**Short Title:** Tomato SlMPK1 negatively regulates thermotolerance

**The tomato mitogen-activated protein kinase SlMPK1 is as a negative regulator of the high temperature stress response**¹

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One sentence summary: Tomato mitogen-activated protein kinase SlMPK1 negatively regulates high temperature tolerance by regulating antioxidant defense and the phosphorylation of substrate SlSPRH1 is involved in this pathway.
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ABSTRACT

High temperature (HT) stress is a major environmental stress that limits plant growth and development. Mitogen-activated protein kinase (MAPK) cascades play key roles in plant growth and stress signaling, but their involvement in the HT stress response are poorly understood. Here, we describe a 47-kDa MBP-phosphorylated protein (p47-MBPK) activated in tomato (Solanum lycopersicum) leaves under HT, and identify it as SlMPK1 by MS/MS analysis. Silencing of SlMPK1 in transgenic tomato plants resulted in enhanced tolerance to HT, while overexpression resulted in reduced tolerance. Proteomic analysis identified a set of proteins involved in antioxidant defense that are significantly more abundant in RNAi-SlMPK1 plants than non-transgenic plants under HT stress. RNAi-SlMPK1 plants also showed changes in membrane lipid peroxidation and antioxidant enzyme activities. Furthermore, using yeast two-hybrid (Y2H) screening, we identified a Serine-Proline-Rich protein Homolog, SISPRH1, which interacts with SlMPK1 in yeast, plant cells, and in vitro. We demonstrate that SlMPK1 can directly phosphorylate SISPRH1. Furthermore, the serine residue Ser-44 of SISPRH1 is a crucial phosphorylation site in the SlMPK1-mediated antioxidant defense mechanism activated during HT stress. We also demonstrate that heterologous expression of SISPRH1 in Arabidopsis led to a decrease in thermotolerance and lower antioxidant capacity. Taken together, our results suggest that SlMPK1 is a negative regulator of thermotolerance in tomato plants. SlMPK1 acts by regulating antioxidant defense, and its substrate SISPRH1 is involved in this pathway.
INTRODUCTION

High temperature (HT) is a major plant stress that disturs cellular homeostasis and leads to severe retardation of crop growth and development, and even death. HT stress can be expected to become increasingly problematic as global warming leads to more adverse climatic changes (Tubiello et al., 2007). Plants have evolved a variety of response mechanisms to elevated temperatures (Kotak et al., 2007). Many HT-responsive genes have been identified and altered gene expression plays an important role in plant HT tolerance (Larkindale and Vierling, 2008; Mittler et al., 2012). Tomato (Solanum lycopersicum) is one of the most important vegetable crops but is susceptible to HT stress, especially in greenhouse facilities.

Nevertheless, the biological functions of most HT-responsive genes in tomato are largely unknown.

The mitogen-activated protein kinase (MAPK) cascades are one of the major and evolutionally conserved signaling pathways by which extracellular stimuli are transduced into intracellular responses in eukaryotic cells. The basic modules consist of three interlinked protein kinase modules (MAPKKK-MAPKK-MAPK). The downstream targets of activated MAPKs can be transcription factors, protein kinases, or cytoskeleton-associated proteins (Nakagami et al., 2005). MAPK gene families show a similar complexity in different species, with 20 genes in Arabidopsis (Arabidopsis thaliana) (Colcombet and Hirt, 2008), 17 genes in rice (Oryza sativa) (Rohila and Yang, 2007), and 16 genes in tomato (Kong et al., 2012). In plants, MAPK cascades have been identified in signal transduction including cell division, hormone responses, development, and disease resistance (Nakagami et al., 2005; Pitzschke et al., 2009; Meng and Zhang, 2013; Xu and Zhang, 2015). It is also well documented that MAPKs play key roles in the regulation of the plant’s response to abiotic stresses like drought, salinity, cold, O3, and heavy metals (Nakagami et al., 2005; Sinha et al., 2011; Moustafa et al., 2014; Pitzschke et al., 2015; De et al., 2016; Zhao et al., 2017). By contrast, there are only a few reports concerning the involvement of MAPKs in the HT response (Evrard et al., 2013).

HT can up-regulate the expression of SlMPK1 in potato (Solanum tuberosum) (Blanco et al., 2006) and induce the activities of 46 kDa HAMK in tobacco (Nicotiana tabacum) cells (Suri and Dhindsa, 2008) and AtMPK6 in Arabidopsis (Li et al., 2012). So far, however, little is known on the molecular mechanisms underlying the role of MAPK in response to HT. Li et al. (2012) showed that AtMPK6-mediated activation of γVPE played an important role in HT-induced programmed cell death (PCD). It was also reported that AtMPK6 negatively regulates the HT response, and AtMPK6–phosphorylated HSFA2 might participate in the response (Evrard et al., 2013). However, the molecular roles of MAPK in response to HT need to be further elucidated. In the tomato genome, 16 MAPK genes, 5 MAPKK genes, and 89 MAPKKK genes have been identified (Kong et al., 2012; Wu et al., 2014). Studies on tomato MAPKs have mainly focused on biotic stresses. The tomato SlMPK1, SlMPK2, and SlMPK3 are activated upon stress responses caused by the wound-signaling, peptide systemin, oligosaccharide elicitors, and aphids (Li et al., 2006b; Kandoth et al., 2007; Stulemeijer et al., 2007). SIMKK2 is a key protein regulating immunity-associated PCD in plants (Melech-Bonfil and Sessa, 2011; Oh et al., 2013). Recent studies showed that silencing of tomato MPKJ/2 by virus-induced gene silencing (VIGS) abolishes plant tolerance to heat, cold, and oxidative stress (Nie et al. 2013; Zhou et al. 2014; Lv et al. 2017). These results suggest an opposite function to AtMPK6, a close homolog to SIMPK1, under HT or cold stress in Arabidopsis (Li et al. 2012; Evrard et al., 2013; Li et al. 2017; Zhao et al., 2017). Therefore, the function of SIMPK1 in abiotic stress resistance in tomato requires further molecular genetic evidence, especially in relation to HT stress.

Here, an HT-activated 47 kDa MBP-phosphorylated protein (p47-MBPK) was identified as SIMPK1 using anion exchange column purification and MS/MS analyses in tomato leaves. Furthermore, the possible molecular mechanisms underlying SIMPK1-mediated responses to HT were investigated. RNA interference (RNAi) silencing of SIMPK1 enhanced HT tolerance in transgenic tomato seedlings. Interestingly, analysis of the proteome using an isobaric tags for relative and absolute quantification (iTRAQ) revealed that several proteins involved in antioxidant defense were significantly up-regulated in RNAi-SIMPK1 plants under HT.
stress. The RNAi-\textit{SIMPK1} plants possessed higher antioxidant defense capacity, while overexpression lines developed opposite phenotypes. In addition, \textit{SISPRH1} was shown to be a substrate of \textit{SIMPK1}, with Ser-44 being the major phosphorylation site. When Ser-44 was mutated, the phosphorylation of \textit{SISPRH1} by \textit{SIMPK1} was almost abolished, showing that Ser-44 phosphorylation is essential for the \textit{SIMPK1}-mediated antioxidant defense involved in HT. Arabidopsis plants overexpressing \textit{SISPRH1} were more sensitive to HT and possessed a lower antioxidant defense capacity. Our results reveal a potential involvement of \textit{SIMPK1} as part of the HT response in tomato, and unravel the molecular mechanisms by which \textit{SIMPK1} negatively regulates the HT response.

\textbf{RESULTS}

\textbf{HT-activated p47-MBPK is identified as \textit{SIMPK1}}

To search for MAPKs involved in the perception of HT signaling, an in-gel kinase activity assay was used. HT caused a significant increase in the activity of a 47-kDa MBP-phosphorylated protein (p47-MBPK, Fig. 1A). The p47-MBPK was also immunoprecipitated with the anti-pTyr monoclonal antibody 4G10, which has been widely used to demonstrate Tyr phosphorylation of MAPKs, an important characteristic of MAPKs. The results suggest that p47-MBPK is a MAPK-like protein. Katou et al. (2005) purified an elicitor-induced 51-kDa MAPK, designated as \textit{StMPK1}. We previously purified an ABA-activated p46-MAPK, identified as \textit{ZmMPK5} (Ding et al., 2009). To reveal the identity and function of the p47-MBPK, we first purified the kinase from HT-treated tomato leaves using different anion exchange columns (Fig. S1). The peak of protein levels and kinase activities was confirmed in each step by silver staining and an in-gel kinase assay, respectively, revealing a 47-kDa kinase in the poly-L-lysine-agarose column fractions with one band (Fig. 1B).

To identify the p47-MBPK, the partly purified protein was silver stained on an SDS-PAGE gel and the band corresponding to 47 kDa was in-gel digested with trypsin, followed by MALDI-TOF/TOF-MS/MS analysis. The search yielded a top score of 341 for Q7Y1Y6, \textit{SIMPK1} \textit{[Solanum lycopersicum]} with four matched peptides using Mascot search engine (Fig. 1C). Furthermore, the selected tryptic peptide (m/z 1777.7845) sequenced by MS/MS revealed an amino acid sequence of ESIAFNPEYQR, corresponding to the specific residues 385–395 of \textit{SIMPK1} (Fig. 1D). To further confirm the identity of p47-MBPK correlated with \textit{SIMPK1}, a C-terminal-specific polyclonal antibody for \textit{SIMPK1} was prepared. The molecular mass of the immunoprecipitated protein kinase from HT-treated tomato leaves was 47 kDa, and this protein was induced by HT stress (Fig. 1E). These results indicate that the HT-activated p47-MBPK is \textit{SIMPK1}.

\textbf{Suppression of \textit{SIMPK1} increased HT tolerance in transgenic plants}

\textit{SIMPK1} (Solyc12g019460), homologous to \textit{AtMPK6}, \textit{ZmMPK5}, and \textit{OsMPK6}, was first isolated from tomato seedlings by Holley et al. (2003). \textit{SIMPK1} can be activated by systemin and several oligosaccharide elicitors (Higgins et al., 2016). In this study, \textit{SIMPK1} activity was induced in HT-treated tomato leaves (Fig. 1, A and E). Given this result, we speculated \textit{SIMPK1} might be involved in the regulation of the HT response. To test this hypothesis, RNA interference (RNAi) was used to suppress the expression of \textit{SIMPK1} in transgenic tomato. Three independent RNAi lines (1-14, 1-23, and 1-24) were selected for HT tolerance testing at the seedling stage (Fig. 2A). The inhibition rate of \textit{SIMPK1} expression by RNAi was over 90%, but the expression of the homologous gene \textit{SIMPK2} was not changed (Fig. S2B), indicating that \textit{SIMPK1} expression was specifically suppressed by RNAi in the transgenic plants. Under normal conditions, the growth of three RNAi lines was similar to that of WT plants (Fig. S2A). When seedlings of WT and RNAi-\textit{SIMPK1} lines grown in soil were treated with HT, the RNAi lines showed more tolerance to HT (Fig. 2B). Consistent with the results, the chlorophyll content of leaf discs from HT-treated plants was lower in...
WT than in RNAi-SIMPK1 lines (Fig. 2, C and D). Similar results of growth inhibition were also found observed in sterile seedlings of RNAi line 1-24 grown in MS media (Fig. 2, E and F). Taken together, these results demonstrate that SIMPK1 has a negative role in HT tolerance in tomato.

The expression of heat shock protein (HSP) and heat shock factor (HSF) genes serves as a master regulator of heat stress responses (HSR). To determine the relationship between SIMPK1 and HSR, we measured the transcript levels of HSFA2 and HSP101, the major heat-inducible HSF and HSP genes (Wu et al., 2017). Our results show that the expression of both genes did not change significantly in SIMPK1 RNAi-lines when compared to WT at normal growth temperature or HT stress (Fig. S2C).

Analysis of SIMPK1-mediated proteins under HT conditions with iTRAQ proteomic assay

To elucidate how SIMPK1 coordinately regulates HT tolerance, the proteins involved in the SIMPK1-mediated HT-responsive pathway were analyzed using iTRAQ. The RNAi line 1-24 was used for the proteomic assay. There was high reproducibility between the iTRAQ analyses according to the coefficient of variation (CV, Fig. S3, A and B). Data analysis detected a total of 2258 and 2433 proteins in the first and second iTRAQ analyses, respectively (Fig. S3C). There were 1787 proteins overlapping between two 8-plex iTRAQ analyses, and only proteins with consistent expression (fold change ratio>1.2 or <0.67, Pval<0.05) in both 8-plex iTRAQ analyses were demonstrated to have statistical significance. Several HT-related proteins such as HSP22.0, HSP101, ATHSP90.1, and HSP70b were highly induced in both 8-plex iTRAQ analyses, suggesting that the HT treatment was effective (Table S1). The results showed that the expression of only a small number of proteins changed in the RNAi line 1-24 plants compared to the WT under normal conditions (Table S2). For example, PPC1 is a phosphoenolpyruvate carboxylase and is part of the adaptation of the plant to salt and drought (Sánchez et al., 2006).

Meanwhile, the expression of 48 proteins was significantly altered in the RNAi-SIMPK1 line 1-24 vs. WT plants under HT conditions (Table S3). An overview of functional protein networks affected in the RNAi-SIMPK1 line 1-24 under HT using STRING 9.0 is shown in Fig. S4. Molecular function enrichment analysis showed that 19 proteins have catalytic activities, including nine proteins with oxidoreductase activity (NRX1, THI1, GLU1, MDAR1, IMD2, SDR5, TKL1, HDS, CRL1) (Table S4). In addition, CAT2 and AOR also have oxidoreductase activities which were not listed in the PANTHER version 12.0 of the Solanum lycopersicum reference genome. Biological process enrichment categorizes these proteins into different metabolic processes including protein folding (e.g., CPN20), amino acid biosynthetic process (e.g., MS1), lipid metabolic process (e.g., ANNAT4), and translation (e.g., emb2394). Interestingly, if we used Bonferroni correction for functional enrichment, oxidoreductase activity (GO:0016491) was the only significantly enriched molecular function (Table S5), indicating that oxidoreductase activity-mediated processes are one of the main processes in the SIMPK1-mediated response to HT. Twenty proteins having significant consistency of expression in at least three of four biological replicates are shown in Table 1, including seven oxidoreductases (NRX1, THI1, GLU1, MDAR1, IMD2, CAT2, AOR).

The expression of HT-related protein-coding genes such as CPN20 and CPN60A1 was highly up-regulated in HT-treated WT plants, suggesting that the HT treatment was effective. The expression pattern of some protein-coding genes, such as CAT2, CPN20, CPN60A1, CPN60B1, and CPN60B2, was consistent with the variations in protein abundance (Fig. S5). Though the expression of HSR master regulator genes HSFA2 and HSP101 did not change, the expression of several heat-inducible genes, such as CHAPERONIN-60A, CPN20, and CPHSC70-2 increased in the SIMPK1 RNAi line 1-14 and 1-24 under HT stress (Fig. S5).

Up-regulation of antioxidant defense in the SIMPK1-suppressed lines under HT

The iTRAQ analysis indicated that the abundance of many proteins involved in cell redox homeostasis increases in the RNAi-SIMPK1 line 1-24 under HT conditions. Plant catalases are part of the major reactive
oxygen species (ROS) scavenging network. For example, a decrease in the activity of the catalase CAT2 in the Arabidopsis cat2-1 mutant correlates with greater accumulation of H$_2$O$_2$ and higher oxidative damage in leaves (Bueso et al., 2007). MDAR1 is a monodehydroascorbate reductase crucial for maintaining a reduced pool of ascorbate (AsA), a major antioxidant and radical scavenger in plants (Eltayeb et al., 2007). The increased oxidoreductase levels in the RNAi-SIMPK1 plants under HT should be reflected in increased antioxidant capacity and decreased levels of ROS. Under HT conditions, the level of H$_2$O$_2$ in RNAi-SIMPK1 lines was lower than in WT plants (Fig. 3A). Lipid peroxidation was estimated by determining malondialdehyde (MDA) content, and showed results similar to those of H$_2$O$_2$ (Fig. 3B).

The generation of ROS is limited or scavenged by antioxidant enzymes like superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), as well as non-enzymatic antioxidants such as AsA and glutathione (GSH; Mittler, 2002). There were significant differences in the activity of APX and accumulation of AsA and GSH between RNAi-SIMPK1 lines and WT (Fig. 3). Concomitantly with the increase in the levels of proteins like CAT2 and MDAR1, our physiological analysis revealed a significant increase in the activity of enzymes involved in AsA-GSH metabolism and regulation in the RNAi-SIMPK1 plants. Therefore, the enhanced HT tolerance observed in RNAi-SIMPK1 plants might be related to activation of the antioxidant defense system, which seems to be the result of de novo synthesis and activation of enzymes.

Overexpression of SIMPK1 decreased HT tolerance in transgenic plants

Genetic and physiological analysis of the RNAi-SIMPK1 line suggests that SIMPK1 plays a negative role in the regulation of HT resistance in tomato. We predicted that overexpression of SIMPK1 might result in decreased HT tolerance. To test this hypothesis, transgenic plants were generated by overexpressing the full-length SIMPK1 under the CaMV 35S promoter. Two SIMPK1-overexpressing lines showed significantly increased SIMPK1 expression (Fig. 4A), indicating that SIMPK1 was successfully overexpressed. We first analyzed the tolerance of transgenic tomato to HT stress and found that SIMPK1-OE lines were nearly dead after HT stress, while all the WT plants survived (Fig. 4C). Furthermore, the SIMPK1-OE lines exhibited more severe growth inhibition than WT plants in MS medium under HT conditions (Fig. 4D). Statistical analysis showed that the SIMPK1-OE transgenic plants suffered significantly more suppression of shoot growth than WT plants after 2 h of HT stress (Fig. 4E). These results suggest that overexpression of SIMPK1 has a negative effect on HT tolerance. We also determined H$_2$O$_2$ levels, MDA content, and two enzyme activities in SIMPK1-OE transgenic plants under HT conditions. Under HT conditions, the accumulation of H$_2$O$_2$ and MDA in SIMPK1-OE transgenic plants was higher than in WT plants (Fig. S6). By contrast, the activities of antioxidant enzymes in SIMPK1-OE transgenic plants were lower than in the WT plants.

SIMPK1 interacts with SISPRH1

MAPKs play important roles in various cellular processes by binding to many types of proteins such as substrates, other protein kinases, protein phosphatases, cytoskeletal proteins, and transcription factors (Tanoue and Nishida, 2003). To identify downstream targets of SIMPK1, we screened a tomato cDNA library using SIMPK1 as the bait in a Y2H assay. The screen identified Solyc06g053700 as a candidate SIMPK1-interacting protein. Solyc06g053700 is a serine-proline-rich protein homolog of unknown function, homologous to Arabidopsis At1g04330 (Fragment). We tentatively named this protein SISPRH1 (Serine-Proline-Rich protein Homolog 1). Cotransformation of SISPRH1 with SIMPK1 confirmed their interaction in yeast (Fig. 5A). SIMKK2 has been reported to interact with SIMPK1 (Kandoth et al., 2007), and was used as a positive control in the present study. The results showed that SIMPK1 interacts with SISPRH1 in a BiFC assay (Fig. 5B). To provide further evidence for such interaction, we analyzed the interaction in vitro using pull-down assays. Using immobilization of recombinant SISPRH1 fusion protein on
GST sepharose beads, we found that GST-SISPRH1, but not GST alone, was able to pull down His-SIMPK1 in vitro (Fig. 5C). Furthermore, the SIMPK1-SISPRH1 interaction was confirmed by co-immunoprecipitation (Co-IP) experiments in vivo on N. benthamiana leaves. Immunoprecipitates of transiently expressed SIMPK1-HA in leaves transformed with SIMPK1-HA and SISPRH1-myc were found to contain SISPRH1 (Fig. 5D). Taken together, these data show that SIMPK1 can interact with SISPRH1 in vitro and in vivo.

**SIMPK1 phosphorylates SISPRH1**

All MAPK interacting proteins use a signature motif known as the D motif (-(R/K)_{1-2}-(X)_{2-6}-L/I/V-X-L/I/V-) to interact with MAPKs (Takuji et al., 2000). A search shows one D motif (RRAPASIQV) located in the N terminus of SISPRH1 (Fig. 6A). To identify the SIMPK1 phosphorylation sites on SISPRH1, recombinant SISPRH1 was phosphorylated in vitro by SIMPK1. MS analysis identified several phosphopeptides (Fig. 6 and Fig. S7). Ser-52 and Ser-94 were found to be phosphorylated exclusively in the SIMPK1-treated SISPRH1. Other sites like Ser-27 and Thr-79 were found to be phosphorylated both in the control and the kinase-treated SISPRH1, which imply that Ser-27 and Thr-79 phosphorylation results from the activity of an *E. coli* kinase (Li et al., 2017). However, the two phosphorylated sites Ser-44 and Ser-49 in SISPRH1 were not identified by MS. To further confirm these SIMPK1-phosphorylated sites, two peptides were synthesized and tested in vitro as substrates for SIMPK1. MS analysis of the peptides showed that Ser-49, Ser-52, and Ser-94 were phosphorylated exclusively by SIMPK1 (Fig. 6A and Fig. S7). While the Ser-49 phosphorylation site was only found in the peptide, the Ser-44 phosphorylation site was not detected by MS.

To further characterize the phosphorylation of SISPRH1, His-tagged SIMPK1 and GST-tagged SISPRH1 were purified and used in kinase assays in vitro. SISPRH1 was phosphorylated in the presence of recombinant SIMPK1 activated by SIMPKK2DD (Fig. 6B). Sequence analysis indicated that there are four potential phosphorylation sites (with a Ser or Thr residue followed by a Pro residue, S/T-P motif) in SIMPK1, (Ser-44, Ser-49, Ser-52, and Ser-94) (Fig. 8A). The phosphorylation sites Ser-49, Ser-52, and Ser-94 were confirmed by MS. Previously, Ser-44 in At1g04330, homologous to Ser-44 in SISPRH1, was examined as the major target of AtMPK6 (Palm-Forster et al., 2012). To identify the phosphorylation site(s) of SISPRH1, Ala exchange of the potential phosphorylation sites was performed. Changes of all four corresponding residues to Ala in SISPRH1 (4xmut) almost completely abolished SISPRH1 phosphorylation (Fig. 6B). We also mutated SISPRH1 at the three predicted phosphosites except Ser-44 (3xmut), and found no effect on the phosphorylation of SISPRH1. By contrast, when only the Ser-44 site was mutated (1xmut), phosphorylation of SISPRH1 was almost abolished (Fig. 6B). The fact that the phosphorylation site Ser-44 was not detected by MS might be a limitation of the MS technique. These results indicate that SISPRH1 is a substrate of SIMPK1, that Ser-44, Ser-49, Ser-52, and Ser-94 are phosphorylation sites, and that Ser-44 is a phosphorylation site conserved between the two homologous proteins (Fig. S8), and is the most preferred site in SISPRH1 for phosphorylation by SIMPK1.

**Overexpression of SISPRH1 decreased HT tolerance in transgenic plants**

To investigate the role of SISPRH1 in HT stress, we first measured the expression of SISPRH1 under HT stress. HT significantly increased the expression of SISPRH1 in tomato leaves (Fig. 7A). At the same time, SIMPK1 expression was also induced by HT (Fig. S10). The BiFC results showed that the interaction between SIMPK1 and SISPRH1 was observed in both nucleus and cytoplasm (Fig. 5B). To further confirm the localization of SISPRH1, a 35S-SIMPK1-GFP construct and a 35S-GFP control were produced, transformed into N. benthamiana (Fig. 7B), and visualized by fluorescence microscopy. GFP fluorescence from SISPRH1-GFP was observed in the nucleus and cytoplasm, but predominantly in the nucleus. To
further explore the molecular function of SISPRH1, full-length *SISPRH1* was heterologously expressed in Arabidopsis plants. Hypocotyl elongation is known to be inhibited by HT, and has been used as a parameter for HT tolerance (Queitsch et al., 2000). The hypocotyl length and growth of HT-stressed, *SISPRH1*-expressing plants were reduced relative to WT when plants were grown in darkness (Fig. 7C). Transgenic Arabidopsis seedlings grown on solid agar MS medium plates treated with 42 °C for 1.5 h were almost completely dead after recovery for 10 days (Fig. 7E). These results suggest that heterologous expression of *SISPRH1* has a negative effect on HT tolerance in Arabidopsis.

The decreased HT tolerance in *SIMPK1*-OE tomato plants might be related to a down-regulation of the antioxidant defense system. To determine whether or not the overexpression of *SISPRH1* has a similar effect, the enzyme activities were measured in *SISPRH1*-expressing Arabidopsis plants under HT conditions. The activities of APX, CAT, and SOD were lower in *SIMPK1*-expressing transgenic plants than in WT plants, while the MDA level was higher under HT conditions (Fig. 7F and Fig. S9). These results suggest that the negative effect of *SISPRH1* heterologous expression on HT tolerance might be related to a decrease in antioxidant defenses.

**Phosphorylation of Ser-44 in SISPRH1 is involved in SIMPK1-mediated antioxidant defense under HT**

To further determine whether SISPRH1 acts downstream of SIMPK1 in the regulation of antioxidant defense under HT, the SISPRH1 mutant (*SISPRH1*<sup>S44A</sup>) alone or *SIMPK1* and *SISPRH1*<sup>S44A</sup> simultaneously were transiently expressed in Arabidopsis mesophyll protoplasts. APX and CAT are two major H<sub>2</sub>O<sub>2</sub>-scavenging enzymes of antioxidant defense. The activities of APX and CAT were lower in protoplasts expressing *SIMPK1* or *SISPRH1*, and even lower in protoplasts co-expressing *SIMPK1* and *SISPRH1* (Fig. 8). However, enzyme activities in protoplasts expressing *SISPRH1*<sup>S44A</sup> or *SIMPK1* and *SISPRH1*<sup>S44A</sup> were the same as those harboring empty vector (EV) or *SIMPK1*, respectively. This results suggests that mutation of S44A (*SISPRH1*<sup>S44A</sup>) cancels the inhibition of enzyme activities, and that SIMPK1-mediated phosphorylation of SISPRH1 at S44 regulates antioxidant defense under HT.

**DISCUSSION**

**SIMPK1 is a negative regulator of HT responses in tomato**

The most extensively studied plant MAPKs are Arabidopsis AtMPK6, AtMPK3, and AtMPK4, all of which are activated by a diversity of stimuli including abiotic stresses, pathogens, and oxidative stress (Pitzschke et al., 2009, 2015; De et al., 2016). The Arabidopsis AtMPK6 and its functional orthologs in other species have been shown to be involved in integrating and transducing several signal stimuli for appropriate cellular response to various stresses (Pitzschke et al., 2009; Kumar and Kirti, 2010; Xu and Zhang, 2015; Lv et al., 2017). It has been well established that MPK6 is a positive regulator of defense responses in plants (Nakagami et al., 2005; Pitzschke et al., 2009; Sinha et al., 2011; Moustafa et al., 2014; Pitzschke et al., 2015; De et al., 2016). For example, overexpression of *OsMAPK5* in rice transgenic plants increased tolerance, while suppression led to hypersensitivity to various stresses including salt, drought, and cold (Xiong and Yang, 2003). However, other studies showed that MPK6 also plays a negative role in defense responses. Silencing WIPK and SIPK in tobacco enhances basal resistance against TMV, but breaks N-mediated resistance (Kobayashi et al., 2010). Cotton GhMPK6a, a homolog of MPK6, negatively regulates osmotic tolerance and bacterial infection in transgenic *N. benthamiana* (Li et al., 2013). Recently, two research groups reported that a MPK3/6 cascade negatively regulates freezing tolerance (Li et al., 2017, Zhao et al., 2017). Interestingly, positive and negative roles in regulating defense responses have been reported recently for the soybean (*Glycine max*). MPK6 (Liu et al., 2014). So far, it is unclear how MAPKs respond and adapt to HT stress. As MAPKs constitute a large gene family, characterization of more HT-responsive MAPKs will
provide a better understanding of the roles of individual members in the stress signaling network. There are only two reports showing that a mpk6 Arabidopsis mutant displays higher HT tolerance (Li et al., 2012; Evrard et al., 2013). We previously purified an ABA-activated p46-MAPK in maize, identified as ZmMPK5 (Ding et al., 2009). In this study, we purified a p47-MBPK using anion exchange columns and identified it as SlMPK1 using MS/MS assay (Fig. 1 and Fig. S1). This study focused on the SIMPK1, an ortholog of AtMPK6, NtSIPK, and OsMPK6. Recent studies showed that silencing of tomato SIMPK1 by VIGS abolishes plant tolerance to heat, cold, and oxidative stress (Nie et al., 2013; Zhou et al., 2014; Lv et al., 2017). According to our results, silencing of SIMPK1 in transgenic tomato enhances HT tolerance (Fig. 2), whereas overexpression of SIMPK1 leads to decreased HT tolerance (Fig. 4). These contrasting results may be related to differences in HT treatments. In our study (long-term trial), the seedlings of WT and RNAi-SIMPK1 transgenic tomato plants were subjected to 38°C/28°C (day/night) for 3 d, then recovered at 25°C/20°C for 10 d. After that, the phenotype of WT and RNAi-SIMPK1 transgenic tomato plants is visible, and RNAi-SIMPK1 tomato plants grow better than WT (Fig. 2B). In their treatment (short-term trial), after 42°C for 10 h, the plants did not display a phenotype, and physical indicators were used to study the roles of the tomato MPK gene (Nie et al., 2013). Therefore, the results of gene function studies using VIGS need to be further verified by stable genetic plant material.

SIMPK1 is involved in HT responses via regulating HT-responsive proteins, including antioxidant defense proteins

To better understand the molecular basis of SIMPK1-mediated HT tolerance in tomato, we first compared the protein abundance profiles of RNAi-SIMPK1 and WT plants under normal or HT conditions. Our results suggest that only 10 proteins show significant changes in abundance in the RNAi-SIMPK1 line 1-24 compared to the WT under normal conditions (Table S2). OPR3 is a peroxisome 12-oxophytodienoate-10,11-reductase and is required for jasmonate synthesis involving in MAPK kinase 2 (MKK2) pathway (Brader et al., 2007). Ariga et al. (2015) showed that AtCSP41B-overexpressing transgenic Arabidopsis lines exhibited heat and salinity stress tolerance, and concluded that maintenance of CSP41B expression under abiotic stresses may alleviate photo inhibition and improve survival under such stresses. ASR (Q2QJT5) is an ABA stress ripening protein related to drought and salt tolerance not found in Arabidopsis (Fischer et al., 2011). AOR is an alkenal/one oxidoreductase, and its homologous protein (AT1G23740) was shown to be down-regulated in the absence of ATMPK6 (mpk6, R6-7) using quantitative proteomics (Miles et al., 2009). Here, AOR (Solyc05g005480) was down-regulated in the RNAi-SIMPK1 line 1-24 compared to WT. Therefore, the results suggest that SIMPK1 suppression improves HT tolerance in RNAi-SIMPK1 tomato plants.

Interestingly, a number of stress-responsive proteins were altered in the RNAi-SIMPK1 line 1-24 vs WT plants under HT conditions. These proteins showed diverse functions such as protein folding, amino acid biosynthetic process, lipid metabolic process, translation, and oxidation-reduction process (Table S3 and S4). The protein-coding gene transcripts noted by others as HT-regulated are, not surprisingly, HSPs/molecular chaperones (CPN60A1, CPN60B1, CPN60B2, CPN20, and CPHSC70-2). CPN-60 activity is most abundant in the developing green tissues, and might be involved in chloroplast biogenesis and plastid division (Ahsan et al., 2010). Salvucci et al. (2008) demonstrated that CPN-60B plays a role in acclimating photosynthesis to HT, possibly by protecting Rubisco Activase from thermal denaturation. Chloroplast CPN20 is a co-chaperonin of CPN60. Moreover, Arabidopsis stromal CPHTC70s are essential for plant development and important for thermotolerance in germinating seeds (Su and Li, 2008). Consistent with these results, the chlorophyll content of RNAi line 1-24 was less affected by HT stress than that of WT leaves (Fig. 2, C and D). These results suggest that SIMPK1-mediated CPN60 accumulation may protect the chloroplast proteins from HT-induced thermal aggregation or denaturation. Moreover, SBPASE is a Calvin cycle enzyme and
functions in photosynthetic carbon fixation. The list also includes three ribosomal family proteins (Solyc09g065270, Solyc01g087730, and Solyc12g100160).

Oxidoreductase activity (GO:0016491) was the only significantly enriched molecular function (Table S5). and seven oxidoreductases (CAT2, MDAR1, NRX1, THI1, GLU1, IMD2, AOR) showed significant consistency of expression in at least three of the four biological replicates (Table 1). This result suggests that the oxidoreductase activity-mediated oxidation-reduction process is one of the main processes in SlMPK1-mediated response to HT. One important response mechanism to HT in plants is the antioxidant defense machinery, including both enzymatic and nonenzymatic antioxidants which work in concert to scavenge HT-caused reactive oxygen species (ROS) and protect plant cells from oxidative stress (Mittler, 2002; Kotak et al., 2007; Miller et al., 2010). In the preliminary proteomic analysis, the protein levels of CAT2 and MDAR1 were up-regulated in the RNAi-SlMPK1 line 1-24 compared with WT under HT conditions (Table 1). In addition, high levels of activity of four enzymes (SOD, POD, CAT, and APX) were detected in the RNAi-SlMPK1 lines under HT conditions (Fig. 3). Both AsA and GSH act as important redox buffers in AsA-GSH cycle. In the present study, the contents of AsA and GSH were also higher in the RNAi-SlMPK1 line 1-24 than in WT under HT stress. These results suggest that RNAi-SlMPK1 lines possess a more efficient antioxidant network than WT. This is corroborated by the accumulation of lower levels of H$_2$O$_2$ in the RNAi-SlMPK1 lines. As an excess of H$_2$O$_2$ results in oxidative stress in plants, our results indicate that SlMPK1 suppression decreases HT-induced oxidative damage, consistent with the reduced levels of MDA, a parameter for determining the membrane lipid peroxidation. An earlier study showed that the ectopic expression of GhMPK6a in N. benthamiana reduced drought and salt tolerance, with elevated MDA and ROS content relative to WT plants (Li et al., 2013). Indeed, it has been reported that CPN20 mediates iron superoxide dismutase (FeSOD) activity independent of its co-chaperonin role in Arabidopsis chloroplasts, supporting a common role for CPN20 in the activation of FeSOD for oxidative stress protection and chloroplast development (Kuo et al., 2013). It is conceivable that the SlMPK1-mediated antioxidant system is effective for removing the ROS produced under HT conditions. In this regard, SlMPK1-OE transgenic plants displayed lower tolerance to HT with low levels of enzyme activities and high levels of H$_2$O$_2$ and MDA (Fig. 4 and Fig. S6). These results indicate that SlMPK1 confers resistance to HT-induced oxidative stress by regulating key enzyme activities. There were no significant differences in the antioxidant system between WT and transgenic lines (RNAi or OE lines) under normal conditions. However, the transgenic plants showed altered activities of antioxidant enzymes after HT stress, suggesting a function for SlMPK1 as a negative regulator in HT stress through regulation of the antioxidant system. NRX1, THI1, GLU1, IMD2, and AOR are five proteins involved in the oxidation-reduction process. NRX1 has thioredoxin-disulfide reductase activity. GLU1 is a ferredoxin-dependent glutamate synthase. IMD2 is a 3-isopropylmalate dehydrogenase involved in leucine biosynthesis. THI1 is a thiamine (vitamin B1) synthase with a dual function in thiamine biosynthesis and mitochondrial DNA damage tolerance. Furthermore, thiamin confers enhanced tolerance to oxidative stress in Arabidopsis (Tunc-Ozdemir et al., 2009). Recent studies suggested that THI1 may play roles in plant abiotic stress responses such as sugar deprivation, high salinity, drought, hypoxia, and oxidative stress (Li et al., 2016a). AOR is a NADPH-dependent reductase involved in the detoxification of reactive carbonyls ($\alpha$,\beta-unsaturated carbonyl compounds) to protect chloroplast function in plants (Yamauchi et al., 2011). Taken together, our results suggest that SlMPK1-mediated HT tolerance in plants might be related to the balance of cellular redox homeostasis.

**SlMPK1 cascades involved in HT response**

MAPKs play important roles in various cellular processes by binding to many types of proteins (Tanoue and Nishida, 2003). Our results indicate that SlMPK1 may be a negative regulator of HT signaling by suppressing the ROS-scavenging pathway in tomato. What is the SlMPK1 pathway in response to HT? As a
first step, we analyzed proteomic data and found that several SIMPK1-mediated homologous proteins were also observed in the Arabidopsis MAPKKK double mutant anp2anp3 (Takáč et al., 2014). A comparative proteomic analysis of anp2anp3 revealed an overabundance of core enzymes such as SOD, DHAR1, and FeSOD1-associated regulatory protein CPN20, which ensure favorable cellular redox conditions as well as accelerated defense against overproduction of ROS (Takáč et al., 2014). In this study, many proteins controlling oxidation-reduction process were also discovered (Table S6). There are about 80 putative MAPKKKs in Arabidopsis and 89 MAPKKKs in tomato, making up of the most complex and the largest group of MAPK pathway components. However, few MAPKKKs have been functionally characterized. At1g73660 encoding a putative MAPKKK was reported to negatively regulate salt tolerance in Arabidopsis (Gao and Xiang, 2008). SIMKK9 (Solyc03g097920), with homology to Arabidopsis AtMKK9, was identified to interact with SIMPK1 using Y2H in our lab (data not shown). Loss of MKK9 activity in Arabidopsis reduces salt sensitivity, indicating that MKK9 negatively regulates salt stress (Xu et al., 2008). MKK9 might be an upstream component of SIMPK1 in response to HT. Recently, the MKK4/5-MPK3/6 cascade was found to negatively regulate cold responses (Zhao et al., 2017). Therefore, we can infer that there may be one or more MAPKKK-MKK cascades upstream of SIMPK1 negatively responding to HT signaling, which still requires further investigation.

The multifunctionality of MPK6 is likely to be conferred by its different substrates. Until now, however, only a few substrates have been reported with functional data (Guo et al., 2016). To gain insight into the interactions downstream of SIMPK1, we used Y2H to screen a tomato cDNA library and obtained a set of candidate proteins. Our data demonstrate that SISPRH1 is a substrate of SIMPK1. SISPRH1 has four serine-proline motifs. SISPRH1 interacts with SIMPK1 in Y2H, BiFC, in vitro pull-down and Co-IP assays (Fig. 5). SISPRH1 is a protein of unknown function, homologous to Arabidopsis At1g04330 (Palm-Forster et al., 2012). Recently, it was also reported as PH2 and shown to be involved in pathogen defense (Palm-Forster, et al. 2017). The first putative phosphorylation site within the primary protein sequence (Ser-44) is predominantly modified by AtMPK6, and is the same site in the conserved region (Ser-44) of SISPRH1. The Pro-x-Ser-Pro (PxSP) motif has been previously touted as a “high-stringency” site for MAPK-directed phosphorylation. AtMPK6 can phosphorylate ERF10 and ACS6 on a serine residue within a PxSP motif (Stulemeijer et al., 2007). The predicted PxSP motif matches for Ser-52 and Ser-94 in SISPRH1. Here, we provide MS data supporting that Ser-49, Ser-52, and Ser-94 can be phosphorylated by SIMPK1 (Fig. 6 and Fig. S7). In vitro phosphorylation assays together with site-directed mutagenesis of the phosphorylated sites indicate that SISPRH1 can be phosphorylated by the activated SIMPK1. Though the Ser-44 phosphorylation site was not found by MS/MS, the site-directed mutagenesis results indicate that Ser-44 is the most preferred site in SISPRH1 for phosphorylation by SIMPK1. This residue (in the motif AIPLLSP) is conserved in closely related homologs of dicotyledonous plants, but not in monocotyledonous plants (Fig. S8). Phosphorylation of MAPK substrates often affect protein stability or turn-over rates. Phosphorylation by MPK4 increases the stability of MYB75 (Li et al. 2016c). By contrast, phosphorylation by MPK3 decreases the stability of WRKY46 (Sheikh et al. 2016). Palm-Forster et al. (2017) showed that the double phosphosite mutant of PH2 displayed enhanced stability compared to the WT. Therefore, we speculate that phosphorylation of Ser-44 by SIMPK1 may affect SISPRH1 stability, which in turn affects downstream signals. The motif AIPLLSP may act as a phosphorylated substrate for MAPK to play an important role in plant growth and development. Whether SISPRH1 is mostly phosphorylated on Ser-44 site after SIMPK1 activation in vivo remains to be established.

To explore the molecular function of SISPRH1 in HT responses, transgenic Arabidopsis plants expressing SISPRH1 were generated. SISPRH1-expressing plants showed shorter growth and lower antioxidant defense capacity than WT under HT stress (Fig. 7 and Fig. S9). These results suggest that SISPRH1 has a negative effect on HT tolerance. Furthermore, we predicted that the phosphorylation of site Ser-44 might be involved.
in the SlMPK1-mediated HT response. The mutation of S44A (SlSPRH1\textsuperscript{S44A}) blocked SlMPK1-mediated inhibition in protoplasts under HT (Fig. 8). These data suggest that SlSPRH1 acts downstream of SlMPK1 in the regulation of antioxidant defenses under HT. Future studies will be required to determine how the phosphorylation of S44A by SlMPK1 affects the function of the SlMPK1-SlSPRH1 cascade in tomato plants.

In summary, our data demonstrate that SlMPK1 responds to HT and plays a negative role in HT tolerance through regulating antioxidant defenses. SlSPRH1 is a phosphorylation substrate of SlMPK1, and the phosphorylation at Ser-44 by SlMPK1 is essential for SlMPK1-mediated responses to HT. These results suggest a possible molecular mechanism involving SlMPK1 in HT responses and provides insight into the MAPK cascade in tomato plants.
METHODS

Plant material and growth condition

Tomato (Solanum lycopersicum cv. OFSN), RNAi-SlMPK1 lines, SlMPK1-OE lines, Tobacco, and Arabidopsis (Col-0 ecotype background, wild-type and SISPRH1-OE lines) were used in this study. The germinated tomato seeds were sown in plastic pots containing compost soil mix and grown under a 14 h-light/10 h-dark photoperiod at 25°C/20°C (day/night) with 70% relative humidity. For the sterile culture, seeds were germinated on Murashige and Skoog (MS) medium under the same conditions. N. benthamiana seedlings were grown under a 16 h-light/8 h-dark photoperiod at 25°C with 70% relative humidity in a growth chamber. About 1-1.5-month-old N. benthamiana seedlings were used for transient expression. Arabidopsis plants were grown under 16 h-light/8 h-dark photoperiod at 23°C on ½ MS plates.

Partial purification of p47-MBPK and identification by mass spectrometry

The purification process was carried out as described in Ding et al. (2009) with slight modifications. Tomato leaves treated with 42°C for 2 h were harvested, frozen, and stored at -80°C. Frozen tomato leaves (1,300 g) were ground to a fine powder in the presence of liquid N2 and mixed with 1000 ml extraction buffer. The crude homogenate was centrifuged at 23,000 g for 1 h, and the resulting supernatant fraction was brought to 30% (NH4)2SO4 saturation. The pellets were then dissolved in buffer A for ultracentrifugation, and the supernatant was loaded onto a Sephadex G 25 M column for desalting. All chromatographic runs were carried out on the AKTA Purifier 100 system and the AKTA Prime System (GE-Healthcare). The fractions were loaded onto the following columns: Q-Sepharose FF column, Phenyl-Sepharose FF column, Q-Sepharose HP column, Mono QTM 5/50 GL column, and Poly-L-lysine-agarose column (see Supplemental Methods). Proteins from different stages of purification were resolved in a 12% polyacrylamide gel containing SDS and stained with silver. The 47-kDa band was excised and digested with trypsin and the tryptic digest was analyzed by LC-MS/MS system. Proteins were identified using MS/MS ion search of Mascot search engine (http://www.matrixscience.com, Matrix Science, London, England).

Generation of transgenic plants

For the RNAi construct, SlMPK1 (Solyc12g019460) was analyzed in the Sol Genomics Network (https://solgenomics.net/search/unigene.pl?unigene_id=576603). RNAi-SlMPK1 construct was made by introducing a fragment targeting the 201-bp 3'UTR of SlMPK1 containing XhoI, SpeI, Ncol, and BamHI restriction sites) into pGSA1285 vector (Adams-Phillips, 2003). For the tomato overexpression construct, the full-length cDNA of SlMPK1 was amplified with specific primers containing BamHI and SacI restriction sites and inserted into binary vector pBI121 driven by CaMV 35S promoter as described previously (Meli and Beachy, 2010). RNAi and overexpression of SlMPK1 in the putative lines were examined by RT-qPCR using specific primers (see Supplemental Method). Positive tomato T0 plants were transplanted to the plastic pots containing compost soil mix soil and grown in the greenhouse for the collection of T0 seeds. The kanamyicn spraying test was used in the genetic segregation analysis (Weide et al., 1989), and the single-copy homozygous T1 seeds were used for further study. To generate SISPRH1-expressing plants, the full-length cDNA of SISPRH1 was inserted into binary vector pBI121. The constructs were introduced into Arabidopsis (Col-0 ecotype) by the floral-dip method (Clough and Bent, 1998). Homozygous T3 seeds were harvested for further analysis.

High-temperature stress

For the HT tolerance assay, seedlings planted in matrix soils or sterile culture were used. The seedlings of WT-type and RNAi-SlMPK1 lines 1-14, 1-23, and 1-24 in the matrix soils at the five-leaf stage were
subjected to 38°C/28°C (day/night) for 3 d and then recovered at 25°C/20°C for 10 d. The RNAi-SlMPK1 transgenic line 1-24 in MS medium were used. After surface-sterilization at 25°C, seeds were allowed to germinate on MS medium at 25°C/20°C for 10 d. Seedlings of the same size were chosen and cut at the bottom of the hypocotyls using a sharp blade (The height of detached plantlets was kept at 2 cm). The detached plantlets were moved to a new MS medium grown at 25°C/20°C for another 3 d, incubated at 45°C for 1 h or 2 h, and then followed by recovery at 25°C/20°C for 14 d.

The seedlings of WT and SlMPK1-OE lines OE11 and OE15 in the matrix soils at the stage of three leaves were subjected to 38°C/28°C (day/night) for 1 d and then recovery at 25°C/20°C for 7 d. For sterile seedling treatment, seedlings of the same size were chosen and cut at the bottom of the hypocotyls using a sharp blade when two cotyledons were expanded completely. The detached plantlets were moved to a new MS medium grown at 25°C/20°C for 3 d, and then incubated at 45°C for 1 h or 2 h, followed by recovery at 25°C/20°C for 7 d.

To measure the hypocotyl length of Arabidopsis, the seeds were plated in rows on 1/2 MS medium, and the plates were covered with foil. After 2 days of cold treatment (4°C), the foil-wrapped plates were placed in a vertical position at 23°C for 3 days. Wrapped plates were then subjected to HT at 45°C for 1 h or 2 h. After that, the plates were incubated in a vertical position at 23°C under light or dark for another 6 days, and the hypocotyl length was measured. For Arabidopsis growth, 10-day-old Arabidopsis seedlings in 1/2 MS medium were subjected to HT treatment at 42°C for 1.5 h, followed by recovery for 10 days.

**Isobaric tag for relative and absolute quantitation (iTRAQ) proteomic assay**

Protein extraction and digestion: Total protein extraction from leaf tissue of three plants grown in different pots of each treatment (WT and RNAl-SlMPK1 (1-24) plants treated with normal conditions or 42°C for 4 h) were performed according to the method of Gong et al. (2014). iTRAQ labeling (Fig. S3) and SCX Fractionation and mass spectrometric analysis (see Supplemental Methods). Protein Identification: The MS/MS spectra were extracted and analyzed with ProteinPilot software (version 4.5, AB SCIEX) searching against an UniProt Solanum lycopersicum protein database (35,812 proteins, update in Sep. 2017). Detected protein threshold [Unused ProtScore (Conf)]: 0.05 (10.0%); FDR Analysis tab checked. All identified proteins had an Unused ProtScore of >1.3 (which corresponds to proteins identified with >95% confidence), as calculated by the software and a global false discovery rate (FDR) of ≤1% determined at the protein level by the PSPEP algorithm. To be considered as differentially expressed, proteins were required to have a p value of P < 0.05, as calculated by the software. The coefficient of variation (CV) was also calculated for each iTRAQ study. For protein abundance ratios measured using iTRAQ, fold-changes >1.2 or <0.67 were considered significant. Only proteins that were identified in two 8-plex iTRAQ were included. Protein ontology classification was performed using PANTHER classification system (http://pantherdb.org, PANTHER Overrepresentation Test/release 20170413, GO Ontology Solanum lycopersicum database Released 2017-10-24).

**Yeast two-hybrid (Y2H) screening**

The desired genes were cloned into the pGBKKT7 vectors and transformed into the Y2HGGold yeast strain (Clontech USA, Mountain View, CA). Then, the competent cells of single clone from the bait transformant (Y2HGgold) were transformed with the cDNA library plasmids constructed with HT-stressed tomato seedlings. The clones were selected on the SD/-Trp/-Leu/-His/-Ade/+AbA/+20 mM 3-AT medium (self-activation of SlMPK1 was effectively suppressed at 20 mM 3-AT), and blue colonies were considered to be potential positive clones. These clones were subsequently tested by PCR for the library plasmids. The full-length CDS of SlMPK1 and SlSPRH1 were cloned into the pGBKKT7 and pGADT7 vectors, respectively. The bait and prey constructs were co-transformed into Y2HGGold yeast strain using the lithium acetate method. Then,
transformants were selected on SD/-Trp/-Leu medium. The colonies were inoculated onto the SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade/+AbA/+X-α-gal/+20 mM 3-AT medium grown at 30 °C for 2-3 days, and photographed. To further confirm the interaction, the full-length CDS of SISPRH1 and SIMPK1 were cloned into the pGMBKT7 and pGADT7 vectors, respectively. pGMBKT7-53 and pGADT7-T were used as positive control.

**Bimolecular fluorescence complementation**

Full-length CDS of SIMPK1 and other genes without a stop codon were cloned into the pSPYCE and pSPYNE vectors, respectively. These constructs were transferred into Agrobacterium EHA105. Then cultures were resuspended in a freshly made solution containing 0.5% glucose, 50 mM MES-KOH, pH 5.6, 10 mM MgCl₂ and 0.1 mM acetosyringone. The OD₆₀₀ of the cell suspensions was adjusted to 0.5, incubated at room temperature for 2 hours before being mixed at a 1:1 ratio, and infiltrated into the leaves of 5-week-old *N. benthamiana*. Protoplasts were extracted after 40 hours of dark culture. Fluorescent signal was detected with a fluorescence microscope (Zeiss, Observer Z1, Germany). Co-expression of SIMPK1-cYFP with SIMPKK2-nYFP was used as positive control.

**Recombinant protein, in vitro pull-down, and in vitro phosphorylation assays**

The DNA fragment encoding full-length SIMPK1 was cloned into pCzn I vector with 6 × His tag at the N terminus of the protein. His-SIMPK1 was overexpressed by adding 0.5 mM of isopropylthio-β-galactoside (IPTG) and purified from *E. coli* BL21 (Plyss) cells by Ni-Agarose Beads. A constitutive-active form of SIMKK2 was developed by replacing the conserved Ser/Thr residues Thr-215 and Ser-221 with Asp (SIMKK2T215D/S221D, referred to hereafter as SIMKK2DD). DNA fragments encoding full-length SIMKK2DD or SISPRH1 were cloned into pGEX-4T-1 vector with GST tag at the N terminus of the protein. GST-SIMKK2DD and GST-SISPRH1 proteins were overexpressed by adding 0.5 mM IPTG and purified from *E. coli* Arctic Express cells by Glutathione Sepharose 4B Beads. In vitro phosphorylation assays were performed as previously described (Pérez-Salamó et al., 2014) with slight modifications. In vitro protein-protein interaction assays (pull-down) were carried out as described in Lin et al. (2014) with slight modifications (see Supplemental Methods).

**In vivo co-immunoprecipitation (Co-IP) assay**

Co-IP was carried out as described in Gou et al. (2015) with slight modifications. The HA and Myc tags are present in the pSPYCE-35S and pSPYNE-35S vector system (Schütze et al., 2009; Gou et al., 2015), so pSPYCE-SIMPK1 and pSPYNE-SISPRH1 can be immunoprecipitated using anti-HA or anti-Myc antibody. Agrobacteria GV3101 harboring each of the two SIMPK1-HA and SISPRH1-Myc constructs was solely or co-infiltrated into the abaxial side of 4-week old tobacco leaves (*N. benthamiana*) using a 1 ml needleless syringe according to http://www.bio-protocol.org/e95 (see Supplemental Method).

**Protein phosphorylation site determination by LC-MS/MS**

GST- SISPRH1 was phosphorylated by His-SIMPK1 in a kinase reaction (25 mM Tris-HCl pH7.5, 1 mM EGTA, 20 mM MgCl₂, 1 mM DTT and 1 × Phosphatase Inhibitor) in the presence of 200 μM ATP and GST-SIMKK2DD at room temperature for 1 h. As control, a parallel reaction without kinases (His-SIMPK1 and GST-SIMKK2DD) was included. After coomassie blue staining, GST- SISPRH1 protein bands were excised, de-stained and dried completely. Tryptic digests were analyzed on a LC-MS system consisting of Easy-nLC 1000 (Thermo Scientific, Bremen, Germany) coupled to a Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Acquired raw data files were processed using Proteome Discoverer software (V2.1, Thermo Fisher Scientific, Bremen, Germany)
and Sequest HT database search engines against a Solanum lycopersicum protein database (see Supplemental
Methods).

**Synthetic peptide phosphorylation site determination by MS/MS**

Five micrograms of each peptide (AIPLLSPSPTSPESDNSLKA or KKWQHPASPFCYEPAP,
synthesized by Ontores Biotechnology, Zhejiang were incubated with His-SIMPK1 and GST-SIMKK2DD in a
kinase reaction (25 mM Tris-HCl pH7.5, 1 mM EGTA, 20 mM MgCl2, 1 mM DTT, 200 µM ATP and 1 ×
Phosphatase Inhibit or) at room temperature for 1 h. The reaction buffer was centrifugally filtered through
a Millipore 5-kDa cutoff filter at 9,000 g at 4°C. The filtrate was purified and determined by LC-MS/MS.

**Subcellular localization**

To determine the subcellular localization of SISPRH1, SIMPK1 was cloned into the pEGAD vector
containing the GFP reporter gene to produce fusion construct pEGAD-SIMPK1-GFP under the control of the
35S. The fusion construct and the control vector (pEGAD) were separately transferred into Agrobacterium
tumefaciens strain EHA105 by heat shock and infiltrated into the leaves of 5-week-old N. benthamiana.
Protoplasts were extracted after 40 hours of dark culture, and fluorescent signals were detected using a
fluorescence microscope (Axio Observer Z1, Zeiss, Germany).

**Arabidopsis mesophyll protoplast transient expression**

SIMPK1, SISPRH1, or SISPRH1S44A were cloned into the pUC19 vector with two 35S enhancer promoters.
Protoplast isolation and transfection were carried out as previously described by Yoo et al. (2007). For
transient expression assays, isolated protoplasts were transfected with pUC19-35S-SIMPK1, pUC19-35S-
SISPRH1 or pUC19-35S-SISPRH1S44A plasmids alone, or co-transfected with pUC19-35S-SIMPK1 and
pUC19-35S-SISPRH1 or pUC19-35S-SISPRH1S44A by polyethylene glycol-mediated transformation at 25°C,
which were then kept at 36 °C for 3 hours. Total proteins were extracted for enzyme activities determination.

**Accession Numbers** SISPRH1 (Solyc06g053700); SIMPK1 (Solyc12g019460)

**Supplemental Material**

- **Fig. S1** Elution profiles of protein concentration and kinase activity from each chromatography step.
- **Fig. S2** Expression level of high-temperature responding genes under high-temperature stress.
- **Fig. S3** iTRAQ proteomics analysis of SIMPK1-mediated response to HT
- **Fig. S4** Prediction of functional protein networks involved in SIMPK1-mediated response to
high-temperature stress using STRING 9.0 applied to proteomic data.
- **Fig. S5** RT-qPCR analysis of transcript levels of genes coding SIMPK1-mediated proteins under HT (HT)
- **Fig. S6** Response of antioxidant defense to HT in the SIMPK1-OE plants.
- **Fig. S7** Summary of mass spectrometry-based mapping of in vitro phosphorylated residues in SISPRH1 by
SIMPK1.
- **Fig. S8** Multiple sequence alignments of conserved motif (AIPLLSP ) in plants.
- **Fig. S9** The response of antioxidant enzymes to HT in SISPRH1-OE plants.
- **Fig. S10** The expression level of SIMPK1 under HT.
- **Table S1** Proteins abundant in the tomato leaves of wild type under high-temperature stress.
- **Table S2** Proteins abundant in the tomato leaves of RNAi-SIMPK1 line 1-24 as compared to the wild type
under normal conditions.
- **Table S3** Proteins abundant in the tomato leaves of RNAi-SIMPK1 line 1-24 as compared to the wild type
under high-temperature conditions.
Table S4 Functional enrichment of identified proteins involved in SlMPK1-mediated response to HT with no Bonferroni correction.

Table S5 Functional enrichment of identified proteins involved in SlMPK1-mediated response to HT with Bonferroni correction.

Table S6 Similar proteins in the RNAi-SlMPK1 line 1-24 and anp2anp3 mutant.
Table 1: Proteins abundant in the leaves of RNAi-SIMPK1 tomato line 1-24 as compared to the wild type under HT conditions using iTRAQ

| UniProt ID | Gene ID | Name | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal | 16:11:14 PVal | 121:11 PVal |
|-----------|---------|------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|
| Q9M5A8    | Solyc07g0422 50 | CPN20 | 2.2284 | 0.00445 | 1.6144 | 0.04330 | 1.7567 | 0.0837 | 2.3335 | 0.02852 |
| C5IU71    | Solyc05g0526 00 | SBPASE | 2.2080 | 4.37E-05 | 1.3183 | 0.00801 | 1.6749 | 0.5166 | 5.8076 | 9.05E-01 |
| K4BAE6    | Solyc02g0827 60 | CAT2 | 1.9770 | 0.00157 | 3.1333 | 0.00022 | 1.3062 | 0.6446 | 1.8707 | 0.00136 |
| K4B1S1    | Solyc01g1034 50 | CPHSC70-2 | 1.9767 | 3.24E-05 | 1.8880 | 0.01473 | 0.7379 | 0.0904 | 4.1687 | 6.57E-02 |
| K4DAD5    | Solyc11g0697 90 | CPN60A | 1.8030 | 0.00306 | 2.7797 | 0.00334 | 1.5754 | 0.0249 | 1.8880 | 0.00038 |
| K4UCF4    | Solyc09g0652 70 | RRF | 1.7539 | 0.00311 | 1.5276 | 0.02924 | 1.7311 | 0.5023 | 1.9770 | 0.04506 |
| K4BW77    | Solyc05g0054 60 | NRX1 | 1.6444 | 0.03503 | 1.5704 | 0.03808 | 1.2022 | 0.0471 | 1.3062 | 0.16041 |
| K4CH99    | Solyc07g0641 60 | THI1 | 1.6293 | 0.00993 | 4.6132 | 0.00785 | 0.9376 | 0.2058 | 1.9589 | 0.00549 |
| K4BHA1    | Solyc03g0635 60 | GLU1 | 1.6144 | 6.86E-05 | 1.2942 | 0.04700 | 1.2803 | 0.9594 | 1.5704 | 1.68E-02 |
| K4CEJ1    | Solyc07g043320 | | 1.6144 | 0.00725 | 1.0186 | 0.65547 | 2.3768 | 0.0003 | 1.2474 | 0.02173 |
| Q672Q6    | Solyc02g0799 50 | PSBQ | 1.5276 | 0.00833 | 1.4723 | 0.00914 | 1.3908 | 0.0604 | 2.8054 | 0.00419 |
| K4AYG3    | Solyc01g0877 30 | PRPL1 | 1.4860 | 0.01086 | 1.7865 | 0.00430 | 0.5058 | 0.0143 | 2.0512 | 0.02356 |
| K4BW79    | Solyc05g0054 60 | AOR | 1.4859 | 0.00513 | 1.5996 | 0.08376 | 1.2909 | 0.0018 | 2.2699 | 0.03217 |
| Q5NE20    | Solyc02g0868 20 | CA1 | 1.4454 | 0.01144 | 1.8197 | 0.03724 | 0.3162 | 0.0137 | 2.5586 | 0.00038 |
| K4B1F9    | Solyc01g102310 | | 1.4191 | 0.00155 | 1.1482 | 0.26353 | 1.6904 | 0.0180 | 1.5849 | 0.01383 |
| K4CQW8    | Solyc09g0093 90 | MDAR1 | 1.3062 | 0.03410 | 1.0765 | 0.99351 | 2.0137 | 0.0321 | 1.3727 | 0.01575 |
| K4AV63    | Solyc01g0288 10 | CPN60B2 | 1.2942 | 0.00080 | 2.6062 | 0.04669 | 0.7178 | 0.5586 | 2.0324 | 0.02828 |
| K4BX77    | Solyc05g0090 30 | IMD2 | 1.2706 | 0.02061 | 1.6293 | 0.03579 | 1.5560 | 0.9938 | 3.0479 | 0.00764 |
| K4BMN4    | Solyc03g1208 50 | CPN60B1 | 1.2482 | 0.03575 | 2.2284 | 0.00645 | 1.0765 | 0.0558 | 3.0479 | 0.00764 |
| K4B0D9    | Solyc01g0975 20 | ANNAT4 | 0.2089 | 0.00403 | 0.7516 | 0.48853 | 0.6546 | 0.0491 | 0.2378 | 0.00022 |
The uniprotKB ID entries were obtained via Uniprot (http://www.uniprot.org) and the GeneIDs were translated using UniProt's ID Mapping.

Name, the name of the detected protein.

I and II indicate two biological replicates using an iTRAQ 8-plex and III and IV indicate another two replicates in another iTRAQ 8-plex.

The values were calculated as the ratio of 116 and 121 to 114 and 118 label, respectively. 116 and 121, RNAi-SlMPK1 line 1-24 under HT; 114 and 118, wild-type (WT). Only proteins identified with Peptides>1 and quantitation results with Pval<0.05 were considered to be statistically different from unity. The proteins were considered to be differentially expressed if their iTRAQ ratios were >1.2 or <0.67. The raw data was shown in Table S3. Proteins significantly different protein ratios (M:W) in at least three of four biological replicates have been shown here. Significant changes are indicated by boldface type.
Fig. 1. The identification of HT-activated SlMPK1. A, HT-activated MBP kinase. Time course of induction of MBP kinase activities by HT (up) and Tyr phosphorylation of HT-activated MBP kinase (down). B, Analysis of purification pools by gel electrophoresis (up) and in-gel kinase assay (down). Lane 1, marker standards; lane 2, pooled fraction from Poly-L-lysine-agarose column; lane 3, pooled fractions from Mono Q TM 5/50 GL column; lane 4, pooled fractions from Q-Sepharose HP column; lane 5, pooled fractions from Phenyl-Sepharose FF column; lane 6, pooled fractions from Q-Sepharose FF column. Proteins from different stages of purification were resolved in a 12% polyacrylamide gel containing SDS and stained with silver (up). The fractions were loaded onto a 12% SDS-polyacrylamide gel embedded with MBP and in-gel kinase assay was performed (down). C, Identification of SlMPK1 by MS/MS. 47-kDa band from SDS-PAGE was cut and protein in gel fragment was digested with trypsin followed by MALDI-TOF/TOF-MS/MS analyses. Proteins were identified by Mascot database searches. Matched peptides were shown in blue. D, MS/MS spectrum of the selected peptide (m/z 1777.7845). The database matching indicated this is a fragment (ESIAFNPEYQR) of SlMPK1. E, The immunoprecipitation kinase analysis of HT-induced SlMPK1. Total proteins were extracted from the HT-treated leaves at the indicated time and the SlMPK1 activity was measured.

Fig. 2. Effects of SlMPK1 suppression on HT tolerance of tomato seedlings. A, Schematic diagram of the SlMPK1-RNAi construct and the expression level of SlMPK1 in the SlMPK1-RNAi plants. The closed green arrows represent the partial sequence of SlMPK1 (up). UTR, untranslated regions; 35S P, 35S promoter; OCS, OCS terminators; Restriction sites used are shown. RT-qPCR analysis of transcript level of SlMPK1 (down) of partial transgenic lines. The numbers indicate independent RNAi transgenic plants. B, Phenotype of RNAi-SlMPK1 lines in the nutritional bowl feeding matrix soils under HT. The seedlings of WT and RNAi-SlMPK1 line 1-14, 1-23, and 1-24 at the stage of five leaves were subjected to 38 °C/28 °C for 3 d and then recovered at 25 °C/20 °C for 10 d. C, Phenotype of disc leaves in RNAi-SlMPK1 lines under HT. The disc leaves from the fourth fully expanded leaves were incubated at 45 °C for 2 h, followed by recovery at 25 °C for 2 d. D, The chlorophyll content of the disc leaves of RNAi-SlMPK1 transgenic plants under HT shown in C. Error bars are ± SD values of three replicates. E, Phenotype of RNAi-SlMPK1 line 1-24 in Murashige and Skoog (MS) medium under HT. F, The fresh weight and plant length of WT and 1-24 shown in E. Statistical differences among the samples shown in A, D, and F are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).

Fig. 3. Response of antioxidant defense to HT in the RNAi-SlMPK1 plants. H2O2 accumulation (A), the accumulation of membrane lipid peroxidation product MDA (B), the activities of SOD(C), POD(D), CAT(E), and APX(F), and the content of non-enzymatic antioxidant AsA (G) and GSH (H) in the wild-type (WT) and RNAi-SlMPK1 line 1-24 under control (25 °C) or HT (42 °C) for 4 h. Error bars are ± SD values of three replicates. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).

Fig. 4. Effects of SlMPK1 overexpression on HT tolerance of tomato seedlings. A, The expression level of SlMPK1 in the SlMPK1-OE plants. RT-qPCR analysis of transcript abundance of SlMPK1 in two independent OE transgenic plants. B, The chart of material combination of C. C, Phenotype of SlMPK1-OE lines in the nutritional bowl feeding matrix soils under HT. The seedlings of wild-type (WT) and SlMPK1-OE line OE11 and OE15 at the stage of three leaves were subjected to 38 °C/28 °C for 1 d and then recovery at 25 °C/20 °C for 1 d (R1), 2 d (R2), 4 d (R4) and 7 d (R7). D, Phenotype of SlMPK1-OE
transgenic line OE11 under HT. The detached plants were planted in a new MS medium grown at 25 °C/20 °C for 3 d and then incubated at 45 °C for 1 h or 2 h, followed by recovery at 25 °C/20 °C for 7 d. E, Fresh weight and plant length of WT and SIMPK1-OE transgenic line OE11 in MS medium under HT as shown in D. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).

**Fig. 5.** Interactions between SIMPK1 and SISPRH1. A, Yeast two-hybrid (Y2H) assay of interactions between SIMPK1 and SISPRH1. SIMPK1 (as bait) was cloned into pGBKTT7 (BD) and SISPRH1 (as prey) was cloned into the pGADT7 (AD). AD-T and BD-p53 was used as a positive control, and AD-EV and SIMPK1-BD was used as a negative control. Simultaneously, SISPRH1 (as bait) was cloned into pGBKTT7 (BD) and SIMPK1 (as prey) were cloned into the pGADT7 (AD). AD-SIMPK1 and BD-EV was used as a negative control. B, Bimolecular fluorescence complementation (BiFC) assay for detecting molecular interactions between SIMPK1 and proteins (SIMKK2 or SISPRH1) transiently co-expressed in tobacco leaf protoplasts. SIMPK1 was fused with the C-terminus of YFP and SIMKK2 and SISPRH1 were fused with the N-terminus of YFP. The images were obtained from the YFP channel, DIC channel, and a merged image of the two channels. The positive control was SIMKK2-nYFP/ SIMPK1-cYFP and the negative control was EV-nYFP and SIMPK1-cYFP. Scale bar = 50 μm. C, SIMPK1 physically interacts with SISPRH1. His-tagged SIMPK1 was incubated with immobilized GST or GST-tagged SISPRH1. Beads were washed, fractionated in 12% (w/v) SDS-PAGE and subjected to immunoblot analysis using an antibody against His (up) or GST (down). Immobilized GST was used as a negative control and GST-tagged SIMKK2DD was used as a positive control. D, In vivo coimmunoprecipitation (Co-IP) assay of SIMPK1 and SISPRH1 interaction in Tobacco leaves. Crude lysates pre-cleared by protein A Sepharose beads (Input) were IP-ed with anti-HA antibody and then detected with anti-HA and anti-Myc antibodies for SIMPK1-HA and SISPRH1-Myc, respectively.

**Fig. 6.** Phosphorylation of SISPRH1 by SIMPK1. A, Identification of phosphorylation sites of SIMPK1-targeted SISPRH1 using LC-MS/MS analysis. Amino acid sequence of SISPRH1 and Arabidopsis homologous AT1G04330 phosphorylation sites marked. The potential MAPK target sites (S/T-P) are boxed in SISPRH1 sequence, where S/T-P detected as being phosphorylated are marked in brown in the SIMPK1-treated SISPRH1 protein or blue in the background SISPRH1 protein and those detected as being phosphorylated by AtMPK6 are shown in orange (Palm-Forster et al., 2012). Part MS/MS spectra are shown in Fig. S7. B, In vitro phosphorylation of purified wild-type and mutant SISPRH1 substrate by SIMPK1. His-tagged SIMPK1 (His-SIMPK1) and GST tagged SISPRH1 (GST-SISPRH1) were used in phosphorylation reactions with 32P labeling. 4x mutant indicates all identified Ser residues Ser-44, -49, -52, -94 changed to Ala, 3xmut indicates all Ser changed to Ala, except the Ser-44, and 1xmut indicates only Ser-44 changed to Ala. Constitutive-active form of SIMKK2DD was used to activate SIMPK1 and also used as a negative control for unspecific phosphorylation of SISPRH1 protein. Phosphorylated SISPRH1 was visualized by Typhoon phosphorimager after gel electrophoresis. The experiment was repeated at least three times with similar results. C, Mass spectrometry analysis of synthetic peptides phosphorylated in vitro by SIMPK1. Synthetic peptides were incubated with His-SIMPK1 and GST-SIMKK2DD in a kinase reaction, which was then centrifugally filtered through a Millipore 5-kDa cutoff filter. The filtrate was determined by LC-MS/MS. The arrow indicates the phosphorylation sites of the peptide. The phosphorylated sites are marked in black in A.

**Fig. 7.** SISPRH1 negatively regulates Arabidopsis tolerance to HT stress. A, SISPRH1 expression is induced
by HT. RT-qPCR analyses of transcript abundance of *SlSPRH1* in the leaves of wild-type (WT) tomato plants during HT (42 °C) for 0, 1, 3, and 6 h. B, Subcellular localization of *SlSPRH1*. The *SlSPRH1*-GFP protein and GFP, driven by CaMV 35S, were separately transformed into tobacco protoplast and visualized by fluorescence microscopy. Images were taken in representative cells expressing GFP (up) or *SlSPRH1*-GFP fusion protein (down) under bright field (middle) or dark field (left). The merged images are shown (right). Scale bar = 50 μm. C, Hypocotyl length of WT and *SlSPRH1*-expressing Arabidopsis lines in ½ MS medium under HT stress. After germination for 3 days, the plates covered with foil were subjected to HT treatment at 45 °C for 1 and 2 h, followed by vertical culturing in the dark for 6 days. D, Expression of *SlSPRH1* in *SlSPRH1*-expressing lines. RT-qPCR analyses of transcript abundance of *SlSPRH1* in the three independent transgenic plants. *ACTIN* was used as internal control. E, Phenotype of *SlSPRH1*-expressing transgenic line 3-13, 6-15 and 2-11 under HT stress. Ten-day-old *Arabidopsis* seedlings in ½ Murashige and Skoog (MS) medium (left) were subjected to HT treatment at 42 °C for 1.5 h, followed by recovery for 10 days (right). F, The response of antioxidant enzymes to HT in the *SlSPRH1*-expressing plants. Two-weeks-old wild-type and transgenic line 3-13 and 6-15 plants were treated with HT for 3 h. The activities of CAT and APX were determined. Error bars are ± SD values of three replicates. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).

**Fig. 8.** The effects of S44 mutation on SIMPK1-mediated antioxidant defense under HT stress. Arabidopsis protoplasts transiently expressing *SIMPK1* alone, *SlSPRH1* alone, *SlSPRH1*<sup>S44A</sup> alone, *SIMPK1* and *SlSPRH1* simultaneously, *SIMPK1* and *SlSPRH1*<sup>S44A</sup> simultaneously or empty vector (EV) were kept at 25 °C for 1 h and then kept at 36 °C for 3 hours. The activities of APX and CAT were measured as described in experimental procedures. Values are means ± SD of three different experiments. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).

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Fig. 1. The identification of HT-activated SlMPK1. A, HT-activated MBP kinase. Time course of induction of MBP kinase activities by HT (up) and Tyr phosphorylation of HT-activated MBP kinase (down). B, Analysis of purification pools by gel electrophoresis (up) and in-gel kinase assay (down). Lane 1, marker standards; lane 2, pooled fraction from Poly-L-lysine-agarose column; lane 3, pooled fractions from Mono QTM 5/50 GL column; lane 4, pooled fractions from Q-Sepharose HP column; lane 5, pooled fractions from Phenyl-Sepharose FF column; lane 6, pooled fractions from Q-Sepharose FF column. Proteins from different stages of purification were resolved in a 12% polyacrylamide gel containing SDS and stained with silver (up). The fractions were loaded onto a 12% SDS-polyacrylamide gel embedded with MBP and in-gel kinase assay was performed (down). C, Identification of SIMPK1 by MS/MS. 47-kDa band from SDS-PAGE was cut and protein in gel fragment was digested with trypsin followed by MALDI-TOF/TOF-MS/MS analyses. Proteins were identified by Mascot database searches. Matched peptides were shown in blue. D, MS/MS spectrum of the selected peptide (m/z 1777.7845). The database matching indicated this is a fragment (ESIAFNPYEYQR) of SIMPK1. Abs. Int., absolute intensity of Y-axis. E, The immunoprecipitation kinase analysis of HT-induced SIMPK1. Total proteins were extracted from the HT-treated leaves at the indicated time and the SIMPK1 activity was measured.
Fig. 2. Effects of \textit{SIMPK1} suppression on HT tolerance of tomato seedlings. A, Schematic diagram of the \textit{SIMPK1}-RNAi construct and the expression level of \textit{SIMPK1} in the \textit{SIMPK1}-RNAi plants. The closed green arrows represent the partial sequence of \textit{SIMPK1} (up). UTR, untranslated regions; 35S P, 35S promoter; OCS, OCS terminators; Restriction sites used are shown. RT-qPCR analysis of transcript level of \textit{SIMPK1} (down) of partial transgenic lines. The numbers indicate independent RNAi transgenic plants. B, Phenotype of RNAi-\textit{SIMPK1} lines in the nutritional bowl feeding matrix soils under HT. The seedlings of WT and RNAi-\textit{SIMPK1} line 1-14, 1-23, and 1-24 at the stage of five leaves were subjected to 38°C/28°C for 3 d and then recovered at 25°C/20°C for 10 d. C, Phenotype of disc leaves in RNAi-\textit{SIMPK1} lines under HT. The disc leaves from the fourth fully expanded leaves were incubated at 45°C for 2 h, followed by recovery at 25°C/20°C for 2 d. D, The chlorophyll content of the disc leaves of RNAi-\textit{SIMPK1} transgenic plants under HT shown in C. Error bars are ± SD values of three replicates. E, Phenotype of RNAi-\textit{SIMPK1} line 1-24 in Murashige and Skoog (MS) medium under HT. F, The fresh weight and plant length of WT and 1-24 shown in E. Statistical differences among the samples shown in A, D, and F are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).
Fig. 3. Response of antioxidant defense to HT in the RNAi-SlMPK1 plants. H$_2$O$_2$ accumulation (A), the accumulation of membrane lipid peroxidation product MDA (B), the activities of SOD(C), POD(D), CAT(E), and APX(F), and the content of non-enzymatic antioxidant AsA (G) and GSH (H) in the wild-type (WT) and RNAi-SlMPK1 line 1-24 under control (25°C) or HT (42°C) for 4 h. Error bars are ± SD values of three replicates. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).
Fig. 4. Effects of *SIMPK1* overexpression on HT tolerance of tomato seedlings. A, The expression level of *SIMPK1* in the *SIMPK1*-OE plants. RT-qPCR analysis of transcript abundance of *SIMPK1* in two independent OE transgenic plants. B, The chart of material combination of C. C, Phenotype of *SIMPK1*-OE lines in the nutritional bowl feeding matrix soils under HT. The seedlings of wild-type (WT) and *SIMPK1*-OE line OE11 and OE15 at the stage of three leaves were subjected to 38°C/28°C for 1 d and then recovery at 25°C/20°C for 1 d (R1), 2 d (R2), 4 d (R4) and 7 d (R7). D, Phenotype of *SIMPK1*-OE transgenic line OE11 under HT. The detached plants were planted in a new MS medium grown at 25°C/20°C for 3 d and then incubated at 45°C for 1 h or 2 h, followed by recovery at 25°C/20°C for 7 d. E, Fresh weight and plant length of WT and *SIMPK1*-OE transgenic line OE11 in MS medium under HT as shown in D. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).
Fig. 5. Interactions between SlMPK1 and SISPRH1. A, Yeast two-hybrid (Y2H) assay of interactions between SlMPK1 and SISPRH1. SlMPK1 (as bait) was cloned into pGBK7 (BD) and SISPRH1 (as prey) was cloned into the pGADT7 (AD). AD-T and BD-p53 was used as a positive control, and AD-EV and SlMPK1-BD was used as a negative control. Simultaneously, SISPRH1 (as bait) was cloned into pGBK7 (BD) and SlMPK1 (as prey) were cloned into the pGADT7 (AD). AD-SlMPK1 and BD-EV was used as a negative control. B, Bimolecular fluorescence complementation (BiFC) assay for detecting molecular interactions between SlMPK1 and proteins (SlMKK2 or SISPRH1) transiently co-expressed in tobacco leaf protoplasts. SlMPK1 was fused with the C-terminus of YFP and SlMKK2 and SISPRH1 were fused with the N-terminus of YFP. The images were obtained from the YFP channel, DIC channel, and a merged image of the two channels. The positive control was SlMKK2-nYFP/ SlMPK1-cYFP and the negative control was EV-nYFP and SlMPK1-cYFP. Scale bar = 50 μm. C, SlMPK1 physically interacts with SISPRH1. His-tagged SlMPK1 was incubated with immobilized GST or GST-tagged SISPRH1. Beads were washed, fractionated in 12% (w/v) SDS-PAGE and subjected to immunoblot analysis using an antibody against His (up) or GST (down). Immobilized GST was used as a negative control and GST-tagged SIMKK2 was used as a positive control. D, In vivo coimmunoprecipitation (Co-IP) assay of SlMPK1 and SISPRH1 interaction in Tobacco leaves. Crude lysates pre-cleared by protein A Sepharose beads (Input) were IP-ed with anti-HA antibody and then detected with anti-HA and anti-Myc antibodies for SlMPK1-HA and SISPRH1-Myc, respectively.
Fig. 6 Phosphorylation of SISPRH1 by SIMPK1. A, Identification of phosphorylation sites of SIMPK1-targeted SISPRH1 using LC-MS/MS analysis. Amino acid sequence of SISPRH1 and Arabidopsis homologous AT1G04330 phosphorylation sites marked. The potential MAPK target sites (S/T-P) are boxed in SISPRH1 sequence, where S/T-P detected as being phosphorylated are marked in brown in the SIMPK1-treated SISPRH1 protein or blue in the background SISPRH1 protein and those detected as being phosphorylated by AtMPK6 are shown in orange (Palm-Forster et al., 2012). Part MS/MS spectra are shown in Fig. S7. B, In vitro phosphorylation of purified wild-type and mutant SISPRH1 substrate by SIMPK1. His-tagged SIMPK1 (His-SIMPK1) and GST tagged SISPRH1 (GST-SISPRH1) were used in phosphorylation reactions with $^{32}$P labeling. 4x mutant indicates all identified Ser residues Ser-44, -49, -52, -94 changed to Ala, 3xmut indicates all Ser changed to Ala, except the Ser-44, and 1xmut indicates only Ser-44 changed to Ala. Constitutive-active form of SIMKKK$^{2\text{DD}}$ was used to activate SIMPK1 and also used as a negative control for unspecific phosphorylation of SISPRH1 protein. Phosphorylated SISPRH1 was visualized by Typhoon phosphorimager after gel electrophoresis. The experiment was repeated at least three times with similar results. C, Mass spectrometry analysis of synthetic peptides phosphorylated in vitro by SIMPK1. Synthetic peptides were incubated with His-SIMPK1 and GST-SIMKKK$^{2\text{DD}}$ in a kinase reaction, which was then centrifugally filtered through a Millipore 5-kDa cutoff filter. The filtrate was determined by LC-MS/MS. The arrow indicates the phosphorylation sites of the peptide. The phosphorylated sites are marked in black in A.
**Fig. 7.** SISPRH1 negatively regulates Arabidopsis tolerance to HT stress. A. *SisPRH1* expression is induced by HT. RT-qPCR analyses of transcript abundance of *SisPRH1* in the leaves of wild-type (WT) tomato plants during HT (42°C) for 0, 1, 3, and 6 h. B. Subcellular localization of SISPRH1. The SISPRH1-GFP protein and GFP, driven by CaMV 35S, were separately transformed into tobacco protoplast and visualized by fluorescence microscopy. Images were taken in representative cells expressing GFP (up) or SISPRH1-GFP fusion protein (down) under bright field (middle) or dark field (left). The merged images are shown (right).
Scale bar = 50 μm. C, Hypocotyl length of WT and SlSPRH1-expressing Arabidopsis lines in ½ MS medium under HT stress. After germination for 3 days, the plates covered with foil were subjected to HT treatment at 45 °C for 1 and 2 h, followed by vertical culturing in the dark for 6 days. D, Expression of SlSPRH1 in SlSPRH1-expressing lines. RT-qPCR analyses of transcript abundance of SlSPRH1 in the three independent transgenic plants. ACTIN was used as internal control. E, Phenotype of SlSPRH1-expressing transgenic line 3-13, 6-15 and 2-11 under HT stress. Ten-day-old Arabidopsis seedlings in ½ Murashige and Skoog (MS) medium (left) were subjected to HT treatment at 42 °C for 1.5 h, followed by recovery for 10 days (right). F, The response of antioxidant enzymes to HT in the SlSPRH1-expressing plants. Two-weeks-old wild-type and transgenic line 3-13 and 6-15 plants were treated with HT for 3 h. The activities of CAT and APX were determined. Error bars are ± SD values of three replicates. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).
Fig. 8. The effects of S44 mutation on SIMPK1-mediated antioxidant defense under HT stress. Arabidopsis protoplasts transiently expressing SIMPK1 alone, SISPRH1 alone, SISPRH1S44A alone, SIMPK1 and SISPRH1 simultaneously, SIMPK1 and SISPRH1S44A simultaneously or empty vector (EV) were kept at 25 °C for 1 h and then kept at 36 °C for 3 hours. The activities of APX and CAT were measured as described in experimental procedures. Values are means ± SD of three different experiments. Statistical differences among the samples are labeled with different letters according to the LSD test (P < 0.05, one-way ANOVA).
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