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Functional analysis of the seven in absentia ubiquitin ligase family in tomato

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Abstract

Seven in absentia (SINA) protein is one subgroup of ubiquitin ligases possessing an N-terminal cysteine-rich really interesting new gene (RING) domain, two zinc-finger motifs, and a C-terminal domain responsible for substrate-binding and dimerization. In tomato (*Solanum lycopersicum*), the *SINA* gene family has six members, and we characterize in this study all tomato *SINA* (*SlSINA*) genes and the gene products. Our results show that *SlSINA* genes are differentially regulated in leaf, bud, stem, flower, and root. All SlSINA proteins possess RING-dependent E3 ubiquitin ligase activity, exhibiting similar specificity towards the E2 ubiquitin-conjugating enzyme. SlSINA1/3/4/5/6 are localized in both cytoplasm and nucleus, whereas SlSINA2 is exclusively localized in the nucleus. Moreover, all SlSINAs can interact with each other for homo- or hetero-dimerization. The functionality of SlSINA proteins has been investigated. SlSINA4 plays a positive role in defense signalling, as manifested by elicitation of E3-dependent hypersensitive response-like cell death; the other SlSINAs are negative regulator and capable to suppress hypersensitive response cell death. Transgenic tomato plants overexpressing *SlSINA2* exhibit pale-green leaf phenotype, suggesting SlSINA2 regulates chlorophyll level in plant cells, whereas transgenic tomato plants overexpressing *SlSINA5* have altered floral structure with exserted stigma, implicating *SlSINA5* plays a role in flower development.

KEYWORDS

NAC transcription factor, plant defense response, UPS-mediated degradation

1 | INTRODUCTION

Ubiquitination is a crucial posttranslational modification with addition of one or more ubiquitin molecules to a substrate protein. Poly-ubiquitination through Lys48 of ubiquitin often results in the degradation of substrate proteins by the 26S proteasome, whereas other Lys-mediated ubiquitination is usually involved in other nondegradative cellular processes, such as signal transduction, subcellular distribution, DNA damage response, and cell cycle control (Chen & Sun, 2009; Husnjak & Dikic, 2012; Swatek & Komander, 2016). Eukaryotes have evolved the ubiquitin system to regulate their proteins to adapt to different developmental stages and environmental changes (Hellmann & Estelle, 2002; Moon, Parry, & Estelle, 2004; Stone, 2014; Teixeira & Reed, 2013). The attachment of ubiquitin to target protein is achieved through a sequential action of three enzymes involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. First, the 76-amino acid ubiquitin molecule is linked to an E1 enzyme in an ATP-dependent manner; second, an E2 takes over the activated ubiquitin from E1 through the cysteine residue at the active site, forming an E2-ubiquitin

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intermediate; finally, the E2-ubiquitin and substrate are brought together by an E3 ligase, so that the ubiquitin is covalently attached to a lysine residue of the substrate protein (Hershko & Ciechanover, 1998).

The ubiquitination pathway is hierarchical in terms of these enzymes. A given eukaryotic genome may have one or two *E1* genes, several dozen *E2* genes, and more than one thousand *E3* genes, which renders the capacity and specificity of the eukaryotic ubiquitin system to modify numerous proteins (Kraft *et al*., 2005, Li *et al*., 2008, Peng *et al*., 2003, Vierstra, 2003, Wagner *et al*., 2011). The difference between the numbers of E2s and E3s suggests that each E2 may serve several E3s as provider of ubiquitin. In contrast, E3 may also be able to function with one or a few E2s in the ubiquitination reaction. In general, the eukaryotic E3 ubiquitin ligases fall into two main families by the presence of either a homology to E6-AP C terminus or a really interesting new gene (RING) domain (Pickart, 2001; Zeng, Vega-Sánchez, Zhu, & Wang, 2006). Significantly, the RING domain is characterized by eight conserved Cys and His residues that coordinate with two zinc ions to help maintain the overall structure of the protein (Deshaies & Joazeiro, 2009). Some RING-containing ubiquitin ligases can work as single subunit protein to gather E2s and substrates for ubiquitination, such as constitutive photomorphogenesis 1 and arm repeat-containing 1, whereas others, such as the anaphase-promoting complex and Skp-Cullin-F-box complex, function as a multisubunit ubiquitin ligase complex with a separate subunit to bind substrates (Hellmann & Estelle, 2002; Moon *et al*., 2004; Stone, Anderson, Mullen, & Goring, 2003).

Seven in absentia (SINA) ubiquitin ligases belong to the RING-type E3 family and contain an N-terminal RING domain that binds to E2, followed by two zinc-fingers and a typical SINA domain devoted to substrate recognition and dimerization (Hu & Fearon, 1999). Although SINA was originally identified in *Drosophila melanogaster* with a significant role in photoreceptor differentiation (Carthew & Rubin, 1990), a growing body of evidence has demonstrated that it plays important roles in many physiological processes in animals, including mitosis (Germani *et al*., 2000), cell growth (Matsuzawa, Takayama, Froesch, Zapata, & Reed, 1998), hypoxia responses (Ma *et al*., 2015), and apoptosis (Christian, Fiandalo, & Schwarze, 2011). In plants, SINA ubiquitin ligases have been demonstrated to play roles in development, abiotic stress response, and plant-microbe interactions. Five SINA members (SINAT1–5) have been identified in *Arabidopsis* (Wang *et al*., 2008). SINAT1 and 2 participate in nutrient starvation response through ubiquitinating the autophagy protein 6 for degradation (Qi *et al*., 2017), whereas SINAT2 by itself is involved in carotenogenesis via ubiquitin-mediated proteolysis of RAP2.2 (Welsch, Maass, Voegel, Dellapenna, & Beyer, 2007). SINAT5 ubiquitinates the NAC1 transcription factor promoting its degradation, thereby attenuating the auxin-induced lateral root formation (Xie *et al*., 2002); SINAT5 may also play a role in the regulation of flowering time through controlling the stability of late elongated hypocotyl and flowering locus C (Park *et al*., 2007; Park *et al*., 2010). In addition, a SINA-like protein SINAL7 has been shown to play an important role in flower development (Peralta, Araya, Nardi, Busi, & Gomez-Casati, 2013). In rice, six *SINA* genes are predicted in the genome, but only one, termed *OsDIS1*, is found to play a negative role in drought stress tolerance, presumably through ubiquitination of the kinase OsNek6 (Ning *et al*., 2011). In *Lotus japonicus*, an SINA ubiquitin ligase (SINA4) has been demonstrated to be responsible for the turnover of symbiosis receptor-like kinase, which is required to initiate both arbuscular mycorrhiza and nodulation (Den Herder, Yoshida, Antolin-Llovera, Ried, & Parniske, 2012).

Recently, we have identified six *SINA* genes in the tomato genome. One of them, termed *SlSINA3*, encodes an E3 ubiquitin ligase that specifically ubiquitinates the defense-related transcription factor SlNAC1 promoting its degradation (Miao *et al*., 2016). In this study, we characterized all six SlSINA proteins. We determined the expression of *SlSINA* genes in different tissues and verified the ubiquitin ligase activity of the encoded SlSINA proteins. Significantly, all SlSINAs exhibit similar preference to E2 ubiquitin-conjugating enzymes in the ubiquitination reaction. In addition, we investigated their roles in diverse physiologic processes, including defense response and development.

2 | MATERIALS AND METHODS

2.1 | Sequence alignment and phylogenetic analysis

The amino acid sequences of SlSINA1, SlSINA2, SlSINA3, SlSINA4, SlSINA5, SlSINA6 (the accession numbers for tomato *SlSINA1–6* genes are AK324518, BT013026, AK322153, AK320390, AK321160, and XM_004248034 in the National Center for Biotechnology Information, respectively), SINAT5, SINA, and SIAH1 were collected in FASTA format. Protein data matrices were aligned using the ClustalX 2.1 multiple sequence alignment program with default gap penalties (Larkin *et al*., 2007). For the phylogenetic analysis, all protein sequences were aligned with ClustalW and entered into MEGA6 to build an unrooted phylogenetic tree (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The evolutionary history was inferred using the neighbour-joining method with 1,000 bootstrap trials, and the support values are labelled on each branch.

2.2 | Plant growth conditions

Tomato *Solanum lycopersicum* cv. Rio Grande (RG) and *Nicotiana benthamiana* (Bombarely *et al*., 2012) plants were grown in a greenhouse with 16-hr light and 8-hr dark, 65% humidity, and a temperature of 26 °C during daytime and 22 °C at night.

2.3 | Quantitative real-time PCR

Total RNAs were isolated from the appropriate plant tissues with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and treated with DNase. Reverse transcription was conducted using SuperScriptII reverse transcriptase (Life Technologies, Carlsbad, CA, USA), and real-time PCR analysis was performed with ABI Prism 7700 sequence detection system using SYBR Green reagents (Life Technologies, Carlsbad, CA, USA). The tomato *Actin 41* gene was used as an internal reference. Relative expression ratios were determined using the REST software (Pfaffl, Horgan, & Dempfle, 2002). Primers used in real-time PCR are listed in Table S1.

2.4 | *Agrobacterium*-mediated transient expression in *N. benthamiana*

Six-week-old *N. benthamiana* plants were used for *Agrobacterium tumefaciens*-mediated transient expression as described previously (Xiao *et al*., 2007). For the subcellular localization assay, *A. tumefaciens* GV2260 strain containing the appropriate green fluorescent protein (GFP) chimera construct was injected into *N. benthamiana* leaves. After 48 hr, the epidermal cell layers were examined using confocal microscope (FV1000; Olympus, Tokyo, Japan) to capture the GFP signal. 4′,6-Diamidino-2-phenylindole staining was performed prior to fluorescence imaging to indicate the nucleus.

For the Western blotting assay, *Agrobacterium*-infected *N. benthamiana* leaf tissues were collected and ground to fine powder with liquid nitrogen, followed by further incubation in 200 µl protein extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 10% [*V*/*V*] glycerol, 1% polyvinylpolypyrrolidone [*W*/*V*], 1 mM phenylmethylsulfonyl fluoride, 10 µl/ml plant protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]) on ice for 10 min and centrifugation at 12,000 g/4 °C for 20 min. The protein extracts were denatured and separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by transferring to the PVDF membrane for Western blotting to detect HA- or Flag-tagged protein using the α-HA (1:2,000 *v*/v; Sigma-Aldrich, St. Louis, MO, USA) or α-Flag (1:5,000 v/v) antibody (Sigma-Aldrich, St. Louis, MO, USA). Primers used in generating the relevant constructs are listed in Table S1.

2.5 | Yeast two-hybrid (Y2H) assay

The LexA-based Y2H system (Golemis *et al*., 2011) was used to determine the interactions between SlSINA proteins. The full-length cDNAs of *SlSINA* genes were cloned into the bait vector pEG202 at the *EcoRI* and *SalI* sites and the prey vector pJG4–5 at the *Eco*RI and *Xho*I sites, respectively. The yeast (*Saccharomyces cerevisiae*) strain EGY48 harbouring the LacZα mark gene was transformed with the bait and prey constructs in the appropriate combinations. The transformed yeast cells were streaked onto X-Gal plates to assess the pair-wise interaction among the SlSINA proteins. Primers used in generating the Y2H constructs are listed in Table S1.

2.6 | Virus-induced gene silencing (VIGS)

The tobacco rattle virus (TRV) vector system was used for gene silencing in *N. benthamiana* plants as described previously (Liu et al., 2002). Acetosyringone-induced *Agrobacterium* cultures containing *TRV2*::*NbSGT1* construct (Peart et al., 2002) or *TRV2* empty vector were used for the inoculation of 2-week-old *N. benthamiana* seedlings.

2.7 | In vitro ubiquitination assay

The in vitro ubiquitination assay was performed following the protocol described in our previous study (Miao *et al*., 2016). The full-length cDNA of *SlSINA* genes were fused to the maltose binding protein (MBP) tag in the pMAL-C2 vector (NEB, Ipswich, MA, USA) at *EcoRI* and *SalI* sites. For SlSINA mutants, site-directed mutagenesis kit was used to introduce cysteine to serine mutation into the conserved RING domain. The recombinant proteins were expressed in the *Escherichia coli* BL21 strain in the presence of 0.5 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified with the Amylose Resin (NEB, Ipswich, MA, USA) following the manufacturer's instructions. To assess the E2–E3 specificity, the purified tomato E2 recombinant proteins were applied to replace the *Arabidopsis* E2 (AtUBC8) in the consistent reaction mixture. The tomato E2s used in this study are as follows: SlUBC1 (KY246895), SlUBC4 (KY246898), SlUBC6 (KY246900), SlUBC7 (KY246901), SlUBC12 (KY246906), SlUBC13 (KY246927), SlUBC17 (KY246911), SlUBC20 (KY246912), SlUBC22 (KY246914), SlUBC27 (KY246919), and SlUBC32 (KY246924). And the expression and purification of the tomato E2 recombinant proteins were performed as described previously (Zhou *et al*., 2017).

2.8 | Generation of transgenic tomato plants

Transgenic tomato plants were generated via the *Agrobacterium*-mediated transformation (Fillatti, Kiser, Rose, & Comai, 1987). The full-length cDNAs of *SlSINA* genes were cloned into the binary vector pBIN-ARS under the control of the cauliflower mosaic virus (CaMV) 35S promoter (van Engelen *et al*., 1995) and transformed into the tomato RG-PtoR. The transgenic tomato lines containing appropriate transgene were verified by PCR using the construct-specific primers. Homozygous T_2 progenies were identified by the segregation pattern of the corresponding T_3 progenies. Individual T_3 homozygous progenies were used for morphological and molecular characterizations.

2.9 | Chlorophyll analysis

Chlorophyll was extracted from representative tomato leaves using 80% (*V*/V) aqueous acetone. The amount of chlorophyll was determined as previous description (Nguyen *et al*., 2014).

3 | RESULTS

3.1 | Bioinformatic analysis of SlSINA proteins

We have recently characterized one tomato SINA ubiquitin ligase (termed SlSINA3) that is involved in defense response (Miao *et al*., 2016). There are six putative *SINA* genes (designated as *SlSINA1–6*) predicted in the tomato genome (Miao *et al*., 2016). In order to further characterize the functionality of SINA ubiquitin ligases in tomato, we cloned the full-length cDNA of all six *SlSINA* genes for the bioinformatic analysis. Alignment of the deduced amino acid sequences of SlSINA1–6 with other representative SINA proteins from Drosophila (SINA), human (SIAH1), and Arabidopsis (SINAT5) indicated SlSINA proteins possesses the conserved domains (RING, zinc-finger, substrate binding, and dimerization) with a variable N-terminal region (Figure 1a). SlSINA1 had a shorter C-terminus lacking 13 amino acids compared to SlSINA2–6 and AtSINAT5 (Figure 1a). The extended C-terminus of SlSINA2–6 was also found in other plant species (Figure S1a). The amino acid identity between SlSINAs and AtSINAT5 varied from 61% to 79%. Among these six SlSINAs, SlSINA1 shared less than

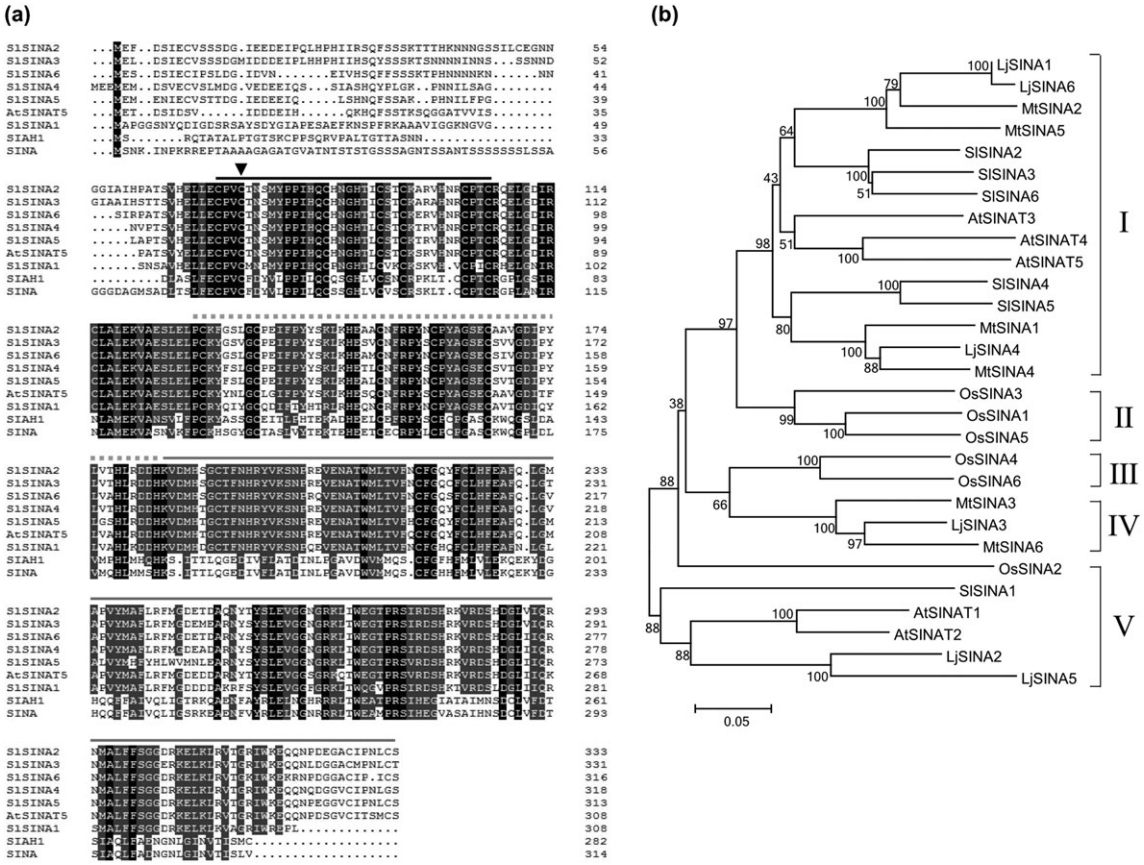

FIGURE 1 Sequence alignment and phylogenetic analysis of seven in absentia (SINA) proteins. (a) Amino acid sequence alignment of SlSINA1–6, AtSINAT5, SINA (Drosophila), and SIAH1 (human). Protein domains are indicated with different underlines, namely, black line (really interesting new gene domain), grey dashed line (zinc-finger motifs), and grey solid line (substrate binding and dimerization domain). The black inverted triangle points the conserved cysteine residue substituted by serine to generate inactive SlSINA proteins. (b) Phylogenetic analysis of SINA proteins from tomato (SlSINA), Arabidopsis (AtSINA), rice (OsSINA), *Medicago truncatula* (MtSINA), and *Lotus japonicus* (LjSINA). The unrooted phylogenetic tree was generated by the neighbour-joining method using the MEGA6 program with 1,000 bootstrap trials

60% identity with others, whereas around 80% identical sequence was found among SlSINA2–6 (Figure S1b).

A phylogenetic analysis was performed on 29 known SINA proteins from five plant species, Arabidopsis, rice (*Oryza sativa*), *Medicago truncatula*, *L. japonicus*, and tomato (*S. lycopersicum*). The phylogram constructed by the neighbour-joining analysis divided all SINA proteins into five groups (Figure 1b). Group I is the largest clade, containing 16 members with a long C-terminus, among which AtSINAT5 participates in lateral root formation and LjSINA4 is indispensable for initiating both arbuscular mycorrhiza and nodulation. Most SlSINAs belong to this group except SlSINA1 that was clustered into Group V. Group V also contains AtSINA1 and 2, which have been uncovered to play a role in nutrient starvation response (Qi *et al*., 2017). In addition, SlSINA3 and 6, as well as SlSINA4 and 5, were grouped in pair, implicating possible functional redundancy between them.

3.2 | Expression pattern of *SlSINA* genes in tomato

Because *SINA* genes, regardless from animals or plants, have been found to play roles in diverse physiological processes, it is necessary to determine the expression of *SlSINA* genes in different tissues to better understand their roles in tomato growth and development. We performed quantitative real-time PCR analysis to examine the expression of *SlSINA* genes in a variety of tissues including root, stem, young leaf, old leaf, bud, and flower. As shown in Figure 2, transcripts of six *SlSINA* genes were detected in all tested tissues. Significantly, *SlSINA3* and *SlSINA4* had similar spatial expression pattern with higher expression levels in old leaf, flower, and root. In addition, most *SlSINAs* were expressed at relatively high levels in root. Thus, the distinct spatial expression pattern displayed by *SlSINA* genes suggests that they are regulated distinctly and may play distinct roles in different tomato tissues.

3.3 | SlSINA proteins are functional ubiquitin ligase whose activity is dependent on the RING domain

Up to date, more than 40 plant *SINA* or *SINA-like* genes have been identified, but only nine have been demonstrated to encode functional E3 ubiquitin ligase (Bao *et al*., 2014; Den Herder *et al*., 2012; Miao *et al*., 2016; Ning *et al*., 2011; Qi *et al*., 2017; Xie *et al*., 2002). Although we have recently determined the ubiquitin ligase activity of SlSINA3 (Miao *et al*., 2016), it is necessary to verify the ubiquitin ligase activity of other five SlSINA proteins. It is generally thought that E3 ubiquitin ligase binds to E2 ubiquitin-conjugating enzyme and often becomes autoubiquitinated as part of its normal function (Pickart, 2001). Therefore, to test the intrinsic ubiquitin ligase activity of SlSINAs, we

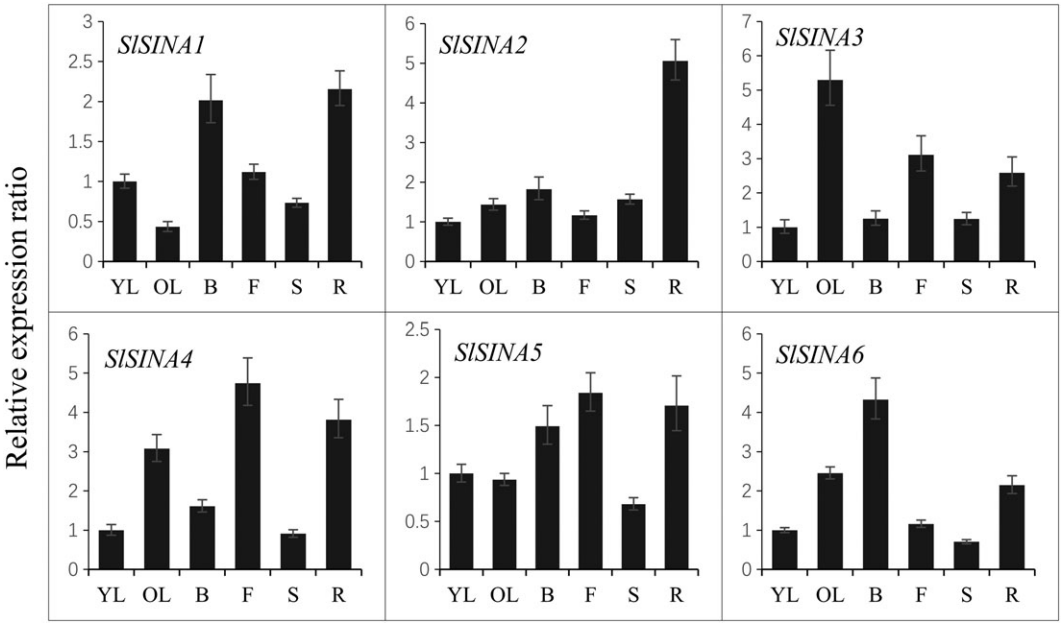

FIGURE 2 The expression pattern of *SlSINA* genes in various tomato tissues. Transcript levels of the six tomato *SlSINA* genes in different tissues (YL, young leaf; OL, old leaf; B, bud; F, flower; S, stem; R, root) were determined by quantitative real-time PCR. The tomato *Actin 41* gene was used for the reference transcript. The transcript values of the *SlSINA* genes determined in young leaf were set at 1. Values are means ± SE of three replicates

conducted the similar in vitro ubiquitination assay on SlSINA3 in our previous publication (Miao *et al*., 2016). Recombinant SlSINA proteins fused with MBP were incubated with recombinant E1 (GST-AtUBA1), E2 (GST-AtUBC8), and FLAG-tagged ubiquitin (FLAG-Ub), with MBP serving as a negative control. As shown in Figure 3a, smear pattern indicating polyubiquitination of protein was detected in reactions with the presence of recombinant E1, E2, FLAG-Ub, and MBP-SlSINAs (Figure 3a, lanes 1–6), but not in any control reaction in which MBP-SlSINA was replaced by MBP or any one of the necessary components was removed (Figure 3a, lanes 7–10). It is notable that these six SlSINA proteins exhibited E3 activity at different levels. SlSINA1/2/6 (Figure 3 a, lanes 1, 2, and 6) had much stronger activity, whereas the activity of SlSINA3/4/5 (Figure 3a, lanes 3, 4, and 5) was significantly weaker.

It has been demonstrated that the RING-type E3 ubiquitin ligase relies on the RING domain for its interaction with the E2 ubiquitin-conjugating enzyme; therefore, this domain is critical for its E3 activity (Den Herder *et al*., 2012; Hu & Fearon, 1999; Ning *et al*., 2011; Xie *et al*., 2002). We next determined whether the E3 activity of SlSINAs is

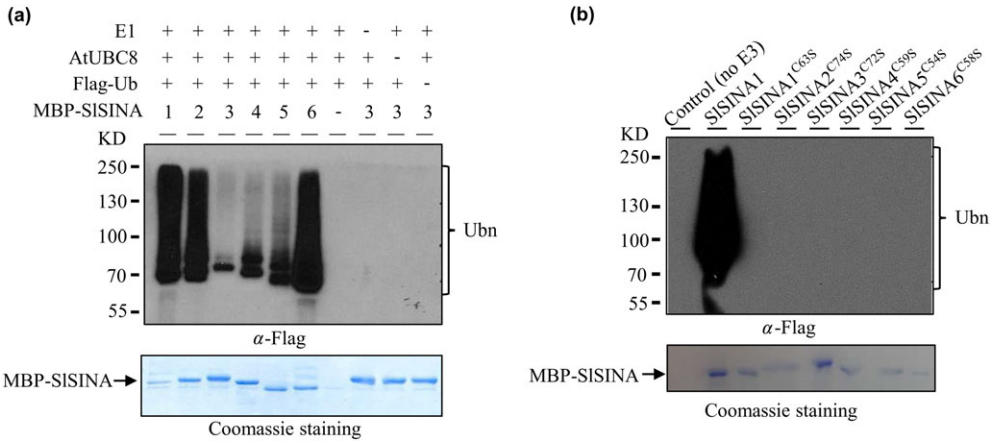

FIGURE 3 The really interesting new gene (RING)-dependent E3 activity of SlSINA proteins. (a) The E3 ubiquitin ligase activity of SlSINA proteins. Maltose binding protein (MBP)-tagged recombinant SlSINA proteins were included in the in vitro ubiquitination assay to determine their activity, which was indicated by polyubiquitination-associated smear detected by Western blotting (WB) using the α-Flag antibody in the presence of E1 (AtUBA1), E2 (AtUBC8), and Flag-Ub. No polyubiquitination-associated smear was detected in negative controls lacking E3 (SlSINA), E1, E2, or Flag-Ub (lanes 7–10). (b) The E3 activity of SlSINA proteins is dependent on the RING domain (the conserved cysteine residue substituted by serine was indicated in Figure 1a). No activity was detected in the in vitro ubiquitination reaction containing individual SlSINA mutants (Cys-to-Ser substitution) with E1 (AtUbA1), E2 (AtUBC8), and Flag-Ub. The WT SlSINA1 protein was included as the positive control, whereas the reaction without E3 served as the negative control. Coomassie staining of the WB indicates the amount of the WT or mutant MBP-SlSINA proteins [Colour figure can be viewed at wileyonlinelibrary.com]

dependent on the conserved RING domain. We generated SlSINA mutants, in which the RING domain was disrupted by substituting a conserved Cys with Ser (Figure 1a). As shown in Figure 3b, none of these RING-destructed mutants exhibited any E3 activity in the in vitro ubiquitination assay. Thus, all SlSINAs are typical RING-type E3 ubiquitin ligase, of which the RING domain is essential for the E3 activity.

3.4 | The E2 specificity of SlSINAs in catalysing ubiquitination

The ubiquitination activity of E3 ubiquitin ligase is fulfilled by transferring of ubiquitin to a substrate protein from an E2-ubiquitin intermediate, which is formed by the E2 ubiquitin-conjugating enzyme. Many studies have shown that the appropriate combination of E2 with E3 is critical for the potential ubiquitination of a given E3 (Kraft, Bostick, Jacobsen, & Callis, 2008; Zhao *et al*., 2013). In Arabidopsis, 37 E2s have been identified, among which AtUBC8 was widely used in in vitro ubiquitination analysis for plant-specific ubiquitin ligases (Kraft *et al*., 2005; Mbengue *et al*., 2010; Stone *et al*., 2005). However, AtUBC8 cannot always guarantee to act as the optional E2 for certain E3s (Mudgil, Shiu, Stone, Salt, & Goring, 2004). Recently, 40 putative E2 ubiquitin-conjugating enzymes have been identified in tomato to form 13 groups based on phylogenetic analysis, among which 10 groups were demonstrated to possess the E2 activity (Zhou *et al*., 2017). Significantly, the unique bacterial ubiquitin ligase AvrPtoB, which is a virulence effector encoded by *Pseudomonas syringae* pv. *tomato* (Abramovitch, Janjusevic, Stebbins, & Martin, 2006; Janjusevic, Abramovitch, Martin, & Stebbins, 2006), specially works with group III E2s for its *in planta* virulence activity (Zhou *et al*., 2017), suggesting that a given E3 ubiquitin ligase may have preference to distinct E2 ubiquitin-conjugating enzyme for its intrinsic activity. Thus, the weak ubiquitination rendered by SlSINA3/4/5 in the in vitro ubiquitination assay (Figure 3a) could be attributable to the intrinsic weak E3 activity or inappropriate E2 used in the assay. To test these possibilities and also determine the E2-preference specificity of six SlSINAs, we included different tomato E2s in our in vitro ubiquitination assay to determine their ability to catalyse ubiquitination together with SlSINAs. One or two E2 enzymes (SlUBC1/4/6/7/12/13/17/20/22/27/32) were randomly selected from 10 groups (Groups II, VI, V, III, IX, X, VIII, XII, I, and IV, respectively) to represent the tomato ubiquitin E2 family. As shown in Figures 4a and S2, six SlSINA proteins confer ubiquitination with a similar preference pattern towards different E2 ubiquitin-conjugating enzymes. In the case of SlSINA1, polyubiquitination was detected in the presence of SlUBC12 or SlUBC17, but not other E2 enzymes; and the E3 activity of SlSINA1 in the combination with SlUBC17 was much weaker than that with SlUBC12 (Figure 4a). Moreover, SlUBC12 was the only one, among all tested E2s, conferring polyubiquitination with SlSINA2/3/4/5/6 (Figure S2). These results indicate that SlSINA ubiquitin ligases have critical preference to the E2 ubiquitin-conjugating enzyme. Next, we sought to determine the E3 activity potential of six SlSINAs by the in vitro ubiquitination assay using the tomato E2 SlUBC12. We found as the case of use of the Arabidopsis E2 AtUBC8 (Figure 3a), SlSINA1, 2, and 6 exhibited higher E3 activity than SlSINA3, 4, and 5 (Figure 4b). Moreover, compared to AtUBC8, SlUBC12 facilitated greater level of polyubiquitination for all six SlSINAs, as indicated by the much more high-molecule mass (around 200kD) detected by Western blotting. This result also reminded us that, if available, the E2 ubiquitin-conjugating enzyme from the same species should be the first choice for the in vitro ubiquitination assay.

So far, for the limited number of plant SINA ubiquitin ligases that have been characterized, the Cys-to-Ser substitution at the RING domain confers a dominant-negative effect on their E3 activity (Den Herder *et al*., 2012; Xie *et al*., 2002). For example, the AtSINAT5^{C49S} and LjSINA1^{C47S} mutants compromise the self-ubiquitination activity of the WT AtSINAT5 and LjSINA1 proteins, respectively (Den Herder *et al*., 2012; Xie *et al*., 2002). Thus, we tested whether tomato SlSINA proteins possess such intrinsic characteristics by conducting the in vitro ubiquitination assay with addition of SlSINA Cys-to-Ser substitution mutants. As expected, all SlSINA Cys-to-Ser substitution mutants no longer possessed E3 activity (Figure 4c). However, to our surprise, the SlSINA1^{C63S} mutant did not show any significant effect on the E3 activity of the WT SlSINA1, even when the amount of SlSINA1^{C63S} protein added to the in vitro ubiquitination reaction was increased to 10-folds of that of the WT SlSINA1 protein (Figure 4d). We then extended our test to other SlSINA Cys-to-Ser substitution mutants and obtained the similar results (Figure S3). Taken together, our results indicate that tomato SlSINA ubiquitin ligases have distinct enzymatic potential, but, unlike other plant-specific SINA ubiquitin ligases (Den Herder *et al*., 2012), their indispensable RING domain does not possess a potential dominant-negative impact.

3.5 | Subcellular localization of SlSINA proteins

Given the fact that ubiquitination can occur in both cytoplasm and nucleus to regulate cytoplasmic and nuclear proteins (Heck, Cheung, & Hampton, 2010; Tanaka, Soriano, & Grusby, 2005; Xie *et al*., 2002; Yoo *et al*., 2013), the ubiquitin ligase must be localized in cytoplasm or nucleus or both. To investigate the subcellular localization of the SlSINA proteins, the full-length cDNA of *SlSINA1–6* genes were cloned into a plant expression vector in fusion with the GFP, and the resulting constructs were expressed in *N. benthamiana* leaves via the *Agrobacterium*-mediated transient expression. Considering the relatively weak leaf-expression of *SlSINA* genes from their native promoters (Figure 2), all *SlSINA* cDNAs were driven by the strong constitutive CaMV 35S promoter. Two days after *Agrobacterium* infiltration, the abaxial epidermis of the *N. benthamiana* leaves was subjected to confocal laser scanning microscopy. As shown in Figure 5, the green fluorescence of free GFP was distributed through cells, with the blue 4′,6-diamidino-2-phenylindole staining indicating the nucleus. Significantly, the green fluorescence of SlSINA1/3/4/5/6-GFP was captured in both cytoplasm and nucleus, whereas the green fluorescence of SlSINA2-GFP was exclusively found in nucleus. These results suggest that SlSINA2 protein is localized in nucleus, whereas SlSINA1/3/4/5/6 proteins are localized in both cytoplasm and nucleus.

3.6 | Interactions among SlSINA proteins

It has been reported that SINA proteins can form homodimer or heterodimer to regulate their own stability and exert biological functions

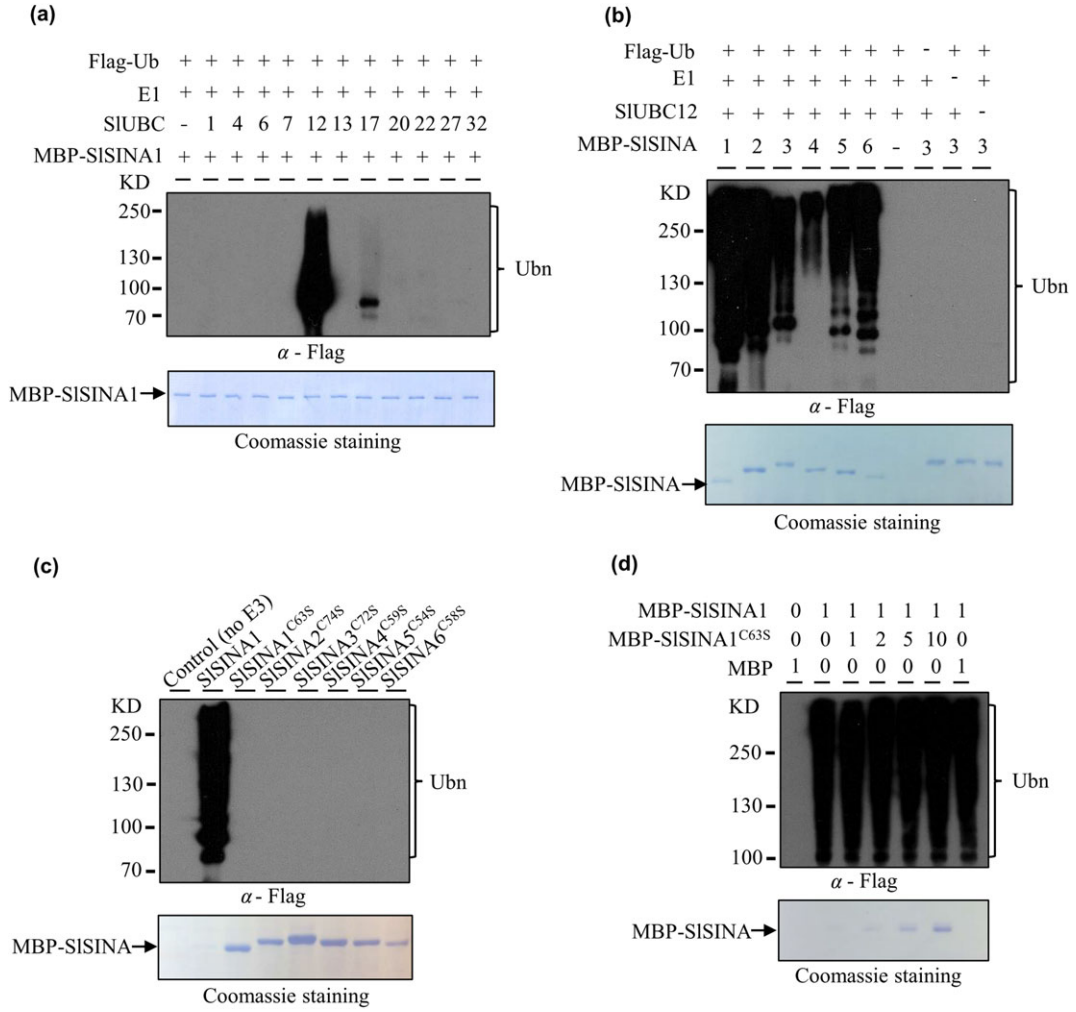

FIGURE 4 The E2 specificity of SlSINA ubiquitin ligases. (a) SlUBC12 and SlUBC17 are two tomato E2s facilitating the E3 activity of SlSINA1. Individual tomato E2 proteins were used to test SlSINA1 activity, which was indicated by the presence of polyubiquitination-associated smear detected by Western blotting using the α-Flag antibody. (b) The in vitro ubiquitination activity of maltose binding protein (MBP)-SlSINAs when acting with tomato SlUBC12. SlSINA3 was included to set up negative control reactions, which were conducted without the presence of E1, E2, or Flag-Ub (lanes 8–10). Similar amount of SlSINA 2, 3, 4, and 5 was used, whereas the amounts of SlSINA1 and SlSINA6 were slightly reduced due to their strong activity. (c) Verification of the dependence of SlSINA E3 activity on the conserved really interesting new gene (RING) domain. The tomato E2 SlUBC12 was used to verify the E3 activity of SlSINA mutants with Cys-to-Ser substitution at the RING domain. The WT SlSINA1 was included as positive control. (d) No effect of SlSINA1^{C63S} mutant on the E3 activity of the WT SlSINA1 protein. Numbers indicate the relative amount of proteins added in the ubiquitination reaction. All experiments were repeated at least two times with similar results. Coomassie staining of the Western blotting indicates the amount of SlNA proteins in the reactions [Colour figure can be viewed at wileyonlinelibrary.com]

(Depaux, Regnier-Ricard, Germani, & Varin-Blank, 2006; Hu & Fearon, 1999; Xie *et al*., 2002). Thus, the six SlSINA proteins were further examined for the ability of homo- and/or hetero-dimerization through pairwise in Y2H assay. To this end, the full-length cDNA of individual *SlSINA* genes was cloned into the bait vector pEG202 or the prey vector pJG4–5, the resulting constructs were cotransformed into yeast cells, and the interactions among individual SlSINA proteins were further examined on the X-Gal-containing medium. As shown in Figure 6, yeast cells harbouring both pEG202::SlSINA and pJG4–5::SlSINA produced blue coloration on the X-Gal-containing medium, indicating that each SlSINA interacts with not only itself but also other SlSINA to form homodimers or heterodimers, respectively. The interaction between unrelated AvrPtoB$_{1-307}$ and Pto protein (Xiao *et al*., 2007) was included as a positive control for the efficiency of Y2H assay, whereas combinations of SlSINA with pJG4–5 vector or pEG202 vector with SlSINA served as negative controls for Y2H assay, in which no blue coloration was observed despite the well-grown yeast cells.

3.7 | SlSINAs play distinct roles in defense signalling

Next, we sought to investigate the functionality of SlSINA proteins, focusing on their roles in different physiological processes, including defense response and development. We took advantage of the *Agrobacterium*-mediated transient assay in *N. benthamiana*, a model nonpathogen system commonly used to study plant defense signalling (Goodin, Zaitlin, Naidu, & Lommel, 2008). In our previous report, we showed that SlSINA3 can suppress the hypersensitive response (HR) cell death caused by three autoactive resistance (R) proteins, Prf^{D1416V}, Rpi-blb1^{D475V}, and Rx^{D460V}, and this cell death suppression (CDS) activity is not caused by degradation of R proteins (Miao *et al*.,

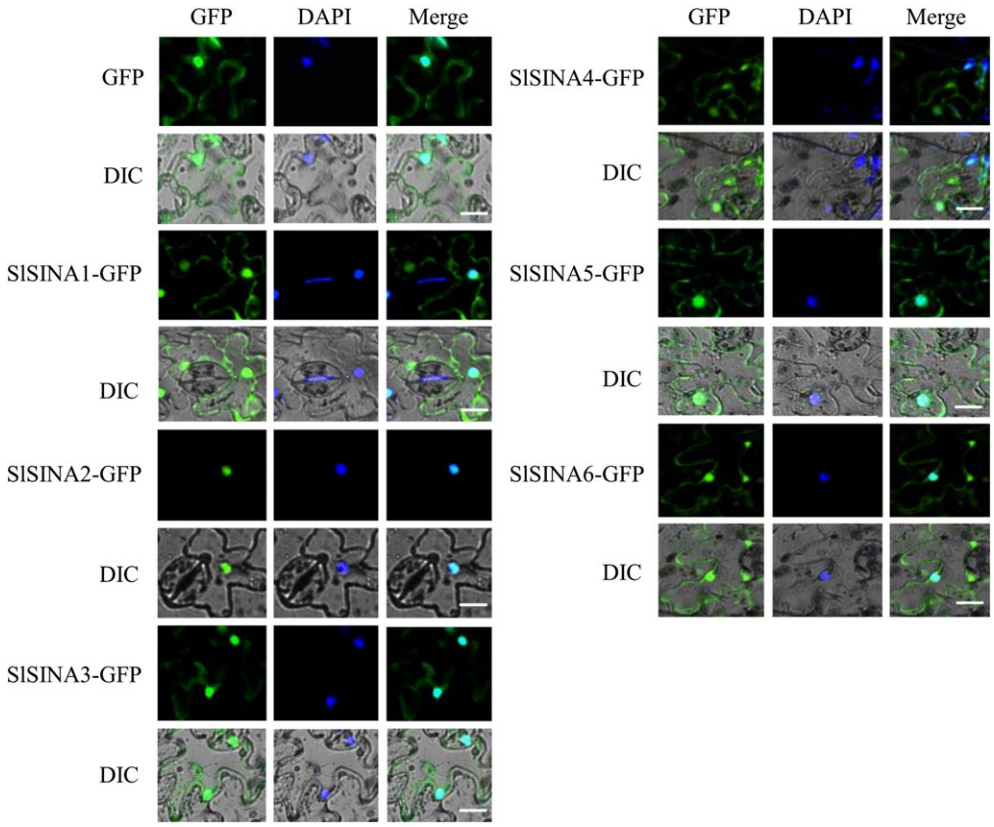

FIGURE 5 Subcellular localization of SlSINA proteins. Appropriate *35S::SlSINA-GFP* fusion constructs were transiently expressed in *Nicotiana benthamiana* leaves by *Agrobacterium*-mediated infiltration. Forty-eight hours after agrobacterial infiltration, the epidermal leaf tissues were peeled for the confocal laser scanning microscopy analysis. Prior staining with 4′,6-diamidino-2-phenylindole (DAPI) located the nucleus. The free green fluorescence protein (GFP) was included as control. Differential interference contrast (DIC) images of the same view were aligned underneath the GFP signal images. Scale bars = 20 μm [Colour figure can be viewed at wileyonlinelibrary.com]

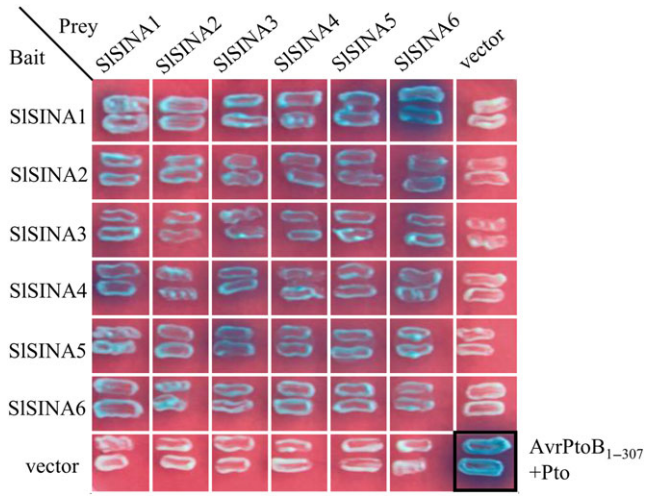

FIGURE 6 Yeast two-hybrid analysis of pair-wise interaction of SlSINA proteins. The full-length cDNA of individual *SlSINAs* were cloned into the bait vector pEG202 or the prey vector pJG4–5 and tested by the LexA-based yeast two-hybrid assay. Blue yeast colonies grown on the X-Gal-containing medium indicate the interaction among SlSINAs. The pEG202::AvrPtoB$_{1-307}$/pJG4–5::Pto pair was included as positive control [Colour figure can be viewed at wileyonlinelibrary.com]

2016). Thus, it is reasonable to examine whether other SlSINA proteins also possess such CDS activity. To this end, we first verified the expression of SlSINAs in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression. To our surprise, transient expression of SlSINA4 triggered cell death in *N. benthamiana* leaves (Figure 7a). Because SlSINA4 is a ubiquitin ligase, we asked whether the SlSINA4-triggered cell death depends on its ligase activity. We examined the SlSINA4^{C59S} mutant and found that the E3 activity is required for elicitation of cell death (Figure 7b). In theory, the SlSINA4-triggered cell death can be attributable to activation of defense-relevant signalling or to general cellular perturbation due to simple overexpression of the SlSINA4 protein. To test these possibilities, we examined whether the SlSINA4-triggered cell death is dependent on SGT1, a known defense signalling component essential for the defense-related cell death signalling in *N. benthamiana* (Kud *et al*., 2013). Similar *Agrobacterium*-mediated transient expression of SlSINA4 was carried out in the *N. benthamiana* leaves in which expression of the *SGT1* gene was suppressed by VIGS. As shown in Figure 7c, SlSINA4 no longer triggered cell death on the *SGT1*-silenced *N. benthamiana* leaves, despite well expression of the SlSINA4 protein. Taken together, these results indicate that overexpression of SlSINA4 can activate the defense-related cell death signalling in an E3 activity-dependent manner and suggest that SlSINA4 is a positive regulator in defense signalling.

Next, we determined the possible CDS activity of SlSINA1/2/5/6. The autoactive R protein Rpi-blb1^{D475V} was used as the cell death elicitor due to its consistency of triggering defense-related HR cell death and its well accumulation in *N. benthamiana* leaves. Following our

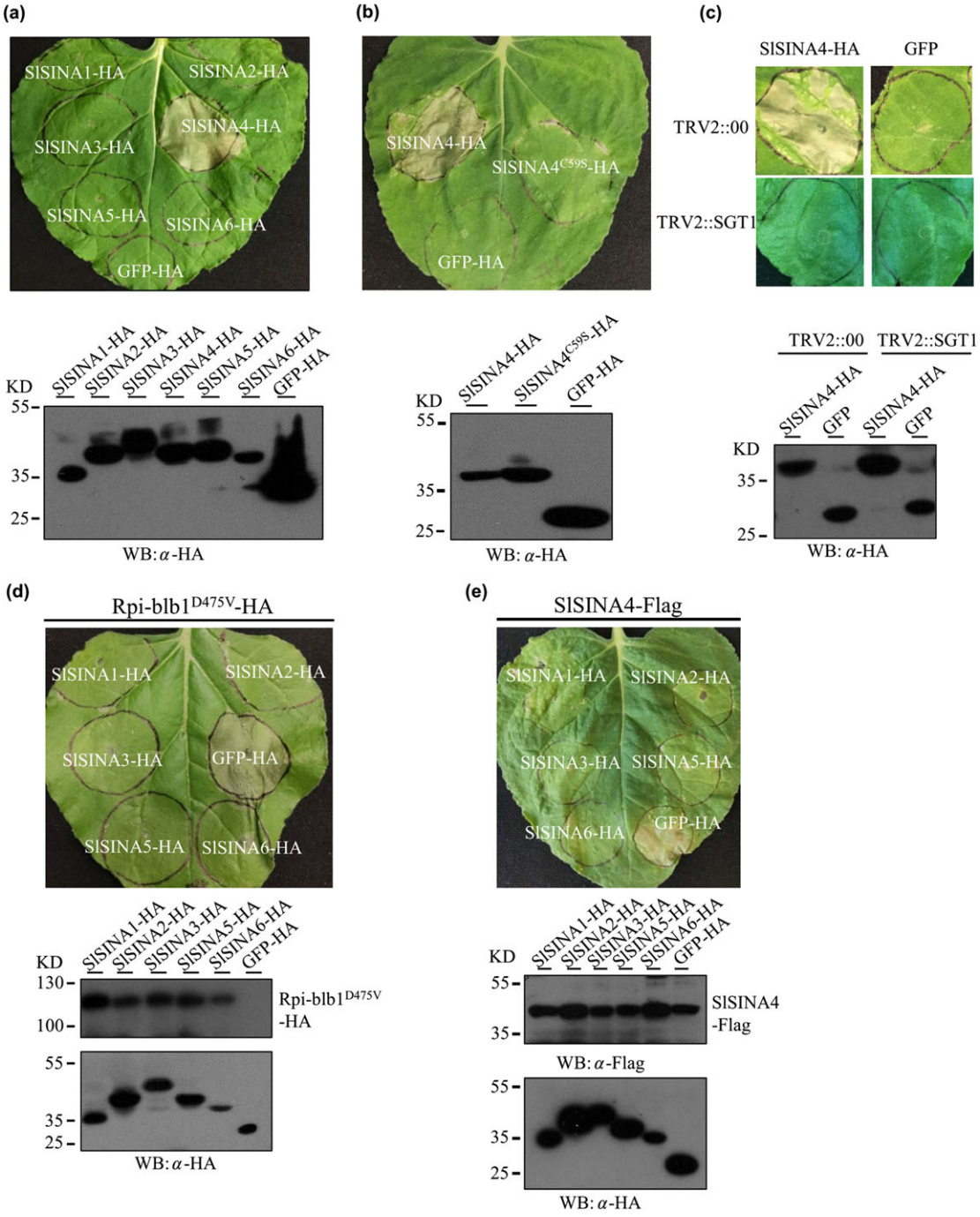

FIGURE 7 Role of SlSINA proteins in plant defense signalling. *Agrobacterium tumefaciens* GV2260 strains carrying indicated constructs, of which all genes were expressed from the 35S CaMV promoter, were syringe-infiltrated into *Nicotiana benthamiana* leaves at appropriate inoculum to determine the development of defense-related HR cell death. Green fluorescence protein (GFP) was included as negative control for cell death elicitor or suppressor. The infiltrated areas on leaf were circled, and photographs were taken 4 days after *Agrobacterium* infiltration. Western blotting using α-HA or α-Flag antibody indicated equal expression and accumulation of the relevant proteins in *N. benthamiana* leaves. All experiments were repeated at least three times with similar results. (a) Among six SlSINA proteins, SlSINA4 was able to elicit cell death when transiently expressed in *N. benthamiana* leaves at inoculum of OD_{600} = 0.6. Western blotting shown at the bottom panel using α-HA antibody indicated equal expression of SlSINA proteins. (b) The E3 activity of SlSINA4 is indispensable to elicit cell death. The E3-deficient SlSINA4^{C59S} mutant was not able to trigger cell death in *N. benthamiana* leaves, despite its well expression in *N. benthamiana* leaves shown by the Western blotting analysis (bottom panel). (c) The cell death triggered by SlSINA4 is defense-related. Silencing of the SGT1, a known component required for defense-related cell death signalling, abolished cell death triggered by SlSINA4 (*Agrobacterium* inoculum: OD_{600} = 0.6). (d) SlSINA1, 2, 3, 5, and 6 negatively regulate defense signalling. Due to weak expression of SlSINAs but extreme overexpression of Rpi-blb1^{D475V}, transient coexpression of HA-tagged SlSINA 1, 2, 3, 5, or 6 with Rpi-blb1^{D475V}-HA was conducted at 16:1 inoculum ratio (OD_{600} = 0.8 for SlSINAs or GFP and OD_{600} = 0.05 for Rpi-blb1^{D475V}-HA). Rpi-blb1^{D475V}-triggered hypersensitive response cell death was suppressed by SlSINA 1, 2, 3, 5, or 6. Note that the Rpi-blb1^{D475V}-HA protein was not detected when expressed with GFP control, presumably due to the non-specific protein degradation caused by extremely strong cell death. (e) SlSINA1, 2, 3, 5, or 6 (OD_{600} = 0.6) were able to suppress cell death-triggered by SlSINA4 (OD600 = 0.6) [Colour figure can be viewed at wileyonlinelibrary.com]

previous experimental method (Miao *et al*., 2016), we demonstrated that the HR cell death triggered by Rpi-blb1^{D475V} can be suppressed by SlSINA1, 2, 5, or 6 (Figure 7d), which resembles the feature of SlSINA3 (Miao *et al*., 2016). Because SlSINAs are functional ubiquitin ligase, we next asked whether SlSINA1/2/5/6 suppress the Rpi-blb1^{D475V}-triggered HR cell death by promoting its ubiquitination-mediated degradation. Western blotting analysis indicated that none of these SlSINAs can trigger degradation of Rpi-blb1^{D475V} (Figure 7 d). In addition, we examined whether CDS activity of SlSINA1/2/3/5/6 act towards the SlSINA4-triggered cell death. As shown in Figure 7e, SlSINA1/2/3/5/6 can suppress the cell death elicited by SlSINA4 without compromising its protein level. Taken together, our data suggest that, like SlSINA3, SlSINA1/2/5/6 may target component(s) downstream of R protein to negatively regulate defense-related cell death signalling.

3.8 | Overexpression of *SlSINA2* results in pale-green phenotype with retarded growth

To further investigate the role of *SlSINA* genes in development, we sought to adopt both loss-of-function and gain-of-function approaches in tomato. Unfortunately, due to highly similar at the DNA level, no unique DNA sequence could be identified to repress each individual *SlSINA* genes by RNA interfere (RNAi) technique, whereas knockout of all *SlSINA* genes by RNAi through the *Agrobacterium*-mediated transformation resulted in lethality in tomato (data not shown), suggesting important role of these SINA ubiquitin ligases in the early development of tomato. We thereby focused on the gain-of-function approach to generate and characterize transgenic tomato plants overexpressing individual *SlSINA* genes from the CaMV 35S promoter. To this end, we introduced the *35S::SlSINA* constructs into the RG-PtoR tomato via *Agrobacterium*-mediated transformation (Fillatti *et al*., 1987). A number of transgenic lines were generated for four of six *35S::SlSINA* constructs, but we did not introduce *35S::SlSINA1* or *35S::SlSINA4* construct into RG-PtoR tomato despite several attempts. We speculated that overexpression of *35S::SlSINA1* or *35S::SlSINA4* construct might also be lethal in RG-PtoR tomato, which is consistent with the observation that overexpression of *SlSINA4* resulted in cell death in *N. benthamiana* leaves (Figure 7a).

Several T_3 homozygous transgenic tomato lines were obtained and overexpression of individual of *SlSINA* genes was verified by quantitative real-time PCR (Figure S4). Among four types of *35S::SlSINA* transgenic tomato plants, the *35S::SlSINA2* and *35S::SlSINA5* transgenic lines exhibited significantly phenotypic alteration and were further characterized. Significantly, *35S::SlSINA2* transgenic tomato plants exhibited pale-green leaves under normal greenhouse growth conditions (Figure 8a,b). The representative leaves of WT and transgenic plants were selected to measure total chlorophyll content. As shown in Figure 8c, the chlorophyll content in the *35S::SlSINA2* transgenic tomato leaves was dramatically reduced to 50% of that in the WT tomato leaves. In consistent with the reduced chlorophyll content, the *35S::SlSINA2* tomato plants exhibited growth retardation (Figure 8 a), presumably due to impaired photosynthesis efficiency associated with the reduced chlorophyll in leaves. Taken together, our results suggest that SlSINA2 plays an important role in development of tomato via regulation of chlorophyll content in leaf tissue.

3.9 | *SlSINA5* plays a role in flower development

We also found that overexpression of *SlSINA5* in transgenic RG-PtoR tomato has significant impact on flower development. Under our greenhouse growth conditions (described in Methods and Materials), more than 90% of the *35S::SlSINA5* transgenic tomato flowers displayed abnormal morphology. As shown in Figure 9a, such abnormal

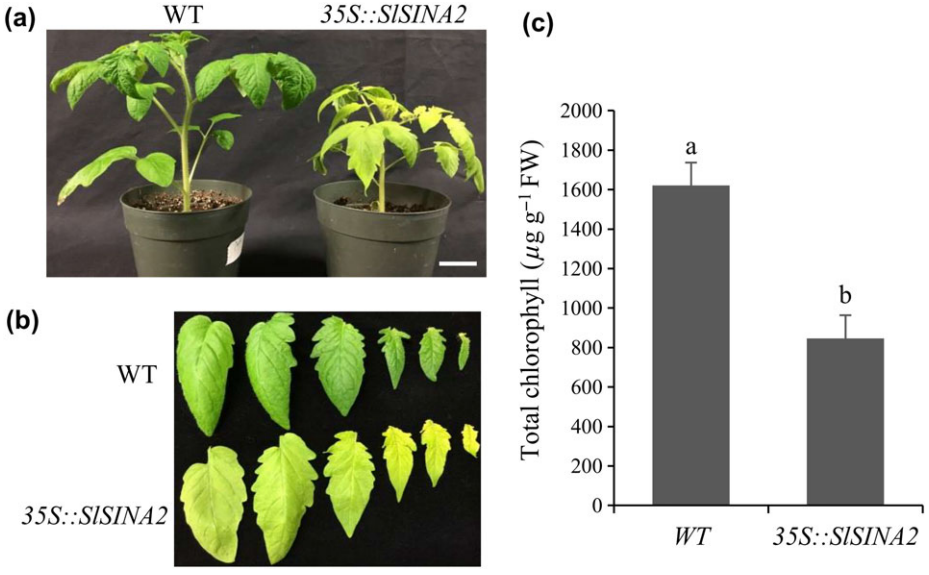

FIGURE 8 Overexpression of *SlSINA2* in transgenic tomato results in pale-green phenotype with retarded growth. (a) Compared to the same age WT tomato plant, 5-week-old *35S::SlSINA2* transgenic tomato plant showed pale-green leaves and retarded growth. Scale bars = 5 cm. (b) The representative leaves of WT and *35S::SlSINA2* tomato plants. (c) Total chlorophyll levels in leaves of WT and *35S::SlSINA2* plants. The experiment was repeated three times with similar results. Data are means ±SD (n = 3). Different letters indicate statistically significant difference as determined by Student's t test ($p \leq 0.01$) [Colour figure can be viewed at wileyonlinelibrary.com]

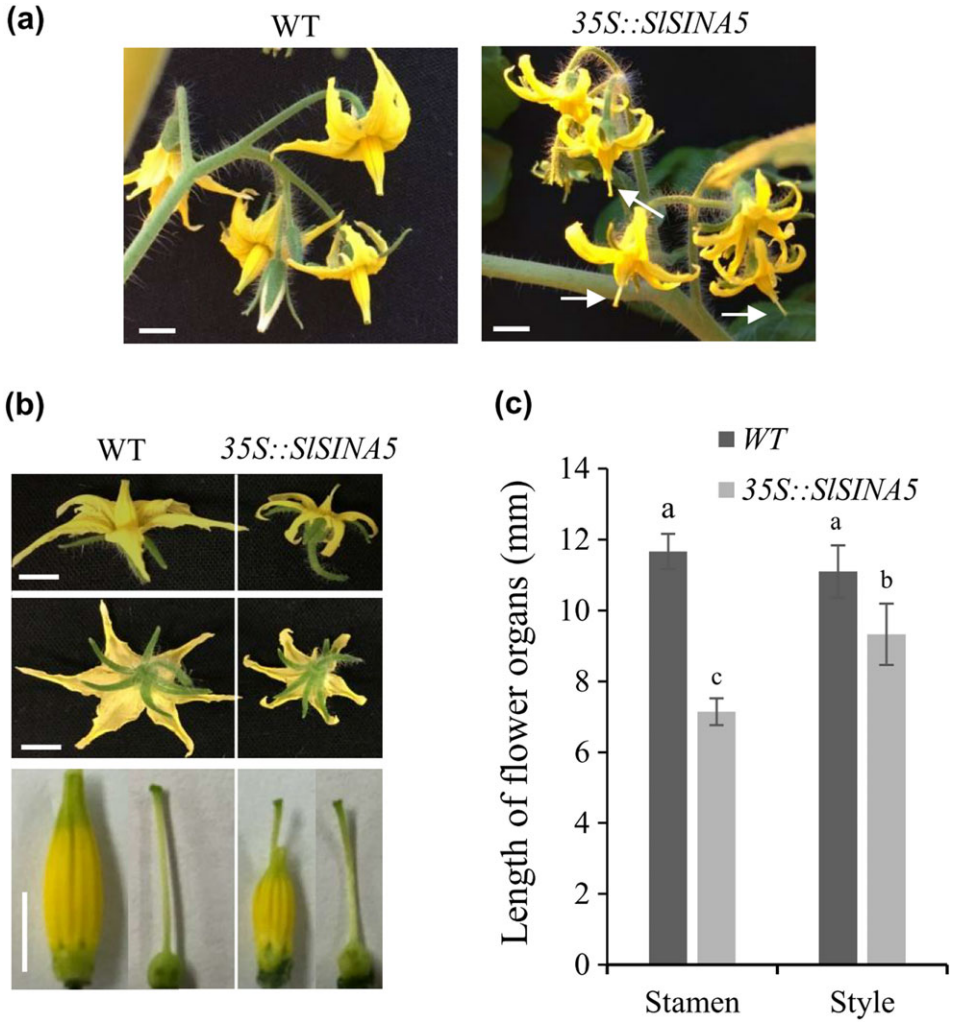

FIGURE 9 Altered floral structure of the *35S::SlSINA5* transgenic tomato. (a) Photograph of inflorescences of the WT and *35S::SlSINA5* tomato plants. White arrows indicate the exserted stigmas. (b) Representative flowers of the WT and *35S::SlSINA5* transgenic tomato plants. The upper panel shows the side view of the whole flower, and the back view is presented in the middle panel. Lower panel presents isolated anthers and pistil. (c) The length of anthers and style of flowers of the WT and *35S::SlSINA5* tomato plant. Data are means ±SD ($n \geq 10$). Different letters indicate statistically significant difference as determined by Student's t test ($p \leq 0.01$). All scale bars = 5 mm [Colour figure can be viewed at wileyonlinelibrary.com]

flower had stigma exserted beyond the anther cone, whereas the stigma of the normal tomato flower was recessed within the anther cone. In addition, the petal tip of the abnormal *35S::SlSINA5* flower was curled up to greater level than that of the normal tomato flower; and the abnormal *35S::SlSINA5* flower was overall smaller in size: The length of sepal, petal, stamen, or style was reduced to certain degree (Figure 9b), with about 39% reduced in stamen and 16% reduced in style (Figure 9c). Thus, the exserted stigma of the abnormal *35S::SlSINA5* flower was attributable to reduced size of the stamen rather than to elongation of the style, which is a novel phenotype found in tomato mutants with altered floral morphology (Carrera, Ruiz-Rivero, Peres, Atares, & Garcia-Martinez, 2012; García-Hurtado *et al*., 2012; Livne *et al*., 2015). Significantly, because of the protruded stigma, such defective flowers were not able to self-pollinate naturally for fruit-set. However, we conducted manual self- and cross-pollination on these defective flowers to test the fertility of their pollens. We found that the pollens were still fertile and the manually pollinated flowers were able to set fruits. Nevertheless, it was notable that, under our greenhouse condition, a small proportion (10%) of *35S::SlSINA5* transgenic tomato flowers were normal and able to self-pollinate to produce fruits and seeds.

4 | DISCUSSION

Our recent genome-wide analysis has identified six members in the tomato *SlNA* (*SlSINA*) gene family (Miao *et al*., 2016). The quantitative real-time PCR analysis conducted in our present study further indicated that these six *SlSINA* genes are differentially regulated at the transcriptional level in tomato tissues. It is notable that, compared to the expression levels in leaf, *SlSINA* genes were expressed at relatively higher levels in root (Figure 2), suggesting that they might play an important role in root physiology in tomato. Although we did not observe any significant alteration in the root of individual *35S::SlSINA* transgenic tomato plants, which implies possible saturation of *SlSINA* transcripts already existed in the WT tomato root, knock-out of *SlSINAs* might cause alteration in root development, which, unfortunately, could not be verified due to the indispensability of *SlSINAs* in

tomato. Nevertheless, two publications have suggested the role of *SINA* genes in root development and defense signalling in root. The *L. japonicus SINA* genes are induced by the rhizobial infection and negatively regulate symbiosis in root of *L. japonicus* (Den Herder *et al*., 2012), whereas, in *M. truncatula*, heterogeneous overexpression of *Arabidopsis SINAT5* affects nodulation in root (Den Herder *et al*., 2008).

It is generally thought the RING-containing ubiquitin ligases use the C-terminus to form dimers, either homozygously or heterozygously, which normally is required for activation of the ligase (Deshaies & Joazeiro, 2009; Dou, Buetow, Sibbet, Cameron, & Huang, 2012; Kozlov *et al*., 2007). In fact, dimerization of SINA ubiquitin ligases has been found to contribute to its stabilization in vivo and is required for the ligase activity (Depaux *et al*., 2006; Hu & Fearon, 1999; Polekhina *et al*., 2002). We found that all six SlSINAs can interact with each other in the Y2H assay (Figure 6), which indicates that a given SlSINA protein can form homodimer with itself or heterodimer with any of other five SlSINA proteins. Thus, SlSINA ubiquitin ligases may function in a homodimeric or heterodimeric complex.

SINA ubiquitin ligases belong to the RING-type E3, of which the conserved RING domain is responsible for binding to the E2 ubiquitin-conjugating enzyme. Mutation at the essential cysteine residue(s) often results in not only loss of the E3 activity but also interference with activity of the WT SINA protein. Such kind of dominant-negative effect is often due to blocking of the activity of the WT SINA protein by forming inactive dimer or/and competition with the WT SINA protein for substrate binding. For example, the *Arabidopsis* SINAT5^{C49S} mutants can act as a dominant-negative protein to poison the E3 activity of the WT SINAT5 protein via hetero-dimerization (Xie *et al*., 2002). However, despite the serine substitution at the equivalent cysteine of SlSINAs completely abolished the E3 potential, it did not render a dominant-negative effect on the E3 activity of the WT SlSINAs (Figure 4d; Figure S3), suggesting that SlSINA proteins are more tolerant to such substitution of the conserved cysteine residue, and further substitutions of multiple cysteine residues may help elucidate this question.

The E2 ubiquitin-conjugating enzymes are often mistakenly considered to play an auxiliary role in ubiquitination process. In fact, E2 has been found to govern the processivity and topology of poly-ubiquitin chain formation and thus determine the fate of ubiquitinated proteins (David, Ziv, Admon, & Navon, 2010; van Wijk & Timmers, 2010; Windheim, Peggie, & Cohen, 2008). It is logical to hypothesize that the most appropriate combination of E3 with E2 will lead to optimization of ubiquitination process. There are 34 distinct functional E2 found in tomato, and they are classified into 13 groups (Zhou *et al*., 2017). Our results indicated that the SlSINAs only function cooperatively with Group III of tomato E2s (Figure 4a; Figure S2). Thus, it is reasonable to speculate this group of E2 also play roles in physiological processes where SlSINAs are involved in. For example, this group of E2 may contribute to plant defense signalling, which is consistent with a recent publication showing that knockdown of this group in plants by VIGS resulted in attenuation of pathogen-associated molecular pattern-triggered defense response (Zhou *et al*., 2017). Significantly, Group III is also the only E2 group used by the *Pseudomonas*-secreted ubiquitin ligase AvrPtoB for its virulence activity. AvrPtoB is a bacterium-specific E3 identified from *Pseudomonas syringae* pv. *tomato*, a bacterial pathogen causing speck disease on tomato plants. In cooperation with SlUBC8, AvrPtoB acts as a virulence effector by ubiquitinating defense-related factors to perturb plant defense system. Such coincidence also implicates that during coevolution between plant and bacterial pathogen, *Pseudomonas syringae* has evolved AvrPtoB ubiquitin ligase to interfere with the plant defense system through utilization of the defense-related E2 ubiquitin-conjugating enzyme. It will be interesting to determine any possible relationship between the bacterium-produced E3 AvrPtoB and the host endogenous E3 SlSINAs. For example, although AvrPtoB does not have a RING domain, it may functionally mimic SlSINA or other host ubiquitin ligase to manipulate the ubiquitination mediated by these endogenous ubiquitin ligases.

Up to date, several plant SINA proteins have been demonstrated to play roles in defense response and development. In Arabidopsis, one SINA ubiquitin ligase, SINAT5, plays roles in both lateral root growth and flower development (Park *et al*., 2010; Xie *et al*., 2002), whereas another, SINAT2, is involved in carotenogenesis (Welsch *et al*., 2007). In *L. japonicus*, the SINA4 negatively regulates *Sinorhizobium meliloti* infection (Den Herder *et al*., 2012). In addition to our previous finding that SlSINA3 is a negative regulator in HR cell death signalling (Miao *et al*., 2016), our present study showed that SlSINA1, 2, 4, 5, and 6 also play roles in defense signalling. SlSINA4 could trigger HR-like cell death signalling when transiently expressed in *N. benthamiana* leaves (Figure 7a), and significantly, this ability was dependent on its ubiquitin ligase activity (Figure 7b), suggesting SlSINA4 might elicit cell death via ubiquitination-mediated degradation of the key negative cell death regulator(s). In contrast, SlSINA1, 2, 3, 5, and 6 negatively regulated defense signalling, as manifested by the suppression of the defense-related HR cell death triggered by the autoactive R protein (Figure 7d; Miao et al., 2016). Significantly, these five SlSINAs likely act at a converged point of cell death signalling, presumably targeting the conserved components for ubiquitination-mediated degradation. We propose this hypothesis based on the following observations and our previous publication: Firstly, SlSINA1, 2, 5, and 6 could suppress Rpi-blb1^{D475V}-triggered HR cell death and SlSINA3 could suppress HR cell death signalling mediated by multiple R proteins (Miao *et al*., 2016); secondly, all five SlSINAs did not trigger degradation of these R proteins, implying that they negatively regulate HR cell death signalling downstream R protein; thirdly, these five SlSINAs also suppressed cell death elicited by SlSINA4, further suggesting SlSINAs target the common signalling component(s) essential for HR cell death. However, given the fact that all SlSINA proteins can interact with each other in the Y2H assay, we cannot completely rule out a possibility that SlSINA1/2/3/5/6 interact with SlSINA4 in plant cells and such interactions may interfere with the latter's functionality, including the SlSINA4-triggered cell death.

Several Arabidopsis mutants have been identified to exhibit pale-green leaves due to reduced production of chlorophyll, which is caused by defective enzyme(s) involved in chlorophyll biosynthesis (Huang & Li, 2009; Lange, Geserick, Tischendorf, & Zrenner, 2008; Maekawa *et al*., 2015). The *35S::SlSINA2* transgenic tomato plants also showed such chlorophyll-deficient pale-green leaf phenotype (Figure 8), which might be associated with down-regulation of chlorophyll biosynthesis. We speculate that, in the *35S::SlSINA2* transgenic tomato plants, the chlorophyll biosynthesis-related genes or their encoded enzymes have been altered due to over production of the SlSINA2 ubiquitin ligase.

For example, enzymes involved in biosynthesis of chlorophyll could be targeted by SlSINA2 for ubiquitination and degradation; or the chlorophyll biosynthesis-related positive regulator(s), such as transcription factor(s) regulating chlorophyll biosynthesis-related genes, could be ubiquitinated by SlSINA2 for degradation. Moreover, these results implicated that the *SlSINA2* gene must be stringently regulated, which is consistent with the observation of relatively low expression of *SlSINA2* gene in the WT tomato leaves (Figure 2).

Tomato possesses flower with recessed stigma in its own anthers in order to self-pollinate. Several tomato mutants with exserted stigma have been identified. These mutants have the elongated style extruding beyond anthers and genes (such as *Style 2.1*) controlling the style length have been identified (Carrera *et al*., 2012; García-Hurtado *et al*., 2012; Livne *et al*., 2015). However, the extruded stigma in the *35S::SlSINA5* transgenic tomato flower was not due to the elongated style, because the style of the *35S::SlSINA5* tomato flower was slightly shorter than that of the normal tomato flower (Figure 9c). In contrast, it was the shortened stamen that rendered style extruding beyond the anther cone. Similar altered floral phenotype of exserted stigma due to shortened stamen has been reported in the transgenic petunia overexpressing a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase mutant, which lacks the target serine residue for phosphorylation by SnRK1 kinase (Hey *et al*., 2006). It is hypothesized that the stamen size in petunia flower is controlled by phytosterol synthesized by the key enzyme HMG-CoA reductase, which is negatively regulated by the SnRK1-mediated phosphorylation. It appears that the phosphorylation-defective HMG-CoA reductase acts as a dominant-negative regulator. Thus, it is possible that SlSINA5 ubiquitin ligase targets stamen-controlling factor(s), such as HMG-CoA or SnRK1, for ubiquitination-mediated degradation, thereby resulting in exserted stigma. Nevertheless, such change in the position of the pollen-bearing anthers usually results in abolishment of self-pollination due to the difficulty to receive pollen from its own anthers.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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