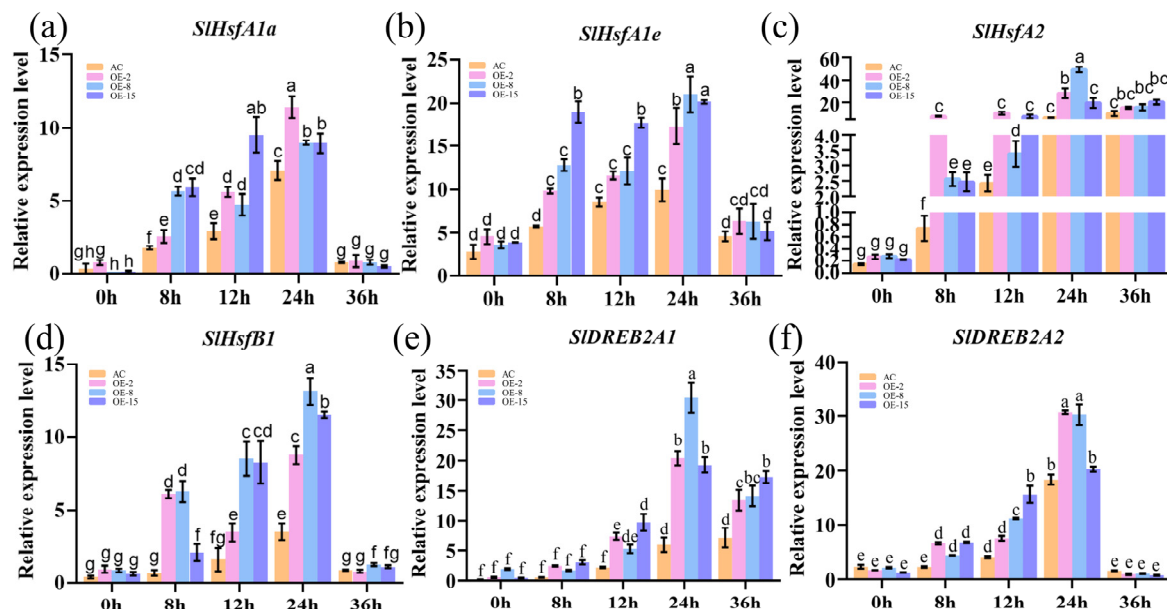


Figure 5. The expression of HSR-related genes. (a–f) The expression of *SlHsfA1a* (a), *SlHsfA1e* (b), *SlHsfA2* (c), *SlHsfB1* (d), *SIDREB2A1* (e) and *SIDREB2A2* (f) in AC and SIFBX38 OE lines at different time points (0, 8, 12, 24 and 36 h). Values represent mean $\pm$ SD, ($n = 3$); different letters indicate significant differences (ANOVA with Duncan's multiple comparisons; $p < 0.05$).

3.5. The Identification of Possible Interacting Proteins

To elucidate the molecular mechanism of *SIFBX38* in heat stress response, a Y2H library screen of the tomato leaf library was performed. No autoactivation of *SIFBX38*-pGADT7 was detected on a QDO/AbA medium (Figure 6a). Thus, yeasts harboring the *SIFBX38*-pGBKT7 vector can be used to screen the library. Colonies growing on the QDO medium were selected for sequencing to identify the corresponding prey genes. A total of 136 candidate interacting proteins were identified (Table S2). Among these, the most frequently recovered genes are *Solyc07g041920.2*, *Solyc12g005630* and *Solyc10g086580*, each appearing in approximately ten independent colonies. Several other genes were found in around five colonies, including *Solyc07g041910*, *Solyc10g055470*, *Solyc06g008910*, *Solyc12g088670*, *Solyc09g009260*, *Solyc07g043420*, *Solyc01g066420*, and *Solyc03g117600*. Symbiotic nitrogen fixation (SNF)-related genes have long been reported to participate in stress response. And there are two SNF-related genes *Solyc02g068100* and *Solyc12g099970* which were identified. Only one transcription factor, *Solyc09g065820*, which encodes a bHLH protein (SlbHLH59), was identified.

To further validate these interactions, the coding sequences of four high-confidence candidates (*Solyc02g068100*, *Solyc09g065820*, *Solyc12g005630*, and *Solyc12g088670*) were cloned to pGADT7 as prey constructs and co-transformed with *SIFBX38*-pGBKT7 into yeast cells. The results showed that all the transformants can grow on a DDO medium, but only these co-expressing *Solyc09g065820* and *SIFBX38* were able to grow on a QDO/AbA medium (Figure 6b). These results indicate that *SIFBX38* may interact with SlbHLH59 to mediate HSR in tomato.

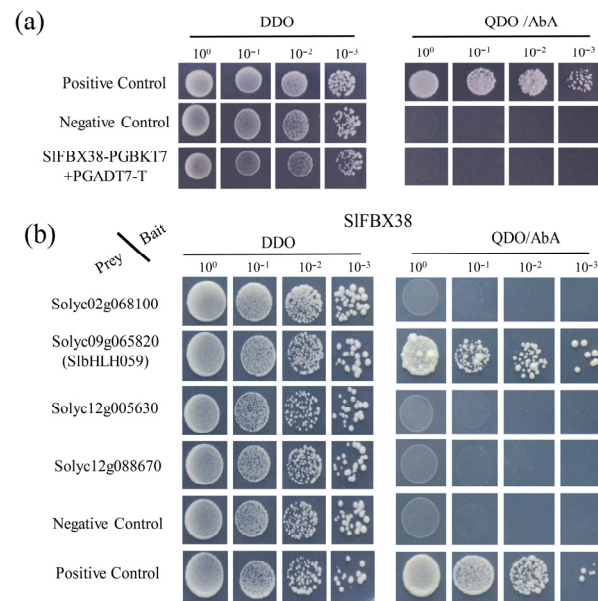


Figure 6. The identification of potential interacting proteins of SIFBX38. (a) Autoactivation analysis of SIFBX38 in yeast cells. (b) Yeast two-hybrid (Y2H) assay between SIFBX38 and several candidates that were identified by Y2H screening.

4. Discussion

4.1. Heat Stress and Various Phytohormones Induced the Expression of SIFBX38 in Leaves

Tomato plants are highly sensitive to heat waves; a slight temperature rise that exceeds the optimal temperature range is detrimental to plant growth and development, thereby posing a great threat to crop productivity [30]. Thus, breeding heat-tolerant varieties is of great significance to combat the extreme heat waves associated with global warming, and this requires in-depth molecular understanding of heat stress responses.

In this study, we first identified and characterized the F-box gene *SIFBX38* in tomato. Phylogenetic analysis showed that instead of clustering with its homologous proteins in tomato, *SIFBX38* is in another branch with FBX proteins from other species. This suggested that the function of *SIFBX38* may be specialized. Phytohormones, especially ABA, play vital roles in heat stress perception and response. ABA-triggered Ca^{2+} influx was observed in guard cells, and cytosolic Ca^{2+} signaling can enhance the efficiency of ABA to close stomata in response to stimuli [31,32]. In addition, multiple cyclic nucleotide-gated channels (CNGCs) are required for ABA-triggered Ca^{2+} influx and ABA-induced stomatal closure in *Arabidopsis* [33]. Except for ABA, other phytohormones hold a potential to crosstalk with ABA in heat stress response [34,35]. In this study, ABA, JA and SA can significantly induce the expression of *SIFBX38* at some time points, while ethylene shows a slight inhibitory role. Though SA and JA are traditionally associated with responses against pathogen invasion or mechanical injuries, they are implicated in response to variable abiotic stimuli as well [36]. In ryegrass, exogenous methyl jasmonate can maintain the content of chlorophyll (Chl) and malondialdehyde (MDA), as well as promote the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), thereby equipping plants with enhanced heat stress tolerance [37]. In cotton (*Gossypium hirsutum*), SA promotes heat tolerance by preserving the photosynthetic system [38]. In maize (*Zea mays* L.), exogenous application of SA can effectively mitigate heat stress-induced leaf senescence by cooperating with lignin and sucrose signal pathways [39]. Thus, it is suggested that *SIFBX38* may be involved in ABA-, SA- and/or JA-mediated thermotolerance response in tomato. In addition, spatiotemporal expression analysis showed that *SIFBX38* is mainly expressed in old leaves and fruits. In addition, drought stress cannot induce the expression

of *SIFBX38*, while heat stress significantly elevated the expression of *SIFBX38* to higher levels at different time points. This showed us that *SIFBX38* may participate in heat stress response.

4.2. *SIFBX38 May Positively Participate in Thermotolerance Response*

Consistent with our hypothesis, overexpression of *SIFBX38* equipped plants with a better growing and photosynthesis performance, yet no significant differences in plant growth, stomata opening or the morphology of guard cells were observed. Moreover, the enhanced activities of antioxidant enzymes—POD, CAT and APX—facilitate the timely scavenge of ROS in *SIFBX38* OE lines, resulting in less ROS-induced damage. Notably, the concentration of ascorbic acid (AsA) was also significantly elevated in *SIFBX38* OE lines. AsA is well documented as a protective compound against drought or oxidative stress in tomato [26,40]. Exogenous application of AsA on leaves has been shown to enhance heat stress tolerance and photosynthetic efficiency in apple leaves under heat stress [41]. Therefore, the increased AsA content in the leaves of *SIFBX38* OE lines likely contributes to their enhanced oxidative stress tolerance under high-temperature conditions.

In line with the enhanced heat stress-tolerant phenotype in *SIFBX38* OE lines, the expression of the transcription factor gene *SIDREB2A* was upregulated, which accounts for the reduced water loss in *SIFBX38* OE lines. *SIDREB2A* genes are vital regulators in thermotolerance response; overexpression of one DREB2A can induce the expression of heat-related genes [42,43]. Additionally, upon heat stress exposure, heat stress transcription factors (HSFs) can be induced quickly. In tomato, HsfA1 and HsfA2 can form hetero-oligomeric super-activation complex in response to heat stress response, and AHA motifs in both contribute to its activation potential [11,44]. In this study, elevated expression of *SIHsfA1s*, *SIHsfA2* and *SIHsfB* in the OE lines also suggests the higher HSR ability in the OE lines. Phytohormones can work synergistically with Hsfs to regulate the thermotolerance in plants [45,46]. It makes sense that the expression of *SIFBX38* can be induced by phytohormones, such as ABA, JA and SA. However, given that neither knock-out nor knock-down lines of *SIFBX38* were successfully generated, we can only infer that *SIFBX38* may positively regulate heat stress response in tomato.

4.3. *The Possible Mechanism of SIFBX38-Mediated Thermotolerance*

Although the function of *SIFBX38* in thermotolerance has been analyzed, the underlying mechanism remains unclear. *SIFBX38* is a typical F-box protein that is located in the nucleus and cytoplasm with no transcriptional ability in tomato. We therefore hypothesized that *SIFBX38* may confer thermotolerance through interacting with other transcription factors or via post-translational regulation. Yeast two-hybrid library screening, followed by a targeted Y2H assay, confirm that *SIFBX38* interacts with the transcription factor SlbHLH59 in yeast. SlbHLH59 promotes the elevated accumulation of AsA by directly binding to the promoter of genes involved in the D-mannose/L-galactose pathway [47]. Accordingly, we propose that *SIFBX38* contributes, at least in part, to heat stress response by facilitating AsA biosynthesis through its interaction with SlbHLH59, although this interaction requires further validation.

However, we cannot exclude other possibilities such as that *SIFBX38* may mediate the protein stability by directly participating in UPR in ER. The mRNA of bZIP60 can be spliced by the ER-located IRE1 (Inositol-requiring enzyme 1) to produce truncated protein that is translocated into the nucleus to activate the HSR-related genes [9]. Another ER-located TF, bZIP28, can move from the ER to the Golgi apparatus, and after subsequent proteolytic modification, bZIP28 can relocate to the nucleus to activate HSR [10]. Like bZIP proteins, NAC transcription factors have been identified in UPR and the translocation

of its coding proteins as well [48]. Thus, though SIFBX38 may work with SlbHLH59 to regulate the accumulation of AsA, whether SIFBX38 functions in UPR in the ER remains to be identified further.

5. Conclusions

In this study, we identified SIFBX38, an F-box protein gene localized to both the nucleus and cytoplasm, which is predominantly expressed in tomato leaves and fruits. Overexpression of *SIFBX38* enhanced thermotolerance associated with improved photosynthetic efficiency, stronger protective enzyme activities, and the upregulation of HSR-related genes. Although SIFBX38 lacks transcription activity, Y2H library screening followed by targeted validation revealed that it interacts with the AsA biosynthesis-related transcription factor SlbHLH059 in yeast. Our findings provide a foundation for studies on SIFBX38-mediated thermotolerance in tomato.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12030343/s1>, Table S1: The sequences of primers used in this study. Figure S1: The alignment of homologous proteins. Figure S2: The abaxial leaves observation of SIFBX38 OE lines. Table S2: The list of proteins identified by Y2H library screening with SIFBX38.

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