Tomato SISAP3, a member of the stress-associated protein family, is a positive regulator of immunity against *Pseudomonas syringae pv. tomato* DC3000

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SUMMARY

Tomato stress-associated proteins (SAPs) belong to A20/AN1 zinc finger protein family, some of which have been shown to play important roles in plant stress responses. However, little is known about the functions and underlying molecular mechanisms of SAPs in plant immune responses. In the present study, we reported the function of tomato SISAP3 in immunity to *Pseudomonas syringae pv. tomato* (Pst) DC3000. Silencing of SISAP3 attenuated while overexpression of SISAP3 in transgenic tomato increased immunity to Pst DC3000, accompanied with reduced and increased Pst DC3000-induced expression of SA signalling and defence genes, respectively. Flg22-induced reactive oxygen species (ROS) burst and expression of PAMP-triggered immunity (PTI) marker genes SPl15 and Sll122 were strengthened in SISAP3-OE plants but were weakened in SISAP3-silenced plants. SISAP3 interacted with two SlBOBs and the A20 domain in SISAP3 is critical for the SISAP3-SlBOB1 interaction. Silencing of SlBOB1 and co-silencing of all three SlBOB genes conferred increased resistance to Pst DC3000, accompanied with increased Pst DC3000-induced expression of SA signalling and defence genes. These data demonstrate that SISAP3 acts as a positive regulator of immunity against Pst DC3000 in tomato through the SA signalling and that SISAP3 may exert its function in immunity by interacting with other proteins such as SlBOBs, which act as negative regulators of immunity against Pst DC3000 in tomato.

Keywords: immune response, NudC, *Pseudomonas syringae pv. tomato* DC3000, stress-associated proteins, tomato.

INTRODUCTION

Plants defend themselves against pathogen attack by deploying a multi-layered immune system, which involve inducible defence responses and constitutive physical barriers (Jones and Dangl, 2006). The first layer of immune response is activated through detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which stimulate PAMP-triggered immunity (PTI) (Boller and Felix, 2009; Macho and Zipfel, 2014; Schwessinger and Ronald, 2012). The second layer of immune response is often initiated after specific recognition of pathogen effectors by intracellular resistance (R) proteins, which are commonly known as effector-triggered immunity (ETI) (Jones and Dangl, 2006). The defence responses associated with PTI and ETI may share some common signalling components and often trigger several early defence responses to restrict pathogen growth and spread (Boiler and Felix, 2009; Meng and Zhang, 2013).

Stress-associated protein (SAP) family is characterized by the presence of A20/AN1 zinc-finger domains and is highly conserved in all plant species (Giri et al., 2013). However, little is known about the function of A20/AN1 proteins in plant disease resistance. Accumulating evidence revealed an important role for SAPs in plant immunity. Overexpression of rice OsSAP1 in tobacco resulted in enhanced resistance against virulent bacterial pathogen, accompanied with up-regulated expression of defence genes (Kothari et al., 2016; Tyagi et al., 2014). By contrast, it was found that overexpression of Arabidopsis AtSAP9, which was induced by pathogen, PAMP molecules and phytohormones, led to increased susceptibility to non-host pathogen *Pseudomonas syringae pv. phaseolicola*, indicating that AtSAP9 plays key roles in basal resistance (Kang et al., 2017). Recently, an orchid SAP protein Pha13 and its Arabidopsis homologue AtSAP5 were reported to serve as an important regulatory hub in plant antiviral immunity (Chang et al., 2018). It was shown that SAPs regulate various stress responses by modulating phytohormone signalling cascades, which are mediated by JA, SA, ET and ABA. Interestingly, Arabidopsis AtSAP5 and AtSAP9 and rice OsSAP7 and OsSAP11 prefer to negatively regulate phytohormone signalling pathways (Kang et al., 2013, 2017, 2013, 2017; Liu et al., 2011; Sharma et al., 2015), while Pha13 positively regulates the expression of two...
SA responsive genes PhaRdR1 and PhaGRX (Chang et al., 2018).

The biochemical function of SAPs has been shown to be associated with the ubiquitin/26S proteasome (UPS)-mediated proteolysis system through acting as E3 ligases or interaction with UPS components. It was found that AtSAP5, acting as E3 ubiquitin ligase, plays a role as a positive regulator of drought stress responses (Choi et al., 2012; Kang et al., 2011, 2013). Besides, some SAPs were found to interact with UPS components, such as ubiquitin receptors RAD23s, which are capable of targeting ubiquitylated substrates to UPS (Farmer et al., 2010; Fu et al., 2010; Saeki, 2017). For instance, Arabidopsis AtSAP5, Prunus PpSAP1 and orchid Pha13 were found to interact with polyubiquitinated proteins (Chang et al., 2018; Choi et al., 2012; Lloret et al., 2017). AtSAP9 was found to interact with RAD23b and RAD23d, which act as shuttling factors of ubiquitin conjugates (Farmer et al., 2010; Guzder et al., 1998; Kang et al., 2017).

Despite these recent studies, a clear scenery of the role of SAPs and the mechanism by which SAPs regulate plant stress responses remain elusive. There are 13 members in tomato SlSAP family (Solanke et al., 2009). In the present study, we performed functional analyses using virus-induced gene silencing (VIGS) for the roles of tomato SlSAPs in disease resistance against Pseudomonas syringae pv. tomato (Pst) DC3000. We found that silencing of SlSAP3 resulted in decreased resistance whereas overexpression of SlSAP3 in transgenic tomato led to enhanced resistance to Pst DC3000, accompanied with decreased and increased Pst DC3000-induced expression of SA signalling and defence genes, respectively. We also found that SlSAP3 interacted with SlBOBBER1 (SlBOB1) and SlBOBBER2 (SlBOB2), tomato orthologues of eukaryotic NudC domain proteins and that silencing of SlBOB1 or co-silencing of three SlBOB genes resulted in enhanced resistance to Pst DC3000. Our data demonstrated that SlSAP3 positively regulates immunity to Pst DC3000 through SA signalling, possibly via the UPS pathway through interacting with SlBOBs.

RESULTS

Silencing of SlSAP3 resulted in reduced resistance to Pst DC3000

To examine the possible involvement of SlSAPs in disease resistance, we performed functional analyses by VIGS approach. For this purpose, a specific fragment for each SlSAP gene (Table S1, see Supporting Information) was chosen to generate VIGS constructs (Liu et al., 2002). The silencing efficiency for a target SlSAP gene was approximately 70% (Fig. S1A, see Supporting Information). Besides, silencing specificity of SlSAP3 was also examined (Fig. S1B, see Supporting Information). The silencing efficiencies for each of the SlSAP genes and specificity for SlSAP3 were satisfied for further experiments.

Next, we examined the changes in resistance of these pTRV-SlSAPs-infiltrated tomato plants to Pst DC3000. In our experiments, necrotic lesions on leaves of the pTRV-SlSAP3-infiltrated and pTRV-SlSAP10-infiltrated plants were larger and denser than those in the pTRV-GUS-infiltrated plants (Fig. 1A). At 3 days post-inoculation (dpi), the bacterial population in the inoculated leaves of the pTRV-SlSAP3-infiltrated and pTRV-SlSAP10-infiltrated plants showed approximately eightfold and 10-fold higher over those in the pTRV-GUS-infiltrated plants, respectively (Fig. 1B). These results indicate that silencing of either SlSAP3 or SlSAP10 resulted in reduced resistance to Pst DC3000. The pTRV-SlSAP10-infiltrated plants showed an earlier yellowing and senescent phenotype and thus, we focused on SlSAP3 to explore its function and mechanism in immune response against Pst DC3000.

The responsiveness of SlSAP3 to Pst DC3000 was also examined. As shown in Fig. 1C, the expression level of SlSAP3 in Pst DC3000-infected plants started to increase at 1 dpi and gradually increased over a period of 3 days. These results indicate that SlSAP3 responds to Pst DC3000.

Overexpression of SlSAP3 led to increased resistance against Pst DC3000

To further confirm the function of SlSAP3 in tomato immunity against Pst DC3000, we transformed 35S promoter driven overexpression SlSAP3 construct fused at the C-terminal with a HA tag (35S:SlSAP3-HA) into tomato cv. Ailsa Craig by Agrobacterium-mediated transformation (Howe et al., 1996). A total of 13 independent transgenic lines were initially obtained and four transgenic homozygous lines were isolated. The transcript levels of SlSAP3 in overexpression lines were validated by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) (Fig. 2A), and the accumulation of SlSAP3-HA fusion protein was detectable using antibody against HA tag (Fig. 2B). Two transgenic lines, SlSAP3-OE-3# and SlSAP3-OE-7#, were chosen for further studies as they had a relatively high level of expression of SlSAP3.

Disease phenotypic analyses showed that Pst DC3000-caused lesions on leaves of SlSAP3-OE plants were smaller and thinner than those in wild-type (WT) plants (Fig. 2C). Accordingly, at 3 dpi, the bacterial population in the inoculated leaves of SlSAP3-OE plants were significantly reduced as compared with those in WT plants (Fig. 2D). These results indicate that overexpression of SlSAP3 intensified tomato resistance against Pst DC3000.

Modification of SlSAP3 expression affected Pst DC3000-induced defence response

To explore whether modification of SlSAP3 expression affected the pathogen-induced defence response, we analysed
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A  pTRV-GUS  pTRV-SISAP1  pTRV-SISAP2  pTRV-SISAP3  pTRV-SISAP4  pTRV-SISAP5  pTRV-SISAP6

pTRV-SISAP7  pTRV-SISAP8  pTRV-SISAP9  pTRV-SISAP10  pTRV-SISAP11  pTRV-SISAP12  pTRV-SISAP13

B

- **Bacterial Growth (log_{10} CFU/cm^2)**

- **Mock**
- **Pst DC3000**

C

- **Relative Expression (folds)**

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and compared the expression of defence genes after infection by *Pst DC3000*. As shown in Fig. 3, the expression levels of some selected defence genes including SlPR1a, SlPR1b, SlPR-P2, SlEDS1, SlLapA1 and SlERF1 in SlSAP3-OE and pTRV-SlSAP3-infiltrated plants were comparable to those in WT or pTRV-GUS-infiltrated plants without *Pst DC3000* infection. At 24 h after infection by *Pst DC3000*, up-regulated expression of SlPR1a, SlPR1b, SlPR-P2 and SlEDS1 in SlSAP3-OE plants were observed as compared with those in WT plants (Fig. 3). By contrast, down-regulated expression of SlPR1a, SlPR1b, SlPR-P2 and SlEDS1 in pTRV-SlSAP3-infiltrated plants was detected as compared with those in pTRV-GUS-infiltrated plants, at 24 h after *Pst DC3000* infection (Fig. 3). However, the *Pst DC3000*-induced expression levels of SlLapA1 and SlERF1 in SlSAP3-OE plants and in pTRV-SlSAP3-infiltrated plants were comparable to those in WT and pTRV-GUS-infiltrated plants, respectively, at 24 h after *Pst DC3000*-infiltrated plants. Taken together, these results indicate that modification of SlSAP3 expression affects the *Pst DC3000*-induced expression of defence genes and thereby modulates immunity against this bacterial pathogen.

**Modification of SlSAP3 expression affected flg22-triggered PTI response**

To explore whether SlSAP3 is involved in tomato PTI response, we compared the flg22-induced reactive oxygen species (ROS) burst and expression of PTI marker genes between SlSAP3-OE and WT.
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In ROS burst assay, no significant ROS burst was detected in leaves of SISAP3-OE and WT plants and in leaves of pTRV-SISAP3- and pTRV-GUS-infiltrated plants without flg22 treatment (Fig. 4A,B). After addition of flg22, ROS burst in leaves of SISAP3-OE plants was relatively earlier and much enhanced as compared with that in WT plants (Fig. 4A). By contrast, a relatively lagged and significantly suppressed flg22-induced ROS burst was observed in leaves of pTRV-SISAP3-infiltrated plants as compared with that in pTRV-GUS-infiltrated plants (Fig. 4B).

Furthermore, the expression changes of SIPT15 and SILRR22, two PTI marker genes in tomato (Kim et al., 2009; Nguyen et al., 2010), in SISAP3-OE plants and in pTRV-SISAP3-infiltrated plants were also examined. At 60 min after treatment, the flg22-induced expression of SIPT15 and SILRR22 in leaves of SISAP3-OE and WT plants and in leaves of pTRV-SISAP3- and pTRV-GUS-infiltrated plants...
plants was detected (Fig. 4C). The flg22-induced expression of SIPTI5 and SILRR22 in leaves of SISAP3-OE plants was significantly strengthened as compared with that in WT plants (Fig. 4C). By contrast, the flg22-induced expression of SIPTI5 and SILRR22 was markedly suppressed in leaves of pTRV-SISAP3-infiltrated plants as compared with those in pTRV-GUS-infiltrated plants (Fig. 4C). Collectively, these results indicate that overexpression of SISAP3 strengthens while suppression of SISAP3 partially attenuates the flg22-induced PTI response.

**SISAP3 did not possess ubiquitin E3 ligase activity in vitro**

Several A20 domain-containing proteins from animals and plants have been shown to possess E3 ubiquitin ligase activity (Kang et al., 2011, 2017; Wertz et al., 2004; Zhang et al., 2017). To determine whether SISAP3 has E3 ubiquitin ligase activity, recombinant GST-SISAP3 protein was produced and tested for E3 ubiquitin ligase activity in vitro. In standard E3 ubiquitin ligase activity assays, no polyubiquitinated products were detected in reactions that lacked ubiquitin, yeast E1, human E2 or GST-SISAP3 while significant polyubiquitinated products were observed in the presence of ubiquitin, yeast E1, human E2 and a positive control Arabidopsis AtPUB13 (Liao et al., 2017) (Fig. 5A). However, in the presence of ubiquitin, yeast E1 and human E2, the GST-SISAP3 fusion protein failed to catalyze the formation of polyubiquitinated products (Fig. 5A). These results indicate that SISAP3 may not possess E3 ubiquitin ligase activity in vitro.

**SISAP3 interacted with SIB081**

To further explore the molecular mechanism of SISAP3 in tomato immunity against Pst DC3000, we tried to identify possible SISAP3-interactors. A cDNA library from Pst DC3000 infected tomato leaves was used as the prey, and the full-length SISAP3 was used as the bait. After screening 2 x 10⁶ yeast cells transformed with a cDNA library prepared from Pst DC3000-infected tomato leaves, 35 positive clones were...
initially obtained. Of these, 21 clones contained in-frame coding sequences coding for six proteins (Table S2, see Supporting Information). Amongst them, the putative SlSAP3-interactor SlBOBBER1 (SlBOB1) (Solyc03g083390), which is a homologue of Arabidopsis non-canonical small heat shock protein required for both development and abiotic stress response (Kahloul et al., 2013; Perez et al., 2009; Silverblatt-Buser et al., 2018), was of our interest for further study. Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (co-IP) assays were conducted to further verify the SlSAP3-SlBOB1 interaction in planta. In BiFC assays, yellow florescent protein (YFP) signal was not detected in leaves co-infiltrated with agrobacteria harbouring p2YN-SlBOB1 and p2YC-EV or p2YN-EV and p2YC-SlSAP3.

Fig. 5 Biochemical activity of SlSAP3 and the interaction between SlSAP3 and SlBOB1. (A) SlSAP3 did not have ubiquitin E3 ligase activity in vitro. Ubiquitination reactions were performed at 30 ºC for 3 h, resolved by SDS-PAGE and detected by immunoblotting using anti-ubiquitin antibody. (B) Bimolecular fluorescence complementation (BiFC) analyses of in planta SlSAP3-SlBOB1 interaction. Agrobacteria carrying different pairs of p2YC and p2YN plasmids were infiltrated into leaves of Nicotiana benthamiana and yellow florescent protein (YFP) signal was visualized under confocal microscopy at 48 h after infiltration. Bar = 50 µm. (C) Co-immunoprecipitation (co-IP) analyses of in planta SlSAP3-SlBOB1 interaction. Agrobacteria harbouring SlSAP3-HA and SlBOB1-GFP were co-infiltrated into N. benthamiana leaves and total proteins were extracted at 48 h after agroinfiltration. Immunoprecipitated proteins were separated on 12% SDS-PAGE and were detected by immunoblotting with anti-GFP-specific antibody or anti-HA-specific antibody as indicated. (D) A20 domain in SlSAP3 is required for the SlSAP3-SlBOB1 interaction. Different truncated mutants of SlSAP3 were generated (left) and examined for their interaction activity with SlBOB1 (right). Yeast cells co-transformed with indicated pairs of pGBK77 and pGADT7 vectors were incubated on SD/-Trp/-Leu and SD/-Ade/-His/-Leu/-Trp + X-α-gal + AbA (QDO/X-α-gal/AbA) plates and interaction activity was judged by the appearance of blue colour. Experiments in (A) and (B) were repeated for three times with similar results.
and p2YC-SiSAP3, whereas significant YFP fluorescence was clearly observed in leaves co-infiltrated with agrobacteria harbouring p2YN-SiBOB1 and p2YC-SiSAP3 (Fig. 5B). The constructs used for confocal observation were all successfully expressed, as revealed by protein gel blot analysis (Fig. S2, see Supporting Information). Notably, the fluorescence generated from the SiSAP3-SiBOB1 interaction was observed in both nuclear and cytoplasmic compartments (Fig. 5B). Similarly, when transiently expressed in leaves of N. benthamiana plants, the GFP-SiSAP3 and GFP-SiBOB1 were localized in both nuclear and cytoplasmic compartments of epidermal cells (Fig. S3, see Supporting Information). Co-IP assays in N. benthamiana after transient coexpression further confirmed that GFP-SiBOB1 immunoprecipitated with SiSAP3-HA but not with the empty vector expressing green fluorescent protein (GFP) alone (Fig. 5C). Taken together, these results demonstrated that SiSAP3 interacts with SiBOB1 in planta.

To determine the domains in SiSAP3 that are crucial for the SiSAP3-SiBOB1 interaction, we created a series of truncated mutants of SiSAP3 (Fig. 5D). In Y2H assays, SiSAP3-A20 did show interaction activity with SiBOB1, although the interaction activity of SiSAP3-A20 seemed relatively weaker than the full-length SiSAP3 (Fig. 5D). By contrast, SiSAP3-AN1 and SiSAP3A20ΔAN1 completely abolished the interaction activity with SiBOB1 (Fig. 5D). These results indicate that the A20 domain in SiSAP3 is crucial for the SiSAP3-SiBOB1 interaction.

Silencing of SiBOB1 resulted in increased resistance to Pst DC3000

Because SiSAP3 interacted with SiBOB1, we then examined whether SiBOB1 played a role in tomato immunity against Pst DC3000. At 3 dpi, Pst DC3000-caused necrotic lesions on leaves of pTRV-SiBOB1-infiltrated plants were less severe than that in pTRV-GUS-infiltrated plants (Fig. 6A) and pTRV-SiBOB1-infiltrated plants supported less bacterial population as compared with that in the inoculated leaves of Pst DC3000 (Fig. 6B). These results suggest that silencing of SiBOB1 resulted in increased resistance to Pst DC3000.

To explore whether silencing of SiBOB1 affected the Pst DC3000-induced defence response, we analysed and compared the expression changes of signalling and defence genes in pTRV-SiBOB1- and pTRV-GUS-infiltrated plants before and after Pst DC3000 infection. In mock-inoculated pTRV-SiBOB1- and pTRV-GUS-infiltrated plants, the expression levels of some selected regulatory genes of the SA and JA/ET signalling pathways and their corresponding defence genes were comparable except for an increase of expression of SITGA1 (Fig. 6C, D). The expression of the SA signalling regulatory genes SIEDS1 and SITGA1 and the corresponding defence genes SIPR1a and SIPR-P2 in pTRV-SiBOB1- and pTRV-GUS-infiltrated plants was significantly up-regulated at 24 h after Pst DC3000 infection (Fig. 6C); however, the expression levels in pTRV-SiBOB1-infiltrated plants were higher than those in pTRV-GUS-infiltrated plants (Fig. 6C). By contrast, the expression levels of the SA signalling defence gene SIPR1b, the JA/ET signalling regulatory genes SIA21, S1ACS1 and SIERF1 and the corresponding defence genes SIPP-II and S1LapA1 were comparable in pTRV-SiBOB1- and pTRV-GUS-infiltrated plants at 24 h after Pst DC3000 infection (Fig. 6C, D). These results indicate that silencing of SiBOB1 enhanced the SA signalling and defence response upon infection of Pst DC3000. Taken together, these data suggest that SiBOB1, unlike the function of SiSAP3, negatively regulates immunity against Pst DC3000.

SiSAP3 interacted with SiBOB2 and co-silencing of SiBOB8s enhanced resistance to Pst DC3000

To test whether SiSAP3 interacted with other SiBOB proteins and whether they are also involved in immunity against Pst DC3000, we characterized the tomato SiBOB family and analysed the interaction of SiSAP3 with other SiBOB members. In addition to SiBOB1, two more SiBOB members were identified and named as SiBOB2 (Solyc02g062410) and SiBOB3 (Solyc06g051950), respectively (Table S4, see Supporting Information). Sequence alignment revealed that the three SiBOB proteins contain conserved characteristic NucD domain at their C-terminals but the regions outside the NucD domain are divergent (Fig. S4A, see Supporting Information). Phylogenetic tree analyses indicated that SiBOB1 and SiBOB3 were clustered into one branch while SiBOB2 was closer to BOB proteins from Arabidopsis, Brassica rapa, rice and soybean (Fig. S4B, see Supporting Information).

We examined the interaction of SiSAP3 with SiBOB2 and SiBOB3 by Y2H, BIFC and Co-IP assays. In Y2H assays, SiBOB2 interacted with both the full SiSAP3 and the SiSAP3-A20 while SiBOB3 did not (Fig. 7A). In BIFC assays, YFP fluorescence was not detected in leaves co-infiltrated with agrobacteria harbouring p2YN-EV and p2YC-EV, p2YN-SiBOB2 and p2YC-EV and p2YN-EV and p2YC-SiSAP3, while significant YFP fluorescence was clearly observed in leaves co-infiltrated with agrobacteria harbouring p2YN-SiBOB2 and p2YC-SiSAP3 (Fig. 7B). Notably again, YFP signal from the SiBOB2-SiSAP3 interaction was observed in both nuclear and cytosolic compartments of epidermal cells (Fig. 7B). Co-IP experiments in N. benthamiana after transient coexpression confirmed that GFP-SiSAP3 immunoprecipitated with SiBOB2-HA but not with the empty vector expressing GFP alone (Fig. 7C). Taken together, these results demonstrated that SiSAP3 interacts with SiBOB1 and SiBOB2 but not with SiBOB3 in planta.

Because the coding sequences of the SiBOB genes are highly conserved, a conserved fragment with high levels of sequence identity amongst SiBOBs (Fig. S4A and Table S1, see...
Supporting Information), designated as SIBOBa, was used to co-silence all SIBOB genes. In pTRV-SIBOBa-infiltrated plants, the transcript levels of SIBOB1, SIBOB2 and SIBOB3 were simultaneously and significantly reduced by 70%, 82% and 95%, respectively, as compared with those in pTRV-GUS-infiltrated plants, at 3 weeks after agroinfiltration (Fig. 7D). We then examined the changes of resistance in pTRV-SIBOBa-infiltrated plants. As shown in Fig. 7E, the Pst DC3000-caused disease severity on leaves of pTRV-SIBOBa-infiltrated plants was less severe than that in pTRV-GUS-infiltrated plants. Accordingly,
the pTRV-SIBOBa-infiltrated plants supported less bacterial growth as compared with that in pTRV-GUS-infiltrated plants, at 3 dpi after Pst DC3000 infection (Fig. 7F). These results indicate that co-silencing of SIBOBs led to a further increase in tomato resistance against Pst DC3000.

**DISCUSSION**

Emerging evidence has indicated that SAPs are involved in plant immunity (Kang et al., 2017; Tyagi et al., 2014). In the present study, we found that SISAP3 plays important roles in resistance to Pst DC3000, as silencing of SISAP3 attenuated while over-expression of SISAP3 strengthened resistance to Pst DC3000 (Figs 1 and 2) and modification of SISAP3 expression markedly affected the expression of Pst DC3000-induced defence genes (Fig. 3). Furthermore, SISAP3 also has functions in tomato PTI response, as revealed by the changes in flg22-induced ROS burst and PTI marker gene expression (Fig. 4). Interestingly, SISAP3 interacted with SIBOB1 and SIBOB2, two of the three tomato SIBOB family members (Figs 5 and 7), and silencing of SIBOB or co-silencing of all SIBOB genes led to enhanced resistance to Pst DC3000 (Figs 6 and 7). These observations demonstrate...
that SISAP3 acts as a positive regulator of immunity against Pst DC3000 in tomato, providing new insights into the biological function of plant SAPs.

It is generally accepted that immune response against (hemi)biotrophic pathogens such as Pst DC3000 is modulated through the SA signalling (Glazebrook, 2005; Grant and Jones, 2009; Mengiste, 2012; Verhage et al., 2010). The Pst DC3000-induced expression of SA signalling regulatory gene SIEDS1 and SA signalling-responsive defence genes SIPR1a, SIPR1b and SIPR-P2 was strengthened in SISAP3-OE plants while partially suppressed in SISAP3-silenced plants (Fig. 3). By contrast, the expression of JA/ET signalling regulatory gene SIERF1 and defence gene SILapA1 was not significantly affected by Pst DC3000 in both SISAP3-OE and SISAP3-silenced plants (Fig. 3). Such different expression patterns imply that the SA signalling pathway is required for the function of SISAP3 in immunity against Pst DC3000. This is consistent with a recent observation that orchid SAP protein Pha13 positively regulates the expression of SA-mediated immune responsive genes (Chang et al., 2018), but is different from a previous observation that AtSAP9 positively acts in JA signalling and negatively acts in SA pathway in response to a non-host pathogen challenge (Kang et al., 2017).

ROS burst is an early response in PTI by serving as an anti-microbial agent and/or as a secondary messenger that triggers downstream defence responses (Kadota et al., 2014; Mengiste, 2012). In our experiment, the flg22-induced ROS burst was relatively earlier and much enhanced in leaves of SISAP3-OE plants while it was relatively lagged and significantly suppressed in leaves of SISAP3-silenced plant (Fig. 4A,B). Meanwhile, the flg22-induced expression of PTI marker genes SIPTIS and SILRR22 was enhanced in SISAP3-OE plants but was weakened in SISAP3-silenced plants (Fig. 4C). These features demonstrate a function for SISAP3, as a positive regulator, in tomato PTI. However, it was previously reported that overexpression of AtSAP9 led to increased susceptibility to a non-host bacterial pathogen, P. syringae pv. phaseolicola, indicating that AtSAP9 is a negative regulator of basal resistance (Kang et al., 2017). The reason for the opposite roles of SISAP3 and AtSAP9 can be partially interpreted that they belong to different clades and may have functionally diverged during evolution based on phylogenetic analysis of SISAP3 with other reported plant SAP proteins (Fig. S5, see Supporting Information). The phylogenetic analysis also revealed the multiple functions of SAPs as most of the clades contain members involved in plant biotic and abiotic stress responses (Fig. S5, see Supporting Information). Despite these contrary results, it seems clear that SAPs play roles in plant PTI/basal resistance.

It has been shown that some of the A20 domain-containing proteins of animal origins possess ubiquitin E3 ligase activity (Mattera et al., 2006; Wertz et al., 2004). In plants, the Arabidopsis AtSAP5 and AtSAP9, rice OsSAP7 and orchid Pha13, all of which contain both A20 and AN1 domains, were found to exhibit ubiquitin E3 ligase activity (Kang et al., 2011, 2017, 2011, 2017; Sharma et al., 2015). Furthermore, the A20 domains in AtSAP5 and Pha13 were responsible for both E3 ligase and ubiquitin binding ability (Chang et al., 2018; Kang et al., 2011). It was also reported that the AN1 domain in AtSAP5 had strong ubiquitin E3 ligase activity (Choi et al., 2012). Although SISAP3 contains typical A20 and AN1 domains, we failed to detect the ubiquitin E3 ligase activity for SISAP3 in our repeated experiments (Fig. 5A), in which the positive control AtPUB13 showed clear ubiquitin E3 ligase activity (Liao et al., 2017). It is therefore likely that ubiquitin E3 ligase activity may not be a common feature for the A20/AN1 domain-containing SAPs. On the other hand, the Arabidopsis AtSAP5 and AtSAP9, Prunus PpSAP1 and orchid Pha13 were found to interact with polyubiquitinated proteins or with UPS shuttling factors such as RADs (Chang et al., 2018; Choi et al., 2012; Farmer et al., 2010; Kang et al., 2017; Lloret et al., 2017). The A20 domains in AtSAP5 and Pha13 are responsible for ubiquitin binding activity (Chang et al., 2018; Choi et al., 2012). In the present study, clones containing genes coding for ubiquitin or ubiquitin-ribosomal fusion protein appeared with high frequency in our efforts towards identification of SISAP3 interactors (Table S3, see Supporting Information), implying that SISAP3 may interact with UPS components in nature. Thus, it is likely that SISAP3 may exert its function in immunity by interacting with other proteins such as UPS components rather than by its ubiquitin E3 ligase activity.

It was previously reported that the Arabidopsis BOBs, acting as protein chaperones and interactors of multiple UPS subunits/components (Gunsalus et al., 2005; Perez et al., 2009; Zheng et al., 2011), are required for growth, development and abiotic stress responses (Jarkuta et al., 2009; Ishibashi et al., 2012; Perez et al., 2009; Silverblatt-Buser et al., 2018). In the present study, SIBOB1 and one of its homologues SIBOB2 appeared as real SISAP3 interacting partners in planta, as verified by BiFC and co-IP approaches (Figs 5 and 7). It is clear that SIBOB1 plays a role in immunity against Pst DC3000, as the SIBOB1-silenced plants displayed enhanced resistance and up-regulated expression of defence genes upon pathogen infection (Fig. 6). However, SIBOBs-co-silenced plants showed less disease severity and supported less bacterial growth, as compared with that in SIBOB1-silenced plants (Figs 6 and 7). For example, a 38-fold decrease vs. a 14-fold reduction in bacterial populations were observed in SIBOBs-co-silenced and SIBOB1-silenced plants (Figs 6 and 7), respectively, as compared with those in GUS-silenced plants, at 3 dpi. This increased level of resistance in SIBOBs-co-silenced plants over that in SIBOB1-silenced plants indicates a function of SIBOB2 and SIBOB3 in immunity against Pst DC3000. Despite the existence of
interaction in planta, SISAP3 and SIBOB1/SIBOB2/SIBOB3 play opposite roles in tomato immunity against Pst DC3000. SISAP3 functions as a positive regulator while SIBOB1 and SIBOB2/SIBOB3 act as negative regulators. Arabidopsis AtBOB1 was previously found to be required for organisinal thermotolerance (Perez et al., 2009). Thus, it seems likely that plant BOB proteins have diverse functions in biotic and abiotic stress responses. Notably, silencing of either SISAP3 or SIBOB1 affected the Pst DC3000-induced SA signalling regulatory and defence genes but not the JA/ET signalling genes (Figs 3 and 6). These observations suggest that a same defence signalling pathway is associated with the functions of SISAP3 and SIBOB1, although they play opposite roles in tomato immunity against Pst DC3000.

In summary, we demonstrated that SISAP3 acts as a positive regulator of immunity against Pst DC3000 in tomato through the SA signalling. We also found that SISAP3 interacted with members of SIBOB family, which act as negative regulators of tomato immunity against Pst DC3000. As SISAP3 does not possess ubiquitin E3 ligase activity in vitro, SISAP3 may exert its function in immunity by interacting with other proteins associated with UPS. However, the mechanism by which the interaction of SISAP3-SIBOBs regulates immunity is an open question to be investigated further. Because both SAP and BOB proteins seem to be associated with UPS via interaction with UPS subunits or components (Choi et al., 2012; Farmer et al., 2010; Fu et al., 2010; Gunsalus et al., 2005; Kang et al., 2017; Lloret et al., 2017; Saeki, 2017), the interaction of SISAP3-SIBOBs in planta might initiate an event that results in the degradation of one or both of them and of other unknown targeting proteins upon pathogen infection. Further characterization of SISAP3 and SIBOB targets of SISAP3 will be helpful to understand the biochemical mechanism of SISAP3-SIBOBs complex in tomato immunity.

**EXPERIMENTAL PROCEDURES**

**Plant growth, treatment and disease assays**

Tomato (Solanum lycopersicum L.) cv. Ailsa Craig was used for all experiments. Plants were grown in a mixture of perlite: vermiculite: plant ash (1:6:2) in a growth room under fluorescent light (200 µmol/m²/s) at 22 °C–24 °C with 60% relative humidity and a 14 h light/10 h dark cycle. Pathogen inoculation, disease assays and measurement of in planta bacterial growth were performed basically according to previously described protocols (Li et al., 2002, 2014). Reactive oxygen species (ROS) assays

ROS assays were carried out as described previously (Shang-Guan et al., 2018). Briefly, leaf discs (0.2 cm²) were incubated overnight in a 96-well plate with water, and 200 mM luminol (Sigma-Aldrich, Saint Louis, MO, USA), 20 mg/mL horseradish peroxidase (Sigma-Aldrich, Saint Louis, MO, USA), or 100 mM flg22 (GenScript, Nanjing, China) were then added. Chemiluminescent signal was recorded at a 2 min interval over 30 min using a Synergy HT plate reader (Biotek Instruments, Inc., Winooski, VT, USA).

**Virus-induced gene silencing (VIGS) assays**

VIGS fragments of 13 SISAP genes (Solank et al., 2009) and three SIBOB genes were amplified using gene-specific primers and cloned into the ptRV2 vector (Liu et al., 2002), yielding plasmids pTRV-SISAPs or pTRV-BOBs. Sequence information for the VIGS fragments is listed in Table S1. In the case of co-silencing of SIBOBs, A 278 bp fragment, designated as SIBOBa that corresponds to the conserved regions in open reading frames (ORFs) of the SIBOB genes, was cloned into the ptRV2 vector, yielding pTRV-SIBOBa. Standard VIGS procedure was applied to 10-day-old tomato seedlings (Li et al., 2014; Liu et al., 2002). Silencing efficiency and specificity were analysed by qRT-PCR at 3 weeks after VIGS manipulation. The primers used are listed in Table S3 (see Supporting Information).

**Generation of SISAP3-OE transgenic lines**

The coding sequence of SISAP3 was amplified with primers SISAP3-OE-HA-F and SISAP3-OE-HA-R (Table S3, see Supporting Information) and cloned into plant transformation vector pFGC1008-HA at Ascl/Kpnl sites under the control of the CaMV 35S promoter. The resulting construct was introduced into tomato cv. Ailsa Craig by Agrobacterium tumefaciens-mediated transformation (Abuqamar et al., 2008; Howe et al., 1996). Transfomants were selected based on their resistance to Hygromycin B. Homozygous T2 or T3 transgenic plants were used for phenotypic and molecular characterization.

**Purification of recombinant GST-SISAP3 protein and ubiquitin E3 ligase activity assay**

The coding sequence of SISAP3 was amplified with a pair of primers (Table S3, see Supporting Information) and cloned into pGEX-4T-3 vector at BamHI/XhoI sites. The resulting plasmid was introduced into the E. coli strain Rosetta DE3 and expression of GST-SISAP3 fusion was induced by adding of 1 mM isopropyl-D-thiogalactoside (IPTG) at 20 °C overnight. Recombinant GST-SISAP3 fusion protein was purified using the Bug-Buster GST-Bind purification kit according to the manufacturer’s protocol (Novagen, Darmstadt, Germany). A GST tag sample was also purified from E. coli cells with the same protocol. Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad, CA, USA) following the recommended method. Ubiquitination assays were performed as described previously (Zhao et al., 2012). Briefly, reactions
(30 μL) contained 5 μg ubiquitin (Boston Biochem, Cambridge, MA, USA), 110 ng E1 (Merck Millipore, Darmstadt, Germany), 100 ng human recombinant UbcH2 (Abcam, Cambridge, UK), and purified 4 μg GST-SISAP3 in buffer (20 mM MOPS, pH 7.2, 100 mM KCl, 5 mM MgCl₂, 5 mM ATP and 10 mM DTT) and were incubated at 30 °C for 3 h. Reactions were stopped by adding SDS sample buffer and analysed by SDS-PAGE. followed by immunoblotting using anti-ubiquitin antibody (CalBiochem, La Jolla, CA, USA). Chemiluminescence signal was detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations.

Y2H screening and verification of SISAP3 interactors

A tomato Y2H library constructed with cDNAs prepared from *Pst* DC3000-infected leaves was screened using SISAP3 as a bait. The co-transformed yeast cells were selected on QDO (SD/-Ade/-His/-Leu/-Trp) medium and the survivors were further screened on QDO medium containing 40 μg/mL X-α-Gal and 125 ng/mL Aureobasidin A. The AD plasmids were rescued from putative positive clones and sequenced. For determining the domains responsible for interaction in SISAP3, the coding sequence of SISAP3 and its deletion mutants (SISAP3-A20, SISAP3-AN1 and SISAP3ΔA20ΔAN1) were PCR amplified with gene-specific primers (Table S3, see Supporting Information) and cloned in-frame into pGBK7 plasmids. Similarly, the coding sequences of SIBOBs were amplified with gene-specific primers (Table S3, see Supporting Information) and cloned in-frame into pGADT7 plasmids. Combined pairs of recombinant pGBK7 plasmids containing SISAP3 or its deletions mutants and pGADT7 plasmids harbouring SIBOBs were co-transformed into yeast cells and the interaction activity was examined by plating yeast cells on DDO and QDO medium containing 40 μg/mL X-α-Gal and 125 ng/mL Aureobasidin A.

Bimolecular fluorescence complementation (BiFC) assays

BiFC assays for determining the interaction between SISAP3 and SIBOBs were performed as described previously (Yang *et al*., 2007). The coding sequence of SISAP3 was amplified with gene-specific primers and cloned into p2YC at *PacI*-AscI sites, yielding plasmid p2YC-SISAP3 that codes for a fusion with the C-terminal fragment of YFP. Similarly, the coding sequences of SIBOB1 and SIBOB2 were amplified with gene-specific primers and cloned into p2YN at the *PacI*-AscI sites, yielding plasmids p2YN-SIBOB1 and p2YN-SIBOB2 that code for fusions with the N-terminal fragment of YFP. BiFC experiments were performed in leaves of 2-week-old *N. benthamiana* plants expressing a known nucleus-localized marker protein RFP-H2B (Chakrabarty *et al*., 2007) as described previously (Yang *et al*., 2007). YFP and red florescent protein (RFP) fluorescence were observed and photographed by a Zeiss LSM780 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) 48 h after agroinfiltration. The primers used for BiFC assays are listed in Table S3 (see Supporting Information).

Co-immunoprecipitation (co-IP) assays

Co-IP assays were conducted according to a previously described procedure (Zhu *et al*., 2014). Briefly, the coding sequences of SISAP3 and SIBOB1 were amplified using gene-specific primers and cloned into pFGC-eGFP vector with a GFP tag at the N-terminus, yielding plasmids pFGC-eGFP-SISAP3 and pFGC-eGFP-SIBOB1. Similarly, the coding sequence of SISAP3 and SIBOB2 were amplified with a pair of gene-specific primers and cloned into pFGC1008-HA, yielding plasmid pFGC1008-SISAP3-HA and pFGC1008-SIBOB2-HA. Agrobacteria harbouring pFGC1008-SISAP3-HA and pFGC-eGFP-SIBOB1 or harbouring pFGC1008-SIBOB2-HA and pFGC-eGFP-SISAP3 were combined and infiltrated into the abaxial air spaces of leaves of 4-week-old *N. benthamiana* plants. The agroinfiltrated leaves were collected at 48 h after agroinfiltration and total proteins were extracted with extraction buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM EDTA, 50 mM EGTA, 25 mM NaF, 1mM NaVO₃, 50mM β-glycerophosphate, 20% [v/v] glycerol, 1mM PMSF, 0.1% [v/v] Triton X-100, 1 mM DTT and 1 x protease inhibitor cocktail [Sigma-Aldrich, Saint Louis, MO, USA]). After centrifugation at 12 000 g for 10 min, 1 mL of supernatant was mixed with GFP-Trap (Chromotr, Planegg-Martinsried, Germany) and rotated overnight at 4 °C. After washing four times with extraction buffer, the GFP-Trap beads were resuspended in 50 μL 2 x SDS sample buffer and boiled for 10 min at 95 °C to dissociate immunoprecipitated protein complex. The dissociated immunoprecipitated proteins were separated on 12% SDS-PAGE and were detected by immunoblotting with anti-GFP-specific antibody (Sigma-Aldrich, Saint Louis, MO, USA) or anti-HA-specific antibody (Sigma-Aldrich, Saint Louis, MO, USA). The primers used are listed in Table S3 (see Supporting Information).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis of gene expression

Total RNA was extracted by Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer’s instructions. RNA was treated with RNase-free DNase and then reverse-transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). The obtained cDNAs were used for gene expression analysis by real-time qPCR. Each qPCR reaction contained 12.5 μL SYBR Premix Ex Taq (TaKaRa, Dalian, China), 0.1 μg cDNA and 7.5 pmol of each gene-specific primer (Table S3, see Supporting Information) in a final volume of 25 μL, and
was run in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). A tomato SIActin gene (Accession No. AB199316) was used as an internal control to normalize the qRT-PCR data and relative expression levels of genes of interest were calculated using the 2^−ΔΔCT method. Three independent biological samples were performed.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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Fig. S1 Silencing efficiency and specificity for target genes. (A) Silencing efficiency of each of the SISAP genes in corresponding virus induced gene silencing (VIGS) infiltrated plants. (B) Silencing specificity in pTRV SISAP3 infiltrated plants. (C) Silencing efficiency and specificity in pTRV SIBO81 infiltrated plants. Two week old tomato seedlings were infiltrated with agrobacteria carrying pTRV SISAPs, pTRV SIBO81 or pTRV GUS constructs and leaf samples were collected at 4 weeks after agroinfiltration. Transcript levels of each of the SISAP and SIBO81 genes in corresponding pTRV SISAP or pTRV SIBO81 infiltrated and pTRV GUS infiltrated plants were analysed by quantitative Reverse Transcription Polymerase Chain Reaction (qRT PCR). SIActin was used as an internal reference gene and relative expression was shown as folds of the transcript value of the SIActin gene. Data presented are the means ± standard errors (SE) from three experiments with independent biological samples. Statistical significance compared with pTRV GUS was determined by Student’s t-tests: *P < 0.05. All experiments were repeated three times with similar results.

Fig. S2 Western blot analysis to detect the expression of bimolecular fluorescence complementation (BiFC) constructs shown in Figs 5b and 7b. Immunoblot analysis of p2YN HA SIBO81, p2YN HA SIBO82 and p2YC HA SISAP3 fusion proteins in Nicotiana benthamiana leaves at 48 h after agroinfiltration. A HA specific antibody was used for detection of HA fusion protein. Equal loading of total proteins was examined by Ponceau staining.

Fig. S3 Subcellular localization of SISAP3 and SIBO8s. Agrobacteria carrying pFGC eGFP SISAP3, pFGC eGFP SIBO8s or pFGC eGFP empty vector were infiltrated into leaves of Nicotiana benthamiana plants expressing a red nucleus marker protein RFP H2B and leaf samples were collected at 48 h after
infiltration for observation under a confocal laser scanning microscope. Images were taken in dark field for green fluorescence (left) and red fluorescence (middle left), white field for cell morphology (middle right) and in combination (right), respectively.

Fig. S4 Sequence alignment and phylogenetic tree analysis of SlBOBs. (A) Alignment of SlBOBs with Arabidopsis AtBOBs. The conserved C terminal NudC domain regions is underlined. Numbers on the right indicate amino acid positions of the BOB proteins. (B) Phylogenetic tree analysis of SlBOBs with other plant BOBs. Phylogenetic tree was constructed by Neighbour joining method using MEGA7 programme. Plant BOBs used and their GenBank accessions are as follows: Arabidopsis thaliana AtBOB1 (NP_200152), AtBOB2 (NP_194518), Oryza sativa OsBOB1 (XP_015640993), Solanum lycopersicum SlBOB1 (XP_004234959), SlBOB2 (XP_004233975), SlBOB3 (XP_025887281), Nicotiana tabacum NtBOB1 (XP_016451285), NtBOB2 (XP_016468156), Brassica rapa BrBOB1 (XP_009132480), BrBOB2 (XP_009108672) and Glycine max GmBOB1 (XP_003526709). Bootstrap values from 1000 replicates are indicated at each node. Bar represents the number of amino acid differences per site.

Fig. S5 Phylogenetic tree analysis of SlSAP3 with other reported plant stress associated proteins (SAPs). Phylogenetic tree was constructed by Neighbour joining method using MEGA7 programme. SAPs involved in plant immunity are indicated by red arrows. Plant SAPs used and their GenBank accessions are as follows: Arabidopsis thaliana AtSAP5 (NP_566429), AtSAP9 (NP_194013), AtSAP10 (NP_194268), AtSAP12 (NP_189461), AtSAP13 (NP_191307), Aeluropus littoralis AISAP (ABK90631), Festuca arundinacea FaZnF (AEZ53300), Leymus chinensis LcSAP (CD808976), Lobularia maritima LmSAP (AUN86611), Malus domestica MdSAP15 (XP_008375158), Medicago truncatula MtSAP1 (XP_024626996), Musa acuminata MusaSAP1 (XP_009411822), Oryza sativa OsSAP1 (XP_015651267), OsSAP7 (XP_015633143), OsSAP8 (XP_015643189), OsSAP9 (XP_015647896), OsSAP11 (XP_015651039), OsSAP16 (XP_015644892), Phalaenopsis aphrodite Pha13 (PATC148746), Prunus persica PpSAP1 (XP_007218502), Saccharum officinarum ShSAP1 (ACT53874), Solanum lycopersicum SISAP3 (ACM68440), Sorghum bicolor SbSAP14 (XP_002466323) and Zea mays ZmAN13 (AQL04999). Bootstrap values from 1000 replicates are indicated at each node. Bar represents the number of amino acid differences per site.

Table S1 Sequence of the virus-induced gene silencing (VIGS) fragments for SISAP and SIBOB genes.

Table S2 Putative SISAP3 interactors identified by Y2H screening.

Table S3 Primers used in this study for different purposes.

Table S4 CDS and amino acid sequences of the SISAP and SIBOB genes.