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SUMO E3 Ligase SlSIZ1 Facilitates Heat Tolerance in Tomato

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Regular Paper

High temperature has become a major abiotic stress that limits crop productivity. Heat shock transcription factors (HSFs) and heat shock proteins (HSPs) play important roles in enhancing thermotolerance of plants. SUMOylation is an important post-translational modification in regulating cellular functions in eukaryotes. SIZ1, a well-characterized SUMO E3 ligase, mediates the process of SUMOylation. In this study, SUMO conjugations were clearly induced by high temperature. Overexpression of SIZ1 SUMO E3 ligase (SlSIZ1) in tomato could enhance the tolerance to heat stress in tomato. The RNA interference (RNAi) plants were more wilted than the wild type with heat treatment. Under heat stress, SlSIZ1 could decrease the accumulation of reactive oxygen species (ROS) and induce some genes of *HSF* and *HSP* transcription. Furthermore, overexpression of SlSIZ1 could increase the level of Hsp70 under high temperature. Yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays showed that SlSIZ1 could interact with SlHsfA1 to mediate the SUMOylation of SlHsfA1 and consequently enhance thermotolerance of tomato. In conclusion, overexpression of SlSIZ1 enhanced heat tolerance by regulating the activities of HsfA1 and increasing the content Hsp70.

Keywords: Heat stress • Heat shock proteins (HSPs) • Heat shock transcription factors (HSFs) • SUMO E3 ligase SlSIZ1 • SUMOylation • Tomato.

Abbreviations: APX, ascorbate peroxidase; BiFC, bimolecular fluorescence complementation; DAB, 3′,3′-diaminobenzidine; H_2O_2, hydrogen peroxide; HSE, heat stress element; HSF, heat shock transcription factor; HSP, heat shock protein; HSR, heat stress response; MDA, malondialdehyde; MS, Murashige and Skoog; MV, methyl viologen; NBT, nitro blue tetrazolium; NLS, nuclear localization sequence; NR, nitrate reductase; $O_2\cdot-$, superoxide anion; ONPG, ortho-nitrophenyl-β-D-galactopyranoside; PEG, polyethylene glycol; PFD, photon flux density; POD, peroxidase; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time PCR; SIZ1, SIZ/PIAS-type SAP and Miz1; SIZ/PIAS, PIAS [protein inhibitor of activated signal transducers and activators of transcription (STAT)] and SIZ [scaffold attachment factor A/B/acinus/PIAS (SAP) and MIZ]; SlSIZ1, tomato SUMO E3 ligase SIZ1; ROS, reactive oxygen species; RNAi, RNA interference; sHSP, small heat shock protein; SUMO, small ubiquitin-like modifier; WT, wild type; YFP, yellow fluorescent protein.

Introduction

High temperature, which causes heat stress, has become a major abiotic stress that limits crop productivity (Kotak et al. 2007). Suitable temperatures for most plants range from 15 to 28°C. Temperatures above or below this range will result in plant damage (Liu et al. 2007). Plant organisms will show membrane injury, protein inactivation, production of reactive oxygen species (ROS) and metabolic destruction under high temperature stress that negatively affects plant growth and yield (Quinn 1988, Iba 2002). In general, many heat shock proteins (HSPs) are up-regulated by heat stress to enhance thermotolerance (Schlesinger 1990). Synthesis of HSPs is necessary for cellular protection under heat stress. HSPs function as molecular chaperones in repairing damaged proteins, reducing protein denaturation and regulating the activities of heat shock transcription factors (HSFs) to enhance the thermotolerance of plants (Schoffl et al. 1998).

In plants, the five major groups of HSPs are Hsp100, Hsp90, Hsp70, Hsp60 and small HSPs (sHSPs) (Jane et al. 2007). These HSPs are highly conserved in different species, and several of them are involved in normal protein functions in the cell (Schlesinger 1990). The Hsp70 family is one of the most characterized and most accumulated HSPs. Approximately 14 Hsp70 proteins were identified in Arabidopsis and localized in different organelles, such as the cytosol, chloroplasts or mitochondria (Jane et al. 2007). Furthermore, these proteins are important for normal cellular functions and involved in protein homeostasis (Young et al. 2004). Hsp70 interaction with different chaperones or factors is also essential for governing them. Hsp90, another important chaperone family, is highly abundant in plant proteins and involved in regulating kinases, signal transduction and transcription factors (Hahn and Scharf 2011). Hsp90 activities are related to some co-chaperones, such as Hsp70 and Hsp40 (DnaJ). A multiprotein complex, which includes Hsp90, Hsp70 and Hsp40, co-operates in protein homeostasis. Seven Hsp90 genes encoding different proteins localized in the chloroplast, mitochondrion and endoplasmic reticulum were also identified in Arabidopsis. Although Hsp90 can be induced by heat stress, it shows no direct relationship to heat tolerance (Jane et al. 2007).

The expression of HSP-encoding genes is regulated by HSFs (Baniwal et al. 2004). Moreover, HSFs are the terminal components of the signal pathway that mediates the responsiveness of

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different genes to heat stress (Morimoto 1998, Schoffl et al. 1998). A conserved motif located in the promoter of heat stress-induced genes is named the heat stress element (HSE; 5′-AGAAnnTTCT-3′). Under heat stress, the activated HSFs recognize the HSE and bind to this element to regulate HSPs (Pelham 1982, Nover et al. 2001). Approximately 17 and 21 different HSF genes were identified in tomato and Arabidopsis, respectively. These HSF genes were classified into three groups (HsfA, HsfB and HsfC) (Nover et al. 2001). Three HSFs, namely HsfA1, HsfA2 and HsfB1, are well known to form a network that regulates the expression of heat stress-induced genes in tomato (Baniwal et al. 2004). HsfA1 is the primary transcriptional regulator of the heat stress response (HSR) in both tomato and Arabidopsis (Mishra et al. 2002, Liu et al. 2011, Yoshida et al. 2011). Furthermore, HsfA2 and HsfB1 are regulated by HsfA1. The activity of HsfA1 is repressed by Hsp70 and Hsp90 under normal conditions and it is active under heat stress (Hahn and Scharf 2011). HsfA2 mainly becomes a HSF, which is related to its high transcriptional activation and continued accumulation during heat stress and recovery (Schramm et al. 2006). HsfA2 is also involved in regulating different environmental stresses (Nishizawa et al. 2006). HsfA1 can interact with HsfA2 to form a HsfA1–HsfA2 hetero-oligomeric complex and become a supertranscriptional factor in activating heat stress-induced genes (Chanschaminet et al. 2009). HsfBs regulate the HSR by repressing the activity of HsfA1 in a feedback pathway, although it is detected downstream of HsfA1 in Arabidopsis (Ikeda and Ohme-Takagi 2011). In tomato, HsfB1 acts as a co-activator of HsfAs, such as HsfA1 (Koskull-Döring et al. 2007).

SUMOylation often occurs in lysine residues within the SUMOylation consensus motif ΨKXD/E, where Ψ is a hydrophobic residue, K is lysine, X is any amino acid and D/E is glutamic acid or aspartic acid. (Schmidt and Muller 2003). The SUMOylation cycle is a conserved process that comprises five steps, namely maturation, E1 activation, E2 conjugation, E3 ligation and deconjugation. Moreover, the SUMOylation pathway requires the sequential action of three enzymes, i.e. E1 SUMO activation enzyme, E2 SUMO conjugation enzyme and E3 SUMO ligase (Colby et al. 2006). SUMO conjugation improves the stress tolerance to heat, cold and drought in Arabidopsis (Kurepa et al. 2003, Miura et al. 2005, Catala et al. 2007, Miura et al. 2007). The DNA binding activity of HsfA2 is regulated by SUMOylation (Hilgarth et al. 2004, Tateishi et al. 2009). In addition, HsfA2 activity is decreased by SUMOylation (Cohen-Peer et al. 2010). Hsf1 is modified by SUMO-1 and SUMO-2 in a stress-inducible manner at Lys298, and Hsf1 phosphorylation at Ser307 and Ser303 could stimulate SUMOylation of Hsf1 in mammals (Hong et al. 2001, Hietakangas et al. 2003, Hilgarth et al. 2003). Similar to HsfA2, SUMOylation may also negatively regulate the activity of HsfA1 during the HSR (Ohama et al. 2016).

SIZ1, a SUMO E3 ligase, mediates the SUMOylation of proteins. SIZ1 influences the conjugation between target proteins and SUMOs to regulate physiological functions. SIZ1 is involved in many signaling pathways, such as exhibiting significantly different sensitivities to low and high temperatures, drought response, salicylic acid signaling, excess copper response, Pi starvation response, ABA response inhibition and nitrogen metabolism in Arabidopsis (Chan and Hasegawa 2006, Yoo et al. 2006, Catala et al. 2007, Miura et al. 2007, Miura et al. 2009, Miura et al. 2010, Chen et al. 2011, Park et al. 2011, Zheng et al. 2012, Miura et al. 2013). The SUMO conjugate and basal thermotolerance levels are decreased in *Atsiz1* mutants under heat stress. However, decreased heat tolerance is not correlated with HSPs (Yoo et al. 2006). A previous study on the role of SIZ1 under heat stress focused on *Atsiz1* mutants, and gain of SIZ1 function in response to heat stress has been investigated (Li et al. 2013, Mishra et al. 2017). The correlation of heat tolerance to HSPs in SIZ1-overexpressing plants is also unclear. Moreover, little information about the relationship between SIZ1 and HSFs was reported. In the present study, we isolated a SIZ1 SUMO E3 ligase (SlSIZ1) from tomato. Overexpression of SlSIZ1 could enhance heat tolerance in tomato, and the enhanced heat tolerance was related to HSPs and HSFs.

Results

Stresses induced SUMO conjugation in tomato

The SUMO conjugation level can be induced by different stresses in rice and apple (Park et al. 2010, Zhang et al. 2016). As described in previous studies, SUMO-1 shows high conversion in different species, and the antibody to AtSUMO1 can be used to detect free and conjugated SUMO in rice, apple and *Dendrobium* (Park et al. 2010, Liu et al. 2015, Zhang et al. 2016). In our study, the SlSUMO1 (Solyc07g064880) sequence determined from BLAST searching is highly homologous to that of AtSUMO1 (Supplementary Fig. S1). Therefore, the AtSUMO1 antibody was used to determine the levels of stress-induced SUMO conjugation in tomato.

Six-week-old seedlings were treated under low temperature (4°C), high temperature (42°C), drought [polyethylene glycol (PEG)] and oxidation [methyl viologen (MV)]. Immunoblot analysis was used to detect SUMO conjugations by the AtSUMO1 antibody (ab5316). **Fig. 1** shows that SUMO conjugation levels were induced by all these stress treatments. SUMO conjugates were clearly induced under temperature treatment, especially at 1 and 2 h under low temperature and 3 h at high temperature. SUMO conjugates can also be induced at 6 and 9 h under drought treatment. Under oxidation stress, the SUMO conjugate level could be induced slightly compared with that in normal conditions.

Overexpression of SlSIZ1 increased the heat tolerance of tomato

The SUMO conjugate level was clearly induced under heat stress. In addition, SIZ1 plays an important role in SUMOylation. Thus, quantitative real-time PCR (qRT-PCR) and immunoblot analysis were used to detect the expression level of SlSIZ1 under heat stress. Subsequently, the *SlSIZ1* expression level was measured under high temperature (42°C) at different time points (0, 3, 6, 9, 12 and 24 h). As shown in Supplementary Fig. S2, the transcript level of *SlSIZ1* was

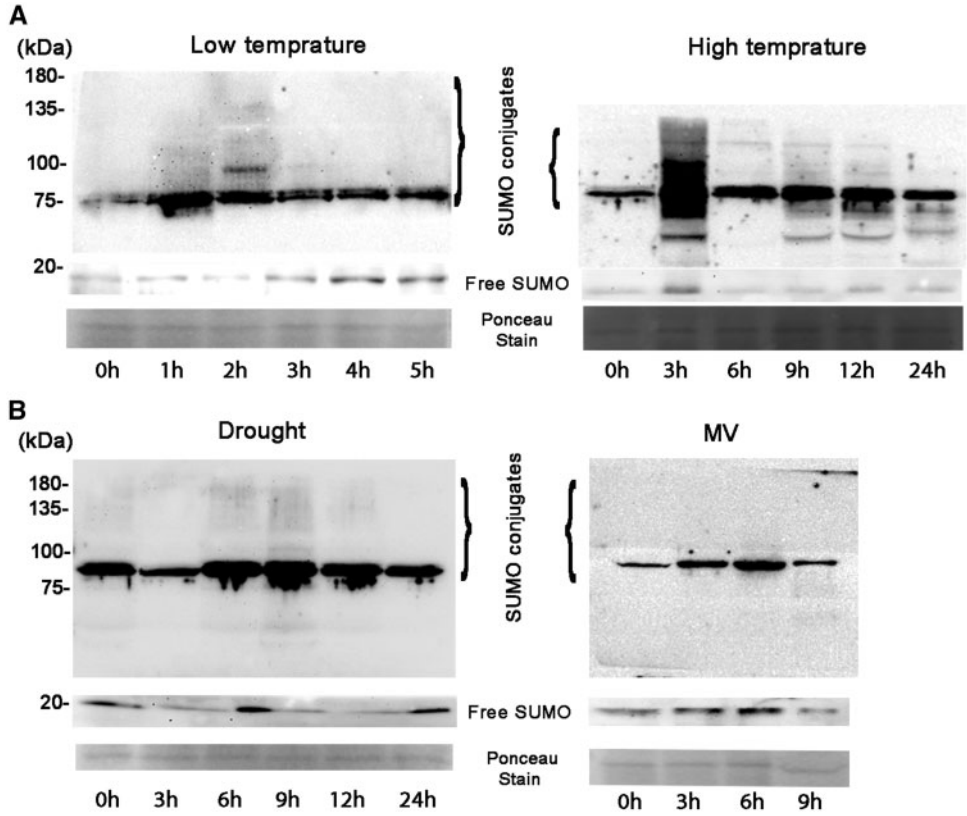

Fig. 1 SUMO conjugates induced by various stresses in tomato. (A) Low and high temperature treatment. (B) Drought and MV treatment. Each lane was loaded with 20 mg of total protein extracted from 6-week-old tomatoes that were exposed to low temperature (4°C), high temperature (42°C), drought (PEG) and oxidation (MV). Tomato leaves were collected at various times after the start of stress treatment (0 h). The resulting crude extracts were subjected to SDS–PAGE, and immunoblot analysis was performed with an antibody against AtSUMO-1. The proteins were transferred on a PVDF membrane and dyed by Ponceau stain. The dyed protein was placed in the Tanon-5500 system for taking photographs and labeled as control.

decreased from 0 to 9 h and then restored. However, immunoblot analysis results showed that the change of SlSIZ1 protein was different from that of the transcript level. The SlSIZ1 protein showed no significant change under high temperature stress.

To reveal the function of SlSIZ1 in vivo, we transformed the full-length coding sequence of *SlSIZ1* into tomato. According to the transcript levels of 11 individual transgenic lines, three sense lines (OE-2, OE-5 and OE-11) were selected for subsequent experiments. We also generated two RNA interference (RNAi) lines (RNAi-16 and RNAi-23) to verify the role of SlSIZ1 at high temperature (Supplementary Fig. S3). Two-week-old wild-type (WT) and transgenic tomato plants were treated at 42°C for 3 d. Furthermore, the growth status was monitored (**Fig. 2**). After 3 d of treatment, the sense plants showed a better growth performance and survival rate than the WT. The cotyledons of sense lines were green while those of the WT turned yellow. WT plants were more wilted than transgenic plants. The total Chl content and fresh weight were also measured. As can be seen in **Fig. 2**B, both the Chl content and the fresh weight were higher in transgenic lines than in the WT, although they decreased in all lines after high temperature treatment.

Six-week-old WT and transgenic tomato plants were treated at 42°C for 24 h to observe the growth status of the fully grown plants. Similar to seedlings, the fully grown WT plants were remarkably wilted compared with the transgenic lines (**Fig. 2**). In additon, the RNAi plants appeared more wilted than the WT (Supplementary Fig. S3). Free proline in transgenic lines was higher than that in the WT after heat treatment, and it increased relative to that in untreated plants. *SlP5CS*, a key gene in the proline synthetic pathway, was clearly up-regulated in transgenic plants with high temperature treatment. After heat treatment, the free proline content in transgenic and WT plants was consistent with the results of qRT-PCR (**Fig. 2**F, G). Heat stress destroyed the stability of plant cell membranes. Consequently, trypan blue staining was detected. After 24 h of high temperature treatment, the sense lines showed weaker blue stains than the WT (**Fig. 2**D). The malondialdehyde (MDA) content, which could indicate cell damage, was also measured. After high temperature treatment, the MDA content was high in the WT, although it increased in all lines (**Fig. 2**E). These results showed that *SlSIZ1* overexpression could enhance the high temperature tolerance of tomato.

Overexpression of SlSIZ1 reduced ROS accumulation

$O_2 \cdot^-$ and H_2O_2 were detected in the 6-week-old WT and transgenic plants (**Fig. 3**A). The color intensities of nitro blue

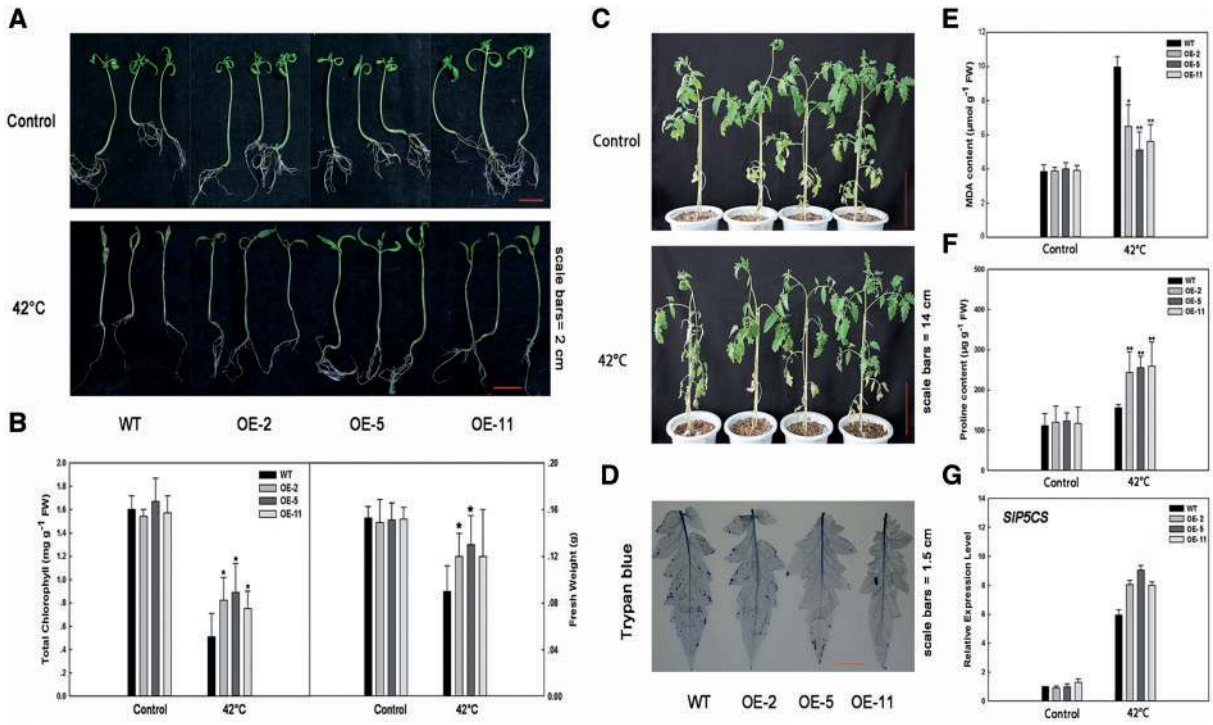

Fig. 2 Performance of seedlings (10 d old) and fully grown plants under high temperature treatment. (A) Phenotype of 10-day-old plants under heat stress. The top panel represents seedlings grown under normal conditions. The bottom panel represents plants treated at 42°C for 3 d. Scale bars = 2 cm. (B) Total Chl content and fresh weight of the young seedlings in (A). (C) Phenotype of 6-week-old plants under heat stress. Scale bars = 14 cm. (D) Trypan blue staining under heat stress. Scale bars = 1.5 cm. (E) MDA content was detected under heat stress. (F) Proline content was detected under heat stress. (G) *SlP5CS* expression was measured after heat stress treatment. The *SlP5CS* transcript level was normalized to *SlEF-1α* expression. Fully grown plants were treated at 42°C for 24 h. For (B), (D), (E), (F) and (G), each column represents an average of three replicates (±SD). $^{**}P < 0.01$) and $^{*}P < 0.05$) show significant differences compared with the control.

tetrazolium (NBT) and 3′3′-diaminobenzidine (DAB) staining in the transgenic seedlings were less than those in the WT. Moreover, the $O_2{\cdot}^-$ and H_2O_2 concentrations were low in the transgenic plants (**Fig. 3**B).

Subsequently, peroxidase (POD; EC 1.11.1.7) and ascorbate peroxidase (APX; EC 1.11.1.11) activities were tested in different 6-week-old lines. These activities were higher in the transgenic plants than in the WT after high temperature treatment (**Fig. 3**C, D). The high POD and APX activities resulted in low accumulation of $O_2{\cdot}^-$ and H_2O_2 in tomato with overexpressed *SlSIZ1*. *SlAPX1* and *SlAPX2* are key genes in the APX enzyme synthetic pathway. *SlPOX1* is a key gene in the POX enzyme synthetic pathway. qRT-PCR detected the transcript levels of *SlAPX1*, *SlAPX2* and *SlPOX1* in the WT and transgenic tomatoes with or without treatment. *SlPOX1* expression was up-regulated under heat stress. Furthermore, the *SlPOX1* level significantly increased in the transgenic lines compared with that in the WT lines after heat treatment (**Fig. 3**E–G). However, the expression of *SlAPX1* and *SlAPX2* was down-regulated in all plants after heat treatment. The transgenic lines showed higher transcript levels of *SlAPX1* and *SlAPX2* than the WT after high temperature treatment, although their expression levels were decreased. Therefore, the increased POD activity may be associated with the expression of *SlPOX1*. Additionally, *SlSIZ1* overexpression decreased ROS accumulation under heat stress, which most probably resulted from the increased actions of POD and APX.

Transcript levels of high temperature-related genes

Three HSFs (SlHsfA1, SlHsfA2 and SlHsfB1) and three HSPs (SlHsp70-3, SlHsp70 and SlHsp90) play important roles in high temperature tolerance. These high temperature-related genes were measured by qRT-PCR after 24 h heat treatment in 6-week-old WT and transgenic lines (**Fig. 4**; Supplementary Fig. S3C). These genes showed different transcript levels after heat treatment in WT and transgenic tomato. The transcript levels of *SlHsfA1* and *SlHsfB1* showed no significant difference among WT, sense and RNAi lines (**Fig. 4**; Supplementary Fig. S3C). Nevertheless, the expression levels of *SlHsfA2*, *SlHsp70-3*, *SlHsp70* and *SlHsp90* demonstrated a noticeable change in transgenic lines. *SlHsfA2*, *SlHsp70-3*, *SlHsp70* and *SlHsp90* were up-regulated in all plants, but their levels in *SlSIZ1* overexpression lines were significantly up-regulated compared with that in the WT after heat stress for 24 h (**Fig. 4**B, D–F). However, these genes were significantly down-regulated in RNAi lines compared with the WT after high temperature treatment for 24 h (Supplementary Fig. S3C). Thus, the enhanced tolerance to heat stress in overexpression lines may be associated with these up-regulated HSFs and HSPs. We also speculated that a

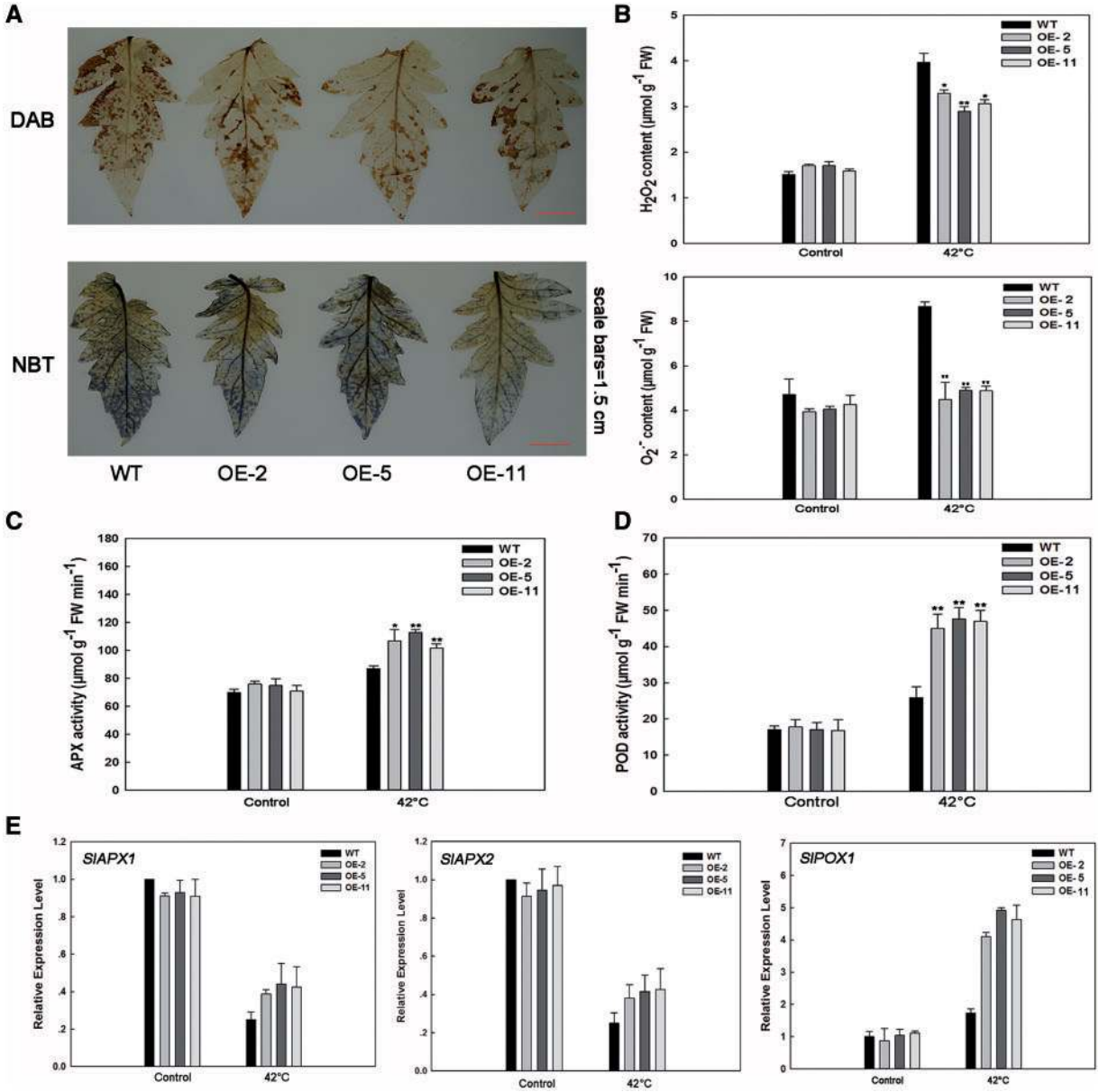

Fig. 3 Contents of ROS in different plants. (A) NBT staining and DAB staining of 6-week-old tomato leaves which were treated at 42°C for 24 h. Scale bars = 1.5 cm. (B) Contents of $O_2^{\cdot-}$ and H_2O_2 in 6-week-old plants. (C and D) Activities of APX and POD. (E–G) Expression of *SlAPX1*, *SlAPX2* and *SlPOX1* was detected in different lines. The transcript levels of these genes were normalized to *SlEF-1α* expression. For (B–D), each column represents an average of three replicates (±SD). $^{**}P < 0.01$ and $^{*}P < 0.05$) display significant differences compared with the control.

connection existed between SlSIZ1 and SlHsfA2, SlHsp70-3, SlHsp70 and SlHsp90.

SlSIZ1 overexpression enhanced the accumulation of SUMO conjugates

To determine the accumulation of SUMO conjugates under heat stress, we detected the SUMO conjugates in the WT and transgenic lines by using anti-SUMO antibody after heat stress. The accumulation of SUMO conjugates was enhanced by heat treatment in all tomato lines, whereas transgenic plants displayed higher conjugation levels than the WT. Moreover, SlSIZ1-overexpressing lines showed higher level of SUMO conjugates than the WT after 2 h of treat stress (**Fig. 5**). Thus, *SlSIZ1* overexpression could enhance the SUMO conjugates, and the high level of SUMO conjugation may be related to SlSIZ1 in response to heat stress.

Hsp70 level was increased in transgenic lines

SlHsp70 expression was clearly up-regulated after 24 h heat treatment (**Fig. 4**). Immunoblot was used to determine the protein level of SlHsp70 in heat shock. The total WT and OE-5 proteins were extracted after treatment at 42°C for 0, 2 and 4 h. Subsequently, SlHsp70 was detected by anti-Hsp70. In **Fig. 6**A, the Hsp70 protein level is shown to be increased after heat treatment. The content of Hsp70 was also higher in transgenic plants. Proteins will be degraded under heat stress in plant

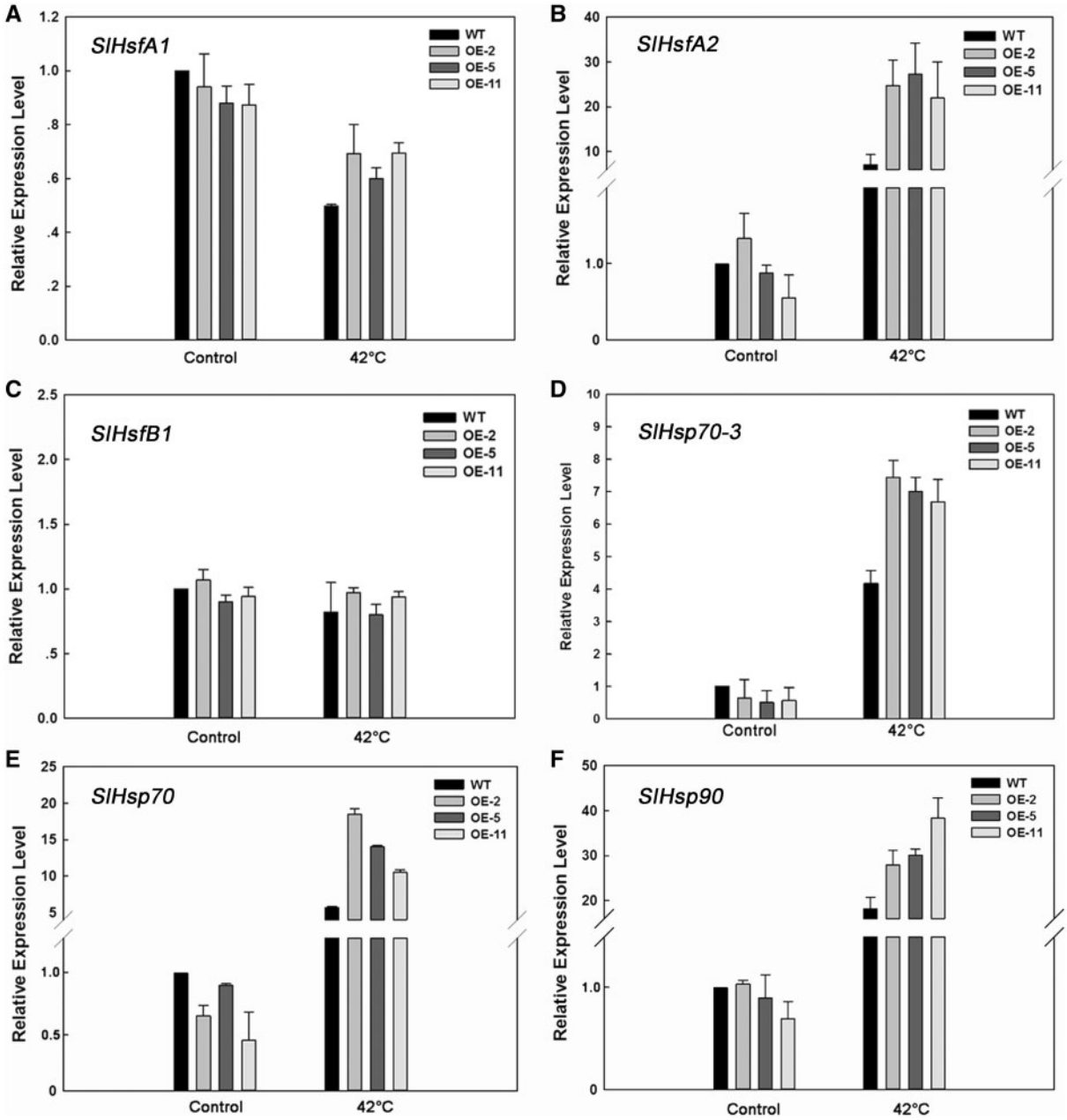

Fig. 4 Transcript levels of several stress-related genes. (A–F) Relative expression levels of *SlHsfA1*, *SlHsfA2*, *SlHsfB1*, *SlHsp70-3*, *SlHsp70* and *SlHsp90*. qRT-PCR was performed using total RNA isolated from 6-week-old wild-type (WT) and transgenic plants which were treated at 42°C for 24 h. The transcript levels of these genes were normalized to *SlEF-1α* expression. Each column represents the means of three replicates, and the bars indicate ± SD.

organisms (Iba 2002). We also treated WT and OE-5 plants at 42°C for 2 and 4 h with cycloheximide. We found that the degree of Hsp70 degradation was higher in the WT than that OE-5 (**Fig. 6**B). Thus, the increased Hsp70 level should be related to both up-regulated expression of *SlHsp70* and protection of Hsp70 from degradation under heat stress.

SlHsfA1 could interact with SlSIZ1

To investigate the potential relationships among SlHsfA1, SlHsfA2, SlHsfB1, SlHsp70 and SlHsp70-3 to SlSIZ1, we first used a yeast two-hybrid system in vitro. The ortho-nitrophenyl-β-D-galactopyranoside (ONPG) assay was used to quantify the interaction. The quantitative yeast two-hybrid assay showed that SlHsfA1 could interact with SlSIZ1, whereas SlHsfB1 and SlHsp70-3 did not bind to SlSIZ1. It seems that SlHsfA2 and SlHsp70 could interact weakly with SlSIZ1 (**Fig. 7**B). Moreover, bimolecular fluorescence complementation (BiFC) assay detected the interaction between SlSIZ1 and SlHsfA1. Both SlSIZ1–nYFP (yellow fluorescent protein) and SlHsfA1–cYFP were co-expressed in *Nicotiana benthamiana* leaves. The YFP fluorescence was clearly demonstrated in the nucleus, whereas no fluorescence was observed in the control

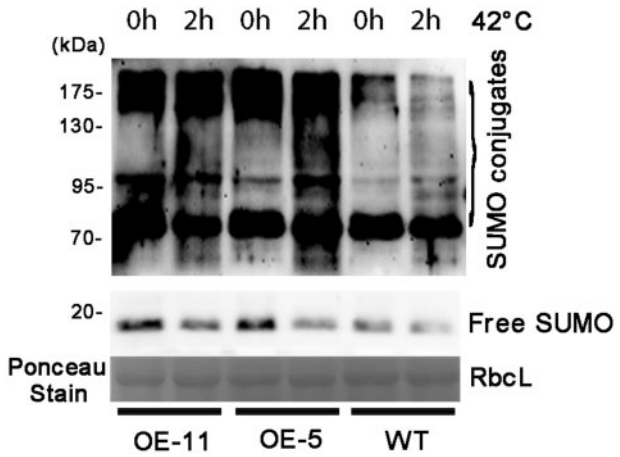

Fig. 5 Accumulation of SUMO conjugates after heat stress. Two-week-old WT, OE-5 and OE-11 lines were subjected to heat stress treatment for 2 h. Total proteins were extracted from untreated or heat-stressed plants. Approximately 20 μg of proteins were loaded for SDS–PAGE, and immunoblot was detected with anti-SUMO antibody. The proteins were transferred on a PVDF membrane and dyed by Ponceau stain. The dyed protein was placed in the Tanon-5500 system for taking photoigraphs and labeled as control.

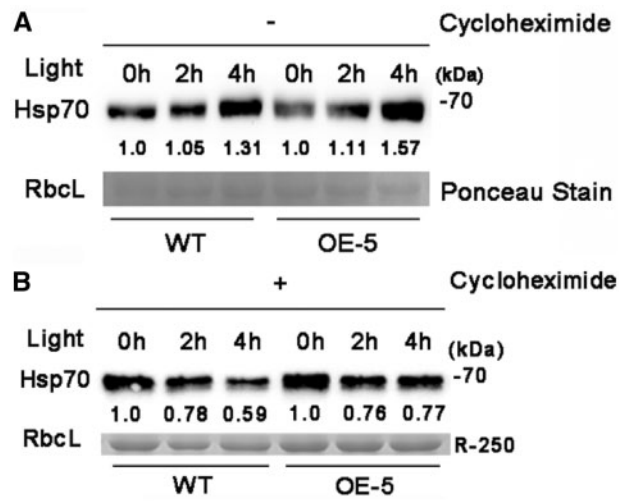

Fig. 6 The Hsp70 level was increased in transgenic lines. (A) Hsp70 level in WT and OE-5 plants with cycloheximide. (B) Hsp70 level in WT and OE-5 plants without cycloheximide. Two-week-old plants were treated at 42°C for 2 and 4 h. RbcL was used as control. Hsp70 quantity was measured by ImageJ software.

(**Fig. 9**A). These results indicated that SlSIZ1 could interact with SlHsfA1.

SlSIZ1 mediated the SUMOylation of SlHsfA1

To confirm that SlSIZ1 mediated the SUMOylation in vivo, we performed an in vivo SUMOylation analysis. We expressed Flag-SlHsfA1 in *Atsiz1-2* mutants and SlSIZ1 complementary *Atsiz1-2* Arabidopsis. The total protein extracts from T_2 WT and transgenic Arabidopsis were immunoprecipitated by anti-Flag antibody and detected by immunoblot with both anti-SUMO1 and anti-Flag antibodies. High molecular weight SUMOylated SlHsfA1 bands were detected when Flag-SlHsfA1 and SlSIZ1 were co-expressed in plants. SUMO conjugation to Flag-SlHsfA1 occurred at higher levels in the Flag-SlHsfA1/SlSIZ1 transgenic Arabidopsis than that when Flag was conjugated to SlHsfA1 alone (**Fig. 8**). Therefore, SlSIZ1 mediated the SUMOylation of the SlHsfA1 protein.

Discussion

It had been reported that the SUMO conjugation level could be induced by several stresses, such as temperature stress, drought stress and ABA in Arabidopsis, rice and apple (Kurepa et al. 2003, Park et al. 2010, Zhang et al. 2016). In our reasearch, SUMO conjugates were induced by high and low temperatures, drought stress and oxidation stress in tomato. Moreover, SUMO conjugation was most obviously induced by high temperature (**Fig. 1**). We suggested that SUMO conjugations induced similarly by several stresses in different plant species could be related to the conserved SUMO and SUMOylation pathway (Park et al. 2010, Liu et al. 2015, Zhang et al. 2016). Nevertheless, the *SlSIZ1* transcript level fluctuated with the duration of heat treatment. In addition, the SlSIZ1 level showed no difference after heat stress (Supplementary Fig. S2). The regulation of transcription and protein levels involves different processes. The protein levels were associated with their accumulation and degradation (Lin et al. 2016). The up-regulated expression of genes could increase the accumulation of proteins in general. However, the down-regulated genes maybe not result in a decrease of proteins which were stable. So, we hypothesized that SlSIZ1 is relatively stable under high temperature. Consequently, we speculated that the quantity of SlSIZ1 was sufficient to mediate SUMOylation under heat stresses, and SlSIZ1 function may be post-translationally controlled.

Chan et al. (2006) showed that AtSIZ1 could facilitate basal thermotolerance in Arabidopsis. *siz1-2* and *siz1-3* mutants displayed thermosensitivity and reduced SUMO conjugation under heat stress. Functional studies of SIZ1 were focused on mutants which were widely available. Many previous studies have shown that *siz1* mutants in Arabidopsis and rice were sensitive to abiotic stresses. However, the enhanced stress tolerance played much more important roles in crops. So, we paid more attention to gain function of SlSIZ1 in tomato; and whether SIZ1 overexpression could enhance the thermotolerance in plants is unknown. Hence, we prepared SlSIZ1 overexpression plants to analyze its role in heat stress. Under normal growth conditions, all plants exhibited no significant difference. Nonetheless, after 42°C heat treatment, the overexpression plants, both young seedlings and fully grown plants, showed better growth and higher Chl content than WT plants (**Fig. 2**), and the transgenic lines showed a lower content of MDA (**Fig. 2**E). However, the SlSIZ1 RNAi lines appeared more wilted than the WT under heat stress (Supplementary Fig. S3). The results indicated that membrane damage was less serious in overexpression plants than in WT plants. We also found that high free proline was related to up-regulated *SlP5CS* in overexpression plants (**Fig. 2**F, G). Moreover, the transcript levels of Hsp70 and Hsp90 involved in thermotolerance were clearly

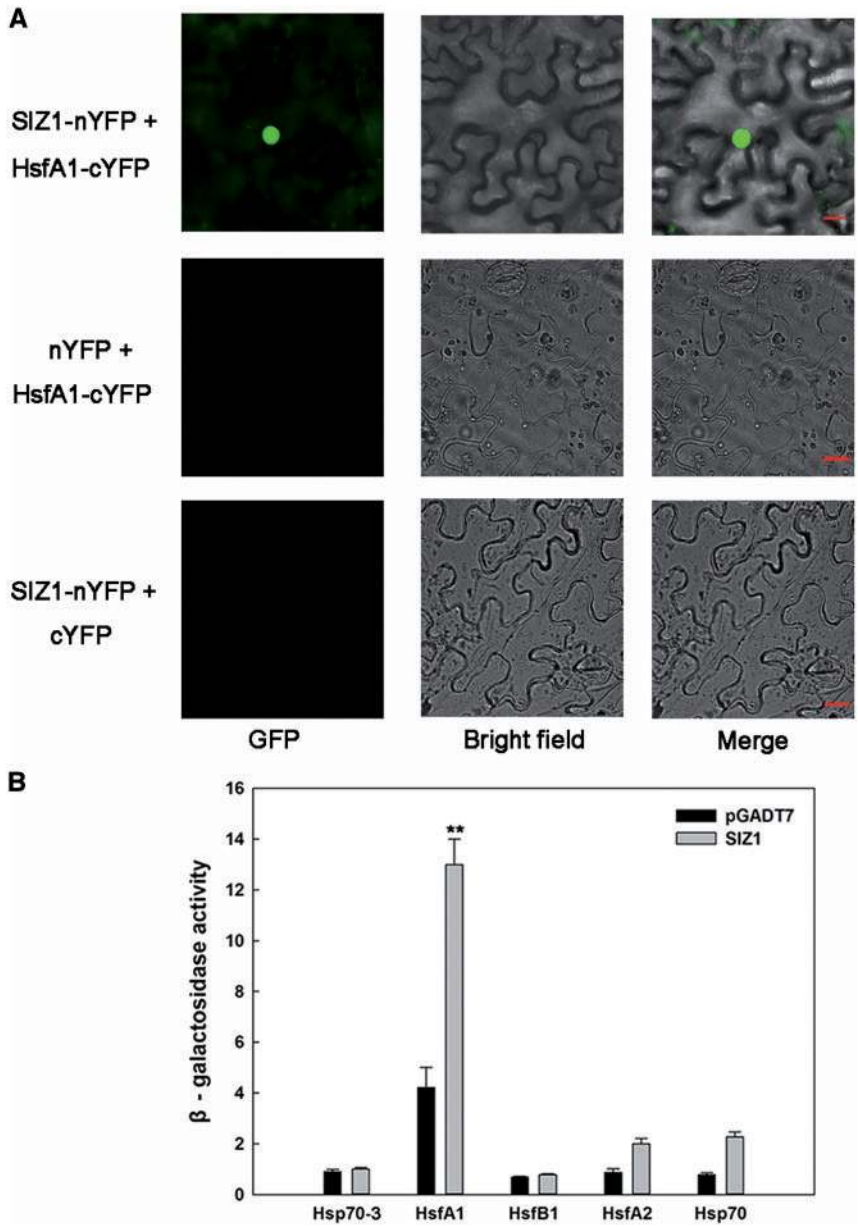

Fig. 7 Interaction between SlSIZ1 and SlHsfA1. (A) BiFC visualization of the interaction between SlSIZ1 and SlHsfA1. *Nicotiana benthamiana* leaves transiently expressing SlSIZ1–nYFP and SlHsfA1–cYFP. Leaf sections were visualized 48 h after transformation under a laser-scanning-meta confocal microscope. The nucleus is indicated by red arrows. Scale bars = 20 μm. (B) Protein–protein interactions were detected by the yeast two-hybrid system and quantified by ONPG assay. The pGADT7 was labeled as the negative control.

up-regulated in sense lines (**Fig. 4**D–F). However, these heat-related genes were down-regulated in SlSIZ1 knock down lines (Supplementary Fig. S3). It has been reported that some HSPs (sHSPs) are involved in protecting the membrane of plants cells (Coucheney et al. 2006, Balogi et al. 2008). Therefore, SlSIZ1 may play an important role in modifying HSPs.

Plant organisms will produce ROS under high temperature stress. SUMO modification influences oxidative stress responses (Feligioni and Nistico 2013), and ROS accumulation in Arabidopsis *siz1-2* mutants increases under drought stress (Miura et al. 2013). However, the relationship between ROS accumulation and SIZ1 under heat stress is rarely reported. In our study, ROS levels increased in all lines after heat stress, but they were low in the overexpression lines (**Fig. 3**). The low ROS content is related to the high activities of some antioxidative enzymes. The activities of POD and APX were higher in sense lines than in the WT after heat stress (**Fig. 3**C, D). In the present study, the expression of *SlPOX1*, which is associated with the POD synthesis pathway, was induced in overexpressing SlSIZ1 plants (**Fig. 3**G). A POD-related gene, At3g28200, was down-regulated in Arabidopsis *siz1-2* mutants (Catala et al. 2007). Thus, the high POD activity may be correlated with the high expression levels of *SlPOX1* regulated by SlSIZ1. The expression levels of *SlAPX1* and *SlAPX2* were slightly higher in

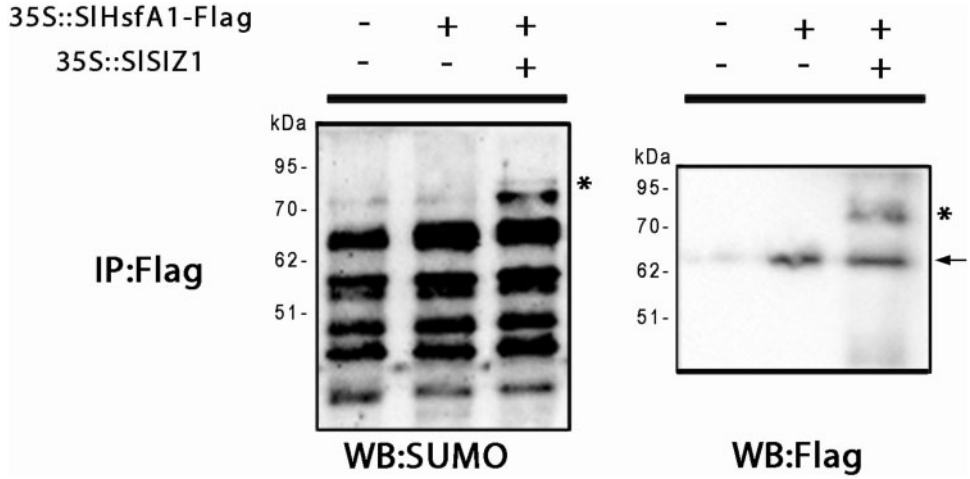

Fig. 8 SlSIZ1 mediated the SUMOylation of SlHsfA1. *Nicotiana benthamiana* leaves transiently infected with *35S: SlHsfA1-Flag* or with both *35S: SlHsfA1-Flag* and *35S-SlSIZ1*. Transient tobacco leaves were collected after 3 d. Anti-Flag antibody was used to immunoprecipitate SlHsfA1-Flag. Anti-SUMO1 antibody was used to determine SUMOylated HsfA1. Input and immunoprecipitated SlHsfA1-Flag were detected with anti-Flag antibody. Asterisks indicate SUMOylated SlHsfA1 bands. The arrowhead indicates the non-SUMOylated SlHsfA1 band.

overexpression plants than in WT plants after 24 h heat stress (**Fig. 3**E, F). The relatively high APX activity in sense plants was independent of its transcription level. In a previous study, *AtAPX1* and *AtAPX2* were induced by heat stress in a short time (Panchuk et al. 2002). Therefore, with 24 h heat treatment, the higher APX activity was irrelevant to its transcription level. The *AtAPX2* transcription level could be induced by H_2O_2 treatment (Nishizawa et al. 2006). Additionally, the *hsfA2* mutants showed high ROS generated in response to heat stress in Arabidopsis and in relation to mitochondrial dynamics (Zhang and Gao 2009). *HsfA2* expression was up-regulated in tomato overexpression plants (**Fig. 4**B). Thus, the high *HsfA2* expression could enhance the capacity for scavenging ROS in transgenic plants. In addition, *AtAPX2* was directly up-regulated by HsfA2 (Schramm et al. 2006). Consequently, *SlHsfA2* induced *SlAPX2* expression in an early stage to produce a large amount of APX to reduce oxidative damage. In addition, a high free proline content also contributed to a low ROS content.

HsfA1, HsfA2 and HsfB1 are considerably important transcriptional factors that regulate heat stress-induced genes in tomato (Baniwal et al. 2004). Thus, the transcript levels of these three genes were tested. HsfA1 was constitutively expressed and defined as the main regulator in the HSR of tomato (Mishra et al. 2002). *SlHsfA1* expression showed no difference before and after 24 h heat treatment (**Fig. 4**A). HsfA2 was regulated by HsfA1. Under heat stress, the active HsfA1 caused subsequent expression of *SlHsfA2* and *HSP* genes (Baniwal et al. 2004). SlHsfA1 was found to interact with SlSIZ1 (**Fig. 7**). We suspected that the high expression level of *SlHsfA2* in sense plants under heat stress may be correlated with high transcriptional activity of SlHsfA1, which is related to SIZ1 mediation. Furthermore, the weak interaction between SlSIZ1 and SlHsfA2 may also improve the transcript level of SlHsfA2. The HsfA1–HsfA2 hetero-oligomeric complex could form a super-transcriptional factor in activating heat stress-induced genes (Chanschaminet et al. 2009). The transcript levels of several *HSP* genes (*SlHsp70-3*, *SlHsp70* and *SlHsp90*) were clearly up-regulated in overexpression *SlSIZ1* lines with heat treatment. Unlike class A HSFs, classes B and C showed no evident functions in Arabidopsis (Czarneckaverner et al. 2000). In tomato, HsfB1 was thought to act as a co-activator of HsfA1 and other transcription factors (Koskull-Döring et al. 2007). So the transcript level of *SlHsfB1* was similar between treated and untreated plants (**Fig. 4**C). Hsp70 is one of the most important and abundant HSPs in plants (Mayer and Bukau 2005). Hsp70s function as primary stabilizers of newly formed proteins, ATP-dependent binding and release. Hsp90 is also an important HSP in protein homeostasis and functions as a co-regulator of signal transduction complexes (Baniwal et al. 2004). Thus, high transcript levels of Hsp70 and Hsp90 could enhance thermotolerance in transgenic tomato.

The heat shock-induced accumulation of SUMO conjugates was reduced in Arabidopsis *siz1-2* and *siz1-3* mutants (Chan and Hasegawa 2006). After 2 h heat treatment, the accumulation of SUMO conjugates clearly increased in the transgenic plants. Such increased accumulation was associated with SlSIZ1 in response to heat stress (**Fig. 5**). SUMOylation could enhance the stability of target proteins (Johnson 2004). Increased accumulation of SUMO conjugates was associated with many SUMOylated proteins. Hence, high APX activity may be involved in its SUMOylation under heat stress. The SUMOylation consensus motif was detected in the SlHsp70 amino acid sequence. SlHsp70 also could interact with SlSIZ1, but the interaction is weak. However, no SUMOylation was detected in SlHsp70. Therefore, the low decrease of SlHsp70 may be associated with SUMOylation, which enhanced its stability under heat stress (**Fig. 6**). A high transcript level of *SlHsp70* and decreased degradation together contributed to a large amount of Hsp70 in sense tomato after heat treatment. SlHsfA2 may also interact weakly with SlSIZ1 (**Fig. 7**B). The interaction may be associated with the HsfA1–HsfA2 hetero-oligomeric complex in activating heat stress-induced genes

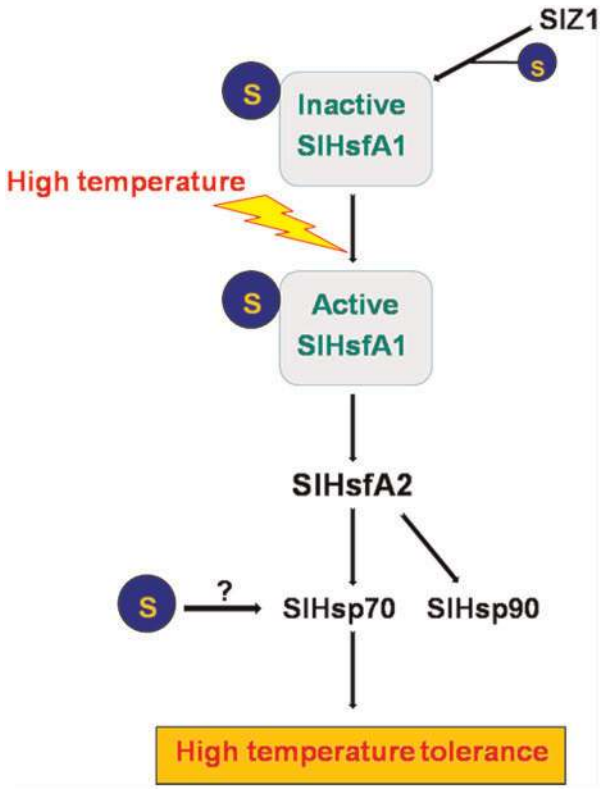

Fig. 9 Proposed model for the function of SlSIZ1 in response to heat stress. SlSIZ1 could interact with SlHsfA1 to facilitate its SUMOylation. Under heat stress, SlHsfA1 was constitutive expressed and defined as the master regulator. The SUMOylation of SlHsfA1 induces the expression of *SlHsfA2*, then SlHsfA2 induces the expression of *SlHsp70* and *SlHsp90* (**Fig. 4**). The stability of SlHsp70 may also be related to SUMO conjugations. SlSIZ1 facilitates tolerance in heat stress though HSFs/HSPs.

(Chanschaminet et al. 2009). In a previous study, HsfA2 activity was found to be decreased by SUMOylation (Cohen-Peer et al. 2010). SIZ1-mediated SUMOylation of HsfA2 reduces the thermotolerance, and overexpression of SlSIZ1 should display heat sensitivity. Nevertheless, the function of the interaction between SlSIZ1 and SlHsfA2 could not be explained.

In summary, the SUMO conjugations could be induced by several stresses in tomato, especially temperature stress. Although the protein and expression levels of tomato SUMO E3 ligase SlSIZ1 changed slightly, SlSIZ1 overexpression could increase SUMO conjugations under heat stress. Such overexpression in tomato could reduce the damage due to high temperature- and heat-induced excess ROS. SlSIZ1-mediated SUMOylation of SlHsfA1 may be of benefit to enhance HsfA1's activity for up-regulating *SlHsfA2*, *SlHsp70* and *SlHsp90* to enhance heat tolerance. Based on the present study and previous research, a model was proposed to explain the function of SlSIZ1 in heat stress (**Fig. 9**). Thus, our study demonstrated the effect of SlSIZ1 overexpression on enhanced heat tolerance in plants. Hence, SlSIZ1 could play an important role in thermotolerance in tomato.

Methods and Materials

Plant materials and treatment conditions

WT tomato seeds (*Solanum lycopersicum* cv. Zhongshu 6 and *S. lycopersicum* cv. M82), T_2 overexpression tomato (Zhongshu 6 background) and SlSIZ1-RNAi tomato (M82 background) seeds were germinated on 1× Murashige and Skoog (MS) medium containing 3% sucrose and 0.8% agar (pH 5.7) at 25°C for 2 weeks. Then tomato seedlings were grown in soil in a greenhouse under the following conditions: photoperiod of 16/8 h (day/night) at 25°C/22°C (day/night), photon flux density (PFD) of 300 μmol m^{-2} s^{-1} and relative humidity of 50–60%. The plants were irrigated with Hoagland nutrient solution once a week.

WT, *siz1-2* mutant (Columbia-0 background) and *SlSIZ1* complementary *Atsiz1-2* Arabidopsis were used in this study. Plant seeds were germinated on 1× MS medium containing 1.5% sucrose and 0.8% agar (pH 5.7) in the dark at 4°C for 4 d to vernalize. Plants grown in plates or soil were transferred to a greenhouse and grown under the following conditions: photoperiod of 16/8 h (day/night) at 22°C/20°C (day/night), PFD of 100 μmol m^{-2} s^{-1} and relative humidity of 60–70%.

For chilling stress, the whole plant was placed in an illuminated incubator at 4°C. For heat stress, the plants were placed in all illuminated incubator at 42°C. For drought stresses, tomato roots were soaked in solution containing 20% PEG-6000 (w/v). For oxidation treatments, plant leaves were sprayed with 10 μmol l^{-1} MV solutions under normal conditions.

Plant transformation

The full-length coding sequence of *SlSIZ1* was cloned from WT tomato cDNA by using a specific primer pair according to the sequence from the NCBI (http://www.ncbi.nlm.nih.gov/) to generate transgenic lines overexpressing SlSIZ1 in WT tomato plants by the leaf disc method. The RNAi special sequence was cloned from WT tomato by using a specific primer pair. SlSIZ1 knock down plants were generated by the leaf disc method. The transgenic lines were selected by PCR and qRT-PCR analyses.

Plasmid constructions

To generate PBI12-SlSIZ1, full-length cDNA was cloned with specific primers *SIZ1*-F and *SIZ1*-R. The PCR fragment was ligated into the pEASY-T1 simple cloning vector (Transgen). The pEASY-T1-*SIZ1* fragment was digested with *Bam*HI and *Sal*I, and this fragment was inserted into the PBI-121 vector.

For generation of pGADT7-SlSIZ1, full-length cDNA was cloned with specific primers AD-*SIZ1*-F and AD-*SIZ1*-R. The PCR fragment was ligated into the pEASY-T1 simple cloning vector (Transgen). The pEASY-T1-AD-*SIZ1* fragment was digested with *Bam*HI and *Xho*I, and this fragment was inserted into the pGADT7 vector.

To generate pGBKT7-SlHsfA1, full-length cDNA without the termination codon was cloned with specific primers BD-*HsfA1*-F and BD-*HsfA1*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-BD-*HsfA1* fragment

was digested with *Nco*I and *Pst*I, and this fragment was inserted into the pGBKT7 vector.

To produce pGBKT7-SlHsfA2, full-length cDNA without the termination codon was cloned with specific primers BD-*HsfA2*-F and BD-*HsfA2*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-BD-*HsfA2* fragment was digested with *Sal*I and *Pst*I, and this fragment was inserted into the pGBKT7 vector.

For generation of pGBKT7-SlHsfB1, full-length cDNA without the termination codon was cloned with specific primers BD-*HsfB1*-F and BD-*HsfB1*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-BD-*HsfB1* fragment was digested with *Nde*I and *Pst*I, and this fragment was inserted into the pGBKT7 vector.

For production of pGBKT7-SlHsp70-3, full-length cDNA without the termination codon was cloned with specific primers BD-*Hsp70-3*-F and BD-*Hsp70-3*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-BD- Hsp70-3 fragment was digested with *Nco*I and *Bam*HI, and this fragment was inserted into the pGBKT7 vector.

To generate pGBKT7-SlHsp70, full-length cDNA without the termination codon was cloned with specific primers BD-*Hsp70*-F and BD-*Hsp70*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-BD-Hsp70 fragment was digested with *Nco*I and *Bam*HI, and this fragment was inserted into the pGBKT7 vector.

For generation of Flag-SlHsfA1, full-length cDNA was cloned with specific primers Flag-*HsfA1*-F and Flag-*HsfA1*-R. The PCR fragment was ligated into the pLB simple cloning vector (TIANGEN). The pLB-Flag-*HsfA1* fragment was digested with *Xba*I and *Kpn*I, and this fragment was inserted into the 1300-Flag vector.

To generate the RNAi-SlSIZ1 vector, the RNAi special sequence was cloned from WT tomato by using specific primers RNAi-*SlSIZ1*-F and RNAi-*SlSIZ1*-F. The PCR fragment was ligated into the pCR^{TM}8/GW/TOPO TA vector (Thermo Fisher). The destination vector was constructed by LR reaction using LR Clonase II (Thermo Fisher). The vector for RNAi was obtained from Professor Daolin Fu in Shandong Agricultural University.

SlSIZ1–nYFP was generated using Gateway^{TM} technology (Thermo Fisher). Full-length cDNA without the termination codon was cloned with specific primers BiFC-*SIZ1*-F and BiFC-*SIZ1*-R. The PCR fragment was ligated into the pCR^{TM}8/GW/TOPO TA vector (Thermo Fisher). The destination vector was constructed by LR reaction using LR Clonase II (Thermo Fisher). The vector for BiFC was obtained from the ABRC (Martin et al. 2009).

HsfA1–cYFP was generated using Gateway^{TM} technology (Thermo Fisher). Full-length cDNA without the termination codon was cloned with specific primers BiFC-*HsfA1*-F and BiFC-*HsfA1*-R. The PCR fragment was ligated into the pCR^{TM}8/GW/TOPO TA vector (Thermo Fisher). The destination vector was constructed by LR reaction using LR Clonase II (Thermo Fisher). The vector for BiFC was obtained from the ABRC (Martin et al. 2009).

Myc-SlSIZ1 was generated using Gateway^{TM} technology (Thermo Fisher). Full-length cDNA without the termination codon was cloned with specific primers Myc-*SIZ1*-F and Myc-*SIZ1*-R. The PCR fragment was ligated into the pCR^{TM}8/GW/TOPO TA vector (Thermo Fisher). The SIZ1 fragment was inserted into pEarleyGate203 by LR reaction using LR Clonase II (Thermo Fisher).

Total RNA isolation and qRT-PCR analysis

Total RNAs were extracted from tomato by using an RNAsimple total RNA kit (TIANGEN). Reverse-transcribed first-strand cDNAs were synthesized using a FastQuant RT kit (with gDNase) (TIANGEN). qRT-PCR was performed on a QuantStudio 6 real-time thermal PCR system (Thermo Fisher) using SYBR-Green Real Master Mix (TIANGEN) according to the manufacturer's instructions. The reaction was conducted under the following conditions: pre-denaturation (95°C for 30 s), 45 cycles of denaturation (95°C for 15 s), annealing (55°C for 15 s), extension (72°C for 15 s) and the final stage (55–95°C) to determine dissociation curves of the amplified products. Results were obtained using three biological replicates from each sample.

BiFC assay in *N. benthamiana*

The growing conditions and method for the BiFC transient assay in *N. benthamiana* were as described in Huang et al. (2013).

Protein extraction and Western blot analysis

Plant total proteins were extracted with extraction buffer [50 mmol l^{-1} Tris pH 7.5, 150 mmol l^{-1} NaCl, 1 mM EDTA, 10 mmol l^{-1} NaF, 25 mmol l^{-1} β-glycerophosphate, 2 mmol l^{-1} sodium orthovanadate, 10% glycerine, 0.1% Tween-20, 0.6% SDS, 1 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 1× Omplete, EDTA-Free Protease inhibitor cocktail (CPIC) (Roche)]. The mixture was placed on ice for 30 min and then centrifuged at 4°C and 10,000 r.p.m. for 10 min. The protein concentration was measured using a BCA Protein Assay Kit (CWBIO). The extracted proteins were separated by SDS–PAGE and transferred on a polyvinylidene fluoride (PVDF) membrane (CWBIO). The membrane was probed with different antibodies and placed in a Tanon-5500 system for analysis. Anti-SlSIZ1 antibody (Genscript) was used to analyze SlSIZ1 in tomato. Quantity of probed protein quantity was measured using ImageJ software (https://imagej.nih.gov/ij/).

SUMOylation assay in vivo

For analysis of stress-induced SUMO conjugations, 6-week-old plants were treated under low temperature (4°C), high temperature (42°C), drought (PEG) and oxidation (MV). For SUMOylation analysis in overexpression tomato, 2-week-old seedlings were treated under 42°C for 2 and 4 h. Afterwards, total protein extraction and Western blot analysis were performed as described above. The proteins were probed with anti-SUMO antibody (ab5316, Abcam) and goat anti-rabbit IgG (CWBIO).

For the SUMOylation assay of HsfA1, the *siz1-2* mutant and SlSIZ1 complementary *Atsiz1-2* Arabidopsis were infiltrated with *Agrobacterium* transformed with *35S: SlHsfA1-Flag*. Transgenic Arabidopsis leaves were collected. The total proteins were extracted and purified according to the method of the Pierce Classic IP Kit (Thermo Fisher). SUMOylation of HsfA1 was probed by anti-SUMO (ab5316, Abcam) and anti-Flag (Transgen) antibodies.

Yeast two-hybrid assays

The yeast two-hybrid assay method was followed using the GAL4-based two-hybrid system (TAKARA, Clontech). Full-length cDNA sequences of *SlHsfA1*, *SlHsfA2*, *SlHsfB1*, *SlHsp70* and *SlHsp70-3* were cloned into pGBKT7, and the full-length cDNA sequence of *SlSIZ1* was cloned into pGADT7 (TAKARA, Clontech) to generate *BD-SlHsfA1*, *BD-SlHsfA2*, *BD-SlHsfB1*, *BD-SlHsp70*, *BD-SlHsp70-3* and *AD-SlSIZ1*.

All constructs were transformed into yeast strain AH109 according to the GAL4-based two-hybrid system (TAKARA, Clontech). Yeast cells were grown on SD medium (−Leu/−Trp). The interactions and ONPG analysis were as described in the Yeast Protocols Handbook (PT3024-1/PR973183, TAKARA, Clontech).

Measurements of MDA, free proline, Chl contents, H_2O_2 and superoxide anion ($O_2\cdot^-$) concentrations and antioxidative enzyme activities

The activities of POD and APX and the concentrations of H_2O_2 and $O_2\cdot^-$ were determined from the leaves (0.5 g) of 8-week-old transgenic and WT plants with or without heat treatment for 24 h. Measurement was performed according to the method of Kong et al. (2014) and Zong et al. (2009). MDA and free proline contents were measured using the method described by Feng et al. (2013). Ten-day-old tomato WT and transgenic seedlings were treated under heat stress for 3 d, and Chl contents were measured using the method described by Arnon (1949).

Statistical alanalysis

Statistical analysis was performed using SPSS13.0 and Sigma Plot 12.5 (https://systatsoftware.com/downloads/download-sigmaplot/).

Primers

All primers used are listed in Supplementary Table S1.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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