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RESEARCH ARTICLE

The heat shock transcription factor SlHSFA3 enhances heat tolerance in tomato by directly modulating both APX activity and *SlAPX1* expression

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Highlights

- The heat shock transcription factor SlHSFA3 positively regulates heat tolerance in tomato.
- SlHSFA3 interacts with SlAPX1 and specifically binds to the *SlAPX1* promoter region.
- The SlHSFA3-SlAPX1 regulatory module improves heat tolerance in tomato by coordinately enhancing ROS-scavenging capacity *via* both enzymatic and transcriptional regulation.

Abstract

Global climate warming and extreme high-temperature events significantly impact crop growth, development, and economic productivity. Plants typically enhance heat tolerance by regulating heat shock transcription factors (HSFs) and antioxidant enzymes to reduce the detrimental impacts of heat stress. However, the precise regulatory mechanisms by which HSFs confer heat tolerance in tomato remain to be elucidated. In this study, we investigated the role of the heat-induced transcription factor SlHSFA3 in tomato heat tolerance. We demonstrated that knockout of *SlHSFA3* increases tomato plants' sensitivity to heat stress, while enhanced expression of *SlHSFA3* improves heat tolerance by promoting reactive oxygen species (ROS) scavenging through increased ascorbate peroxidase (APX) activity. Similarly, overexpression of *SlAPX1* enhances heat tolerance in tomato by reducing ROS accumulation, whereas its knockout increases heat stress sensitivity. Furthermore, SlHSFA3 interacts with SlAPX1 to further augment APX activity. Molecular analysis revealed that SlHSFA3 directly upregulates the expression of *SlAPX1* by binding to its promoter region. In summary, our findings elucidate the molecular mechanism of the SlHSFA3-SlAPX1 regulatory pathway in tomato heat tolerance, providing a theoretical foundation for developing advanced biotechnological and breeding strategies to improve crop adaptation under elevated temperature conditions.

Keywords: SlHSFA3, heat tolerance, *SlAPX1*, ROS, tomato

1. Introduction

In recent years, severe climatic events have progressively reduced crop yields, presenting a major challenge to worldwide food stability (Challinor *et al.* 2014). Studies indicate that approximately 50% of crop yield losses worldwide can be attributed to abiotic stress (Wang *et al.* 2004). Among these stressors, rising global temperatures present a particularly serious challenge by exposing plants to frequent heat stress. Mild temperature increases generally cause less damage, typically resulting in hypocotyl elongation, altered flowering times, and other morphological and developmental changes associated with thermomorphogenesis (Casal and Balasubramanian 2019). However, excessively high temperatures severely impair plant growth and development, leading to substantial crop yield reductions and considerable economic impacts at the national level (Challinor *et al.* 2014).

Plants have acquired sophisticated physiological and

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molecular strategies to adapt and thrive under conditions of elevated temperatures. Upon exposure to heat stress, plants accumulate various thermoprotectants, phytohormones and signaling molecules. Heat stress signals are transmitted into cells through intricate pathways, leading to the accumulation of unfolded proteins and reactive oxygen species (ROS). These changes will trigger the expression of heat shock transcription factors (HSFs), thereby regulating the expression of heat response genes and enhancing heat tolerance (Kaushal *et al.* 2016; Ohama *et al.* 2017).

HSFs are pivotal transcriptional regulators present across eukaryotes, contributing significantly to growth, development, and stress responses. Their expression is triggered by various environmental stresses, including high temperatures, salinity, oxidative conditions, drought, and intense light (Kotak *et al.* 2007; Wei *et al.* 2016). Extensive research has been conducted on the role of HSFs in regulating plant responses to heat stress (Baniwal *et al.* 2004). Under high-temperature conditions, HSFs specifically bind to heat shock elements (HSEs) in the promoters of heat responsive genes, and interact with other transcription factors to form a transcriptional complex for inducing the expression of targeted heat responsive genes (Scharf *et al.* 2012). As crucial transcriptional regulators, HSFs modulate the activation of various stress-responsive genes, including heat shock proteins (HSPs), other molecular chaperones, antioxidant defense components, metabolic protection systems and osmoprotectants (Guo *et al.* 2016). The HSFs across different plants exhibit relative evolutionary conservation, with their gene structures showing certain similarities. Most plant HSF proteins contain five functional domains: the DNA-binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES) and activator motifs (AHA) (Guo *et al.* 2016). Although the number of HSF members varies among plant species, their classification remains relatively stable. Currently, all known HSFs in plants are classified into three primary groups: A, B, and C (von Koskull-Döring *et al.* 2007). The DBD domain, located at the N-terminus of HSFs, is the most conserved domain responsible for binding to HSEs, while the OD and AHA domains exhibit greater variability, suggesting their involvement in functional differences among HSFs (Wang *et al.* 2018).

HSF genes are ubiquitously present in plants and play crucial roles in thermal tolerance. In tomato, the genome encodes at least 26 HSF members, which are classified into three classes: 17 members of Class A, 8 members of Class B, and 1 member of Class C (Yang *et al.* 2016). Class A HSFs, especially HSFA1 and HSFA2, serve as key molecular components in the heat tolerance signaling cascade, orchestrating essential functions during plant thermal stress responses (Liu *et al.* 2014). Additionally, HSFA3, in conjunction with HSFA2, have been implicated in heat stress memory. HSFA3 activates the hypermethylation of histone H3K4 to sustain continuous expression of heat stress memory-related genes (Friedrich *et al.* 2021). Silencing the *HSFA1a* gene in tomato leads to increased sensitivity to heat stress, with downregulation of stress responsive genes; conversely, plants overexpressing *HSFA1a* exhibit improved heat tolerance (Mishra *et al.* 2002). In poplar, salt stress induces *HSFA5a* expression, which promotes key enzyme genes in the flavonol biosynthesis pathway, significantly boosting flavonol accumulation and reducing ROS levels in roots, ultimately enhancing the plant's adaptive capacity under saline conditions (Song *et al.* 2024). Scientific investigations have revealed that the inability of HSFB transcription factors to activate gene expression is due to the absence of the AHA domain in their protein structure, which is crucial for binding to certain basic transcription elements (Czarnecka-Verner *et al.* 2000). However, subsequent studies have found that LpHSFB1 in tomato can cooperate with class A HSFs, acting as a co-transcriptional activator to enhance the transcription of target genes (Bharti *et al.* 2004). Similarly, recent studies also show that LlHSFC2 can interact with HSFA to activate its transcriptional activity, and co-expression of *LlHSFC2/LlHSFA3A* further improve plant heat stress tolerance (Wu *et al.* 2024). Thermal stress triggers the activation of *SlHSFA3* expression in tomato plants, and when *SlHSFA3* is overexpressed in *Arabidopsis*, it confers enhanced heat tolerance to the transgenic lines (Li *et al.* 2013). Nevertheless, the specific function and regulatory mechanism of *SlHSFA3* in tomato remain to be elucidated.

As a key component of the ascorbate-glutathione pathway, ascorbate peroxidase (APX) exhibits high substrate specificity for ascorbic acid while facilitating the conversion of H_2O_2 into H_2O (Chen and Asada 1989). In plants, APXs are distributed in various organelles, such as the cytoplasm, mitochondria, chloroplasts, and peroxisomes (Li *et al.* 2007). APX plays a pivotal role in plants' response to abiotic stress by regulating intracellular redox homeostasis. Research indicates that *ZmAPX1* enhances maize resistance to small spot disease by clearing H_2O_2 and activating the JA signaling pathway (Zhang *et al.* 2022). In *Manihot esculenta*, the direct interaction of MeTDC2 and MeASMT2, two enzymes involved in melatonin synthesis, with MeAPX2 activates APX activity and enhances antioxidant capacity, thereby protecting cassava from oxidative stress (Bai *et al.* 2020). In rice, a knockout mutant of the cytoplasmic APX enzyme *OsAPX2* exhibits reduced APX activity and heightened sensitivity to abiotic stress (Zhang *et al.* 2013). APX plays a central role in the ROS scavenging network and responds to multiple stress factors. In terms of heat tolerance, *APX1* knockout mutants in *Arabidopsis thaliana* exhibit increased sensitivity to both heat and oxidative stress (Davletova *et al.* 2005; Koussevitzky *et al.* 2008). In tomato, CPK28 enhances heat tolerance by phosphorylating APX2, which scavenges heat-induced ROS accumulation to maintain cellular redox homeostasis. Conversely, *APX2* knockout mutants exhibit increased sensitivity to heat stress (Hu *et al.* 2021).

Tomato (*Solanum lycopersicum*) represents a globally important horticultural crop valued for its substantial agricultural productivity and rich nutrient composition. However, tomato plants exhibit vulnerability to environmental stresses, particularly heat and drought during cultivation and growth. Consequently, elucidating the molecular mechanisms governing tomato growth and development under these stress conditions is essential for enhancing crop productivity. In this study, we found that heat-induced *SlHSFA3* positively

regulates heat tolerance in tomato since plants with *SlHSFA3* knockout become more sensitive to heat stress. Specifically, plants with *SlHSFA3* knockouts exhibit increased sensitivity to heat stress. Further investigation revealed that SlHSFA3 positively regulates the expression of the ascorbate peroxidase-encoding gene *SlAPX1* and interacts with the SlAPX1 protein to enhance its activity, thereby facilitating ROS reduction. These findings provide valuable insights into the molecular mechanisms by which SlHSFA3 modulates heat tolerance in tomato plants.

2. Materials and methods

2.1. Plant materials and growth conditions

The tomato plant material investigated in our study was AC (*Solanum lycopersicum* cv. Ailsa Craig), which was also used as the genetic transformation recipient. And *Nicotiana benthamiana* was used for transient expression. All WT and transgenic lines were grown in the greenhouse of Huazhong Agricultural University, China (16-h light/8-h dark cycle was used, with temperatures of 25°C during the day and 20°C at night, and 60% humidity). Transgenic seeds of the *SlAPX1* overexpression (OE) and CRISPR/Cas9 knockout (CR) lines were obtained from a previous experiment (Li *et al.* 2024).

2.2. Gene isolation, vector construction and genetic transformation

SlHSFA3 homologous protein sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and Sol Genomics Network (https://solgenomics.net/). Multiple sequence alignment and phylogenetic tree construction were performed using N-J method in MEGA11 software, and gene family evolution analysis was performed using TBtools software. *SlHSFA3* full-length coding sequence (CDS) was amplified from AC cDNA and cloned into the pHELLSGATE8 hyperexpression vector using CloneExpress® II One-Step Cloning Kit (Vazyme, Nanjing, China). CRISPR/Cas9 technology was used to generate the *SlHSFA3* knockout mutant, as described in a previous study (Yang *et al.* 2019). Two sites in the first exon of *SlHSFA3* were chosen as targets for designing the CRISPR/Cas9 vector. The recombinant plasmids were introduced into *Agrobacterium* strain C58 *via* liquid nitrogen transformation for subsequent tomato transformation. To screen for positive transformants and detect homozygous mutations, genomic DNA was isolated from the leaves of transgenic plants. All primers used in these experiments are listed in Appendix A.

2.3. RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from various tissues of transgenic and WT tomato plants using TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized from total RNA using a HiScript® II First-Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The tomato *Actin* gene (Solyc11g005330) was used as a reference gene. qRT-PCR was performed using gene-specific primers on the QuantStudio™ 6 Flex Real-Time PCR System. All experiments were conducted with three biological replicates, and the $2^{-\Delta\Delta Ct}$ method was applied to calculate relative gene expression.

2.4. Heat shock treatment and phenotype identification of transgenic plants

SlHSFA3 transgenic lines (OE-8, OE-30, CR-20, and CR-39), *SlAPX1* transgenic lines (OE-11, OE-25, CR-6, and CR-7), and wild-type AC were germinated and grown in substrate soil under greenhouse conditions with a constant temperature of 25°C and a 16-h light/8-h dark photoperiod. At the five-leaf stage, uniformly grown seedlings were selected for heat shock treatment. For heat stress, control plants remained under normal conditions, while the heat shock treatment group was exposed to 42°C in an incubator. During the experiment, plants were photographed and leaf samples were collected, flash-frozen in liquid nitrogen, and stored at –80°C for further physiological traits measurement. To analyze leaf responses to heat stress, leaves were stained with diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) to detect the content of H_2O_2 or $O_2^{-}\cdot$, respectively, following established protocols (Liu *et al.* 2019). Relative electrical conductivity, malondialdehyde (MDA) content, and antioxidant enzyme activity, including APX, catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), were measured in both control and heat-treated plants (Li *et al.* 2016). All measurements of MDA content and enzyme activities were reported on a fresh weight basis. The activities of APX, POD, CAT, and SOD represent the total enzymatic activity in plant leaves. All experiments were conducted in at least three independent biological replicates, with each replicate consisting of pooled tissue from 3 plants.

2.5. RNA sequencing (RNA-seq)

RNA-seq was performed using total RNA isolated from transgenic plants (*SlHSFA3* knockout lines CR-9, CR-20, and CR-39) and WT (AC) control plants subjected to heat stress at 42°C for 6 h. The experiments were conducted with three independent biological replicates. RNA-seq was conducted on the Illumina platform, and the sequencing data were processed using the tomato genome (https://solgenomics.net/) as a reference. The average FPKM (expected number of fragments per kilobase per million base pairs of sequenced transcription sequences) was used to quantify gene expression levels (Trapnell *et al.* 2010). The significance level for differentially expressed genes (DEGs) is typically set at a P-value<0.05, and the False Discovery Rate (FDR) is used to correct for false positives.

2.6. Transcriptional activation analysis in yeast cells and yeast two hybrid (Y2H) analysis

For transcriptional activation analysis, we amplified the complete *SlHSFA3* CDS from AC cDNA and ligated it into the pGBKT7 vector (Clontech, USA) to obtain the BD-SlHSFA3 recombinant plasmid. The plasmid was transformed into yeast strain AH109 and cultured on SD/–Trp plates at 30°C. Diluted positive clones were spotted on SD/–Trp and SD/–Trp/–His/–Leu/-X-α-gal media, and growth and color development were analyzed after 3 d at 30°C.

For Y2H, we amplified the complete *SlHSFA3* CDS from

AC cDNA and ligated it into the pGADT7 vector as bait protein AD-SlHSFA3, and the full-length CDs of *SlAPX1* was cloned into the pGBKT7 vector to generate prey protein BD-SlAPX1. The bait and prey constructs were co-transformed into the yeast strain AH109, forming the following combinations: BD-SlAPX1+AD and BD-SlAPX1+AD-SlHSFA3. The transformed yeast cells were cultured in SD/–Trp/–Leu medium at 30°C, and colonies were subsequently transferred to SD/–Trp/–Leu/–His/–Ade medium for screening of protein-protein interactions.

2.7. Subcellular localization and co-localization analysis

The full-length *SlHSFA3* and *SlAPX1* CDs (excluding the stop codon) were amplified and ligated into pCAMBIA-1304-mCherry and pRI101-eGFP vectors, respectively, to obtain fusion proteins. These plasmids were transformed into *Agrobacterium* strain GV3101 separately. For subcellular localization, SlHSFA3-GFP was co-infiltrated with a nuclear localization marker (H-mCherry) into *N. benthamiana* leaves using *Agrobacterium*-mediated transformation (Zheng *et al.* 2022). For co-localization analysis, SlHSFA3-mCherry and SlAPX1-GFP was co-infiltrated into *N. benthamiana* leaves. Tobacco plants following infiltration were incubated at 25°C for 2 d. Fluorescence signals were observed using a confocal laser scanning microscope (SP8, Leica), with GFP excited at 488 nm and mCherry at 561 nm.

2.8. Bimolecular fluorescence complementation (BiFC) assay

BiFC experiments were performed following previously described methods (Walter *et al.* 2004). The full-length CDS of *SlHSFA3* was amplified and inserted into the N-terminal YFP (nYFP) vector to generate SLHSFA3-nYFP fusion protein. Similarly, the full-length CDS of *SlAPX1* was cloned into C-terminal YFP (cYFP) vector to generate SlAPX1-cYFP fusion protein. *Agrobacterium* strain GV3101 was transformed with the constructed vectors, which were then injected into tobacco leaves. The SlHSFA3-nYFP construct was co-infiltrated with SlAPX1-cYFP, and an empty vector was used as a negative control. After 48 h of inoculation, the YFP fluorescence signal at 515nm was observed using a laser confocal microscope (SP8, Leica).

2.9. Luciferase complementation assay

The CDS of the amplified *SlHSFA3* without the stop codon were ligated into the pJW771-nLUC vector to express the SlHSFA3-nLUC fusion protein. Similarly, the CDS of *SlAPX1* was ligated into the pJW772-cLUC vector for expression of fusion protein SlAPX1-cLUC. Each recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101, and different combinations of *Agrobacterium* suspensions were co-infiltrated into *N. benthamiana* leaves. After 48 h, reconstituted luciferase activity was detected on infiltrated leaves using an *in vivo* molecular imaging system (IndiGo^{TM}).

2.10. Co-Immunoprecipitation

The CDS of *SlHSFA3* and *SlAPX1* without the stop codon were inserted into the pH7LIC-FLAG and pH7LIC-HA vector, respectively, to generate FLAG- and HA-tagged recombinant proteins. The resulting constructs were transformed into *A. tumefaciens* GV3101. *Agrobacterium* strains containing the FLAG- or HA-tagged constructs were mixed and infiltrated into *N. benthamiana* leaves for transient expression of tagged proteins. After 48 h, total leaf protein was extracted and incubated with anti-FLAG antibody-conjugated magnetic agarose beads (anti-DDDDK-tag mAb-Magnetic Agarose, MBL) at 4°C with gentle shaking for 2–4 h. After incubation, the magnetic-agarose beads were precipitated, and bound proteins were analyzed *via* SDS-PAGE. Immunoblot detection of interacting proteins was performed using an anti-HA antibody. Total leaf protein extract prior to immunoprecipitation was used as the input control.

2.11. Yeast one-hybrid assay (Y1H)

The full-length CDS sequence of *SlHSFA3* was amplified and cloned into the PGADT7 vector to generate a prey vector. The 3-kb promoter region of *SlAPX1* was amplified from tomato gDNA and cloned into the pabai vector to generate the bait vector. The linearized bait vector was first transformed into yeast strain Y1H gold and cultured on SD/-Ura medium at 30°C. Subsequently, the prey plasmid was transformed into the yeast strain Y1H gold containing the bait plasmid. The pGADT7 empty vector served as a negative control. Transformants were inoculated on SD/–Ura/–Leu medium. Positive transformants were diluted to an OD600 of 0.1 and then plated on SD/–Ura/–Leu medium with different Aureobasidin A (AbA) concentrations for screening, the concentrations of AbA were 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 80 ng mL^{-1}, respectively.

2.12. Prokaryotic expression and protein purification and EMSA

The full-length of *SlHSFA3* CDs was amplified from AC cDNA and cloned into the prokaryotic expression vector PGEX-4T-1 containing a GST-tag. The recombinant GST-SlHSFA3 fusion protein was expressed using *Escherichia coli* BL21 (DE3) cells. When the cells were cultured in LB medium to reach OD_{600}=0.6, protein expression was induced by adding IPTG and incubating at 16°C for 18–20 h. Protein purification was performed following previously described protocols (Li *et al.* 2021).

EMSA probe was a 35 bp sequence containing the *SlAPX1* promoter binding element. The sense oligonucleotide, labeled with FAM at the 5′ end, was used to synthesize the probe, which was then diluted to a concentration of 10 mmol L^{-1}. A competitor probe was similarly annealed. The purified GST-tagged SlHSFA3 protein was incubated with the probe at 4°C in the dark for 30 min, followed by electrophoresis on a 6% non-denaturing polyacrylamide gel with 0.5× TBE buffer, protected from light.

2.13. Dual-luciferase assay

The complete CDS of *SlHSFA3* was amplified and ligated into the effector vector pGreen II 62SK. Concurrently, the *SlAPX1* promoter region was amplified from AC gDNA and integrated into the reporter vector pGreen II 0800. The resulting recombinant plasmids were transformed into *Agrobacterium*

GV3101 (containing pSoup) (Hellens *et al.* 2005). A mixture of *Agrobacterium* cultures carrying the effector vector and reporter constructs (at a ratio of 8:2) was infiltrated into *N. benthamiana* leaves for transient expression. pGreen II 62SK empty vector was used as a negative control. After 48 h, fluorescence resulting from recombinant luciferase activity was detected on the infiltrated leaves using an *in vivo* molecular imaging system (IndiGo^{TM}). Quantification of firefly and Renilla luciferase activities was performed using the dual luciferase reporter detection system kit (Vazyme, Nanjing, China) and Infinite M200 (Tecan) tablet reader.

2.14. Statistical analysis

Results from three biological replicates are expressed as mean±SD. GraphPad Prism was used for statistical analysis, and a two-tailed Student's *t*-test was employed to evaluate differences between groups. Asterisks denote significant differences: *, P<0.05; **, P<0.01 and ***, P<0.001.

3. Results

3.1. SlHSFA3 functions as a heat shock response transcription factor induced under heat stress

To elucidate the roles of *HSF* genes in tomato under heat stress, we performed comprehensive bioinformatic and transcriptomic analyses of the tomato *HSF* gene family. Evolutionary analysis of HSF protein sequences revealed that this family comprises 26 members (Appendix B), all of which possess the conserved HSF domains and highly conserved motifs (Appendix C). To investigate the response of HSFs to heat stress, transcriptome profiling was carried out on tomato plants treated at 42°C for 6 h. A substantial number of genes were differentially expressed, predominantly associated with oxidative phosphorylation, photosynthesis, sucrose metabolism, and glycan degradation pathways (Appendix D). Notably, the most significantly upregulated genes included those responsible for encoding HSFs and antioxidant-related proteins. Specifically, we identified ten HSF-encoding genes that exhibited increased expression following heat stress and may play crucial roles in the heat stress response (Fig. 1-A; Appendix E). To validate the transcriptome data, we selected representative genes, including those encoding HSFs, and previously identified heat stress-related genes were selected for qRT-PCR expression analysis (Appendix F). The qRT-PCR results were consistent with transcriptome results in terms of their expression patterns (Appendix G), confirming the reliability of our sequencing data. These results suggest that tomato plants cope with heat stress by adjusting the expression of *HSFs* and antioxidant-related genes. Among all *HSFs*, *SlHSFA3* exhibited the high up-regulation in response to heat stress. Phylogenetic analysis revealed that SlHSFA3 shares high similarity with HSFA3 from *Arabidopsis*, tobacco, and potato (Appendix H). Consequently, we selected SlHSFA3 for further investigation.

Transcriptional factors are distinguished by their nuclear localization and transcriptional activation capability. To validate SlHSFA3 as a potential heat shock responsive transcription factor, we analyzed its subcellular localization and transcriptional activation properties. To analyze localization, tobacco leaves were used to transiently express the SlHSFA3-GFP fusion protein under the control of the CaMV 35S promoter. Confocal microscopy revealed that GFP alone exhibited a diffuse cytoplasmic distribution, whereas the SlHSFA3-GFP fusion protein colocalized with the red fluorescence of a nuclear marker. This confirms that SlHSFA3 is predominantly localized in the nucleus, although some SlHSFA3 fluorescent signals were also observed in the cytoplasm (Fig. 1-B). To assess the transcriptional activation activity of SlHSFA3, we constructed a BD-SlHSFA3 vector and expressed it in yeast AH109 cells. Yeast cells containing empty pGBKT7 (BD, as negative control) and BD-SlHSFA3 grew well on SD/–Trp medium, but only those harboring BD-SlHSFA3 could grow normally and produce a blue reaction on SD/–Trp/–His/–Leu/-X-α-gal medium (Fig. 1-C), indicating that SlHSFA3 possesses transcriptional activation activity in yeast AH109 cells.

Expression pattern analysis revealed that *SlHSFA3* is ubiquitously expressed across all tissues, with notably higher expression observed in leaves and flowers (Fig. 1-D). To investigate the response of SlHSFA3 to abiotic stresses, tomato seedlings were subjected to heat, high salt, and drought stress treatments. *SlHSFA3* expression was significantly induced under all stress conditions, particularly showing continuous upregulation in response to heat stress treatment (Fig. 1-E–G). These findings indicate that *SlHSFA3* functions as a transcription factor that is markedly upregulated in response to heat stress.

3.2. SlHSFA3 positively regulates tomato heat tolerance by increasing ascorbic acid activity

To investigate the function of SlHSFA3 in tomato heat stress responses, we generated overexpressed (OE) and CRISPR/Cas9 (CR) tomato plants. The expression levels of *SlHSFA3* were quantified in the OE lines (Fig. 2-A). Specific primers designed for the target region were used to identify mutations in CRISPR/Cas9-generated mutants (CR lines). Specifically, the CR-9 mutant exhibiting a four-base deletion (AGCC), the CR-20 mutant had a nine-base deletion (TGCCGCAGC), and the CR-39 mutant showed a two-base deletion (GC) (Fig. 2-B). These mutations resulted in premature termination of SlHSFA3 in CR-9 and CR-39, while CR-20 led to amino acid substitutions. Subsequently, we sowed T_2 generation seeds of OE-8, OE-30, CR-20, and CR-39 lines. At the five-leaf stage, seedlings were then subjected to 42°C treatment. In normal growth environments, no apparent phenotypic differences were found among wild-type (WT), OE, and CR lines. However, after a 10-h heat treatment, the CR lines exhibited the most severe wilting, whereas the OE lines showed enhanced heat tolerance relative to the WT (Fig. 2-C). The accumulation of H_2O_2 and $O_2^{\cdot-}$ in transgenic and WT plants after heat stress were assessed using NBT and DAB staining techniques. The results indicated that CR line leaves stained more intensely, while OE line leaves stained less intensely compared to WT, suggesting higher ROS accumulation in CR lines while lower levels in OE lines (Fig. 2-D). The relative conductivity (REC) of the leaves was similar across all lines under normal conditions. After heat treatment,

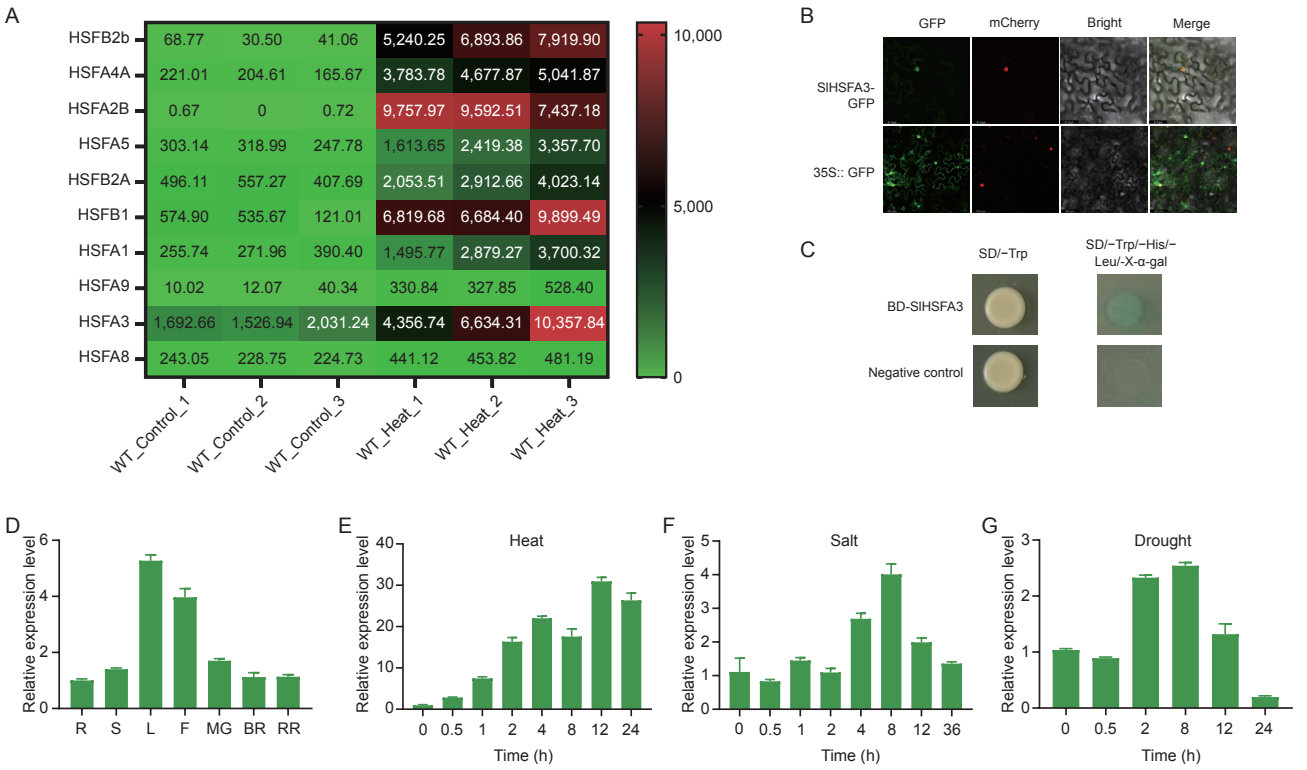


Fig. 1 Gene features of *SlHSFA3*. A, heatmap illustrating the expression levels of *SlHSFs* in tomato under normal growth conditions (WT-control) and following heat treatment (WT-heat). The color scale indicates FPKM values. B, subcellular localization of SlHSFA3. The SlHSFA3-GFP fusion protein was transiently co-expressed with H-mCherry (a nuclear localization marker) in tobacco leaves. The GFP empty vector was used as a control. Fluorescence signals were observed 48 h after *Agrobacterium* infiltration. C, transcriptional activation activity analysis of SlHSFA3. The BD-SlHSFA3 fusion protein was introduced into the yeast strain AH109 and cultured on SD/–Trp and SD/–Trp/–His/–Leu/-X-α-gal media, respectively. The pGBKT7 empty vector served as a negative control. D, qRT-PCR analysis to determine the transcription levels of *SlHSFA3* in different tissues and developmental stages. R, root; S, stem; L, leaves; F, flowers; MG, mature green stage fruit; BR, breaker stage fruit; RR, red ripe fruit stage. E–G, expression patterns of *SlHSFA3* under stresses of heat, salt and drought analyzed using qRT-PCR. Samples were collected at different time points, with expression levels normalized to the 0-h time point (set to 1). The error bars represent SD from three independent biological replicates (n=3).

the REC increased in all lines, with CR lines exhibiting a significantly greater increase compared to WT (Fig. 2-E). A similar trend was also noted for changes in malondialdehyde (MDA) content among these lines (Fig. 2-F). These findings suggest that CR lines experienced more pronounced cell membrane damage relative to WT. We also evaluated the activities of antioxidant enzymes (SOD, POD, and CAT). Under normal growth conditions, no significant differences in enzyme activities were observed among the WT, OE, and CR lines. However, heat stress significantly increased SOD and POD activities in all lines, with the final activity levels ranking as follows: OE>WT>CR (Fig. 2-G and H). Notably, CAT activity showed a distinct response to heat stress, increasing significantly only in the OE lines but decreasing in the CR lines after high-temperature treatment (Fig. 2-I). These findings suggest that the CR lines exhibit a markedly reduced capacity for ROS scavenging compared to WT plants.

To elucidate how SlHSFA3 regulates heat tolerance at the molecular level, RNA-Seq analysis was conducted on WT and CR leaves after 6 h of 42°C heat treatment. When exposed to heat stress, CR leaves exhibited 2,591 DEGs, with 1,287 genes upregulated and 1,304 genes downregulated (Fig. 3-A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that *SlHSFA3* significantly influenced multiple pathways, including ascorbate and aldarate metabolism, glutathione metabolism, and plant hormone signal transduction (Fig. 3-B). Additionally, Gene Ontology (GO) enrichment analysis demonstrated that DEGs were significantly enriched in biological processes (BP), such as organic hydroxy compound metabolic process, lipid metabolic process, and defense response, as well as molecular functions (MF), including endopeptidase inhibitor activity, oxidoreductase activity, and microtubule binding (Fig. 3-C). Further investigation indicated that a substantial proportion of these DEGs were HSFs or HSPs. Under unstressed conditions, WT and CR lines exhibited comparable expression levels for these genes. Following heat treatment, their expression levels increased markedly; however, the CR lines displayed lower expression levels compared to WT (Appendix I). These results indicate that SlHSFA3 plays a role in the ascorbate and aldarate metabolism pathways (Fig. 3-B).

Ascorbate, the ionic form of ascorbic acid (AsA), and aldonate are crucial metabolic intermediates in plants, playing essential roles in antioxidation, energy metabolism, and stress responses (Foyer and Noctor 2011). After heat treatment,

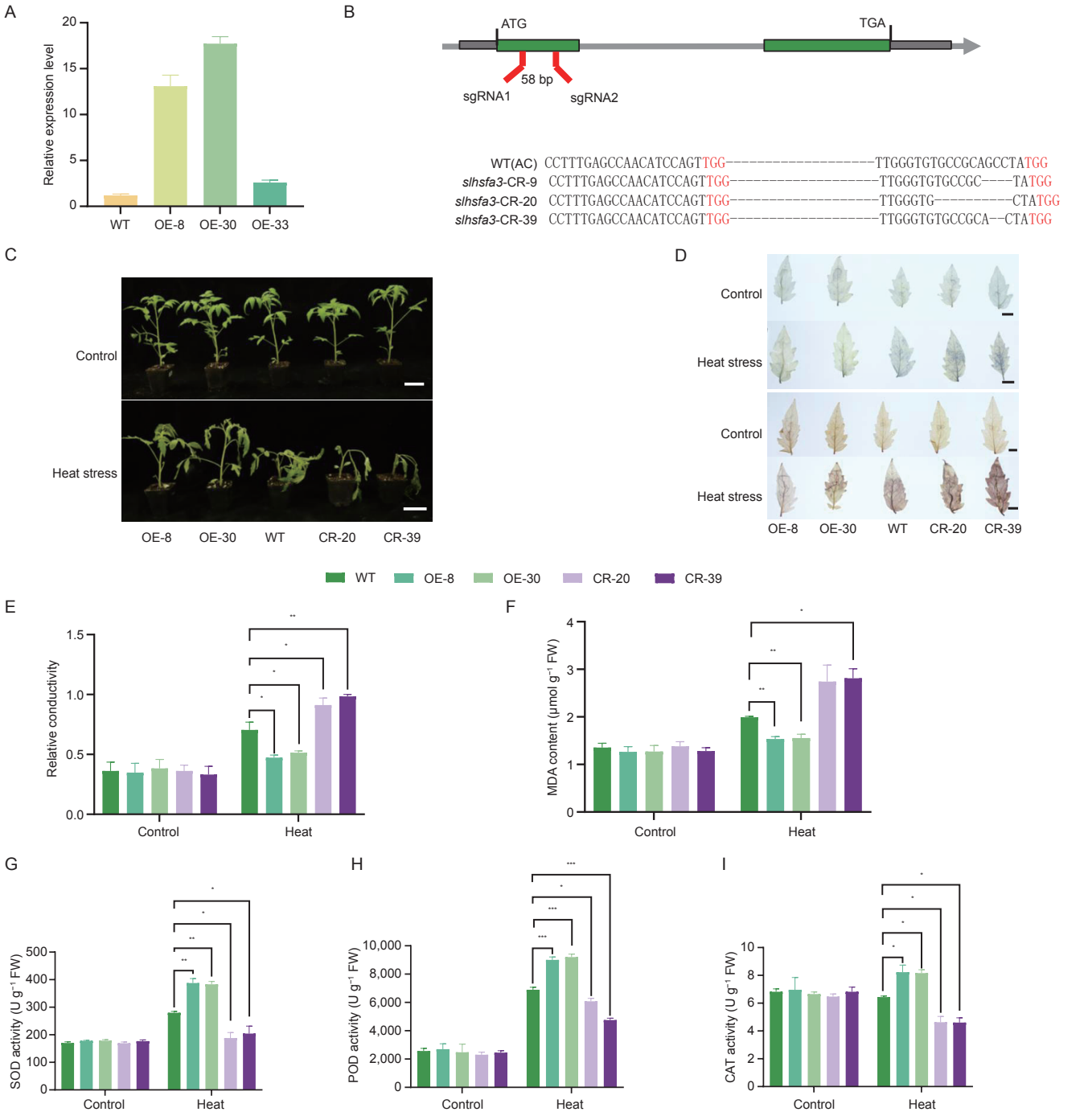


Fig. 2 SlHSFA3 enhances heat tolerance in tomato. A, qRT-PCR analysis was used to determine the relative expression levels of *SlHSFA3* in three overexpression (OE) lines and WT (AC) plants. WT was used to normalize the expression levels. B, mutation types in three CRISPR/Cas9-edited (CR) lines. C, phenotypes of OE, CR, and WT plants. Scale bar=8 cm. D, ROS accumulation detected by NBT and DAB staining. Scale bar=1 cm. E, relative conductivity (REC). F, MDA content. G–I, activities of antioxidant enzymes SOD (G), POD (H), and CAT (I) in the leaves. Panels C–I show results obtained under normal (control) and 42°C (heat stress) conditions using OE and CR lines of *SlHSFA3* and WT plants. All experiments included three biological replicates, with each replicate consisting of pooled tissue from 3 plants. Data are presented as means±SD (n=3). *, **, and ***, significant differences at P<0.05, P<0.01 and P<0.001, respectively.

we observed significantly higher expression levels of several key genes involved in ascorbate and aldarate metabolism in WT compared to CR lines. These genes include aldehyde dehydrogenase genes (Solyc02g084640, Solyc06g060250, and Solyc03g122310), *APX1* (Solyc06g005160) and *APX6* (Solyc11g018550) genes. In contrast, the expression of *APX3a* (Solyc02g083630) and *APX3b* (Solyc02g083620) was significantly downregulated in CR leaves compared to WT after heat treatment (Fig. 3-D). To elucidate the role of APX under heat stress, we measured APX enzyme activity in both WT and *SlHSFA3* transgenic plants. Under unstressed conditions, WT, OE, and CR plants exhibited comparable APX

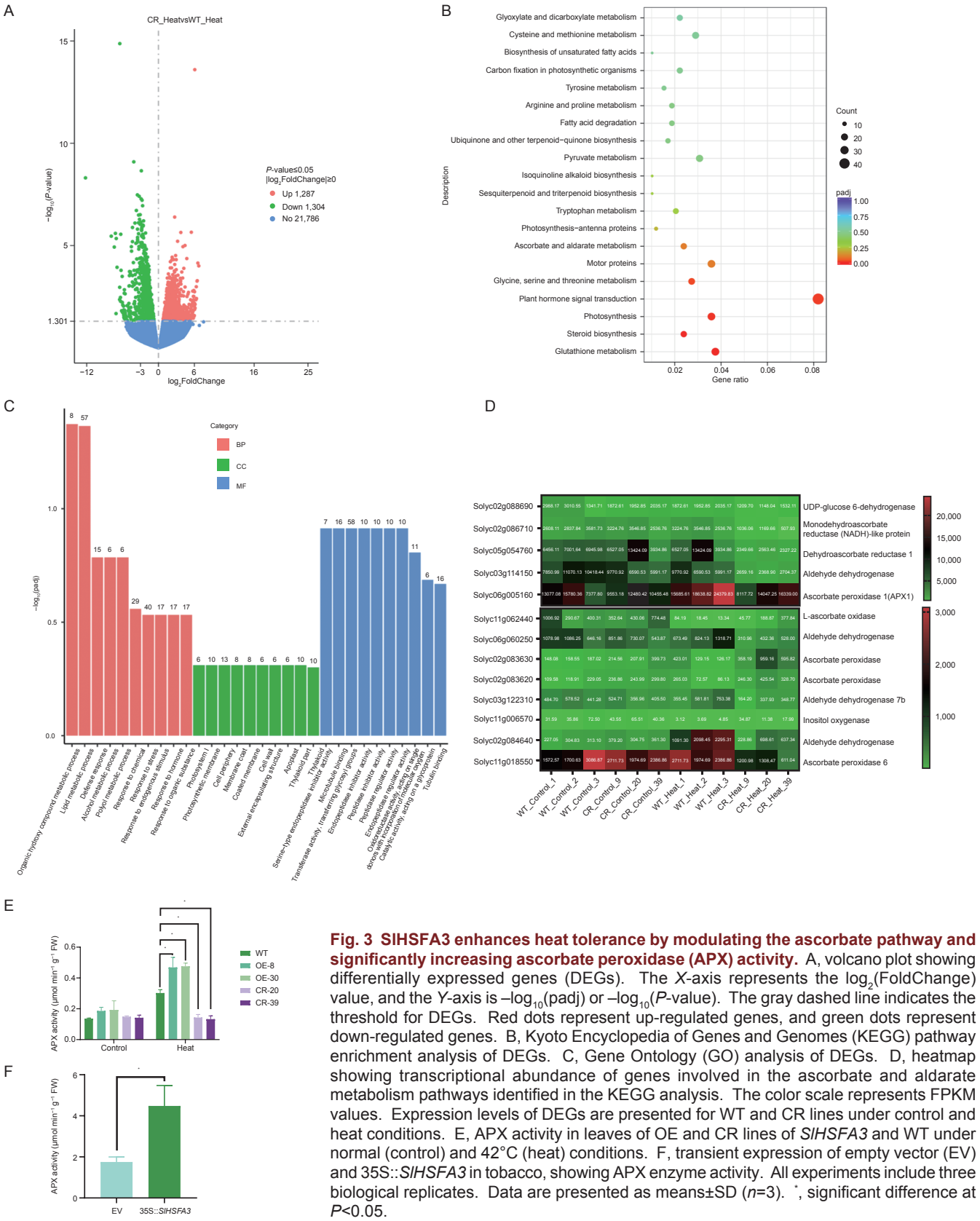


Fig. 3 SlHSFA3 enhances heat tolerance by modulating the ascorbate pathway and significantly increasing ascorbate peroxidase (APX) activity. A, volcano plot showing differentially expressed genes (DEGs). The *X*-axis represents the $\log_2$(FoldChange) value, and the *Y*-axis is $-\log_{10}$(padj) or $-\log_{10}$(*P*-value). The gray dashed line indicates the threshold for DEGs. Red dots represent up-regulated genes, and green dots represent down-regulated genes. B, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. C, Gene Ontology (GO) analysis of DEGs. D, heatmap showing transcriptional abundance of genes involved in the ascorbate and aldarate metabolism pathways identified in the KEGG analysis. The color scale represents FPKM values. Expression levels of DEGs are presented for WT and CR lines under control and heat conditions. E, APX activity in leaves of OE and CR lines of *SlHSFA3* and WT under normal (control) and 42°C (heat) conditions. F, transient expression of empty vector (EV) and 35S::*SlHSFA3* in tobacco, showing APX enzyme activity. All experiments include three biological replicates. Data are presented as means±SD (n=3). *, significant difference at P<0.05.

activity levels. However, following heat stress, APX activity increased in OE lines while it decreased in CR lines relative to WT (Fig. 3-E). To verify whether SlHSFA3 enhances APX enzyme activity, we overexpressed *SlHSFA3* (*35S::SlHSFA3*) in tobacco. The results demonstrated a significant increase in APX activity in the transgenic tobacco leaves (Fig. 3-F). These findings indicate that SlHSFA3 improves tomato heat tolerance by enhancing APX enzyme activity, thereby strengthening oxidative stress defense mechanisms under high-temperature conditions.

3.3. SlHSFA3 interacts with SlAPX1

To investigate the mechanisms by which *SlHSFA3* regulates tomato heat tolerance, we predicted its potential interacting proteins using the STRING database (https://cn.string-db.org/). A total of nine candidate proteins were identified (Appendix J). Based on our previous transcriptome analysis (Fig. 3-D) and findings on enhanced APX enzyme activity (Fig. 3-E and F), APX1 was selected for further verification. Preliminary yeast two-hybrid analysis confirmed interactions between SlHSFA3 and SlAPX1 (Fig. 4-A). APXs are key antioxidant enzymes involved in ROS metabolism. Our previous studies have demonstrated that SlAPX1 positively regulates drought tolerance in tomato (Li *et al.* 2024). Further luciferase complementation assays demonstrated that SlHSFA3 interacts with SlAPX1, as evidenced by significant luciferase activity detected in tobacco leaves co-transformed with SlHSFA3-nLUC and SlAPX1-cLUC (Fig. 4-B). Bimolecular fluorescence complementation (BiFC) assays further revealed that SlHSFA3 and SlAPX1 interact within both the nucleus and cytoplasm (Fig. 4-C). Additionally, SlHSFA3 was expressed as a Flag-tagged protein (SlHSFA3-Flag), while SlAPX1 was fused with an HA tag (SlAPX1-HA). Immunoprecipitation using anti-Flag antibodies followed by detection with an anti-HA antibody confirmed the interaction between SlHSFA3 and SlAPX1 (Fig. 4-D). Co-localization experiments in cells co-expressing SlHSFA3-mCherry and SlAPX1-eGFP showed overlapping red (SlHSFA3-mCherry) and green fluorescence (SlAPX1-eGFP) signals in both the nucleus and cytoplasm (Fig. 4-E). To evaluate whether SlHSFA3 enhances APX activity through its interaction with SlAPX1, APX enzyme activity was measured in tobacco leaves co-expressing both proteins. The results indicated that APX enzyme activity was significantly higher in the presence of SlHSFA3 compared to the control (Appendix K). These findings suggest that SlHSFA3 not only interacts with SlAPX1 but also modulates its enzymatic activity.

3.4. SlHSFA3 activates *SlAPX1* expression to improve heat tolerance

Based on the transcriptome data, *SlAPX1* and *SlHSFA3* exhibited co-expression under heat treatment (Fig. 3-D). To further elucidate their potential regulatory relationship, we analyzed the promoters of *SlAPX1* and identified an HSE (5′-AGAAnnTTCT-3′) within the *SlAPX1* promoter. Specifically, this motif, with the precise sequence 5′-AGAACCTTCT-3′, is located 877 base pairs upstream of the start codon. Y1H assay confirmed that SlHSFA3 indeed binds to this promoter region (Fig. 5-A and B). To verify the *in vitro* binding of SlHSFA3 to the *SlAPX1* promoter, we conducted electrophoretic mobility

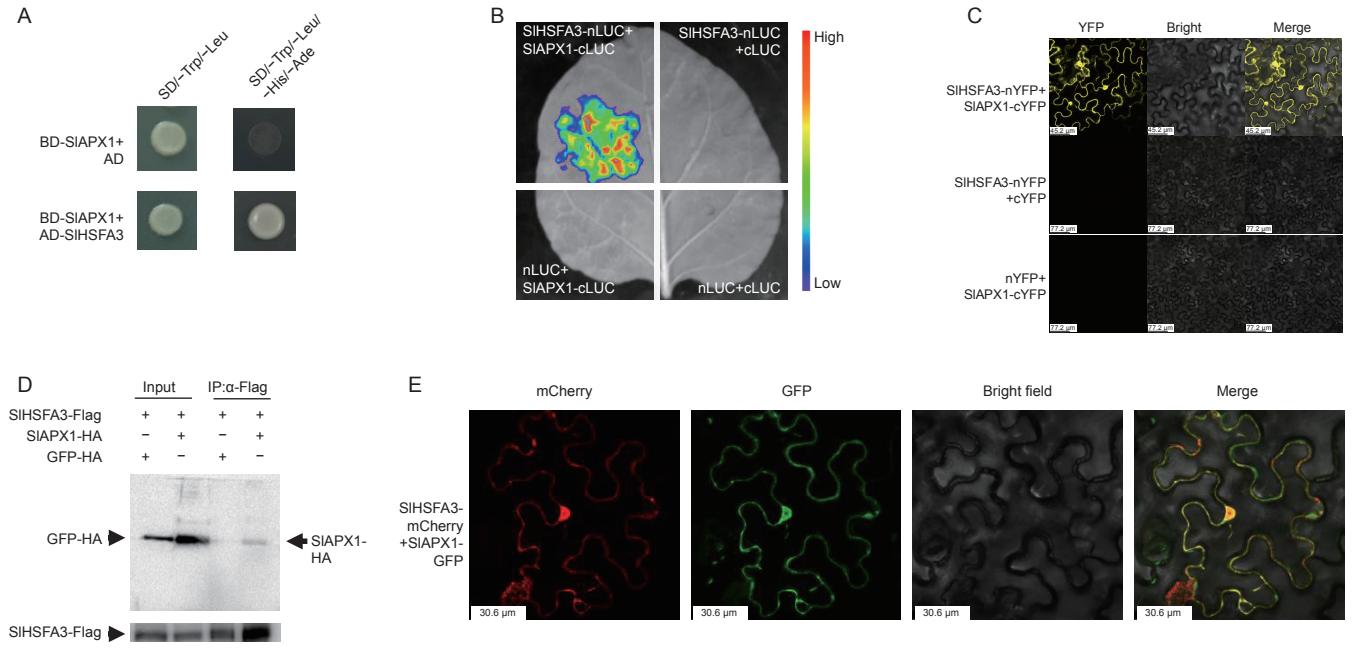


Fig. 4 SlHSFA3 interacts with SlAPX1. A, yeast two hybrid assay validating the interaction between SlHSFA3 and SlAPX1. Yeast cells were cultured on SD/–Trp/–Leu medium, and interactions were examined on SD/–Trp/–Leu/–His/–Ade solid medium. B, luciferase complementation assay in *Nicotiana benthamiana* leaves. SlHSFA3 was fused to the N-terminal fragment of luciferase (nLUC), while SlAPX1 was fused to the C-terminal luciferase (cLUC). Empty nLUC and cLUC vectors served as controls. C, bimolecular fluorescence complementation assay demonstrating SlHSFA3 interaction with SlAPX1. The N-terminal half of YFP was fused to SlHSFA3 (SlHSFA3-nYFP), while the C-terminal of YFP was fused to SlAPX1 (SlAPX1-cYFP). The interaction of the proteins resulted in YFP reconstitution and detectable yellow fluorescence in leaf cells. Individual protein expression vectors were used as negative controls. D, co-immunoprecipitation to verify the interaction between SlHSFA3 and SlAPX1. SlHSFA3 was expressed as a Flag-tagged protein (SlHSFA3-Flag) in tobacco (*N. benthamiana*) leaf cells, and SlAPX1 was expressed as an HA-tagged fusion protein (SlAPX1-HA). SlHSFA3-Flag and SlAPX1-HA were co-expressed as the experimental group, while SlHSFA3-Flag and GFP-HA were co-expressed as a negative control. Total protein extract (input) was immunoprecipitated using anti-Flag antibodies, and the presence of SlAPX1-HA in the immunoprecipitated complex was detected *via* anti-HA antibodies. E, co-localization analysis of SlHSFA3 with SlAPX1. Red and green fluorescence represent mCherry and GFP-fused proteins, respectively. “bright” represents the bright-field image, and “merge” shows the overlay of all three channels.

shift assay (EMSA) using a 35 bp double-stranded DNA fragment containing the *SlAPX1* HSF binding motif and its flanking sequences, which was synthesized and labeled with fluorescein dye (5′-FAM) (Fig. 5-B). The EMSA results demonstrated a distinct gel shift in the presence of SlHSFA3 protein, indicating that the DNA probes containing the AGAACCTTCT *cis*-element formed a complex with SlHSFA3, resulting in a band shift to a higher molecular weight region (Fig. 5-C). To further validate this interaction in planta, we fused the *SlAPX1* promoter to a luciferase (LUC) reporter gene and assessed transcriptional activation through a dual luciferase assay. Co-expression of SlHSFA3 with the *SlAPX1* promoter in tobacco leaves significantly increased luminescence intensity and LUC enzyme activity compared to the control group (Fig. 5-D and E). These data indicate that SlHSFA3 can bind to the *SlAPX1* promoter and enhance *SlAPX1* expression. This conclusion is supported by the observation that *SlHSFA3* overexpression also increased *SlAPX1* expression under heat stress conditions (Fig. 5-F).

To investigate whether *SlAPX1* influences heat tolerance, we generated *SlAPX1* CR and OE tomato lines (Li *et al.* 2024), followed by a comprehensive heat tolerance analysis. The results demonstrated that, under normal growth conditions, all lines exhibited uniform growth (Fig. 6-A). After 24 h of

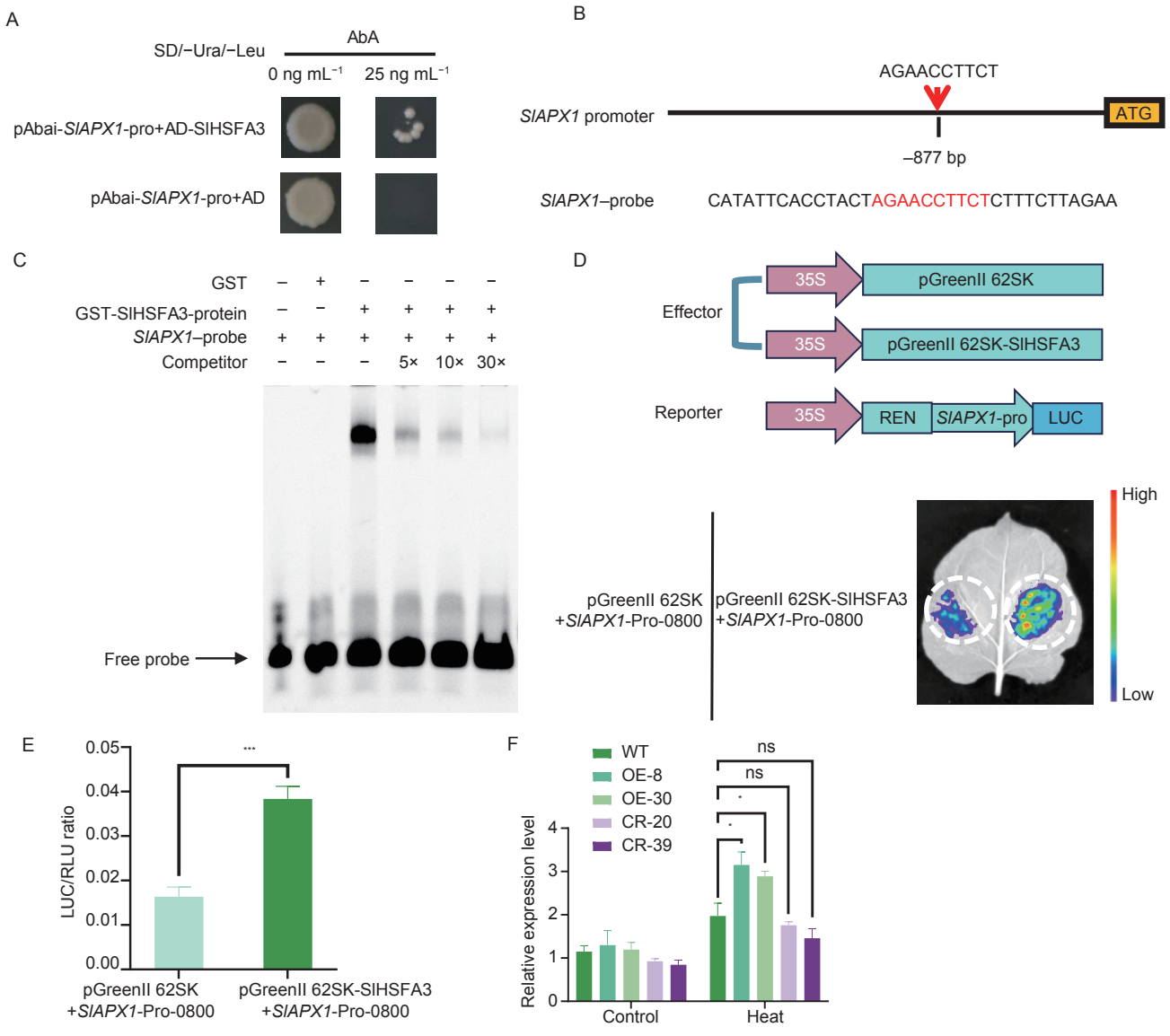


Fig. 5 SlHSFA3 positively regulates the expression of *SlAPX1*. A, yeast one hybrid analysis showing the interaction between SlHSFA3 and *SlAPX1* promoter. Enhanced resistance to Aureobasidin A (AbA) indicates interaction between the bait and prey. B, distribution of heat shock element (HSE) binding elements on the promoter of *SlAPX1* and probe sequence used in the EMSA assay. A *cis*-element located at 2 kb in the *SlAPX1* promoter region is indicated by red arrows. The sequence including HSE binding element and its flanking fragments is designed as the *SlAPX1* probe. The red letters in the probe sequence represent the binding motif. C, EMSA confirming the binding of SlHSFA3 to *SlAPX1* probe. The "+" and "–" represent the presence or absence of the binding interaction, respectively, and 5×, 10×, and 30× represent different competitive concentrations. D and E represent LUC fluorescence imaging (D) and intensity quantification (E) in tobacco leaves, respectively. Data are presented as mean±SD (n=6). F, qRT-PCR analysis of *SlAPX1* expression in the leaves of OE and CR lines of *SlHSFA3* as well as WT under normal (control) and 42°C (heat) conditions. Data are presented as mean±SD (n=3). * and ***, significant differences at P<0.05 and P<0.001, respectively.

exposure to a high temperature of 42°C, the CR and WT plants displayed severe and mild wilting, respectively, whereas the OE plants remained vigorous. To evaluate oxidative stress levels, DAB and NBT staining of mature leaves was performed. Following heat treatment, H_2O_2 and $O_2^{\cdot-}$ accumulation was lower in the leaves of OE plants compared to WT and CR lines (Fig. 6-B). Under normal growth conditions, relative leaf conductivity measurements demonstrated no significant differences across all lines. Nevertheless, after heat treatment, the relative conductivity increased, with significantly higher levels in CR plants and significantly lower levels in OE plants compared to the WT (Fig. 6-C). APX enzyme activity analysis revealed that *SlAPX1* overexpression significantly increased APX activity under normal growth conditions. Under heat stress, APX activity increased in all plants, but was significantly lower in the CR lines while significantly higher in the OE plants compared to the WT (Fig. 6-D). These results collectively demonstrate that *SlAPX1* positively regulates heat tolerance by enhancing antioxidant enzyme activity and further promoting ROS scavenging.

4. Discussion

With global climate change, high-temperature events are becoming increasingly frequent, posing a serious threat to crop yield and quality. Understanding the molecular mechanisms underlying plant responses to high temperature conditions is of great significance for developing strategies to enhance crop resilience through genetic engineering. The plant HSF family consists of multiple members, forming a complex regulatory network. These factors play a pivotal role in heat stress tolerance in addition to responding to various abiotic stresses. HSFs interact and coordinate to transduce thermal signals, forming a highly intricate and hierarchical regulatory network (von Koskull-Döring *et al.* 2007). The plant HSF family members are categorized into Class A, Class B, and Class C, each with evolutionarily conserved core transcriptional regulatory functions. Class A members, particularly HSFA1, HSFA2, and HSFA3, are primarily responsible for activating heat shock reactions (HSRs) (Scharf *et al.* 2012). Among them, HSFA1s is the key transcription activator to regulate the expression of heat shock-related genes in plants (Yoshida *et al.* 2011). AtHSFA2, induce a variety of defense genes in the response to heat stress. AtHSFA2 is normally not expressed but is rapidly induced under environmental stresses (Nishizawa-Yokoi *et al.* 2009). In *Arabidopsis*, HSFA1 predominantly induces the expression of genes involved in transcriptional regulation, while HSFA2 primarily activates genes related to metabolism and redox balance (Liu *et al.* 2013). Class B and Class C HSFs primarily regulate stress responses and development-related gene expression by interacting with Class A HSFs or other factors to enhance or suppress transcriptional activity (Bharti *et al.* 2004; Wu *et al.* 2024).

In our study, transcriptome analysis following heat treatment revealed a significant upregulation of multiple transcription factors and stress-responsive genes, including HSFA3 and DREB1 (dehydration-responsive element binding protein). DREB is a transcription factor unique to plants that primarily regulates responses to abiotic stresses

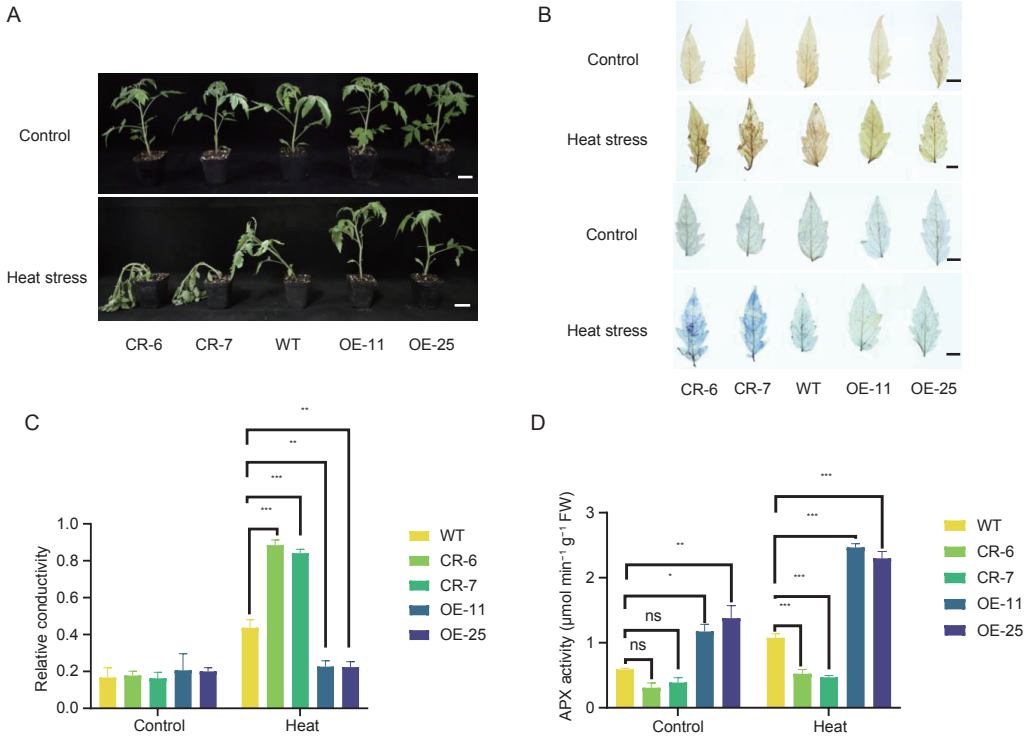


Fig. 6 *SlAPX1* enhances heat tolerance in tomato plants. A, phenotypes. Scale bar=5 cm. B, DAB and NBT staining. Scale bar=1 cm. C, relative conductivity. D, APX activity. All panels represent data from CR and OE lines of *SlAPX1* and WT plants under control and 42°C heat stress treatments. Data are presented as mean±SD (n=3). *, **, and ***, significant differences at P<0.05, P<0.01 and P<0.001, respectively.

such as cold, salinity, and drought. It specifically binds to the dehydration-responsive element (DRE) *cis*-acting element to modulate the expression of stress-responsive genes, thereby enhancing overall plant stress tolerance (Zhao *et al.* 2014). *SlHSFA3* is significantly up-regulated in tomato plants under heat shock conditions. It exhibits the highest homology with *Arabidopsis AtHSFA3* and is classified as a member of the class A heat shock transcription factor family (Appendix H). In tomato, *HSFA3* functions similarly to *HSFA1a* and *HSFA2*, responding to heat stress by inducing the expression of downstream stress-related genes (Bharti *et al.* 2000). We investigated the expression dynamics of *SlHSFA3* in tomato leaves under heat, high salt, and drought stress at various time points. Under drought and high-salt stress, *SlHSFA3* was mildly induced but rapidly declined or ceased expression altogether (Fig. 1-F and G). Conversely, exposure to high temperatures markedly upregulated *SlHSFA3* expression (Fig. 1-E), underscoring its critical role in tomato's adaptation to heat stress. The expression of *SlHSFA3* displayed a dynamic temporal pattern characterized by initial increases followed by decreases (Fig. 1). This phenomenon may be attributed to the early induction of multiple stress proteins, some of which potentially suppress *SlHSFA3* expression. As heat stress persists, these inhibitory proteins are gradually depleted, thereby alleviating their suppressive effects and enabling *SlHSFA3* expression to rebound. In line with this observation, previous studies in *Arabidopsis* have demonstrated that thermally induced HSF binding protein AtHSBP interacts with AtHSFs, reducing the DNA-binding activity of AtHSFA1b and consequently diminishing the transactivation capacity of HSFs and the protein levels of HSPs (Hsu *et al.* 2010). Overexpression of *AtHSFA3* in *Arabidopsis* increases heat tolerance, whereas T-DNA insertion mutants exhibit reduced heat tolerance (Yoshida *et al.* 2008). These findings are consistent with our results, which demonstrate that *SlHSFA3* positively regulates thermal tolerance in tomato (Fig. 2). Nevertheless, the target genes of HSFA3 in tomato and its regulatory mechanisms during the heat stress response remain largely unexplored.

4.1. SlHSFA3 enhances heat tolerance in tomato by regulating ROS scavenging enzyme activity

Plants subjected to heat stress may suffer from membrane damage, protein denaturation, ROS accumulation, metabolic disturbances, and diminished growth and yield (Ingram 1984) (Iba 2002). The cell membrane is particularly vulnerable to abiotic stresses, with heat stress often leading to membrane integrity loss (Blum and Ebercon 1981). Excessive ROS production accelerates membrane lipid peroxidation, reduces membrane thermal stability, and ultimately disrupts cell membrane function (Xu *et al.* 2006). Plant membrane damage and oxidative balance can be assessed by measuring REC, MDA content, and ROS content. The *SlHSFA3*-OE transgenic tomato plants displayed lower REC and MDA content than WT plants after heat treatment, which is in contrast to the situation in *SlHSFA3* mutant materials (Fig. 2-E and F). These results indicate that *SlHSFA3* contributes to maintaining tomato membrane system integrity under heat stress. To evaluate ROS levels, DAB and NBT staining were performed to qualitatively detect $O_2^{-}\cdot$ and H_2O_2, respectively. Our staining results showed that $O_2^{-}\cdot$ and H_2O_2 levels were significantly higher in *slhsfa3*-CR mutants than in wild type and OE lines after heat stress in tomato plants (Fig. 2-D). This suggests that overexpression of *SlHSFA3* suppresses excessive ROS accumulation, consequently alleviating heat-induced cell membrane damage and maintaining cellular homeostasis.

Through long-term natural selection, plants have evolved various mechanisms to counteract oxidative stress, including enzymatic and non-enzymatic antioxidant systems. The enzymatic system comprises SOD, POD, CAT, and APX, while the non-enzymatic system is composed of antioxidants, including ascorbic acid, flavonoids, vitamin E, glutathione and carotenoids. Under heat stress, plants enhance their ROS scavenging ability by increasing antioxidant enzyme activities and antioxidant contents, thereby improving heat tolerance. For instance, SlWRKY50 in tomatoes activates JA synthase under low-temperature stress, thereby promoting JA accumulation. This increase in JA levels enhances antioxidant enzyme activity and strengthens the plant's capacity to scavenge ROS, ultimately improving its cold tolerance (Wang *et al.* 2024). In our study, the presence of *HSFA3* significantly enhances the APX activity mediated by APX1 (Appendix K). This finding aligns closely with previous research, which demonstrated that the interaction between BBX18 and APX1 inhibits APX activity, while BBX18 binding to the promoter region of *APX1* suppresses its expression (Li *et al.* 2024). In addition, the regulatory role of HSFA3 in *APX1* expression might contribute to the enhancement of APX activity when HSFA3 binds to *APX1*. SlHSFA3-mediated enhancement of APX activity may involve both transcriptional activation of *SlAPX1* and potential post-translational regulation through their physical interaction. However, the precise contribution of each mechanism warrants further investigation. In conclusion, the reduced heat tolerance observed in *SlHSFA3* mutants could be attributed to excessive ROS accumulation due to impaired antioxidant enzyme activity, which exacerbates oxidative damage under heat stress.

4.2. SlHSFA3 positively regulates *SlAPX1* expression and promotes heat tolerance

SlHSFA3 mediates heat-induced ROS clearance by transcriptionally regulating the expression of antioxidase genes. APX exhibits the greatest affinity for H_2O_2 compared to other metabolic enzymes and can efficiently remove H_2O_2 (Huang *et al.* 2018). Tomatoes possess seven APX-coding genes (Najami *et al.* 2008), with *SlAPX1* and *SlAPX2* being key players in APX enzyme synthesis. Our previous studies have demonstrated that *SlAPX1* is involved in regulating drought tolerance in tomatoes. In this study, we identified that SlHSFA3 interacts with SlAPX1 (Fig. 4). Prior research has shown that SlAPX1 is localized in the cytoplasm (Li *et al.* 2024), co-localization experiments revealed that SlAPX1 exhibits fluorescence in both the nucleus and cytoplasm (Fig. 4-E), indicating that a portion of SlAPX1 relocates to the nucleus upon interaction with SlHSFA3. This indicates that the interaction may influence

the subcellular localization of both proteins. Previous studies have reported that OsNAC2 is localized in the nucleus, OsXb22a resides in the cytoplasm, and OsEREBP1 exhibits dual localization in both the nucleus and cytoplasm. In experiments examining interaction-induced localization shifts, co-expression of OsEREBP1 with OsNAC2 proteins resulted in significant nuclear accumulation of OsEREBP1 fluorescence signals, whereas co-expression of OsEREBP1 with OsXb22a proteins caused OsEREBP1 fluorescence signals to predominantly remain in the cytoplasm (Zhong *et al.* 2024). In addition, post-translational modifications (such as phosphorylation, ubiquitination, acetylation, etc.) that occur between interacting proteins can significantly influence the subcellular localization of their interaction partners. For example, the phosphorylation of BIN2, facilitated by 14-3-3 proteins, promotes the nuclear export of BZR1, leading to the accumulation of phosphorylated BZR1 in the cytoplasm (Wang *et al.* 2021). In conclusion, the mechanisms underlying the changes in subcellular localization following the interaction between SlAPX1 and SlHSFA3 warrant further investigation.

SlAPX1 promoter analysis showed that the *SlAPX1* promoter contains HSEs, and we further verified through EMSA and Y1H assays that SlHSFA3 binds to the *SlAPX1* promoter to regulate its expression (Fig. 5). LUC assay demonstrated that SlHSFA3 activates the expression of *SlAPX1* (Fig. 5-D and E). Consistently, overexpression of *SlAPX1* enhanced heat tolerance in plants (Fig. 6). However, in *SlHSFA3* knockout lines, the expression of *SlAPX1* was not significantly suppressed (Fig. 5-F), possibly due to the high endogenous expression level of *SlAPX1* in tomato and functional redundancy among HSFs. Even with *SlHSFA3* knockout, other HSFs may maintain partial *SlAPX1* activation. It is well-documented that HSFs typically form oligomers to function effectively(Baniwal *et al.* 2007). For instance, in tomatoes, SlHSFA2 interacts with SlHSFA1a to achieve robust transcriptional activation (Chan-Schaminet *et al.* 2009). In *Arabidopsis*, AtHSFA5 specifically interacts with AtHSFA4b, which inhibits AtHSFA4b oligomerization and DNA-binding ability (Baniwal *et al.* 2007). In lily, LlHSFC2 enhances the transcriptional activation ability of HSFs by acting as a coactivator through interaction (Wu *et al.* 2024). Furthermore, other transcription factors may also regulate the expression of *SlAPX1* under stress conditions. Through the analysis of DEGs in the RNA-seq data, it was found that in the heat stress condition, the knockout of *SlHSFA3* also affected the expression of *POD*, *CAT*, and *GPX* (glutathione peroxidase) genes. Notably, the expression of *GPX7* (Solyc08g006720) was significantly lower in *slhsfa3*-CR mutants compared to the WT. Since its promoter region contains an HSE, we speculate that SlHSFA3 may directly regulate *GPX7* expression. In summary, the mechanism by which SlHSFA3 modulates other antioxidant enzyme genes remains to be further investigated.

Our results elucidate a potential mechanism by which *SlHSFA3* regulates heat stress responses in tomatoes (Fig. 7). Specifically, the overexpression of *SlHSFA3* enhances the activity of the antioxidant enzyme APX through its interaction with SlAPX1, thereby improving ROS clearance efficiency. Moreover, SlHSFA3 upregulates the expression of *SlAPX1*, which further facilitates ROS reduction and enhances heat tolerance in tomato. These findings offer significant insights into the development of stress-tolerant crops *via* genetic engineering or breeding strategies focused on increasing APX expression and activity.

5. Conclusion

In this study, we elucidated the mechanism by which the heat shock transcription factor SlHSFA3 significantly enhances heat tolerance in tomato. Our findings reveal that SlHSFA3 not only modulates antioxidant enzyme activity but also directly binds to and activates the expression of ascorbate peroxidase gene (*SlAPX1*), thereby enhancing ROS scavenging capacity and ultimately improving heat stress tolerance.

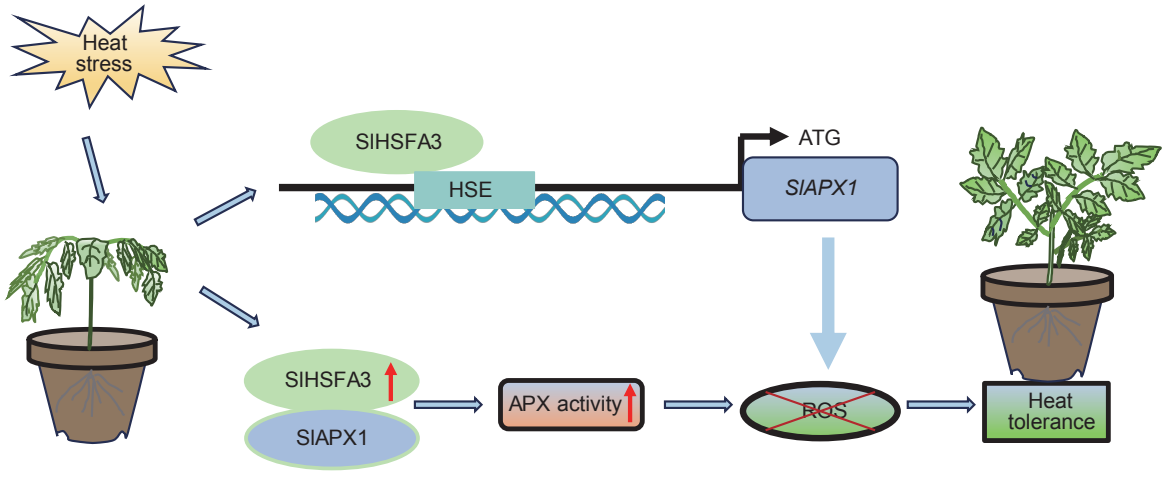


Fig. 7 Proposed molecular mechanism of SlHSFA3 regulation of tomato heat tolerance. SlHSFA3 interacts with SlAPX1 to enhance the activity of the antioxidant enzyme APX, thereby improving reactive oxygen species (ROS) clearance. Furthermore, SlHSFA3 upregulates *SlAPX1* expression by directly binding to the heat shock element (HSE), a *cis*-acting element in the promoter region, which enhances ROS scavenging capacity in tomatoes and strengthens their tolerance to heat stress.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used DeepSeek-R1 to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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References

Bai Y J, Guo J R, Reiter R J, Wei Y X, Shi H T. 2020. Melatonin synthesis enzymes interact with ascorbate peroxidase to protect against oxidative stress in cassava. *Journal of Experimental Botany*, **71**, 5645–5655.

Baniwal S K, Bharti K, Chan K Y, Fauth M, Ganguli A, Kotak S, Mishra S K, Nover L, Port M, Scharf K D, Tripp J, Weber C, Zielinski D, von Koskull-Döring P. 2004. Heat stress response in plants: A complex game with chaperones and more than twenty heat stress transcription factors. *Journal of Biosciences*, **29**, 471–487.

Baniwal S K, Chan K Y, Scharf K D, Nover L. 2007. Role of heat stress transcription factor HsfA5 as specific repressor of HsfA4. *Journal of Biological Chemistry*, **282**, 3605–3613.

Bharti K, von Koskull-Döring P, Bharti S, Kumar P, Tintschl-Körbitzer A, Treuter E, Nover L. 2004. Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. *Plant Cell*, **16**, 1521–1535.

Bharti K, Schmidt E, Lyck R, Heerklotz D, Bublak D, Scharf K D. 2000. Isolation and characterization of HsfA3, a new heat stress transcription factor of *Lycopersicon peruvianum*. *Plant Journal*, **22**, 355–365.

Blum A, Ebercon A. 1981. Cell-membrane stability as a measure of drought and heat tolerance in wheat. *Crop Science*, **21**, 43–47.

Casal J J, Balasubramanian S. 2019. Thermomorphogenesis. *Annual Review of Plant Biology*, **70**, 321–346.

Challinor A J, Watson J, Lobell D B, Howden S M, Smith D R, Chhetri N. 2014. A meta-analysis of crop yield under climate change and adaptation. *Nature Climate Change*, **4**, 287–291.

Chan-Schaminet K Y, Baniwal S K, Bublak D, Nover L, Scharf K D. 2009. Specific interaction between tomato HsfA1 and HsfA2 creates hetero-oligomeric superactivator complexes for synergistic activation of heat stress gene expression. *Journal of Biological Chemistry*, **284**, 20848–20857.

Chen G X, Asada K. 1989. Ascorbate peroxidase in tea leaves — Occurrence of 2 isozymes and the differences in their enzymatic and molecular-properties. *Plant and Cell Physiology*, **30**, 987–998.

Czarnecka-Verner E, Yuan C X, Scharf K D, Englich G, Gurley W B. 2000. Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential. *Plant Molecular Biology*, **43**, 459–471.

Davletova S, Rizhsky L, Liang H J, Zhong S Q, Oliver D J, Coutu J, Shulaev V, Schlauch K, Mittler R. 2005. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. *Plant Cell*, **17**, 268–281.

Foyer C H, Noctor G. 2011. Ascorbate and glutathione: The heart of the redox hub. *Plant Physiology*, **155**, 2–18.

Friedrich T, Oberkofler V, Trindade I, Altmann S, Brzezinka K, Lämke J, Gorka M, Kappel C, Sokolowska E, Skirycz A, Graf A, Bäurle I. 2021. Heteromeric HSFA2/HSFA3 complexes drive transcriptional memory after heat stress in *Arabidopsis*. *Nature Communications*, **12**, 3426.

Guo M, Liu J H, Ma X, Luo D X, Gong Z H, Lu M H. 2016. The plant heat stress transcription factors (HSFs): Structure, regulation, and function in response to abiotic stresses. *Frontiers in Plant Science*, **7**, 114.

Hellens R P, Allan A C, Friel E N, Bolitho K, Grafton K, Templeton M D, Karunairetnam S, Gleave A P, Laing W A. 2005. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*, **1**, 13.

Hsu S F, Lai H C, Jinn T L. 2010. Cytosol-localized heat shock factor-binding protein, AtHSBP, functions as a negative regulator of heat shock response by translocation to the nucleus and is required for seed development in *Arabidopsis*. *Plant Physiology*, **153**, 773–784.

Hu Z J, Li J X, Ding S T, Cheng F, Li X, Jiang Y P, Yu J Q, Foyer C H, Shi K. 2021. The protein kinase CPK28 phosphorylates ascorbate peroxidase and enhances thermotolerance in tomato. *Plant Physiology*, **186**, 1302–1317.

Huang L P, Jia J, Zhao X X, Zhang M Y, Huang X X, Ji E, Ni L, Jiang M Y. 2018. The ascorbate peroxidase APX1 is a direct target of a zinc finger transcription factor ZFP36 and a late embryogenesis abundant protein OsLEA5 interacts with ZFP36 to co-regulate *OsAPX1* in seed germination in rice. *Biochemical and Biophysical Research Communications*, **495**, 339–345.

Iba K. 2002. Acclimative response to temperature stress in higher plants: Approaches of gene engineering for temperature tolerance. *Annual Review of Plant Biology*, **53**, 225–245.

Ingram D L. 1984. Effects of temperature and exposure time interactions on thermostability of woody plant-root cell-membranes. *Hortscience*, **19**, 566.

Kaushal N, Bhandari K, Siddique K H M, Nayyar H, Tejada Moral M. 2016. Food crops face rising temperatures: An overview of responses, adaptive mechanisms, and approaches to improve heat tolerance. *Cogent Food & Agriculture*, **2**, 1134380.

von Koskull-Döring P, Scharf K D, Nover L. 2007. The diversity of plant heat stress transcription factors. *Trends in Plant Science*, **12**, 452–457.

Kotak S, Vierling E, Bäumlein H, Von Koskull-Döring P. 2007. A novel transcriptional cascade regulating expression of heat stress proteins during seed development of *Arabidopsis*. *Plant Cell*, **19**, 182–195.

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R. 2008. Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. *Journal of Biological Chemistry*, **283**, 34197–34203.

Li G B, Wang J F, Zhang C L, Ai G, Zhang D D, Wei J, Cai L Y, Li C B, Zhu W Z, Larkin R M, Zhang J H. 2021. L2, a chloroplast metalloproteinase, regulates fruit ripening by participating in ethylene autocatalysis under the control of ethylene response factors. *Journal of Experimental Botany*, **72**, 7035–7048.

Li H B, Qin Y M, Pang Y, Song W Q, Mei W Q, Zhu Y X. 2007. A cotton ascorbate peroxidase is involved in hydrogen peroxide homeostasis during fibre cell development. *New Phytologist*, **175**, 462–471.

Li J H, Ai G, Wang Y L, Ding Y, Hu X M, Liang Y, Yan Q X, Wu K, Huang R, Chen C R, Ouyang B, Zhang X G, Pan Y, Wu L, Hong Z L, Zhang J H. 2024. A truncated B-box zinc finger transcription factor confers drought sensitivity in modern cultivated tomatoes. *Nature Communications*, **15**, 8013.

Li J H, Ouyang B, Wang T T, Luo Z D, Yang C X, Li H X, Sima W, Zhang J H, Ye Z B. 2016. HyPRP1 gene suppressed by multiple stresses plays a negative role in abiotic stress tolerance in tomato. *Frontiers in Plant Science*, **7**, 967.

Li Z J, Zhang L L, Wang A X, Xu X Y, Li J F. 2013. Ectopic overexpression of *SlHsfA3*, a heat stress transcription factor from tomato, confers increased thermotolerance and salt hypersensitivity in germination in transgenic *Arabidopsis*. *PLoS ONE*, **8**, e54880.

Liu H C, Charng Y Y. 2013. Common and distinct functions of *Arabidopsis* class A1 and A2 heat shock factors in diverse abiotic stress responses and development. *Plant Physiology*, **163**, 276–290.

Liu H C, Charng Y Y. 2014. Acquired thermotolerance independent of heat shock factor A1 (HsfA1), the master regulator of the heat stress response. *Plant Signaling & Behavior*, **7**, 547–550.

Liu Y, Yang T Y, Lin Z K, Gu B J, Xing C H, Zhao L Y, Dong H Z, Gao J Z, Xie Z H, Zhang S L, Huang X S. 2019. A WRKY transcription factor PbrWRKY53 from *Pyrus betulaefolia* is involved in drought tolerance and AsA accumulation. *Plant Biotechnology Journal*, **17**, 1770–1787.

Mishra S K, Tripp J, Winkelhaus S, Tschiersch B, Theres K, Nover L, Scharf K D. 2002. In the complex family of heat stress transcription factors, HSfA1 has a unique role as master regulator of thermotolerance in tomato. *Genes & Development*, **16**, 1555–1567.

Najami N, Janda T, Barriah W, Kayam G, Tal M, Guy M, Volokita M. 2008. Ascorbate peroxidase gene family in tomato: Its identification and characterization. *Molecular Genetics and Genomics*, **279**, 171–182.

Nishizawa-Yokoi A, Yoshida E, Yabuta Y, Shigeoka S. 2009. Analysis of the regulation of target genes by an *Arabidopsis* heat shock transcription factor, HsfA2. *Bioscience Biotechnology and Biochemistry*, **73**, 890–895.

Ohama N, Sato H, Shinozaki K, Yamaguchi-Shinozaki K. 2017. Transcriptional regulatory network of plant heat stress response. *Trends in Plant Science*, **22**, 53–65.

Scharf K D, Berberich T, Ebersberger I, Nover L. 2012. The plant heat stress transcription factor (Hsf) family: Structure, function and evolution. *Biochimica et Biophysica Acta-Gene Regulatory Mechanisms*, **1819**, 104–119.

Song Q, He F, Kong L F, Yang J R, Wang X J, Zhao Z J, Zhang Y Q, Xu C Z, Fan C F, Luo K M. 2024. The IAA17.1/HSFA5a module enhances salt tolerance in *Populus tomentosa* by regulating flavonol biosynthesis and ROS levels in lateral roots. *New Phytologist*, **241**, 592–606.

Trapnell C, Williams B A, Pertea G, Mortazavi A, Kwan G, Van Baren M J, Salzberg S L, Wold B J, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, **28**, 511–515.

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, Kudla J. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant Journal*, **40**, 428–438.

Wang L H, Chen H, Chen G Y, Luo G B, Shen X Y, Ouyang B, Bie Z L. 2024. Transcription factor SlWRKY50 enhances cold tolerance in tomato by activating the jasmonic acid signaling. *Plant Physiology*, **194**, 1075–1090.

Wang R J, Wang R X, Liu M M, Yuan W W, Zhao Z Y, Liu X Q, Peng Y M, Yang X R, Sun Y, Tang W Q. 2021. Nucleocytoplasmic trafficking and turnover mechanisms of BRASSINAZOLE RESISTANT1 in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, **118**, e2101838118.

Wang W X, Vinocur B, Shoseyov O, Altman A. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science*, **9**, 244–252.

Wang X M, Shi X, Chen S Y, Ma C, Xu S B. 2018. Evolutionary origin, gradual accumulation and functional divergence of heat shock factor gene family with plant evolution. *Frontiers in Plant Science*, **9**, 71.

Wei Y X, Hu W, Xia F Y, Zeng H Q, Li X L, Yan Y, He C Z, Shi H T. 2016. Heat shock transcription factors in banana: Genome-wide characterization and expression profile analysis during development and stress response. *Scientific Reports*, **6**, 36864.

Wu Z, Li T, Ding L P, Wang C P, Teng R D, Xu S J, Cao X, Teng N J. 2024. Lily LlHSFC2 coordinates with HSFAs to balance heat stress response and improve thermotolerance. *New Phytologist*, **241**, 2124–2142.

Xu S, Li J L, Zhang X Q, Wei H, Cui L J. 2006. Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. *Environmental and Experimental Botany*, **56**, 274–285.

Yang T X, Deng L, Zhao W, Zhang R X, Jiang H L, Ye Z B, Li C B, Li C Y. 2019. Rapid breeding of pink-fruited tomato hybrids using the CRISPR/Cas9 system. *Journal of Genetics and Genomics*, **46**, 505–508.

Yang X D, Zhu W M, Zhang H, Liu N, Tian S B. 2016. Heat shock factors in tomatoes: Genome-wide identification, phylogenetic analysis and expression profiling under development and heat stress. *PeerJ*, **4**, e1961.

Yoshida T, Ohama N, Nakajima J, Kidokoro S, Mizoi J, Nakashima K, Maruyama K, Kim J M, Seki M, Todaka D, Osakabe Y, Sakuma Y, Schöffl F, Shinozaki K, Yamaguchi-Shinozaki K. 2011. *Arabidopsis* HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Molecular Genetics and Genomics*, **286**, 321–332.

Yoshida T, Sakuma Y, Todaka D, Maruyama K, Qin F, Mizoi J, Kidokoro S, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K. 2008. Functional analysis of an Arabidopsis heat-shock transcription factor HsfA3 in the transcriptional cascade downstream of the DREB2A stress-regulatory system. *Biochemical and Biophysical Research Communications*, **368**, 515–521.

Zhang J H, Jia X M, Wang G F, Ma S J, Wang S X, Yang Q, Chen X Y, Zhang Y Q, Lyu Y J, Wang X X, Shi J W, Zhao Y T, Chen Y H, Wu L J. 2022. Ascorbate peroxidase 1 confers resistance to southern corn leaf blight in maize. *Journal of Integrative Plant Biology*, **64**, 1196–1211.

Zhang Z G, Zhang Q, Wu J X, Zheng X, Zheng S, Sun X H, Qiu Q S, Lu T G. 2013. Gene knockout study reveals that cytosolic ascorbate peroxidase 2 (OsAPX2) plays a critical role in growth and reproduction in rice under drought, salt and cold stresses. *PLoS ONE*, **8**, e57472.

Zhao T, Xia H, Liu J Y, Ma F W. 2014. The gene family of dehydration responsive element-binding transcription factors in grape (*Vitis vinifera*): Genome-wide identification and analysis, expression profiles, and involvement in abiotic stress resistance. *Molecular Biology Reports*, **41**, 1577–1590.

Zheng F Y, Cui L, Li C X, Xie Q M, Ai G, Wang J Q, Yu H Y, Wang T T, Zhang J H, Ye Z B, Yang C X. 2022. Hair interacts with SlZFP8-like to regulate the initiation and elongation of trichomes by modulating *SlZFP6* expression in tomato. *Journal of Experimental Botany*, **73**, 228–244.

Zhong Q, Yu J T, Wu Y D, Yao X F, Mao C J, Meng X Z, Ming F. 2024. Rice transcription factor OsNAC2 maintains the homeostasis of immune responses to bacterial blight. *Plant Physiology*, **195**, 785–798.

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