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The ubiquitin ligase SEVEN IN ABSENTIA (SINA) ubiquitinates a defense-related NAC transcription factor and is involved in defense signaling

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Summary

- We recently identified a defense-related tomato (*Solanum lycopersicum*) NAC (NAM, ATAF1,2, CUC2) transcription factor, NAC1, that is subjected to ubiquitin–proteasome system-dependent degradation in plant cells. In this study, we identified a tomato ubiquitin ligase (termed SEVEN IN ABSENTIA3; SINA3) that ubiquitinates NAC1, promoting its degradation.
- We conducted coimmunoprecipitation and bimolecular fluorescence complementation to determine that SINA3 specifically interacts with the NAC1 transcription factor in the nucleus. Moreover, we found that SINA3 ubiquitinates NAC1 *in vitro* and promotes NAC1 degradation via polyubiquitination *in vivo*, indicating that SINA3 is a ubiquitin ligase that ubiquitinates NAC1, promoting its degradation. Our real-time PCR analysis indicated that, in contrast to our previous finding that *NAC1* mRNA abundance increases upon *Pseudomonas* infection, the *SINA3* mRNA abundance decreases in response to *Pseudomonas* infection.
- Moreover, using *Agrobacterium*-mediated transient expression, we found that overexpression of SINA3 interferes with the hypersensitive response cell death triggered by multiple plant resistance proteins.
- These results suggest that SINA3 ubiquitinates a defense-related NAC transcription factor for degradation and plays a negative role in defense signaling.

Introduction

NAC (NAM, ATAF1,2, CUC2) transcription factors belong to one of the largest families of plant-specific transcription factors and consist of a conserved N-terminal NAC domain involved in DNA binding and a highly variable C-terminal domain responsible for transcriptional activation (Puranik *et al.*, 2012). Although NAC transcription factors were originally identified as a result of their role in developmental processes, multiple lines of evidence have shown that they are also largely involved in plant defense response. In both the model plant *Arabidopsis thaliana* and crop plants such as rice, pepper, wheat, potato and rapeseed, numerous *NAC* genes are induced in response to pathogen infection (Puranik *et al.*, 2012). Genetic impairing or overexpression of certain *NAC* genes resulted in attenuated or enhanced resistance to pathogens (Delessert *et al.*, 2005; Wang *et al.*, 2009; Wu *et al.*, 2009; Huang *et al.*, 2013), suggesting that NAC transcription factors could positively or negatively regulate plant defense response.

The ubiquitin–proteasome system (UPS)-mediated protein degradation is an important post-translational regulatory mechanism and plays a significant role in many physiological processes by removal of intracellular proteins (Harper & Schulman, 2006; Ravid & Hochstrasser, 2008). The UPS pathway contains E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) enzymes that function in concert to covalently link ubiquitin to the substrate protein (Harper & Schulman, 2006). The ubiquitin moieties are consecutively added to a lysine residue of the substrate protein through linkages at one of seven lysine residues (K48, in most cases) in each ubiquitin to form a polyubiquitin chain, which will consequently be recognized by the 26S proteasome for proteolytic degradation (Harper & Schulman, 2006). The ubiquitin E1s and E2s are relatively conserved, whereas the E3s are highly diverse and determine the substrate specificity (Harper & Schulman, 2006). In plants, UPS-mediated

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degradation not only cleans up misfolded and/or damaged proteins to avoid potential toxicity, but also adjusts the amount of regulatory proteins, in particular transcription factors and signaling kinases, which allows for tight control of many physiological processes such as senescence, development, stress tolerance and defense response in a temporally and spatially specific mannar (Sadanandom *et al.*, 2012).

The SEVEN IN ABSENTIA (SINA) ubiquitin ligase was first identified in *Drosophila melanogaster* and is required for formation of the R7 photoreceptor involved in eye development (Carthew & Rubin, 1990). The SINA ubiquitin ligase contains a conserved N-terminal cysteine-rich C_3H_4 RING domain, two zinc finger motifs and a C-terminal domain responsible for substrate-binding and dimerization (Hu & Fearon, 1999). It is generally thought that, like many other monomeric RING-containing ubiquitin ligases, SINA and SINA homologs (Siah) directly interact with their substrates through the substrate-binding domain (SBD) and facilitate the ubiquitination via the RING domain (House *et al.*, 2003). In plants, despite the fact that a genome-wide analysis suggests the existence of SINA ubiquitin ligases in many plant species (Wang *et al.*, 2008), only five SINA ubiquitin ligases have been identified and their functions are not fully understood (Xie *et al.*, 2002; Welsch *et al.*, 2007; Den Herder *et al.*, 2008, 2012; Park *et al.*, 2010; Ning *et al.*, 2011a). In Arabidopsis, one SINA ubiquitin ligase, SINAT5, plays roles in both lateral root growth and floral development (Xie *et al.*, 2002; Park *et al.*, 2010), whereas another, SINAT2, is involved in carotenogenesis (Welsch *et al.*, 2007). In rice, the SINA ubiquitin ligase OsDIS1 functions as a negative regulator in drought stress response (Ning *et al.*, 2011b). In *Lotus japonicas*, the *Lotus* SINA4 negatively regulates *Sinorhizobium meliloti* infection (Den Herder *et al.*, 2012). Finally, in *Medicago truncatula*, heterogeneous overexpression of *Arabidopsis* SINAT5 affects nodulation (Den Herder *et al.*, 2008).

Although a few SINA-interacting proteins have been identified as putative ubiquitinating substrates in plants, including the *Arabidopsis* transcription factors AtNAC1 (Xie *et al.*, 2002), LHY (Park *et al.*, 2010), and RAP2.2 (Welsch *et al.*, 2007), the *Lotus* symbiosis receptor-like kinase SYMRK (Den Herder *et al.*, 2012) and the rice tubulin complex-related serine-threonine protein kinase OsNek6 (Ning *et al.*, 2011a), only the *Arabidopsis* AtNAC1 and LHY have been shown to be ubiquitinated by SINAT5 (Xie *et al.*, 2002; Park *et al.*, 2010). Nevertheless, these studies suggest that plant SINA ubiquitin ligases may regulate physiological and cellular processes through targeting transcription factors and/or signaling kinases for promoting their degradation.

We recently demonstrated that the defense-related tomato (*Solanum lycopersicum*) NAC transcription factor NAC1 (Selth *et al.*, 2005) is fine-tuned at both transcriptional and post-translational levels (Huang *et al.*, 2013). The tomato NAC1 transcription factor was originally identified as a host protein interacting with the geminivirus replication enhance (REn) protein of tomato leaf curl virus in a yeast two-hybrid (Y2H) screen and the relevance of this interaction is unclear (Selth *et al.*, 2005). A role for tomato NAC1 in the defense response is supported by our recent findings of rapid induction of *NAC1* gene expression in tomato during *Pseudomonas* infection and enhanced susceptibility to *Pseudomonas* infection in *Nicotiana benthamiana* whose *NAC1* homologs were silenced (Huang *et al.*, 2013). Furthermore, we found that the tomato NAC1 is ubiquitinated and degraded by the UPS in plant cells (Huang *et al.*, 2013). Given a significant role of NAC1 in the defense response, we have speculated that tomato plants have evolved a mechanism to fine-tune the abundance of NAC1 transcription factor. Under normal conditions without pathogen challenge, the *NAC1* gene is expressed at a basal level and the encoded NAC1 protein is rapidly degraded by the UPS to prevent autoactivation of stress response signaling. Upon *Pseudomonas* infection, the expression of the *NAC1* gene is rapidly up-regulated to produce more NAC1 transcription factor to compensate for its degradation, thereby activating downstream defense-related genes (Huang *et al.*, 2013).

In this study, we identified a tomato SINA ubiquitin ligase SINA3 that targets NAC1 for ubiquitination and degradation. In contrast to up-regulation of the *NAC1* gene, the expression of the *SINA3* gene is down-regulated during defense response to *Pseudomonas* infection. Moreover, overexpression of SINA3 interferes with hypersensitive response (HR), a localized defense-related cell death, triggered by multiple R proteins. Together, our experimental data suggest that SINA3 targets the defense-related NAC1 transcription factor for degradation and plays a negative role in defense response.

Materials and Methods

Yeast two-hybrid assay

A LexA Y2H system was used to test protein interactions (Golemis *et al.*, 2001). The N-terminal 260 amino acids of NAC1 ($NAC1_{1\text{-}260}$), which did not exhibit autoactivation, were cloned into the bait vector pEG202 at the *Eco*RI and *Sal*I sites, whereas the SINA1-6 (the National Center for Biotechnology Information's accession numbers for tomato *SINA1-6* genes are AK324518, BT013026, AK322153, AK320390, AK321160 and XM_004248034) were cloned into the prey vector pJG4-5 at *Eco*RI and *Xho*I sites, respectively. The resulting bait and prey constructs were introduced into yeast (*Saccharomyces cerevisiae*) EGY48 and transformed yeast cells were streaked onto X-Gal plates to assess the interactions between NAC1 and SINA1-6. Photographs were taken at 2 d after incubation at 30°C. Primers used in generating the Y2H constructs are listed in Supporting Information Table S1.

In vitro ubiquitination assay

SINA3 and hemagglutinin (HA) epitope-tagged NAC1-HA were PCR-amplified from tomato leaf cDNA and pTEX::NAC1-HA (Huang *et al.*, 2013), respectively, and cloned into the pMAL-C2 vector (NEB, USA) at *Eco*RI and *Sal*I to generate the maltose binding protein (MBP)-fusion proteins. The resulting constructs were introduced into *Escherichia coli* BL21 where the recombinant proteins were expressed with the presence of 0.5 μM isopropyl β-D-1-thiogalactopyranoside. The *in vitro* ubiquitination assay was performed as described previously (Abramovitch

et al., 2006) with minor modifications. The ubiquitination reaction mixture (30 μl) contained 40 ng GST-E1 (AtUBA1), 100 ng GST-E2 (AtUBC8), 1 μg MBP-SINA3, 2 μg FLAG-Ub (Boston Biochem, Cambridge, MA, USA) in the ubiquitination buffer (50 mM Tris HCl, pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 30 mM creatine phosphate (Sigma-Aldrich) and 50 ng μl^{-1} creatine phosphokinase (Sigma-Aldrich)). The reaction mixture was incubated at 30°C for 2 h and terminated by sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated with 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by western blotting using the α-FLAG (Sigma-Aldrich) antibody.

The *in vitro* ubiquitination of NAC1 by SINA3 was performed using 500 ng MBP-NAC1-HA as substrate. After incubation, 15 μl α-HA affinity matrix (Roche Applied Science, Indianapolis, IN, USA) was added to the incubation mixture to immunoprecipitate the ubiquitinated MBP-NAC1-HA protein. After washing three times with the washing buffer (20 mM Tris HCl, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, 0.05% Tween 20), proteins were separated by 7.5% SDS-PAGE and the ubiquitinated NAC1-HA was determined by western blotting using the α-FLAG or α-HA antibody (Sigma-Aldrich). Primers used in generation of the MBP fusion constructs are listed in Table S1.

Agrobacterium-mediated transient expression and coimmunoprecipitation

Agrobacterium-mediated transient expression was carried out as described previously (Xiao *et al.*, 2007), with the minor modification that freshly transformed *Agrobacterium tumefaciens* GV2260 and relatively young plant leaves with a light green color were used for agrobacterial injection. The Rpi-blb1^{D475V}-HA and Rx^{D460V}-HA constructs were generated in the same way as Prf^{D1416V}-HA, as described in our previous publication (Du *et al.*, 2012). For the coIP assay, *Agrobacterium*-infected *N. benthamiana* (Domin) leaf tissues were collected at 48 h after infiltration and ground to a fine powder with liquid nitrogen. The lysate was resuspended in 1.0 ml protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol (DTT), 10% glycerol, 1% polyvinylpolypyrolidone, 1 mM phenylmethylsulfonyl fluoride (PMSF), plant protease inhibitor cocktail (Sigma-Aldrich)) and centrifuged at 12 000 ***g***/4°C for 20 min. The supernatant was incubated with 15 μl α-HA affinity matrix (Roche Applied Sciences) at 4°C for 2 h to capture the epitope-tagged protein. After washing four times with washing buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF), the immunoprecipitated protein complex was separated by SDS-PAGE and then subjected to western blotting analysis using the α-HA or α-FLAG antibody. Primers used in generating the relevant construct are listed in Table S1.

Bimolecular fluorescence complementation (BiFC) analysis

NAC1 was PCR-amplified from tomato leaf cDNA (see Table S1 for primers used) and cloned into the pBSPYNE vector containing the N-terminal 155 amino acids of yellow fluorescence protein (YFP) protein to generate NAC1-NYFP, whereas SINA3 or SINA3$_{1\text{-}181}$ was cloned into the pBSPYCE vector containing the C-terminal 83 amino acids of YPF to generate SINA3-CYFP and SINA3$_{1\text{-}181}$-CYFP (Walter *et al.*, 2004), respectively. BiFC assay was carried out via the *Agrobacterium*-mediated transient expression of NAC1-NYFP with SINA3-CYFP or SINA3$_{1\text{-}181}$-CYFP in *N. benthamiana* leaves as described previously (Walter *et al.*, 2004). Two 4-wk-old *N. benthamiana* leaves were injected with *Agrobacterium* GV2260 strains containing individual BiFC construct pairs and a binary plasmid expressing the p19 protein to suppress gene silencing. After 4′,6-diamidino-2-phenylindole (DAPI) staining, the epidermal cell layers were examined using confocal microscope to simultaneously capture DAPI and YFP signals.

Results

The tomato defense-related transcription factor NAC1 interacts with the ubiquitin ligase SINA3

We recently characterized the involvement of the tomato transcription factor NAC1 in the plant defense response and its UPS-mediated degradation (Huang *et al.*, 2013). However, the corresponding ubiquitin ligase was not identified at that time. Previous research demonstrated that the *Arabidopsis* SINAT5 protein, a RING-finger type ubiquitin ligase homologous to the *Drosophila* SEVEN IN ABSENTIA (SINA) ubiquitin ligase, interacts with and ubiquitinates the *Arabidopsis* NAC transcription factor AtNAC1, promoting its degradation (Xie *et al.*, 2000, 2002). Although tomato NAC1 is not orthologous to AtNAC1, which regulates *Arabidopsis* root hair development through the auxin signaling (Xie *et al.*, 2000; Selth *et al.*, 2005), it is possible that tomato NAC1 can be ubiquitinated via a similar RING-containing ubiquitin ligase. Thus, we investigated whether a SINAT5-like ubiquitin ligase in tomato can target NAC1 to promote its degradation. We applied a BLAST search for SINAT5 homologs in the tomato genome database (http://solgenomics.net/) and found six tomato genes showing significant homology with *SINAT5*. The full-length cDNAs of these six *SINAT5-like* genes were cloned and named *SINA1-6*. The deduced SINA1-6 proteins share 78.6% identity at the amino acid level, containing a highly conserved RING domain, a typical SINA-specific Zn-finger domain and an SBD (Hu & Fearon, 1999) (Fig. S1). To test the possible direct interaction between SINA1-6 and NAC1, which reflects an enzyme-substrate relationship, we carried out a Y2H assay in which SINA1-6 were expressed as prey and NAC1$_{1\text{-}260}$ (containing the N-terminal 260 amino acids of NAC1; the full-length NAC1 exhibits autoactivation in yeast (Selth *et al.*, 2005)) was expressed as bait. Protein–protein interaction in Y2H was assessed on X-Gal-containing medium and medium lacking leucine. As shown in Fig. 1(a), only yeast cells containing SINA3 and NAC1$_{1\text{-}260}$ exhibited blue coloration on the X-Gal plate, despite normal protein accumulation for all combinations of NAC1$_{1\text{-}260}$ and SINAs. However, these yeast cells failed to grow on medium lacking leucine (Fig. S2), suggesting there might be a weak interaction between NAC1 and SINA3 when expressed in yeast.

The apparent weak interaction between SINA3 and NAC1 in the Y2H assay led us to determine the possible *in vivo* interaction of NAC1 with SINA3 in plant cells using the coIP assay established in *N. benthamiana* by our laboratory (Huang *et al.*, 2013). To this end, *Agrobacteria* containing the epitope-tagged full-length NAC1 (NAC1-FLAG) and SINA1-6-HA constructs, controlled by the cauliflower mosaic virus (CaMV) 35S promoter, were infiltrated into *N. benthamiana* leaves for transient expression. To prevent NAC1 degradation, the proteasome inhibitor MG132 was added to the *Agrobacterium* inoculum harboring appropriate constructs including a vector control. As shown in Fig. 1(b), NAC1 protein accumulated to a considerable level in *N. benthamiana* leaves in the presence of MG132. After protein extraction and immunoprecipitation with the α-HA antibody matrix, the immunoprecipitated protein complex was verified by western blotting using the α-FLAG antibody. The NAC1-FLAG protein was detected in the α-HA antibody-immunoprecipitated complex from the leaf tissue expressing NAC1-FLAG and SINA3-HA (Fig. 1b), but not in the immunoprecipitated complex from the leaf tissue expressing NAC1-FLAG and any other SINA-HAs or the vector control. This indicates that NAC1 specifically interacts with SINA3 in plant cells and suggests that SINA3 could be the ubiquitin E3 ligase responsible for NAC1 ubiquitination.

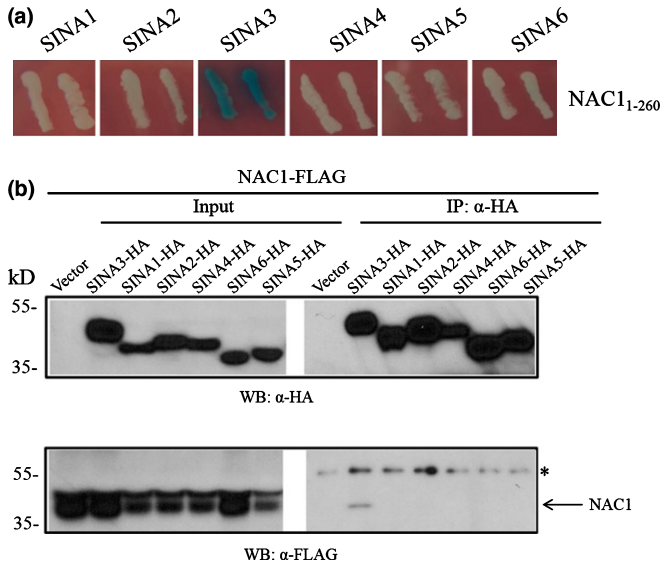

Fig. 1 NAC1 interacts with a tomato SEVEN IN ABSENTIA (SINA) ubiquitin ligase. (a) NAC1 interacts with SINA3 in the LexA-based yeast two- assay. The N-terminal 260 amino acids (NAC1$_{1-260}$), which did not exhibit self-activation, were expressed as bait and SINA1-6 were expressed as prey. Blue yeast colonies grown on the X-Gal-containing medium indicate the interaction between NAC1 and SINA3. The expression and accumulation of NAC1$_{1-260}$ and SINA proteins in yeast were verified by western blotting using α-LexA and α-HA antibody, respectively (lower panel). (b) *In vivo* interaction of NAC1 with SINA3 determined by coimmunoprecipitation. *Agrobacterium tumefaciens* GV2260 strains containing the cauliflower mosaic virus 35S promoter-driven epitope-tagged SINA1-6 constructs (SINA1-6-HA), NAC1 (NAC1-FLAG) construct or empty vector were syringe-infiltrated into *Nicotiana benthamiana* leaves at a concentration of $OD_{600} = 0.4$. *A. tumefaciens* containing the empty vector was used as a negative control. MG132 (100 μM) was coinjected with the *Agrobacterium* suspension to prevent the degradation of NAC1-FLAG protein. Two days after *Agrobacterium* infiltration, proteins were extracted for immunoprecipitation (IP) with α-HA affinity matrix, followed by western blotting (WB) using the α-FLAG antibody to determine the association of NAC1 with SINA3. The presence of NAC1 protein (indicated by an arrow in the bottom right) in the immunoprecipitated complex of NAC1 coexpressed with SINA3 suggests specific interaction between them. The asterisk indicates a nonspecific cross-reaction band detected by the antibody.

NAC1 and SINA3 interact in the nucleus

Next, we used confocal microscopy and BiFC (Walter *et al.*, 2004; Kanaoka *et al.*, 2008) to determine where NAC1 and SINA3 are localized within the plant cell and where NAC1-SINA3 complex formation occurs. As shown in Fig. 2(a), under overexpression conditions in the presence of MG132 to prevent NAC1 degradation, NAC1-GFP was exclusively localized in the nucleus, whereas SINA3-GFP was observed in both cytoplasm and nucleus. It is notable that, unlike free GFP, the SINA3-GFP was not evenly distributed in the cytoplasm. To determine the localization of NAC1–SINA3 interaction, NAC1, SINA3 and SINA3$_{1-181}$, the N-terminal 181-amino-acid region of SINA3 lacking the SBD, were each cloned into BiFC vectors (Walter *et al.*, 2004). The resulting constructs were expressed in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression in the presence of MG132 and the BiFC analysis was performed 2 d after agrobacterial infiltration. As shown in Fig. 2(b), a strong YFP signal was observed when NAC1-NYFP was coexpressed with SINA3-CYFP, but not with the control SINA3$_{1-181}$-CYFP, which lacks the SBD, and was limited in the nucleus of the *N. benthamiana* cells, suggesting that NAC1 interacts with SINA3 in the nucleus.

SINA3 possesses ubiquitin ligase activity and ubiquitinates NAC1 *in vitro*

The specific *in planta* interaction of NAC1 with SINA3 suggests that SINA3 may directly ubiquitinate the NAC1 substrate. To verify this notion, we first determined that the ubiquitin ligase activity of SINA3 was capable of self-ubiquitination in the presence of ubiquitin E1 and E2 enzymes. We conducted the *in vitro* ubiquitination assay (Abramovitch *et al.*, 2006) using recombinant E1 (GST-AtUBA1) and E2 (GST-AtUBC8) enzymes, FLAG-tagged ubiquitin (FLAG-Ub), and MBP-SINA3. Self-ubiquitination of SINA3 was observed when FLAG-Ub, E1, and E2 were present (Fig. 3A, lane 1), but not in any control reaction in which any one of the necessary components was missing (Fig. 3a upper panel, lanes 2–5). Thus, SINA3 is a functional ubiquitin ligase.

To define NAC1 as a substrate of the SINA3 ubiquitin ligase, the recombinant MBP-NAC1-HA protein was included in the *in vitro* ubiquitination reaction mixture described earlier. After incubation, the ubquitination reaction mixture was immunoprecipitated with the α-HA antibody matrix to purify the substrate MBP-NAC1-HA. The purified MBP-NAC1-HA was subjected to western blotting analysis using α-FLAG or α-HA antibody to

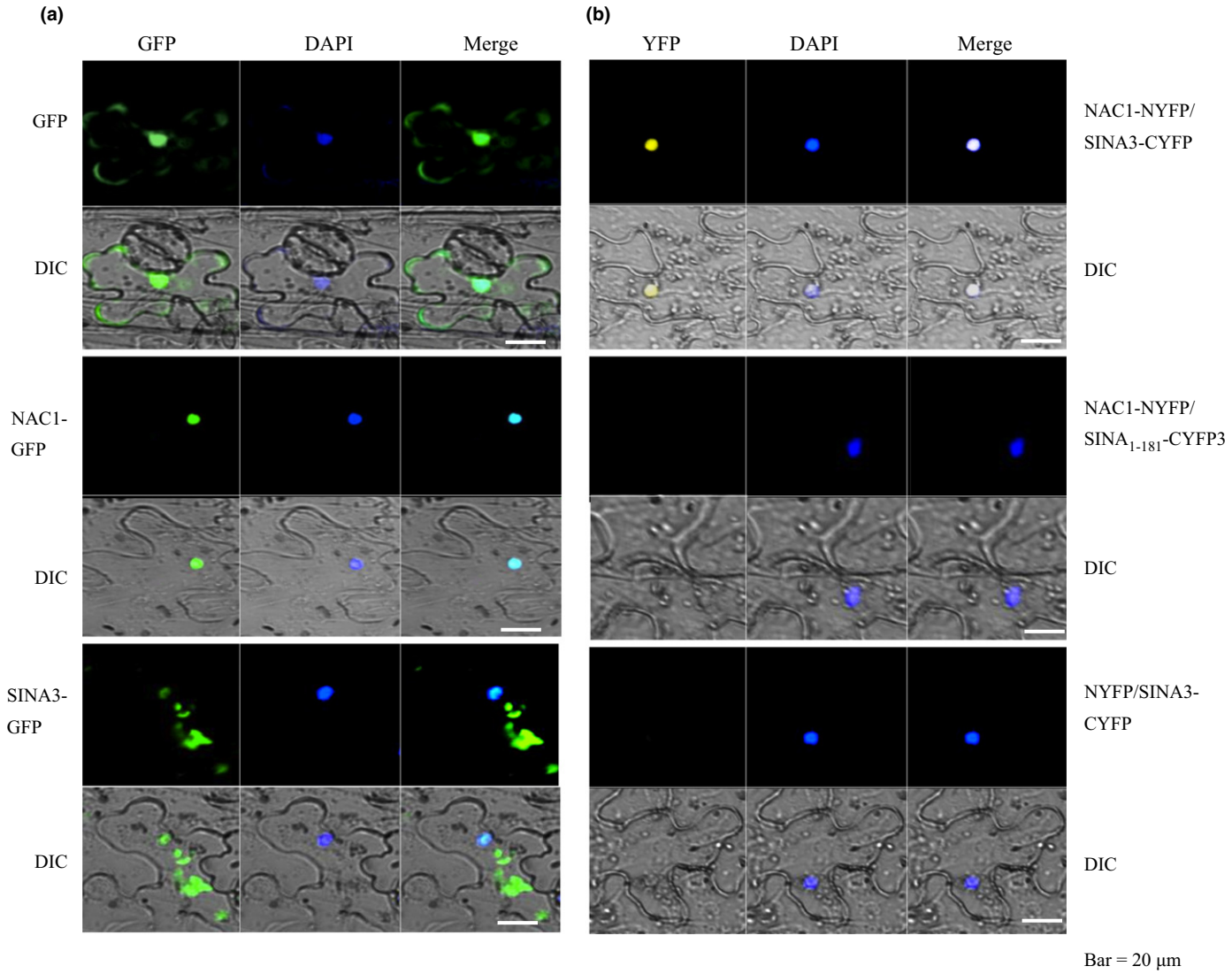

Fig. 2 NAC1 and SEVEN IN ABSENTIA3 (SINA3) interact in the nucleus. (a) Subcellular localization of NAC1 and SINA3. *Agrobacterium tumefaciens* GV2260 strains harboring the cauliflower mosaic virus (CaMV) 35S promoter-driven NAC1-GFP, SINA3-GFP or free green fluorescent protein (GFP) were infiltrated into *Nicotiana benthamiana* leaves at inoculums of $OD_{600} = 0.4$. Two days after agroinfiltration, the infiltrated leaf tissue was subjected to confocal microscopy, which indicates that the NAC1-GFP is exclusively localized in the nucleus, whereas the SINA3-GFP is localized in both cytoplasm and nucleus. Preceding 4′,6-diamidino-2-phenylindole (DAPI) staining of the leaf tissue verifies the nucleus. Differential interference contrast (DIC) images of the same view are aligned underneath the GFP signal images. (b) NAC1 interacts with SINA3 in the nucleus. The CaMV 35S promoter-driven NAC1-NYFP and SINA3-CYFP constructs were coexpressed in *N. benthamiana* leaves as in (a). After DAPI staining of leaf tissue to locate the nucleus, epidermal cell layers were examined using a confocal microscope to capture the yellow fluorescent protein (YFP) signal resulting from interaction between NAC1 and SINA3. Coexpression of NAC1-NYFP with $SINA3_{1-181}$-CYFP or NYFP with SINA3-CYFP did not result in production of YFP signal and served as negative controls, in which no restored YFP signal was observed. DIC images of the same view are aligned underneath the YFP signal images.

detect the presence of FLAG-Ub, MBP-NAC1-HA and FLAG-Ub-MBP-NAC1-HA protein conjugates. As expected, a ubiquitin-associated ladder-like smear, indicating the presence of polyubiquitination of both MBP-SINA3 (self-ubiquitination) and MBP-NAC1-HA, was detected by the α-FLAG antibody (Fig. 3b upper panel, lane 1). The identity of polyubiquitinated MBP-NAC1-HA was further verified by the α-HA antibody (Fig. 3b lower panel, lane 1), suggesting that SINA3 can ubiquitinate NAC1 *in vitro*. Thus, we conclude SINA3 is a ubiquitin ligase capable of ubiquitinating NAC1.

SINA3 promotes NAC1 degradation in planta via polyubiquitination

Next we sought to determine whether SINA3 can promote NAC1 degradation *in vivo* through ubiquitination. As NAC1 is rapidly degraded in tomato and cannot be detected by western blotting without the presence of MG132 (Huang *et al.*, 2013), we heterogeneously expressed NAC1 in *N. benthamiana*, in which the *Agrobacterium*-mediated transiently expressed NAC1 accumulated to certain level that can be detected by western

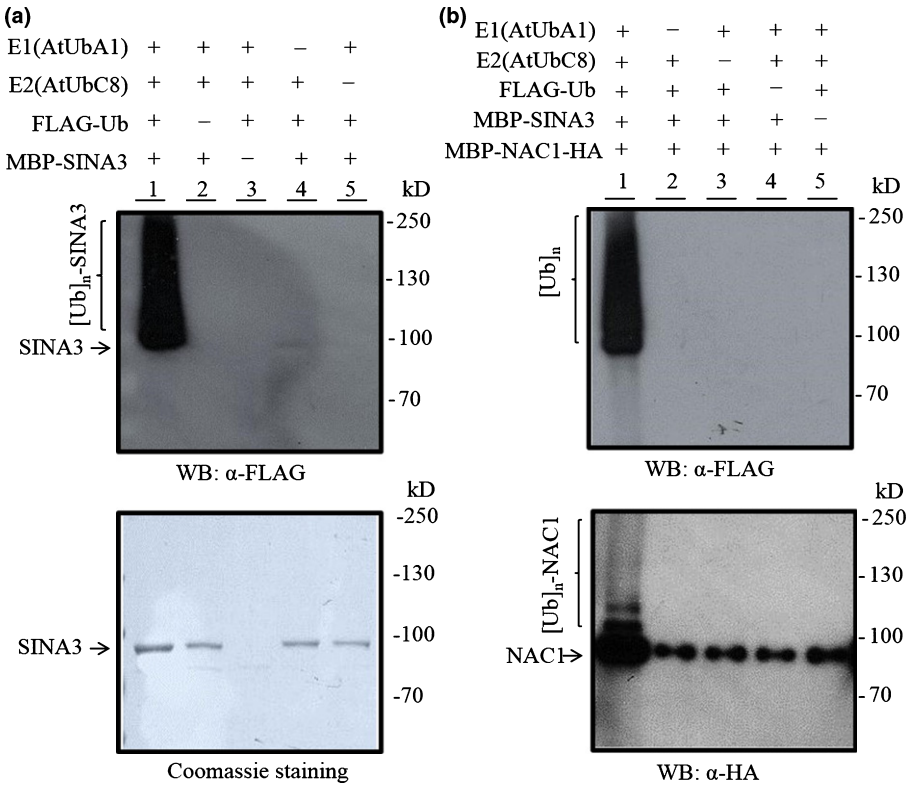

Fig. 3 SEVEN IN ABSENTIA3 (SINA3) ubiquitinates NAC1 *in vitro*. (a) The ubiquitin E3 activity of SINA3. Polyubiquitination of SINA3 (E3 ligase) was observed in the reaction in the presence of recombinant E1, E2 and FLAG-Ub (lane 1), but not in any control reaction in which any of the necessary components was missing (lanes 2–5). Polyubiquitinated forms of SINA3, caused by self-ubiquitination of SINA3, verify the ubiquitin ligase activity of SINA3. Coomassie staining of the western blotting (WB) indicates an equal amount of SINA3 present in the reactions (lower panel). (b) Polyubiquitination of NAC1 by SINA3 in the presence of recombinant E1, E2, maltose binding protein (MBP)-SINA3, MBP-NAC1-hemagglutinin (HA) and FLAG-Ub (lane 1). The *in vitro* ubiquitination reaction mixture was immunoprecipitated with α-HA antibody matrix to purify the MBP-NAC1-HA substrate, followed by WB using α-FLAG antibody (top panel) or α-HA antibody (lower panel) to determine all polyubiquitination forms (including self-ubiquitination of SINA3, which represents the majority of the polyubiquitinated protein) and the presence of polyubiquitinated MBP-NAC1-HA protein, respectively.

blotting (Fig. 4a). We found that the NAC1 level was dramatically attenuated when coexpressed with SINA3, while its accumulation was not significantly affected when coexpressed with the vector control or the nonfunctional mutant $SINA3^{C72S}$ (Fig. 4a), in which the conserved Cys was substituted with a Ser in the RING domain (Den Herder *et al.*, 2012). This result indicates that SINA3 promotes NAC1 degradation *in vivo*. In general, the 26S proteasome-mediated degradation is dependent on polyubiquitination of the target protein. Thus, we next sought to examine the potential ubiquitination of NAC1 by SINA3 *in vivo*. Again, NAC1-FLAG and HA-tagged ubiquitin were coexpressed in *N. benthamiana* leaves with or without the presence of SINA3-HA, and the possible ubiquitination of NAC1-HA triggered by SINA3 was investigated. In order to observe the ubiquitinated NAC1 protein, the proteasome-specific inhibitor MG-132 was included in the agrobacterial inoculum to prevent NAC1 degradation. Total protein was extracted at 36 h post agroinfiltration, followed by western blotting using α-FLAG antibody to detect NAC1 protein. As shown in Fig. 4(b), the polyubiquitinated NAC1-HA moieties, indicated as the smear banding pattern above the NAC1-HA, were detected in the presence of SINA3, suggesting that SINA3 may ubiquitinate NAC1 *in vivo*. It is notable that in the absence of SINA3, a slow-migrated NAC1 form was observed (indicated by the asterisk in Fig. 4b). The identity of this NAC1 band was not determined. Significantly, when SINA3 was coexpressed, this slow-migrated form was abolished and the NAC1 was heavily polyubiquitinated instead. Taken together, these results suggest that SINA3 is a ubiquitin E3 ligase promoting the ubiquitination-mediated degradation of NAC1 in plant cells.

In contrast to up-regulation of the NAC1 gene, the expression of the SINA3 gene is down-regulated during the defense response to *Pseudomonas* infection

Given the fact that NAC1 plays an important role in the defense response and is subjected to UPS-mediated degradation, up-regulation of *NAC1* gene transcription during the defense response could be a strategy used by plants to produce more NAC1 protein to compensate for its degradation by ubiquitin ligases (Huang *et al.*, 2013). Alternatively, plants might adopt other mechanisms to interfere with the ubiquitination of NAC1 protein, such as repressing transcription of the cognate ubiquitin ligase genes. To test the latter hypothesis, we determined *SINA3* mRNA abundance during the response to *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) infection. Following the experimental regime used in our previous studies on *NAC1*(Huang *et al.*, 2013), we examined *SINA3* mRNA abundance in three different interactions between tomato and *Pst*: the R-mediated immune interaction in which resistant RG-*PtoR* plants (expressing the resistance gene *Prf*) were inoculated with *Pst*DC3000; the disease interaction in which susceptible RG-*prf3* plants (containing a 1 kb deletion in the *Prf* gene; Salmeron *et al.*, 1996) were inoculated with *Pst*DC3000; and the pathogen-associated molecular pattern-triggered immune interaction in which susceptible RG-*prf3* plants were inoculated with the nonpathogenic *Pst*DC3000 *hrcC* mutant strain (Deng *et al.*, 1998). RG-*prf3* plants infiltrated with 10 mM $MgCl_2$ served as a mock

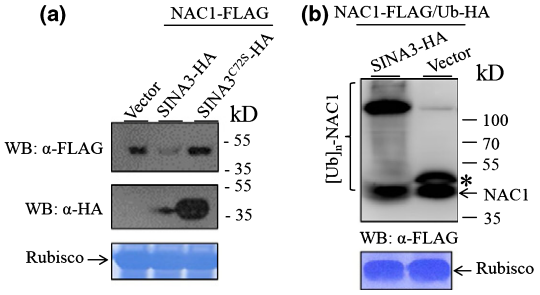

Fig. 4 SEVEN IN ABSENTIA3 (SINA3) promotes NAC1 degradation *in planta* via polyubiquitination. (a) *Agrobacterium tumefaciens* GV2260 strains harboring the cauliflower mosaic virus (CaMV) 35S promoter-driven FLAG-tagged NAC1 (NAC1-FLAG), in combination with the HA-tagged SINA3 (SINA3-HA), SINA3^{C72S} mutant (SINA3^{C72S}-HA), or empty vector, were infiltrated into *Nicotiana benthamiana* leaves at a concentration of OD_{600} = 0.3. Leaf tissue was harvested at 32 h after infiltration for western blotting (WB) using the α-FLAG or α-HA antibody. (b) *Agrobacterium tumefaciens* GV2260 strains harboring the CaMV 35S promoter-driven constructs of epitope-tagged ubiquitin (HA-Ub) or NAC1 (NAC1-FLAG) were infiltrated into *N. benthamiana* leaves at a concentration of OD_{600} = 0.4. *A. tumefaciens* containing the empty vector (Vector) was used as a control. MG132 (100 μM) was coinjected with the *Agrobacterium* suspension to prevent the degradation of NAC1 protein. Thirty-six hours after *Agrobacterium* infiltration, proteins were extracted for WB assay using α-FLAG antibody to determine NAC1-associated polyubiquitin chain, which appears as a smear. The asterisk indicates an unidentified modification form of NAC1.

control. By real-time reverse transcription polymerase chain reaction analysis, we monitored the transcript abundance of the *SINA3* and *NAC1* genes at different time points after bacterial infiltration to determine the correlation between the expression patterns of these two genes in response to pathogen infection. As shown in Fig. 5, *NAC1* mRNA abundance was increased in all three interactions, which was consistent with our previous publication (Huang *et al.*, 2013), whereas the expression of the *SINA3* mRNA was reciprocally repressed. Significantly, the down-regulation pattern of the *SINA3* gene was inversely correlated with the up-regulation pattern of the *NAC1* gene in the three different tomato–*Pst* interactions. Taken together, our results suggest that tomato plants have evolved a complex mechanism to regulate the defense-related NAC1 transcription factor in response to *Pseudomonas* infection: up-regulation of the *NAC1* gene to produce more NAC1 protein and down-regulation of the *SINA3* gene encoding the ubiquitin ligase to compensate for NAC1 degradation.

Overexpression of SINA3 represses the R protein-mediated HR cell death in *N. benthamiana*

We next sought to determine the biological significance of the SINA3 ubiquitin E3 ligase in plant defense signaling. We first examined the effect of SINA3 on HR cell death signaling mediated by the tomato resistance protein Prf, which confers resistance to *Pst* (Salmeron *et al.*, 1996). The Prf^{D1416V} mutant is an autoactive form of Prf and can trigger the HR cell death when transiently expressed in *N. benthamiana* leaves (Du *et al.*, 2012). We coexpressed SINA3 and Prf^{D1416V} in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression at a 4 :1 (SINA3:Prf^{D1416V}) inoculum ratio. We found that cell death triggered by Prf^{D1416V} was abolished in the presence of SINA3 (Fig. 6a), suggesting that SINA3 negatively regulates the HR cell death signaling. As SINA3 is a ubiquitin ligase, one possible reason for this suppression of cell death is that SINA3 ubiquitinates Prf^{D1416V}, resulting in its degradation. To test this possibility, we examined Prf^{D1416V} accumulation *in planta* with or without the presence of SINA3. Prf^{D1416V} causes a strong cell death reaction that might trigger nonspecific protein degradation, so we coexpressed Prf^{D1416V} with SINA3 at a slightly higher concentration of inoculum, OD_{600} = 0.3. Western blotting analysis indicated that coexpression of SINA3 with Prf^{D1416V} did not affect Prf^{D1416V} accumulation (Fig. 6a), suggesting that SINA3 does not lead to Prf^{D1416V} protein degradation.

We next asked whether the cell death suppression activity of SINA3 is specific to Prf-mediated HR cell death signaling, or whether SINA3 acts as a general negative regulator for HR cell death signaling. We assessed the ability of SINA3 to interfere with HR cell death triggered by two other autoactive R proteins, Rpi-blb1^{D475V} and Rx^{D460V}, both of which cause HR cell death when overexpressed in *N. benthamiana* leaves (Bendahmane *et al.*, 2002; van Ooijen *et al.*, 2008). SINA3 was coexpressed with Rpi-blb1^{D475V} or Rx^{D460V} in *N. benthamiana* leaves at the same 4 : 1 *Agrobacterium* inoculum ratio as used for Prf^{D1416V}. In around 50% of experimental repetitions, we found that Rpi-blb1^{D475V}- or Rx^{D460V}-triggered cell death was suppressed by overexpression of SINA3, and SINA3 indeed does not trigger Rpi-blb1^{D475V} or Rx^{D460V} degradation (Fig. 6b,c). Representative leaf tissue showing SINA3-mediated cell death suppression is shown in Fig. 6(b). We note that the cell death suppression activity of SINA3 for Rpi-blb1^{D475V}- or Rx^{D460V}-induced HR was less effective than that observed for the suppression of Prf^{D1416V}-induced HR, which is probably a result of extremely strong cell death elicited by overexpression of Rpi-blb1^{D475V} or Rx^{D460V} (van Ooijen *et al.*, 2008). The Rx^{D460V} protein was not detected when expressed with the empty vector control (Fig. 6c), presumably as a result of the nonspecific protein degradation caused by such extremely strong cell death.

Discussion

As NAC transcription factors are one of the largest families of plant-specific transcription factors and they have significant roles in diverse physiological processes, it is not surprising to us that the regulation of NAC transcription factors is complex and occurs at multiple levels. First, NAC transcription factors can be regulated at the transcriptional level: up- or down-regulated in response to internal or external stimuli, which is the case for numerous NAC transcription factors (Olsen *et al.*, 2005; Puranik *et al.*, 2012). Second, a few *Arabidopsis* NAC transcription factors, including CUC1/2 and AtNAC1, are regulated at the post-transcriptional level via miRNA-mediated cleavage (Olsen *et al.*, 2005; Puranik *et al.*, 2012). Third, several NAC transcription factors are subjected to post-translational modifications, such as phosphorylation of *Arabidopsis* NTL6 and rice OsNAC2 by the

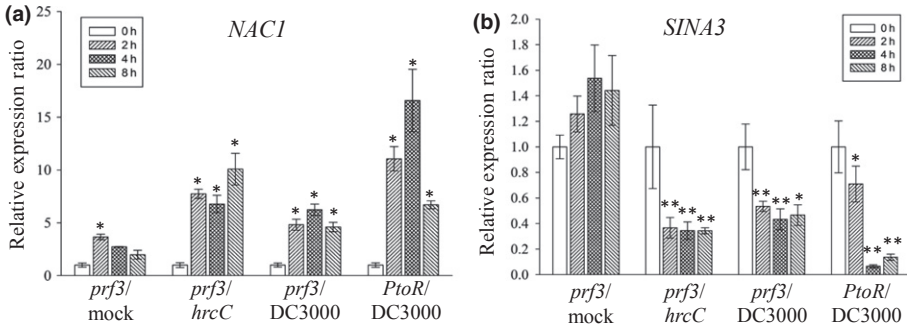

Fig. 5 Down-regulation of the *SEVEN IN ABSENTIA3* (*SINA3*) gene by *Pseudomonas* infection. (a, b) Up-regulation of the *NAC1* gene (a) but down-regulation of the *SINA3* gene (b) during different plant–pathogen interactions. Resistant *RG-PtoR* or susceptible *RG-prf3* tomato plants were inoculated with appropriate *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 strains at an inoculum of 2×10^7 colony-forming units ml^{-1} or mock solution (10 mM $MgCl_2$). In all cases, total RNA was isolated at the indicated time points after *Pst* infiltration. The relative expression level of *NAC1* or *SINA3* genes were analyzed by real-time reverse transcription polymerase chain reaction using the gene-specific primers. The expression of the tomato *EF1-α* gene served as an internal control for normalization. Values are means ± SE of three replicates. Asterisks represent significant differences (**, $P < 0.001$; *, $P < 0.05$, by two-tailed Student's *t*-test) in *NAC1* or *SINA3* gene expression between 0 h and other time points after *Pst* or mock treatment. The experiment was repeated three times with similar results.

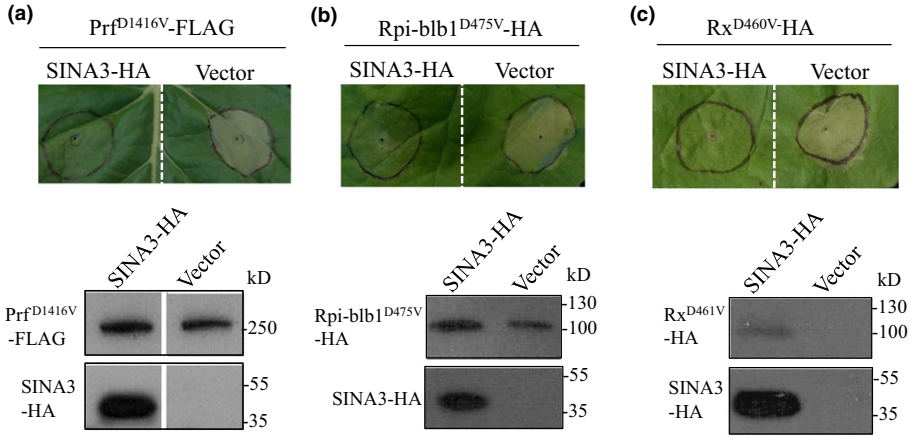

Fig. 6 SEVEN IN ABSENTIA3 (SINA3) represses hypersensitive response (HR) cell death mediated by multiple resistance proteins in *Nicotiana benthamiana*. Overexpression of SINA3 interferes with HR cell death mediated by multiple R proteins. (a–c) *Agrobacterium tumefaciens* GV2260 strains carrying the indicated constructs were syringe-infiltrated into *N. benthamiana* leaves at $OD_{600} = 0.2$ for Prf^{D1416V}-FLAG (a), Rpi-$blb1^{D475V}$ (b) or Rx^{D460V} (c) and $OD_{600} = 0.8$ for SINA3-HA or vector control. All genes were expressed from the 35S cauliflower mosaic virus (CaMV) promoter. Photographs were taken 3 d after *Agrobacterium* infiltration. The assay results provided below indicate that SINA3 does not trigger degradation of Prf^{D1416V}, Rpi-$blb1^{D475V}$ or Rx^{D460V}.

SnRK2.8 kinase and an as-yet-unidentified kinase (Kaneda *et al.*, 2009; Kim *et al.*, 2012), respectively, and ubiquitination of *Arabidopsis* AtNAC1 by the SINAT5 ubiquitin ligase (Xie *et al.*, 2002). We previously found that the tomato NAC1 transcription factor is regulated at both the transcriptional and post-translational levels, as manifested by increased *NAC1* mRNA abundance in response to *Pseudomonas* infection and the ubiquitination of NAC1 protein (Huang *et al.*, 2013). In this work, we sought to identify a ubiquitin ligase that regulates NAC1 protein accumulation. We examined six tomato SINA ubiquitin ligases (SINA1-6) and determined that SINA3 specifically interacts with NAC1 in plant cells. We also found that SINA3 ubiquitinates NAC1 *in vitro* and promotes NAC1 degradation via polyubiquitintion *in vivo*. In addition, we demonstrated that SINA3 interferes with HR cell death signaling mediated by multiple R proteins. Our results support the hypothesis that SINA3 ubiquitin ligase negatively regulates cell death signaling by promoting the ubiquitination-mediated degradation of defense-related proteins.

Among the six highly homologous tomato SINAs (SINA1-6) identified, SINA3 interacts with and ubiquitinates the defense-related NAC1 transcription factor in Y2H assay. Although the SINA3–NAC1 interaction was found to be weak in yeast (we could only detect the interaction on X-Gal-dependent, but not leucine-dependent, assay, which in fact suggests the C-terminal 261–301 amino acid region of NAC1 is required for the interaction), it was also further verified by coIP assay *in planta*. The ubiquitination of NAC1 by SINA3 was determined by polyubiquitination of NAC1 when ubiquitin E1, E2, SINA3 and NAC1 were incubated with the ubiquitin molecule. It is generally thought that polyubiquitination of a substrate protein results in proteasomal proteolysis, and thus coexpression of the ubiquitin ligase with the substrate may promote the degradation of the substrate. This notion was substantiated by the degradation of the

exogenously expressed NAC1 in the presence of coexpression of SINA3 (Fig. 4a), which is consistent with the *in vivo* polyubiquitination of NAC1 triggered by SINA3 in *N. benthamiana* (Fig. 4b). Taken together, our data suggest that SINA3 ubiquitinates NAC1 transcription factor to promote its degradation. It is interesting to note that, without coexpression of SINA3, NAC1 protein also accumulated as a slow-migrated form (Fig. 4b), representing an unidentified modification of NAC1. We speculate that this could be a monoubiquitinated and/or phosphorylated form of NAC1, a topic to be explored in future experiments. Significantly, such modification was abolished when SINA3 was applied and the main modification of NAC1 changed to polyubiquitination.

Our previous publication demonstrated that the defense-related NAC1 transcription factor is fine-tuned at both transcriptional and post-translational levels and is important for plant disease resistance (Huang *et al.*, 2013). It is reasonable to speculate that under normal conditions without pathogen infection, plants need to tightly control transcription factors like NAC1 to prevent autoactivation of defense signaling. At the post-translational level, a rapid turnover of NAC1 protein may help to maintain the signaling balance, while at the transcriptional level, the *NAC1* gene is expressed at a basal level to produce a limited amount of NAC1 protein. Upon pathogen infection, plants could employ mechanisms to counteract the degradation of NAC1 protein. One is rapid expression of the *NAC1* gene in order to produce more protein, ideally overcoming the degradation of NAC1, while the other would be interference with ubiquitination of NAC1 to prevent its degradation, which can be achieved by transcriptional down-regulation of the *SINA3* ubiquitin ligase gene. Reduced mRNA abundance of the *SINA3* gene in response to *Pst*DC3000 infection supports this hypothesis. Unfortunately, because of a lack of a NAC1-specific antibody, we were unable to determine the dynamic change of ubiquitination and degradation of native tomato NAC1 protein during the defense response to *Pseudomonas* infection. Generation of transgenic tomato plants expressing epitope-tagged NAC1-HA under the control of a constitutive promoter (such as the CaMV 35S promoter instead of the stress-inducible native promoter) will avoid protein accumulation as a result of transcriptional induction of the *NAC1* gene and should enable us to address dynamic regulation of NAC1 protein levels in future work.

Given the fact that NAC1 plays a positive role in the plant defense response (Huang *et al.*, 2013) and SINA3 ubiquitinates NAC1 for promoting its degradation (Figs 3, 4), it is logical to hypothesize that SINA3 functions as a negative regulator in plant defense signaling. This was substantiated by the down-regulation of SINA3 gene expression in response to *Pseudomonas* infection (Fig. 5) and interference of overexpressed SINA3 with HR cell death triggered by multiple R proteins (Fig. 6). However, it is possible that, besides targeting the NAC1 transcription factor, SINA3 also ubiquitinates other positive defense regulator(s) to promote its degradation, thereby repressing HR cell death signaling during the defense response. In fact, the SINA3–NAC1 interaction is exclusively restricted in the nucleus (Fig. 2b), suggesting that SINA3 ubiquitinates NAC1 in the nucleus for proteasome-mediated degradation. Additionally, NAC1 is exclusively localized in the nucleus, whereas SINA3 is localized in both the nucleus and cytoplasm. Therefore, it is possible that the cytoplasm-localized SINA3 also targets other unknown factors, thereby promoting their degradation in the cytoplasm. Thus, identification of additional SINA3 ubiquitin ligase substrates will be helpful to elucidate the mechanistic basis of SINA3-mediated regulation in defense response.

It is interesting to note that all plant SINA ubiquitin ligases identified to date from four plant species (SINAT5 from *Arabidopsis*, SINA4 from *Lotus japonicus*, OsDIS1 from rice and SINA3 from tomato) function as negative regulators of certain physiological processes: the Arabidopsis SINAT5 negatively controls lateral root growth by targeting the transcription factor AtNAC1 essential for the auxin-mediated lateral root development (Xie *et al.*, 2002); the *L. japonicus* SINA4 negatively regulates *Sinorhizobium* infection (Den Herder *et al.*, 2012) and is speculated to exert a negative effect on nodulation as it apparently targets the symbiosis receptor-like kinase (SYMRK), a positive regulator in symbiotic signal transduction, for promoting its degradation (Den Herder *et al.*, 2012); the rice OsDIS1 plays a negative role in drought stress tolerance, presumably by targeting the tubulin complex-related kinase OsNek6 despite lack of evidence for the ubiquitination of OsNsk6 by OsDIS1(Ning *et al.*, 2011a); and in this study we demonstrate that SINA3, and possibly other SINA isoforms, acts as a negative regulator for cell death signaling. Therefore, it seems that plant SINA ubiquitin ligases generally act as negative regulators by targeting important positive components in certain signaling pathways. In particular, we found in this study that SINA3 targets a defense positive regulator SlNAC1. As SINA3 interferes with HR cell death triggered by multiple R proteins without affecting their accumulation level, it is likely that SINA3 also targets a conserved cell death positive regulator(s), functioning downstream of the R protein at a convergence point of HR cell death signaling, for ubiquitination and degradation. It will be interesting to test whether SINA3 can ubiquitinate the known cell death regulators (e.g. MAPKKKα; del Pozo *et al.*, 2004) and/or explore other putative ubiquitination targets of SINA3 using a biochemical approach such as Y2H screening, which will be a subject of our future research.

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Author contributions

M.M., X.N., J.K. and X.D. performed the research; Y.L. and F.X. designed the research; J.A. and T.P.D. contributed new reagents; Y.L., J.C.K. and F.X. analyzed the data; and Y.L. and F.X. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Alignment of six tomato SINA ubiquitin ligases.

Fig. S2 A weak interaction between NAC1 and SINA3 identified by the LexA-based Y2H assay.

Table S1 List of primers used in this study

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