



Article

SIMDH3 Interacts with Autophagy Receptor Protein SLATI1 and Positively Regulates Tomato Heat Tolerance

Sitian Wang ¹, Li Zhang ¹, Linyang Zhang ¹, Kang Yong ¹, Tao Chen ¹, Lijun Cao ^{2,3} and Minghui Lu ^{1,*} 

¹ College of Horticulture, Northwest A&F University, Yangling 712100, China; 15191021199@163.com (S.W.); zl236426@163.com (L.Z.); 17869725925@163.com (L.Z.); yongkang@nwfau.edu.cn (K.Y.); chengtao61@163.com (T.C.)

² Department of Biology, Duke University, Durham, NC 27708, USA; lc353@duke.edu

³ Howard Hughes Medical Institute, Duke University, Durham, NC 27708, USA

* Correspondence: lmhdick@nwsuaf.edu.cn

Abstract: Autophagy, a highly conserved protein degradation system, plays an important role in protecting cells from adverse environmental conditions. ATG8-INTERACTING PROTEIN1 (ATI1) acts as an autophagy receptor, but its functional mechanisms in plants' heat stress tolerance remain unclear. In this study, using LC-MS/MS, we identified malate dehydrogenase (SIMDH3) as a SLATI1-interacting protein. Further studies showed that heat stress induced the expression of *SIMDH3* and *SIMDH3* co-localized with SLATI1 under both 22 °C and 42 °C heat treatment conditions. Moreover, silencing of *SIMDH3* increased the sensitivity of tomato to heat stress, as evidenced by exacerbated degradation of chlorophyll; accumulation of MDA, H₂O₂, and dead cells; increased relative conductivity; and inhibition of stress-related gene expression. Conversely, overexpression of *SIMDH3* improved tomato's heat tolerance, leading to opposite effects on physiological indicators and gene expression compared to *SIMDH3* silencing. Taken together, our study suggests that *SIMDH3* interacts with SLATI1 and positively regulates tomato heat tolerance.

Keywords: malate dehydrogenase; heat stress; autophagy; ATI1; tomato



Citation: Wang, S.; Zhang, L.; Zhang, L.; Yong, K.; Chen, T.; Cao, L.; Lu, M. *SIMDH3* Interacts with Autophagy Receptor Protein SLATI1 and Positively Regulates Tomato Heat Tolerance. *Int. J. Mol. Sci.* **2024**, *25*, 7000. <https://doi.org/10.3390/ijms25137000>

Academic Editor: Hunseung Kang

Received: 29 May 2024

Revised: 20 June 2024

Accepted: 20 June 2024

Published: 26 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Global warming increases the frequency of extreme high temperatures [1], with heat stress emerging as one of the most severe abiotic stresses that impede plants' growth, development, and reproduction. Heat stress induces protein misfolding, denaturation, oxidation, and aggregation, disturbing crucial biological processes within plant cells [2]. Unlike mobile organisms, plants cannot escape the adverse effects of high temperatures. Instead, they have evolved a complex set of mechanisms at the physiological, biochemical, and molecular levels to mitigate heat stress [3], with the efficient degradation of aggregated proteins playing a vital role in this process [4].

Protein degradation serves essential functions during plants' development and in their responses to environmental stimuli, primarily occurring via two pathways: the ubiquitin-proteasome system (UPS) and autophagy. While the UPS plays a dominant role in the degradation of soluble and short-life proteins, autophagy is responsible for the aggregated and long-life proteins, non-protein substances such as nucleic acids and lipid bodies, and even entire organelles [5]. Autophagy is executed by sequential autophagy-related proteins (ATGs), through which the cellular cargoes are sequestered into autophagosome, a double-membrane engulfing vesicle, and then transported to the vacuole for degradation [6,7]. The involvement of autophagy in plants' responses to heat stress has attracted many investigations. For example, in *Arabidopsis*, autophagy contributes to heat stress memory by NBR1-mediated degradation of HSP90.1 and ROF1 [8], while in tomato, the heat tolerance responses were compromised in autophagy-suppressed plants [9].

Autophagy is a selective degradation mechanism targeting specific cellular components or proteins under different cellular conditions [10]. The recognition of specific cargo in the autophagy pathway relies on cargo receptors, which directly or indirectly identify and tether cargos into autophagosomes by interacting with ATG8, the core protein of autophagy, through the Atg8-interacting motif (AIM) or LC3-interacting region (LIR) [11]. Studies have shown that plant ATG8s and ATG8-interacting proteins are associated with plants' growth and responses to hormones and abiotic stresses [11–15]. Multiple autophagy receptors have been identified in plants, such as NBR1, DSK2A/B, MAPR5, ORM1, ATI1, and ATI2 [16]. During carbon starvation in Arabidopsis, ATG8-interacting protein1 (AtATI1) localizes to the endoplasmic reticulum (ER) and plastids to form ATI-ER and ATI-PS bodies, which are then transported to the central vacuole for selective turnover of specific proteins [17,18]. In addition, the expression of *AtATI1* in Arabidopsis can rescue the salt-hypersensitive phenotype displayed by the knockdown double mutant of *ati1ati2* [18].

Malate dehydrogenase (MDH) serves as a pivotal enzyme in the tricarboxylic acid (TCA) cycle and C4 photosynthesis, facilitating the reversible conversion between malic acid and oxaloacetic acid (OAA) [19]. MDH plays a key role in plant growth and development by participating in multiple cellular physiological activities, including mitochondrial energy metabolism, the malate–aspartate shuttle system, reactive oxygen species metabolism, and disease resistance [20]. Furthermore, MDH significantly contributes to plants' responses to environmental stresses by regulating the cellular redox balance, photosynthesis, and antioxidative defense mechanisms [21,22]. However, the involvement of MDH in plants' heat tolerance remains unclear.

Tomato (*Solanum lycopersicum* L.) is a widely cultivated vegetable crop around the world; however, it is highly sensitive to high temperatures, resulting in plant wilting, pollen abortion, and reduced fruit yield and quality [23]. In this study, a tomato SIATI1-interacting protein, tomato malate dehydrogenase 3 (SIMDH3 Solyc02g063490.2), was identified via the liquid chromatography–tandem mass spectrometry (LC-MS/MS) technique. Subsequent studies demonstrated that silencing *SIMDH3* rendered tomato plants more sensitive to heat stress, while overexpressing *SIMDH3* enhanced tomato's heat tolerance. Our findings shed light on the role of *SIMDH3* in improving the heat tolerance of tomato plants, contributing to the understanding of the mechanisms underlying plant resilience to heat stress and the development of tomato varieties with higher heat tolerances.

2. Results

2.1. *SIMDH3* Interacts with *SIATI1*

To identify the interacting proteins of SIATI1 in plants' heat tolerance, we employed immunoprecipitation coupled with LC-MS/MS, using SIATI1 as the bait to screen for its interacting proteins. Six candidate proteins were identified (Table S1), among which MDH was chosen for further validation due to its known role in plant tolerance to abiotic stress [24]. The finding was further confirmed using Y2H and Co-IP assays in vitro. Specifically, the yeast cells co-transformed with SIMDH3, and SIATI1 could grow on a SD/-Leu/-Trp/-His/-Ade medium (Figure 1A), whereas growth was not observed in other combinations, and SIMDH3-GFP was coimmunoprecipitated with SIATI1-Myc in vitro (Figure 1B). In addition, we detected that SIATI1 interacts with SIMDH3 in vivo through BiFC and split luciferase assay. Strong fluorescent (Figure 1C) and luminescence (Figure 1D) signals were detected when SIATI1 and SIMDH3 were co-expressed in tobacco (*Nicotiana benthamiana*) leaves. These results demonstrated interactions between SIATI1 and SIMDH3.

2.2. *SIMDH3* Responds to Heat Treatment and Co-Localizes with *SIATI1* under Heat Stress

To investigate whether *SIMDH3* responds to heat stress, tomato seedlings were exposed to heat stress at 42 °C, and the leaves were sampled at different timepoints after the treatment. The *SIMDH3* expression was significantly increased at each timepoint of heat treatment compared to 0 h (Figure 2), which is consistent with the findings of Imran [25]. These data suggest that *SIMDH3* does indeed respond to heat stress.

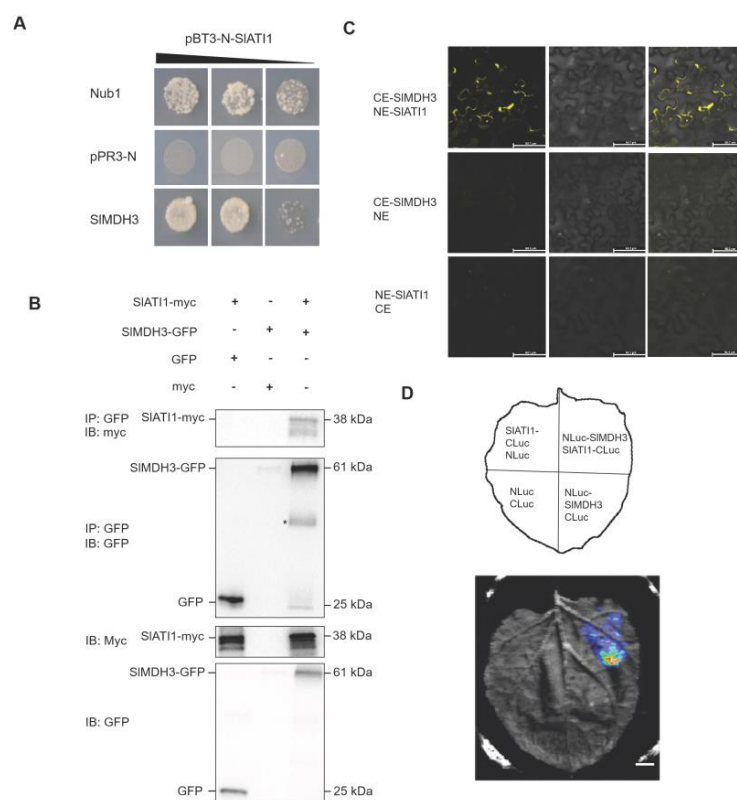


Figure 1. SIMDH3 interacts with SIAT11. **(A)** Interaction of SIAT11 and SIMDH3 in Y2H assay. Nub1, positive control; pPR3-N, negative control. **(B)** Co-IP assay of in vivo interaction between SIMDH3 and SIAT11. Constructs of SIMDH3-GFP and SIAT11-Myc were co-transfected in *N. benthamiana* leaves. Protein samples of inputs and outputs were immunoprecipitated with anti-GFP or anti-Myc antibodies. * represents unknown protein. **(C)** Interactions of SIMDH3 and SIAT11 in bimolecular fluorescence complementation (BiFC) assays. SIAT11 and SIMDH3 were fused to N-terminal or C-terminal portions of yellow fluorescent protein (NE or CE) and then transiently co-expressed after agroinfiltration in *N. benthamiana* and incubated at 22 °C for 3 d under light conditions. Construct pair CYFP-SIMDH3 + nYFP was co-transfected as negative control. Microscope's magnification: 10 × 40. **(D)** Split luciferase (Luc) assay of SIAT11-SIMDH3 interaction. SIMDH3 and SIAT11 were fused to CLuc and NLuc, respectively, and were transiently co-expressed in *N. benthamiana* leaves. Construct pairs SIAT11-CLuc + NLuc, NLuc-SIMDH3 + CLuc, and NLuc + CLuc were co-transfected as negative controls.

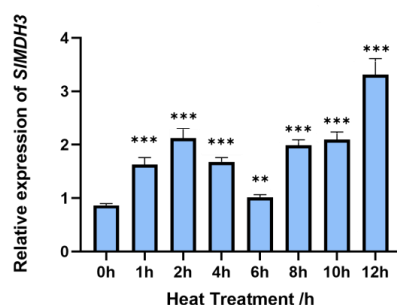


Figure 2. Analysis of SIMDH3 expression during heat stress in tomato (cv. Micro-Tom). Plants were subjected to heat treatment at 42 °C with three biological replications, each consisting of three plants at 5–6 leaf stage. **, $p < 0.01$; ***, $p < 0.001$.

Subsequently, we investigated the subcellular localization of SIMDH3 by transiently expressing it in the epidermal cells of tobacco leaves. The GFP signal was observed in the cytoplasm of leaves injected with *agrobacterium* harboring the plasmid of pART27-

GFP-SIMDH3, while in leaves injected with the empty vector pART27, the signal was uniformly distributed across the entire cell (Figure 3A). Given that SIMDH3 interacts with SIAT11 and responds to heat stress, we further explored their subcellular localization under a high temperature. When pART27-GFP-SIMDH3 and pART27-mCherry-SIAT11 were transiently co-expressed in the epidermal cells of tobacco leaf, the green fluorescence of the SIMDH3:GFP fusion protein was overlapped with the red fluorescence of the SIAT11:mCherry fusion protein under a normal temperature, while a more pronounced overlap was observed under heat stress (Figure 3B). These results indicate that SIMDH3 co-localizes with SIAT11.

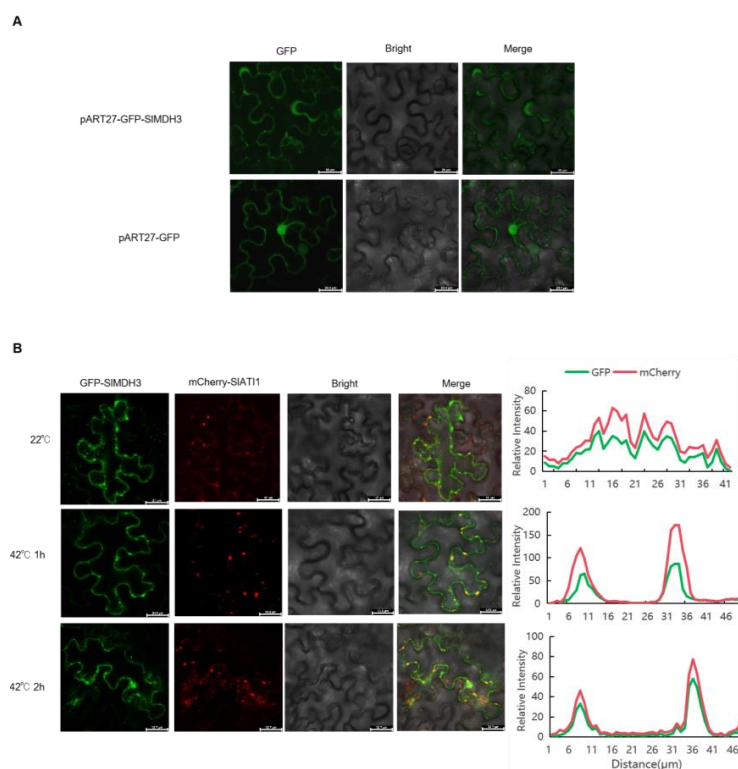


Figure 3. SIMDH3 co-localizes with SIAT11. (A) Subcellular localization of SIMDH3 in cytoplasm. (B) SIAT11 co-localizes with SIMDH3 under normal and high temperatures. All constructs were infiltrated into *N. benthamiana* leaves using *Agrobacterium* strain GV3101. Green, red, and yellow colors show signals of GFP-SIMDH3, mCherry-SIAT11, and their merged protein, respectively. Microscope's magnification: 10×40 .

2.3. SIMDH3-Silenced Plants Display Decreased Tolerance to Heat Stress

In order to investigate the involvement of *SIMDH3* in plants' heat stress tolerance, we generated *SIMDH3*-silenced tomato plants (TRV2:*SIMDH3*) by using virus-induced gene silencing (VIGS) technology. Upon observing the photo-bleaching phenotype in the leaves of positive control plants TRV2:SiPDS, we measured the expression level of *SIMDH3* in TRV2:*SIMDH3* and the control plants TRV2:00. Plants with a silencing efficiency over 70% (Figure S1) were chosen for further analysis.

Under normal temperature conditions, the activity of MDH in TRV2:*SIMDH3* was significantly lower than that in TRV2:00. After heat treatment (42 °C for 8 h), the MDH activity increased in both plants but remained obviously lower in TRV2:*SIMDH3* compared to TRV2:00 (Figure 4A), confirming the successful knockdown of *SIMDH3* in TRV2:*SIMDH3* plants. After the heat stress treatment, the growing points of TRV2:*SIMDH3* wilted, whereas those of the control plants TRV2:00 showed no significant wilting symptoms (Figure 4B). Additionally, both the relative conductivity and MDA content of the tomato leaves increased after heat treatment, with significantly higher levels observed in TRV2:*SIMDH3* than

in TRV2:00 (Figure 4C). However, after heat treatment, the total chlorophyll content of tomato leaf decreased, with markedly lower levels in TRV2:SIMDH3 than in control plants (Figure 4C). In addition, using DAB- and Trypan blue-staining methods, we measured the accumulation of H_2O_2 and dead cells, respectively. Under a normal temperature, there was minimal H_2O_2 and dead cells in tomato leaves. Following heat treatment, the accumulation of both H_2O_2 and dead cells increased in the leaves of both TRV2:SIMDH3 and TRV2:00, with more severe accumulation observed in the former compared to the latter (Figure 4D).

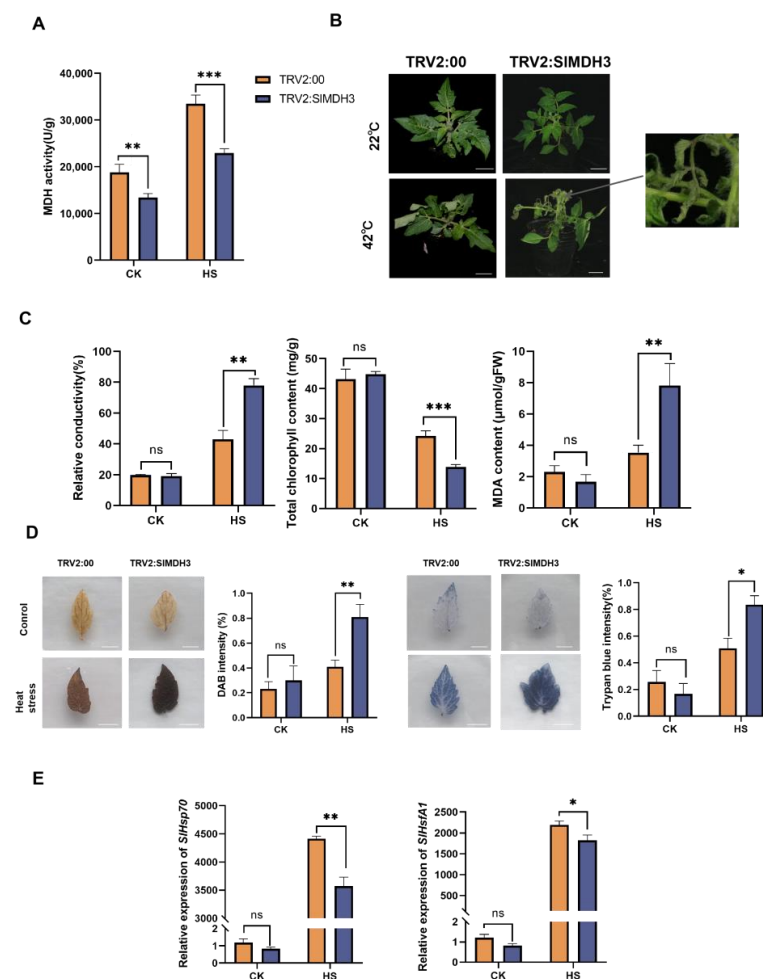


Figure 4. *SIMDH3* silencing increases tomato's sensitivity to heat stress. (A) MDH activity in TRV2:00 and TRV2:SIMDH3 plants. (B) Phenotypes of tomato seedlings. (C) Relative conductivity, total chlorophyll content, and MDA content in TRV2:00 and TRV2:SIMDH3 plants. (D) Accumulation of dead cells (stained using Trypan blue) and H_2O_2 (stained using DAB) after heat stress in tomato leaves. (E) Changes in relative expression levels of heat tolerance marker *SIHsp70* and *SIHsfA1* after heat stress in tomato leaves. TRV2:SIMDH3, *SIMDH3*-silenced tomato plants; TRV2:00, negative control plants. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significance.

Heat shock proteins (HSPs) play important roles in facilitating protein refolding to restore cellular homeostasis and protect plants from abiotic stress [26], and heat shock transcription factors (Hsfs) are responsible for the transcriptional regulation of HSPs and serve as key components in the plant's response to heat stress. Under normal temperature conditions, there was no significant difference in the expression levels of the heat tolerance marker genes *SIHSP70* and *SIHsfA1* in the leaves of TRV2:SIMDH3 and TRV2:00. However, after heat stress treatment, the expression level of both *SIHSP70* and *SIHsfA1* increased in the leaves of both tomato plants. Notably, in TRV2:SIMDH3 leaves, the expression levels of these two genes were significantly lower than those in TRV2:00 leaves (Figure 4E).

2.4. *SIMDH3* Overexpression Plants Exhibit Enhanced Heat Tolerance

To further validate the impact of *SIMDH3* on the heat tolerance of tomato plants, we generated six *SIMDH3* overexpression transgenic tomato lines (OE1–OE6). The relative expression levels of *SIMDH3* in all the six *SIMDH3*-OE lines were significantly higher than that in wild-type (WT) ones (Figure S2), and the two lines, OE3 and OE6, were selected for subsequent experiments.

Under normal temperature conditions, the MDH activities in the two *SIMDH3*-OE lines were notably higher than in WT. There was no significant difference in the relative conductivity, MDA level, accumulation of dead cells and H₂O₂, and expression levels of *SIHSP70* and *SIHsfA1* of the two lines. After heat stress treatment (42 °C for 10 h), both the WT and *SIMDH3*-OE lines displayed leaf wilting; however, the wilting degrees in the *SIMDH3*-OE lines were milder compared to WT plants (Figure 5A), and the MDH activity in the tested tomato lines increased by approximately 1.4 times; however, the activity levels in the two *SIMDH3*-OE lines were still significantly higher than that of WT (Figure 5B). Furthermore, the *SIMDH3*-OE lines showed obviously lower MDA levels (Figure 5B), higher accumulation of dead cells and H₂O₂ (Figure 5C), and higher expression levels of *SIHSP70* and *SIHsfA1* (Figure 5D) compared to WT.

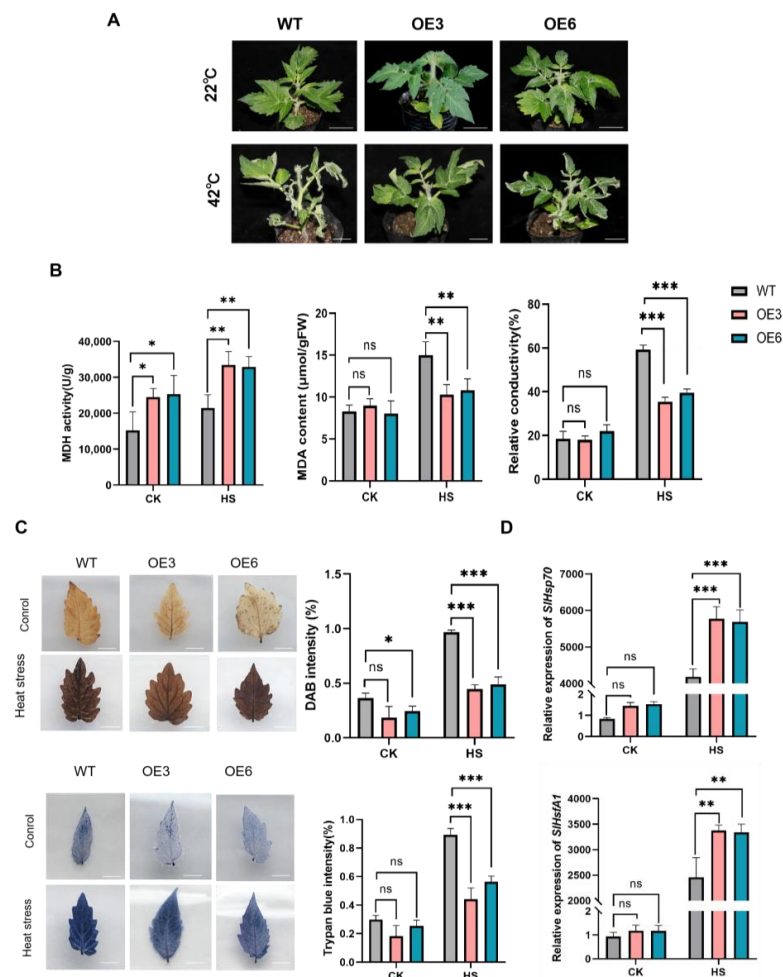


Figure 5. *SIMDH3* overexpression enhances tomato's tolerance against heat stress. (A) Phenotypes of tomato seedlings after exposure to heat stress (42 °C for 10 h). (B) MDH activity, relative conductivity, and MDA content. (C) Accumulation of dead cells (stained using Trypan blue) and H₂O₂ (stained using DAB) in tomato leaves. (D) Changes in relative expression levels of heat tolerance marker *SIHsp70* and *SIHsfA1* after heat stress in tomato leaves. WT, wild type; OE3 and OE6, *SIMDH3* overexpression lines. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significance.

3. Discussion

The aggregation of denatured protein caused by high temperature imposes toxicity on plant cells, which can be degraded through selective autophagy via the cargo recognition by autophagy receptors. Although autophagy is initially viewed as a non-selective mechanism for the bulk degradation of cytosolic components under acute stress, it has evolved into a recognized selective process known as selective autophagy [15]. This paradigm shift has unveiled specific mechanisms where pivotal proteins such as Autophagy-Related Protein8 (ATG8) play indispensable roles in both bulk and selective autophagy [27]. As an autophagy receptor, ATI1 has been reported to play important roles in plants' responses to carbon starvation [17,18,28], salt stress [18], viral infection [18], and exogenous phytohormone [17] by targeting various cargoes for their degradation in vacuole.

In our previous study, we discovered that SLATI1 plays a positive role in regulating the heat tolerance of tomato. To explore the underlying mechanism, in this study, we identified SIMDH3 as an SLATI1-interacting protein (Figure 1) and found that the expression of *SIMDH3* was induced by heat treatment (Figure 3). To further elucidate the involvement of *SIMDH3* in the tomato's response to heat stress, we developed *SIMDH3*-silenced plants and *SIMDH3*-overexpressed lines. Heat stress often induces alterations in malondialdehyde (MDA) levels, serving as a marker for lipid peroxidation and oxidative stress [29]. Plant cell membranes play a pivotal role in maintaining the cell's microenvironment and normal metabolic activities. Under optimal conditions, cell membranes selectively permeate substances. However, exposure to adversities such as extreme temperatures, drought, salinity, and pathogenic infections can damage the cell membrane, leading to increased permeability, electrolyte leakage, and heightened conductivity. Numerous studies have evidenced that chlorophyll biosynthesis diminishes under high-temperature stress [30], thereby impairing chlorophyll biosynthesis in plastids [31]. Thus, we assessed pertinent physiological parameters under various environmental conditions (Figures 4 and 5). Our findings revealed a notable decrease in chlorophyll content in silenced plants compared to the wild-type (WT) plants. However, conductivity and MDA levels exhibited an inverse trend, suggesting that gene silencing potentially exacerbates tomato plants' susceptibility to heat stress. Additionally, we quantified dead cells in plant samples using Trypan Blue staining and evaluated hydrogen peroxide accumulation in leaves through DAB staining to assess oxidative stress levels. As anticipated, the silenced genotype displayed more intense staining in both methods compared to the WT leaves, indicating that gene silencing heightens the plant's sensitivity to heat stress to some extent. Conversely, overexpression of the gene resulted in a complete reversal of all indicators (Figure 5), suggesting a potential role of this gene in enhancing tomato plants' resistance to heat stress.

A high temperature leads to the accumulation of ROS in plant cells, disturbing the redox homeostasis and causing protein denaturation, cytomembrane damage, and even cell death [32]. MDH catalyzes the conversion of malate to oxaloacetate, coupled with the conversion of NAD⁺ to NADH, which is important for plants to produce reductant equivalents in response to developmental requirements and changing environments [33]. In our study, compared to normal conditions, after heat stress treatment, we observed the accumulation of ROS and dead cells, peroxidation of membrane lipids, and damage to the cytomembrane's integrity in tomato leaves (Figures 4 and 5). All these adverse effects imposed by heat stress were exacerbated by *SIMDH3* silencing but mitigated by *SIMDH3* overexpression, suggesting that *SIMDH3* contributes to tomato's heat tolerance by controlling redox homeostasis. Kandoi also observed that the overexpression of maize chloroplast NADP-MDH in Arabidopsis enhanced salt tolerance by promoting the malate valve to maintain redox homeostasis inside and outside the plastid [21]. Wang suggested that the apple cytosolic MDH conferred higher tolerance to cold and salt stresses in transgenic apple plants by producing more reductive redox states [34]. However, Wu found that Arabidopsis mitochondrial MDH2 negatively regulated tolerance to Cd stress by modulating ROS levels [35]. Nan reported that rice chloroplast MDH1 negatively regulated tolerance to salt stress by affecting vitamin B6 content and ROS accumulation [36]. These contradictory

results may be related to the subcellular locations and functional diversity of MDHs, and further investigation is needed.

The subcellular translocation of proteins is a countermeasure employed by plants in response to heat stress, altering their interaction with other molecular components [37]. Upon exposure to heat stress, the Arabidopsis glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (AtGAPC) and rice NAC transcription OsNTL3 (NTM1-like3) relocate from the cytoplasm to the nucleus, enabling them to transmit the heat signal and trigger downstream heat responses [38,39]. In this study, we observed the co-localization of SIMDH3 with SLATI1 (Figure 3B), suggesting that SIMDH3 may function together with SLATI1 in tomato to combat heat stress. ATI1, as an autophagy receptor protein, interacts with the autophagy core protein ATG8. When participating in autophagy, it typically first forms a distinct ATI body before being fused or engulfed by autophagosomes. Interestingly, the punctate co-localization of SIMDH3 and SLATI1 that we observed appears to be dynamic, suggesting the possibility of ATI1 involvement in the formation of ATI bodies and its participation in autophagy. However, whether these structures do, indeed, represent autophagosomes and whether SIMDH3 is involved in the autophagy process remains unclear. Therefore, further research is needed to delve deeper into the relationship between them in future studies.

In summary, we identified the SLATI1 interaction protein SIMDH3, which plays an active role in regulating tomato's tolerance to heat stress

4. Methods and Materials

4.1. Plant Materials and Growth Conditions

The tomato (*Solanum lycopersicum* L. cv. Micro-Tom) and tobacco (*Nicotiana benthamiana*) used in this study were grown with a thermoperiod of 16 h light/8 h darkness at 22 °C/20 °C and 22 °C/18 °C, respectively. The light intensity was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the relative humidity was approximately 70%.

4.2. LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) Assay

The coding sequence (CDS) of *SLATI1* was cloned into pART27 with a GFP-tag using homologous recombination Assembly Mix (638947, TaKaRa, Kyoto, Japan). The resulting expression vector, pART27-GFP-SLATI1, was then transformed into an *Agrobacterium* strain GV3101. Overnight-cultured *Agrobacterium* suspension ($\text{OD}_{600} = 0.8$) was injected into tobacco leaves. The inoculated plants were grown for 3 d and then subjected to a 3 h treatment of 42 °C, and the untreated plants were used as control. The total protein was extracted with GTEN buffer as described by Tameling and Baulcombe [40] and purified following the method of Fan [41]. The purified protein was subjected to enzymatic hydrolysis and desalination and then analyzed using LC-MS/MS (API 2000, AB Sciex, Framingham, MA, USA). The number of peptides and the peptide spectrum matches (PSMs), as well as the protein false discovery rate (FDR) Confidence: Combined, were used to screen for candidate proteins of interest. The final candidate proteins were further filtered by comparing the treated and control groups.

4.3. Yeast Two-Hybrid (Y2H) Assay

The CDS of *SIMDH3* and *SLATI1* were individually cloned into the pPR3-N vector and pBT3-N vector, and the recombinant plasmids, pBT3-N-SLATI1 and pPR3-N-SIMDH3, were co-transformed into Y2H Gold yeast cells according to the manual of the *Saccharomyces cerevisiae* transformation kit (PT1199, Pytbio, Wuhan, China). The transformed yeast cells were grown on SD/Leu/-Trp and SD/-Ade/-Hiss/-Leu/-Trp medium.

4.4. Co-IP (Coimmunoprecipitation) Assay

The CDS of *SIMDH3* or *SLATI1* were cloned into pART27-GFP and pART27-Myc, respectively. The resulting vector was then transformed into the *Agrobacterium* strain GV3101. The bacterial suspension was injected into tobacco leaves, which were cultured

for 3 d. Leaves of transformed tobaccos were collected, and then, total proteins were extracted as described above. Two protein pairs, pART27-Myc-SIAT11 + pART27-GFP and pART27-GFP + pART27-Myc, were used as negative controls. Samples were ground in liquid nitrogen and homogenized in immunoprecipitation (IP) buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 10% glycerol) with 0.3% Triton X-100. A portion of the total lysis solution was reserved as the input, and the remaining lysate was incubated with anti-GFP Affinity beads (SA070001, Smart-Lifesciences, Changzhou, China) for 2 h at 4 °C. The beads were then collected and washed three times with balanced solution (50 mM Tris, 500 mM NaCl, pH 7.4) and subsequently loaded with loading buffer before being incubated at 95 °C for 5 min. IP products and the input samples were separated using 12% SDS-PAGE, and the target proteins were detected by Western blot using anti-GFP or anti-Myc antibodies.

4.5. Split Luciferase Assay

The CDS without a termination codon of *SIMDH3* and the full CDS with the termination codon of *SIAT11* were cloned into the expression vector pCAMBIA1300 to generate the expression vectors pCAMBIA1300-NLuc-SIMDH3 and pCAMBIA1300-SIAT11-CLuc, respectively, which were then individually transformed into *Agrobacterium* strains GV3101. The bacterial suspensions were co-injected into tobacco leaf and cultured for 3 days, and the fluorescence was observed according to the protocol described by Chen [42].

4.6. BiFC Assay

The CDSs of *SIAT11* and *SIMDH3* were cloned into the pSPYNE and pSPYCE vector, respectively, and the resulting constructs were transformed into the *Agrobacterium* strain GV3101. The bacterial suspensions were co-injected into the lower epidermis of tobacco leaves, which was then cultured in darkness for 3 d. The observation of YFP fluorescence was executed with a Laser Scanning Confocal Microscope (FV3000, Olympus, Japan) at excitation/emission (ex/em) 514/530–555 nm, and the tobacco co-transformed with CE-SIMDH3 + NE was used as the negative control.

4.7. Subcellular Localization of SIMDH3

The vector, pART27-GFP-SIMDH3, was transformed into the *Agrobacterium* strain GV3101. The empty vector, pART27, served as the control. The resulting *Agrobacteria* solution was injected into the back surface of 40-day-old tobacco leaves, and the plants were incubated overnight at 22 °C in darkness and then transferred to normal conditions for 2–3 d. The expression vectors pART27-GFP-SIMDH3 and pART27-mCherry-SIAT11 were co-transformed into tobacco leaf for co-localization analysis. The fluorescence signal was observed using Laser Scanning Confocal Microscope (FV3000, Olympus, Tokyo, Japan).

4.8. Analysis of SIMDH3 Expression of Tomato Seedlings during Heat Stress

Tomato seedlings at the 5- to 6-leaf stage were subjected to a 42 °C treatment. Leaves were sampled at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h post-treatment, immediately frozen in liquid nitrogen, and stored at −80 °C for total RNA extraction.

4.9. Generation of SIMDH3-Silenced Tomato Plants

The *SIMDH3*-silenced tomato plants were generated using virus-induced gene silencing (VIGS) as described [43]. The gene-silencing fragment of *SIMDH3* was selected using the SGN VIGS Tool (<https://vigs.solgenomics.net/> accessed on 19 February 2023.) and amplified using the primer pair TRV2:SIMDH3-F/R (Table S2). The PCR product was ligated to pTRV2:00 to construct the gene-silencing vector pTRV2:SIMDH3. Tomato seedlings transiently transformed with pTRV2:SIMDH3 were used as *SIMDH3*-silenced plants (TRV2:SIMDH3), and those with pTRV2:SIPDS and pTRV2:00 were used as positive and negative controls, respectively (TRV2:SIPDS and TRV2:00). Upon observation of albino symptoms in the leaves of TRV2:SIPDS plants, the relative expression levels of *SIMDH3*

in TRV2:SIMDH3 and TRV2:00 were tested, and the silencing efficiency of *SIMDH3* was calculated. Tomato plants with silencing efficiencies greater than 70% were chosen for subsequent experiments [44].

4.10. Generation of Tomato Lines with *SIMDH3* Overexpression

Tomato cotyledons were used as explants for the generation of *SIMDH3* overexpression (*SIMDH3*-OE) lines. The healthy cotyledons were inoculated with the *Agrobacterium* strain GV3101, transformed with pART27-GFP-*SIMDH3*. The expression level of *SIMDH3* in the tomato *SIMDH3*-OE lines was assessed using a qRT-PCR assay with the primer pair qSIMDH3-F/R (Table S2).

4.11. Experimental Treatments

For heat stress treatment, tomato seedlings were subjected to a high temperature of 42 °C treatment for 8 h (for TRV2:00 and TRV2:SIMDH3) or 10 h (for WT and *SIMDH3*-OE lines). Following the treatments, the phenotypes of tomato plants were recorded through photography, and tomato leaves were sampled for the measurements of physiological indices and gene expressions. Each treatment was replicated three times, with each replicate comprising six tomato seedlings.

4.12. Total RNA Extraction, cDNA Synthesis, and qRT-PCR Analysis

Total RNA was isolated from tomato leaves using the Trizol kit (Invitrogen, Waltham, MA, USA), and the synthesis of first-strand cDNA was conducted using the PrimeScript Kit (TaKaRa, Kusatsu, Japan). The qRT-PCR assay was carried out using SYBR Premix Ex Taq II (TaKaRa, Japan). The relative gene expression levels were calculated following the method of $2^{-\Delta\Delta CT}$ [45], in which *SlUbi3* served as the internal control. All the sequences of primer pairs are listed in Table S2.

4.13. Determination of Physiological Indexes

The MDH enzyme activity was determined using NAD-MDH Activity Detection Kit (Solarbio BC1040) following the manufacturer's instructions. The lipid peroxidation in cell membranes of tomato leaf discs were quantified by measuring the MDA content as previously described by Luo [46]. The total chlorophyll content was assessed using a spectrophotometric method following extraction into 80% acetone (*v/v*) [47]. The relative conductivity of tomato leaves was measured according to the method of Luo [46]. The accumulation of H₂O₂ and dead cells was observed using the methods of Zimmermann [48] and Wang [49], respectively. All experiments were performed with three biological replicates.

4.14. Statistical Analysis

The experimental data were plotted using Graphpad 9.5 software, and the significant difference in data between different treatments was analyzed using *t*-test in SPSS 23.0 software at the 0.05, 0.01, and 0.001 levels.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25137000/s1>.

Author Contributions: S.W. designed and conducted the experiments and wrote the draft of the manuscript. L.Z. (Li Zhang), L.Z. (Linyang Zhang), K.Y., and T.C. conducted the experiments and analyzed the data. L.C. revised the manuscript. M.L. designed the experiments and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the National Natural Science Foundation of China (No. 32172552) and the earmarked fund for CARS (CARS-23-G22).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Zhu, T.; Yang, S.-L.; De Smet, I. It is time to move: Heat-induced translocation events. *Curr. Opin. Plant Biol.* **2023**, *75*, 102406. [[CrossRef](#)] [[PubMed](#)]
2. Hemantaranjan, A.; Bhanu, A.N.; Singh, M.N.; Yadav, D.K.; Patel, P.K.; Singh, R.; Katiyar, D. Heat Stress Responses and Thermotolerance. *Adv. Plants Agric. Res.* **2014**, *1*, 1–10. [[CrossRef](#)]
3. Chinnusamy, V.; Zhu, J.; Zhu, J.K. Cold stress regulation of gene expression in plants. *Trends Plant Sci.* **2007**, *12*, 444–451. [[CrossRef](#)] [[PubMed](#)]
4. Zhou, Y.; Xu, F.; Shao, Y.; He, J. Regulatory Mechanisms of Heat Stress Response and Thermomorphogenesis in Plants. *Plants* **2022**, *11*, 3410. [[CrossRef](#)] [[PubMed](#)] [[PubMed Central](#)]
5. Klionsky, D.J. Autophagy. *Curr. Biol.* **2005**, *15*, R282–R283. [[CrossRef](#)] [[PubMed](#)]
6. Kamada, Y.; Funakoshi, T.; Shintani, T.; Nagano, K.; Ohsumi, M.; Ohsumi, Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* **2000**, *150*, 1507–1513. [[CrossRef](#)]
7. Noda, N.N.; Ohsumi, Y.; Inagaki, F. Atg8-family interacting motif crucial for selective autophagy. *FEBS Lett.* **2010**, *584*, 1379–1385. [[CrossRef](#)]
8. Thirumalaikumar, V.P.; Gorka, M.; Schulz, K.; Masclaux-Daubresse, C.; Sampathkumar, A.; Skirycz, A.; Vierstra, R.D.; Balazadeh, S. Selective autophagy regulates heat stress memory in Arabidopsis by NBR1-mediated targeting of HSP90.1 and ROF1. *Autophagy* **2021**, *17*, 2184–2199. [[CrossRef](#)] [[PubMed](#)] [[PubMed Central](#)]
9. Zhou, J.; Wang, J.; Yu, J.-Q.; Chen, Z. Role and regulation of autophagy in heat stress responses of tomato plants. *Front. Plant Sci.* **2014**, *5*, 174. [[CrossRef](#)] [[PubMed](#)] [[PubMed Central](#)]
10. Avin-Wittenberg, T.; Honig, A.; Galili, G. Variations on a theme: Plant autophagy in comparison to yeast and mammals. *Protoplasma* **2012**, *249*, 285–299. [[CrossRef](#)]
11. Kirisako, T.; Ichimura, Y.; Okada, H.; Kabeya, Y.; Mizushima, N.; Yoshimori, T.; Ohsumi, M.; Takao, T.; Noda, T.; Ohsumi, Y. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J. Cell Biol.* **2000**, *151*, 263–275. [[CrossRef](#)] [[PubMed](#)]
12. Yoshimoto, K.; Hanaoka, H.; Sato, S.; Kato, T.; Tabata, S.; Noda, T.; Ohsumi, Y. Processing of ATG8s, Ubiquitin-like Proteins, and Their Deconjugation by ATG4s Are Essential for Plant Autophagy. *Plant Cell* **2004**, *16*, 2967–2983. [[CrossRef](#)] [[PubMed](#)]
13. Slavikova, S.; Ufaz, S.; Avin-Wittenberg, T.; Levanony, H.; Galili, G. An autophagy-associated Atg8 protein is involved in the responses of Arabidopsis seedlings to hormonal controls and abiotic stresses. *J. Exp. Bot.* **2008**, *59*, 4029–4043. [[CrossRef](#)] [[PubMed](#)]
14. Zientara-Rytter, K.; Lukomska, J.; Moniuszko, G.; Gwozdecki, R.; Surowiecki, P.; Lewandowska, M.; Liszewska, F.; Wawrzynska, A.; Sirko, A. Identification and functional analysis of Joka2, a tobacco member of the family of selective autophagy cargo receptors. *Autophagy* **2011**, *7*, 1145–1158. [[CrossRef](#)] [[PubMed](#)]
15. Zhou, J.; Wang, J.; Cheng, Y.; Chi, Y.-J.; Fan, B.; Yu, J.-Q.; Chen, Z. NBR1-Mediated Selective Autophagy Targets Insoluble Ubiquitinated Protein Aggregates in Plant Stress Responses. *PLoS Genet.* **2013**, *9*, e1003196. [[CrossRef](#)] [[PubMed](#)]
16. Stephani, M.; Dagdas, Y. Plant Selective Autophagy—Still an Uncharted Territory with a Lot of Hidden Gems. *J. Mol. Biol.* **2020**, *432*, 63–79. [[CrossRef](#)] [[PubMed](#)]
17. Honig, A.; Avin-Wittenberg, T.; Ufaz, S.; Galili, G. A new type of compartment, defined by plant-specific Atg8-interacting proteins, is induced upon exposure of Arabidopsis plants to carbon starvation. *Plant Cell* **2012**, *24*, 288–303. [[CrossRef](#)] [[PubMed](#)]
18. Michaeli, S.; Honig, A.; Levanony, H.; Peled-Zehavi, H.; Galili, G. Arabidopsis ATG8-INTERACTING PROTEIN1 Is Involved in Autophagy-Dependent Vesicular Trafficking of Plastid Proteins to the Vacuole. *Plant Cell* **2014**, *26*, 4084–4101. [[CrossRef](#)] [[PubMed](#)] [[PubMed Central](#)]
19. Ocheretina, O.; Scheibe, R. Cloning and sequence analysis of cDNAs encoding plant cytosolic malate dehydrogenase. *Gene* **1997**, *199*, 145–148. [[CrossRef](#)]
20. Musrati, R.A.; Kollárová, M.; Mernik, N.; Mikulášová, D. Malate dehydrogenase: Distribution, function and properties. *Gen. Physiol. Biophys.* **1998**, *17*, 193–210.
21. Kandoi, D.; Mohanty, S.; Tripathy, B.C. Overexpression of plastidic maize NADP-malate dehydrogenase (ZmNADP-MDH) in Arabidopsis thaliana confers tolerance to salt stress. *Protoplasma* **2018**, *255*, 547–563. [[CrossRef](#)] [[PubMed](#)]
22. Tesfaye, M.; Temple, S.J.; Allan, D.L.; Vance, C.P.; Samac, D.A. Overexpression of Malate Dehydrogenase in Transgenic Alfalfa Enhances Organic Acid Synthesis and Confers Tolerance to Aluminum. *Plant Physiol.* **2001**, *127*, 1836–1844. [[CrossRef](#)] [[PubMed](#)]
23. Hoshikawa, K.; Pham, D.; Ezura, H.; Schafleitner, R.; Nakashima, K. Genetic and Molecular Mechanisms Conferring Heat Stress Tolerance in Tomato Plants. *Front. Plant Sci.* **2021**, *12*, 786688. [[CrossRef](#)]
24. Yao, Y.-X.; Dong, Q.-L.; Zhai, H.; You, C.-X.; Hao, Y.-J. The functions of an apple cytosolic malate dehydrogenase gene in growth and tolerance to cold and salt stresses. *Plant Physiol. Biochem.* **2011**, *49*, 257–264. [[CrossRef](#)] [[PubMed](#)]

25. Imran, M.; Munir, M.Z.; Ialhi, S.; Abbas, F.; Younus, M.; Ahmad, S.; Naeem, M.K.; Waseem, M.; Iqbal, A.; Gul, S.; et al. Identification and Characterization of Malate Dehydrogenases in Tomato (*Solanum lycopersicum* L.). *Int. J. Mol. Sci.* **2022**, *23*, 10028. [[CrossRef](#)] [[PubMed](#)]
26. Morán Luengo, T.M.; Kityk, R.; Mayer, M.P.; Rüdiger, S.G. Hsp90 Breaks the Deadlock of the Hsp70 Chaperone System. *Mol. Cell* **2018**, *70*, 545–552.e9. [[CrossRef](#)] [[PubMed](#)]
27. Johansen, T.; Lamark, T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* **2011**, *7*, 279–296. [[CrossRef](#)] [[PubMed](#)]
28. Wu, J.; Michaeli, S.; Picchianti, L.; Dagdas, Y.; Galili, G.; Peled-Zehavi, H. ATI1 (ATG8-interacting protein 1) and ATI2 define a plant starvation-induced reticulophagy pathway and serve as MSBP1/MAPR5 cargo receptors. *Autophagy* **2021**, *17*, 3375–3388. [[CrossRef](#)] [[PubMed](#)] [[PubMed Central](#)]
29. Mathur, S.; Agrawal, D.; Jajoo, A. Photosynthesis: Response to high temperature stress. *J. Photochem. Photobiol. B* **2014**, *137*, 116–126. [[CrossRef](#)]
30. Li, C.; Ma, M.; Zhang, T.; Feng, P.; Chen, X.; Liu, Y.; Brestic, M.; Galal, T.M.; Al-Yasi, H.M.; Yang, X. Comparison of photosynthetic activity and heat tolerance between near isogenic lines of wheat with different photosynthetic rates. *PLoS ONE* **2021**, *16*, e0255896. [[CrossRef](#)]
31. Dutta, S.; Mohanty, S.; Tripathy, B.C. Role of temperature stress on chloroplast biogenesis and protein import in pea. *Plant Physiol.* **2009**, *150*, 1050–1061. [[CrossRef](#)]
32. Hendrix, S.; Dard, A.; Meyer, A.J.; Reichheld, J.-P. Redox-mediated responses to high temperature in plants. *J. Exp. Bot.* **2023**, *74*, 2489–2507. [[CrossRef](#)] [[PubMed](#)]
33. Selinski, J.; Scheibe, R. Malate valves: Old shuttles with new perspectives. *Plant Biol.* **2019**, *21* (Suppl. S1), 21–30. [[CrossRef](#)] [[PubMed](#)]
34. Wang, Q.; Sun, H.; Dong, Q.; Sun, T.; Jin, Z.; Hao, Y.; Yao, Y. The enhancement of tolerance to salt and cold stresses by modifying the redox state and salicylic acid content via the cytosolic malate dehydrogenase gene in transgenic apple plants. *Plant Biotechnol. J.* **2016**, *14*, 1986–1997. [[CrossRef](#)]
35. Wu, X.; Han, Y.; Zhu, X.; Shah, A.; Wang, W.; Sheng, Y.; Fan, T.; Cao, S. Negative regulation of cadmium tolerance in Arabidopsis by MMDH2. *Plant Mol. Biol.* **2019**, *101*, 507–516. [[CrossRef](#)] [[PubMed](#)]
36. Nan, N.; Wang, J.; Shi, Y.; Qian, Y.; Jiang, L.; Huang, S.; Liu, Y.; Wu, Y.; Liu, B.; Xu, Z. Rice plastidial NAD-dependent malate dehydrogenase 1 negatively regulates salt stress response by reducing the vitamin B6 content. *Plant Biotechnol. J.* **2020**, *18*, 172–184. [[CrossRef](#)] [[PubMed](#)]
37. Kan, Y.; Mu, X.-R.; Gao, J.; Lin, H.-X.; Lin, Y. The molecular basis of heat stress responses in plants. *Mol. Plant* **2023**, *16*, 1612–1634. [[CrossRef](#)]
38. Kim, S.-C.; Guo, L.; Wang, X. Nuclear moonlighting of cytosolic glyceraldehyde-3-phosphate dehydrogenase regulates Arabidopsis response to heat stress. *Nat. Commun.* **2020**, *11*, 3439. [[CrossRef](#)] [[PubMed](#)]
39. Liu, X.; Lyu, Y.; Yang, W.; Yang, Z.; Lu, S.; Liu, J. A membrane-associated NAC transcription factor OsNLT3 is involved in thermotolerance in rice. *Plant Biotechnol. J.* **2020**, *18*, 1317–1329. [[CrossRef](#)]
40. Tameling, W.I.; Baulcombe, D.C. Physical Association of the NB-LRR Resistance Protein Rx with a Ran GTPase-Activating Protein Is Required for Extreme Resistance to Potato virus X. *Plant Cell* **2007**, *19*, 1682–1694. [[CrossRef](#)]
41. Fan, G.; Yang, Y.; Li, T.; Lu, W.; Du, Y.; Qiang, X.; Wen, Q.; Shan, W. A Phytophthora capsici RXLR Effector Targets and Inhibits a Plant PPIase to Suppress Endoplasmic Reticulum-Mediated Immunity. *Mol. Plant* **2018**, *11*, 1067–1083. [[CrossRef](#)]
42. Chen, H.; Zou, Y.; Shang, Y.; Lin, H.; Wang, Y.; Cai, R.; Tang, X.; Zhou, J.-M. Firefly Luciferase Complementation Imaging Assay for Protein-Protein Interactions in Plants. *Plant Physiol.* **2008**, *146*, 323–324. [[CrossRef](#)] [[PubMed](#)]
43. Zhao, Y.; Tian, X.; Wang, F.; Zhang, L.; Xin, M.; Hu, Z.; Yao, Y.; Ni, Z.; Sun, Q.; Peng, H. Characterization of wheat MYB genes responsive to high temperatures. *BMC Plant Biol.* **2017**, *17*, 208. [[CrossRef](#)] [[PubMed](#)]
44. Liang, M.; Li, H.; Yong, K.; Huang, G.; Guo, Y.; Lu, M. Pepper autophagy related gene CaATG8e differentially regulates plant tolerance to heat and salt stress. *Sci. Hortic.* **2023**, *308*, 111559. [[CrossRef](#)]
45. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
46. Luo, Y.; Xie, Y.; Li, W.; Wei, M.; Dai, T.; Li, Z.; Wang, B. Physiological and Transcriptomic Analyses Reveal Exogenous Trehalose Is Involved in the Responses of Wheat Roots to High Temperature Stress. *Plants* **2021**, *10*, 2644. [[CrossRef](#)] [[PubMed](#)]
47. Arkus, K.A.; Cahoon, E.B.; Jez, J.M. Mechanistic analysis of wheat chlorophyllase. *Arch. Biochem. Biophys.* **2005**, *438*, 146–155. [[CrossRef](#)] [[PubMed](#)]
48. Zimmermann, P.; Heinlein, C.; Orendi, G.; Zentgraf, U. Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. *Plant Cell Environ.* **2006**, *29*, 1049–1060. [[CrossRef](#)]
49. Wang, M.; He, C.; Shi, G.; Yin, Q.; Zhang, H.; Yang, W.; Yue, A.; Wang, L.; Du, W. Genome-wide analysis of the SCAMPs gene family of soybean and functional identification of GmSCAMP5 in salt tolerance. *BMC Plant Biol.* **2023**, *23*, 628. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.