Sumoylation E3 Ligase SIZ1 Modulates Plant Immunity Partly through the Immune Receptor Gene *SNC1* in *Arabidopsis*

Mingyue Gou,¹ Quansheng Huang,^{1,2} Weiqiang Qian,¹ Zemin Zhang,^{1,3} Zhenhua Jia,^{1,4} and Jian Hua¹

¹Plant Biology Section, School of Integrated Plant Science, Cornell University, Ithaca, NY 14853, U.S.A.; ²Xinjiang Key Laboratory of Crop Biotechnology, Institute of Nuclear and Biological Technology, Xinjiang Academy of Agricultural Sciences, Urumqi 830091, China; ³State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Agriculture, South China Agricultural University, Guangzhou, 510642, China; and ⁴Institute of Biology, Hebei Academy of Sciences, Shijiazhuang, Hebei, 050081, China

Accepted 6 March 2017.

The small ubiquitin-like modifier E3 ligase SIZ1 regulates multiple processes in *Arabidopsis*, including salicylic-acid-dependent immune responses. However, the targets of SIZ1 in plant immunity are not known. Here, we provide evidence that the plant immune receptor nucleotide-binding leucine-rich repeat gene *SNC1* partially mediates the regulation of plant immunity by SIZ1. The *siz1* loss-of-function mutant has an autoimmune phenotype that is dependent on *SNC1* and temperature. Overexpression of *SIZ1* partially rescues autoimmune mutant phenotypes induced by activation or overaccumulation of *SNC1*, and the *SNC1* protein amount is attenuated by *SIZ1* overexpression. In addition, overexpression of the F-box protein CPR1 that degrades the *SNC1* protein inhibits the growth defects and disease resistance of the *siz1* mutant. Furthermore, we found that the *SNC1* protein is sumoylated in planta. Although it remains to be determined whether SIZ1 primarily modulates the *SNC1* protein via sumoylation or affects *SNC1* transcript level, our data indicate that *SNC1* is a major mediator of defense response modulated by *SIZ1* and that *SNC1* is a crucial target for fine-tuning plant defense responses.

Eukaryotic cells use a variety of small polypeptides, including ubiquitin and ubiquitin-like proteins (ULP), to posttranslationally modify proteins. The small ubiquitin-like modifier (SUMO) is one ULP that shows three-dimensional structures very similar to those of ubiquitin but shares only approximately 18% identity in amino acid sequences (Bayer et al. 1998). Like ubiquitin, SUMO can be covalently attached to its substrate protein via the sequential actions of three enzymes (E1, E2, and E3) in the so-named sumoylation process (Colby et al. 2006; Kurepa et al. 2002; Melchior 2000). From

sequence comparisons of various SUMO targets, an aKxE motif was identified as the sumoylation site with K (lysine) as the SUMO conjugation site (Melchior 2000). SUMO can also noncovalently interact with other proteins through targeting specific SUMO-interacting motifs (SIM) (Hecker et al. 2006; Kerscher 2007). In mammals and *Saccharomyces cerevisiae*, sumoylation affects protein–protein interaction, protein subcellular localization, protein–DNA interactions, and enzymatic activity (Verger et al. 2003). Sumoylation regulates multiple biological processes, including the cell cycle, DNA repair, and transcriptional activity (Hay 2005). In many cases, sumoylation was considered an antagonist to ubiquitination because the same lysine residue can be modified by either SUMO or ubiquitin (Desterro et al. 1998; Hoege et al. 2002). However, sumoylation facilitates ubiquitination in some cases, because it could serve as a targeting signal for ubiquitination and ubiquitin-dependent degradation (Manente et al. 2011; Miteva et al. 2010; Praefcke et al. 2012; Prudden et al. 2007; Uzunova et al. 2007).

SUMO in *Arabidopsis thaliana* is encoded by the *SUM* gene family that comprises eight members, among which *SUM1*, -2, -3, and -5 have detected expression and are likely functional (Kurepa et al. 2002). *Arabidopsis* also has three SUMO activating enzyme (SAE) coding genes (*SAE1a*, *SAE1b*, and *SAE2*) and two SUMO conjugating enzyme (SCE) coding genes (*SCE1a* and *SCE1b*) (Kurepa et al. 2002). Although a small family of SUMO E3 genes has been identified in both yeast and animals, *SIZ1* and *HPY2* are the only two SUMO E3 orthologous genes found in *Arabidopsis* (Ishida et al. 2009; Kurepa et al. 2002). These two genes act independently and their combined function is essential for plant development (Ishida et al. 2012). Most of the SIZ1 sumoylation targets isolated to date are transcription factors, including PHR1 in phosphate starvation-response (Miura et al. 2005), ICE1 in freezing tolerance (Miura et al. 2007), as well as ABI5 and MYB30 in abscisic acid signaling (Miura et al. 2009; Zheng et al. 2012). SIZ1 was also shown to regulate FLD, a histone demethylase that represses the expression of flowering gene *FLC* via reducing H4 acetylation at the *FLC* locus (He et al. 2003; Jin et al. 2008). In addition, SIZ1 was shown to sumoylate and activate CHROMOMETHYLASE 3 and SLEEPY1 that are key regulators of DNA methylation and gibberellic acid signaling, respectively (D. Y. Kim et al. 2015a; S. I. Kim et al. 2015b).

The *siz1* mutants also show drastic growth defects and salicylic acid (SA)-dependent defense responses (Lee et al. 2007). These defects appear to be independent of defects in processes

M. Gou and Q. Huang contributed equally to the work.

Current address of M. Gou: Biology Department, Brookhaven National Laboratory, Upton, NY 11973, U.S.A.

Current address of W. Qian: State Key Laboratory of Protein and Plant Gene Research, School of Life Science, Beijing University, Beijing 100871, China.

Corresponding author: J. Hua; E-mail: jh299@cornell.edu

© 2017 The American Phytopathological Society

described above and *SIZ1* may target some as-yet-unidentified components to regulate plant defense response. The *siz1-2* mutant is more resistant to bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 expressing AvrRPS4 but not AvrRpt2, suggesting that *SIZ1* negatively regulates resistance induced by some immune receptor genes but not the others (Lee et al. 2007). Consistent with the negative role of *SIZ1* in plant defense regulation, sumoylation and desumoylation are shown to be an important protein modification process in plant–pathogen interactions. For instance, the tomato SUMO (T-SUMO) was found to interact with ethylene-inducing xylanase (EIX), a strong elicitor from the fungus *Trichoderma viride* that triggers rapid defense response. Overexpression of *T-SUMO* in tobacco suppressed EIX induction of ethylene accumulation and cell death (Hanania et al. 1999). In addition, the type III effector *AvrBsT* from the plant pathogen *Xanthomonas campestris* was identified as a potential SUMO protease. *AvrBsT* enters its hosts and interferes with the host defense response, possibly by desumoylating a key defense regulator (Orth et al. 2000).

Similar to *siz1*, many autoimmune mutants are dependent on SA and some of them such as *bon1* and *cpr1/cpr30* are dependent on *SNC1*, a Toll interleukin-1 receptor nucleotide-binding site-type gene (Gou et al. 2009; Yang and Hua 2004; Zhang et al. 2003). The *SNC1* gene is under a complex regulation at transcriptional and posttranscriptional levels (Gou and Hua 2012; Johnson et al. 2012). For instance, the H3K4 methyltransferase gene *TRITHORAX-RELATED 7* (*ATXR7*) and E3 ubiquitin ligase genes *HISTONE MONOUBIQUITINATION1* (*HUB1*) and *HUB2* are involved in the regulation of *SNC1* at a transcriptional level (Xia et al. 2013; Zou et al. 2014). At a posttranscriptional level, the *SNC1* protein is regulated by the F-box protein *CPR1/CPR30* and an E4 ubiquitin ligase *MUSE3* through ubiquitination and subsequent 26S-proteasome dependent degradation (Cheng et al. 2011; Gou et al. 2009, 2012; Huang et al. 2014). Here, we show that the regulation of *SIZ1* on plant immunity is largely through the plant immunity receptor gene *SNC1* and that *SIZ1* affects *SNC1* protein accumulation in planta. In addition, sumoylation was observed in *SNC1* protein in planta. Thus, this study reveals an important target of *SIZ1* in plant immunity and indicates a complex regulation of *SNC1* to achieve fine tuning of plant immunity.

RESULTS

The *siz1*-related phenotype is temperature sensitive and *SNC1* dependent.

The *siz1-3* mutant shows a growth defect resembling that of *bon1-1* and *snc1-1*. The mutant is dwarf when grown at 22°C but was largely wild type looking at 28°C (Fig. 1A and B). Quantification of biomass revealed that the biomass ratios of *siz1* and the wild type were 15% at 22°C and 55% at 28°C (Fig. 1C and D), indicating an inhibition of the growth defect by high temperature. Because high temperature can suppress immunity mediated by nucleotide-binding leucine-rich repeat (NB-LRR) genes such as *SNC1* (Yang and Hua 2004; Zhu et al. 2010), we tested whether or not the growth defect in *siz1* is dependent on *SNC1*. We first analyzed the transcript level of *SNC1* in *siz1-3* by a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiment. The *SNC1* transcript was three times more in *siz1-3* compared with the wild-type Col-0 (Fig. 2A). We then crossed *siz1-3* with *snc1-11*, the loss-of-function mutant of *SNC1*, to obtain the *siz1-3 snc1-11* double mutant. The double mutant exhibited a partially rescued growth phenotype at 22°C, with a biomass threefold more than the *siz1-3* single mutant (Fig. 2B and C). The *siz1-3 snc1-11* double mutant grew to a similar size as the wild-type Col-0 at 28°C (Fig. 2C). These data indicate that the growth defect of *siz1-3* is temperature sensitive and is partially *SNC1* dependent.

Because *SNC1* upregulation was shown to confer enhanced disease resistance, we determined whether or not *siz1*-related defense response is dependent on *SNC1*. To this end, we monitored the expression of defense marker gene *PR1* and assayed the growth of virulent *P. syringae* pv. *tomato* strain DC3000 in *siz1-3* and *siz1-3 snc1-11*. RNA blot analysis showed that *PR1* gene expression was dramatically upregulated in *siz1-3*, as previously reported (Lee et al. 2007), and this upregulation was greatly reduced in the *siz1-3 snc1-11* double mutant (Fig. 2D). We subsequently used both vacuum and dipping inoculation methods to test pathogen growth in different genotypes. With both methods, *siz1-3* exhibited significantly more resistance to strain DC3000 than the wild type, as previously reported (Lee et al. 2007). The *snc1-11* mutation largely abolished the enhanced disease resistance phenotype of *siz1-3*, and the *siz1-3 snc1-11* mutant was as susceptible to the pathogen as the wild type (Fig. 2E and F). These data indicate that *siz1*-induced enhanced disease resistance to strain DC3000 is mainly dependent on *SNC1*.

Overexpression of *SIZ1* partially suppresses *bon1-1* and *snc1-1* phenotype.

Because the loss-of-function mutation of *SNC1* suppresses the *siz1-3* mutant phenotype, *SIZ1* is a negative regulator of *SNC1*. To determine whether *SIZ1* has a rather direct modulation of *SNC1* amount or activity, we overexpressed *SIZ1* in *bon1-1* and *snc1-1*, where *SNC1* transcript and *SNC1* protein, respectively, are elevated or activated (Zhang et al. 2003; Yang

Fig. 1. Growth defect of *siz1-3* can be largely suppressed by a high temperature of 28°C. **A**, Growth phenotypes of Col-0 wild type (WT) and *siz1-3* mutant at 22°C. **B**, Growth phenotypes of Col-0 WT and *siz1-3* mutant at 28°C. **C**, Quantification of biomass of Col-0 WT and *siz1-3* grown at 22 and 28°C. Twenty plants of each genotype were used for biomass quantification. Asterisks indicate statistical differences of *siz1-3* compared with Col-0 WT (Student's *t* test, ****P* < 0.001). **D**, Ratio of biomass for *siz1-3* to that of Col-0 WT at 22 and 28°C. Asterisks indicate statistical differences of 28 compared with 22°C (Student's *t* test, ****P* < 0.001).

and Hua 2004). Interestingly, 12 of 16 *SIZ1* overexpression lines in *bon1-1* showed a close to wild-type phenotype, indicating a partial rescue of *bon1* by *SIZ1* overexpression (Fig. 3A). Similarly, 10 of 16 *SIZ1* overexpression lines in *snc1-1* showed a partial rescued phenotype (Fig. 3B). These genetic data indicate an important role of *SIZ1* in *SNC1*-related growth regulation.

Overexpression of *SIZ1* reduced *SNC1* transcripts and protein accumulation.

To determine whether *SIZ1* regulates *SNC1* at the level of transcript or protein, we overexpressed *SIZ1* in a *pSNC1::SNC1:green fluorescent protein* (GFP) transgenic line, where the *SNC1* protein can be detected by the GFP tag (Zhu et al. 2010). This line exhibits a phenotype similar to that of *bon1-1* and *snc1-1* due to higher expression of *SNC1* in transgene than the endogenous gene (Zhu et al. 2010). Similar to that in *bon1-1* and *snc1-1*, overexpression of *SIZ1* resulted in a rescued phenotype in 8 of the 10 *pSNC1::SNC1:GFP* transgenic lines (Fig. 3C). It appeared that the rescue was more complete for *pSNC1::*

SNC1:GFP than for *snc1-1* and *bon1* but this would need to be verified via side-by-side growth comparison. Nevertheless, the *SIZ1* overexpression lines in *pSNC1::SNC1:GFP* allowed us to analyze both the RNA and the protein levels of *SNC1*. Shown by qRT-PCR analysis, the *SNC1* transcript was downregulated in the *SIZ1* overexpression lines to approximately 50% of the *pSNC1::SNC1:GFP* transgenic level in two representative *SIZ1* overexpression lines (Fig. 3D). In addition, the *PR1* gene is drastically downregulated to 50 and 5% of the *pSNC1::SNC1:GFP* transgenic level in the two *SIZ1* overexpression lines, respectively (Fig. 3E). Western blotting revealed that the *SNC1:GFP* protein level was downregulated in the transgenic lines overexpressing *SIZ1*, and it was hardly detected in one of the transgenic lines (Fig. 3F). These data indicate that *SIZ1* impacts both RNA and protein levels of *SNC1*. Because of a feedback regulation between the *SNC1* protein activity and the *SNC1* transcription (Li et al. 2007), it remains to be determined whether the protein or RNA of *SNC1* is more directly modulated by *SIZ1*.

Fig. 2. The *siz1-3* defect is partially rescued by *snc1-11* mutation. **A**, Relative expression level of *SNC1* in Col-0 wild type and *siz1-3* mutant quantified by quantitative reverse-transcription polymerase chain reaction. Asterisks indicate statistical differences of *siz1-3* compared with Col-0 (Student's *t* test, *** $P < 0.001$). **B**, Growth phenotypes of Col-0, *siz1-3*, *snc1-11*, and *siz1-3 snc1-11* grown at 22°C. **C**, Quantification of the biomass of Col-0, *snc1-11*, *siz1-3*, and *siz1-3 snc1-11* grown at 22 and 28°C. Twenty plants of each genotype were used for biomass quantification. **D**, *PR1* gene expression pattern in Col-0, *snc1-11*, *siz1-3*, and *siz1-3 snc1-11* detected by Northern blot. Ethidium bromide-stained RNA served as loading control. **E** and **F**, Pathogen growth assay of Col-0, *snc1-11*, *siz1-3*, and *siz1-3 snc1-11* by vacuum (E) and dipping (F) inoculation. For C, E, and F, letters indicate statistical differences ($P < 0.01$; one-way analysis of variance, Tukey's honestly significant difference test) of different genotypes.

Overexpression of *CPR1* partially rescues *siz1*-related growth defect and disease resistance.

Because the F-box protein *CPR1* directly controls *SNC1* protein accumulation (Cheng et al. 2011; Gou et al. 2012), we tested whether the defect of *siz1* could be rescued when the overaccumulation of *SNC1* protein is attenuated by *CPR1* overexpression. Indeed, 15 of 20 lines of *siz1-3* harboring the *CPR1* overexpression (*CPR1-OE*) construct showed significantly rescued growth phenotype (Fig. 4A and B). It was noted that the growth rescue of *siz1-3* by *CPR1* overexpression was significantly greater than by the *snc1-11* mutation. The *siz1-3 CPR1-OE* lines were 25 to 30% larger in biomass compared with the *siz1-3 snc1-11* double mutant (Fig. 4B). Because a few NB-LRR genes were shown to be negatively regulated by *CPR1* (Cheng et al. 2011; Gou et al. 2012), this more complete rescue by *CPR1-OE* suggests that NB-LRR genes other than *SNC1* might be activated in *siz1-3*.

Similar to the growth phenotype, the disease resistance phenotype of *siz1-3* is also affected by *CPR1* overexpression. We performed pathogen growth assay of *P. syringae* pv. *tomato* DC3000 using both vacuum and dipping inoculation methods.

All of the three *CPR1-OE* lines in *siz1-3* background displayed more pathogen growth (>10-fold) compared with the *siz1-3* single mutant in both methods (Fig. 4C and D). Therefore, *CPR1* overexpression is able to largely rescue both the growth and disease resistance defects of *siz1-3*.

The *siz1-3* growth phenotype is independent of *FLD* or *ATXR7*.

It has been shown that *SIZ1* controls flowering time by sumoylating *FLD* that deacetylates histone H4 at the *FLC* chromatin and, thus, regulates *FLC* expression (Jin et al. 2008). Therefore, we explored the possibility that *SIZ1* negatively regulates *SNC1* expression through histone deacetylation enzyme *FLD* as it does for the *FLC* expression. We introduced a loss-of-function mutation *fld-3* and an *FLD* overexpressing construct into *siz1-3*. If *FLD* regulates *SNC1* negatively, we would expect to see an enhanced phenotype of *siz1-3* by *fld-3* and a rescued phenotype of *siz1-3* by *FLD* overexpression. However, no obvious growth difference was observed between *siz1-3* and *siz1-3 fld-3* before bolting (Fig. 5A), although *siz1-3 fld-3* did show delayed flowering similar to that of the *fld-3* single mutant. In

Fig. 3. *SIZ1* negatively regulates *SNC1*. **A**, Growth phenotypes of wild-type Col-0, *bon1-1*, and two *bon1-1* lines harboring the *p35S::SIZ1* construct. **B**, Growth phenotypes of Col-0, *snc1-1*, and two *snc1-1* lines harboring the *p35S::SIZ1* construct. **C**, Growth phenotypes of Col-0, *pSNC1::SNC1:GFP*, and two *pSNC1::SNC1:GFP* lines harboring the *35S::SIZ1* construct. **D**, Relative expression level of *SNC1* gene in Col-0, *pSNC1::SNC1:GFP*, and two *pSNC1::SNC1:GFP* lines harboring the *35S::SIZ1* construct detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). **E**, Relative expression level of *PR1* gene in Col-0, *pSNC1::SNC1:GFP*, and two *pSNC1::SNC1:GFP* lines harboring the *35S::SIZ1* construct detected by qRT-PCR. For **D** and **E**, letters indicate statistical differences ($P < 0.01$; one-way analysis of variance, Tukey's honestly significant difference test) of different genotypes. **F**, *SNC1:GFP* protein level in Col-0, *pSNC1::SNC1:GFP*, and two *pSNC1::SNC1:GFP* lines harboring the *35S::SIZ1* construct detected by Western blot.

addition, all 25 *FLD* overexpression lines in *siz1-3* had the same growth defect as the *siz1-3* single mutant (Fig. 5B).

We also analyzed the role of the histone methylation gene *ATXR7* in the *SIZ1* function in immunity because *ATXR7* has been shown to regulate expression of both *FLC* and *SNC1* (Tamada et al. 2009; Xia et al. 2013). The double mutant of *siz1-3 atr7-1* was constructed, and the *atr7-1* mutation did not suppress the growth phenotype of *siz1-3* as it did for *snc1-1* (Fig. 5C). We subsequently generated the *bon1-1 atr7-1* double mutant and found that *atr7-1* suppresses the *bon1-1* growth defect to a large extent (Fig. 5D). These data suggest that *BON1*

and *SIZ1* regulate *SNC1* through different mechanisms, and *SIZ1* does not seem to regulate *SNC1* and *FLC* in a similar manner.

The *SNC1* proteins are sumoylated in planta.

To determine whether or not *SNC1* itself can be directly sumoylated, we used GPS-SUMO software (Zhao et al. 2014) to predict sumoylation sites and SUMO-interaction motifs in the *SNC1* protein. Four putative sumoylation sites and four SUMO-interaction motifs were detected in the *SNC1* protein (Fig. 6A). We subsequently analyzed potential sumoylation

Fig. 4. Overexpression of *CPR1/CPR30* rescues the *siz1* defects. **A**, Growth phenotypes of Col-0, *siz1-3*, *siz1-3 snc1-11*, and four lines of *siz1-3* harboring *p35S::CPR1* construct (*CPR1-OE/siz1-3*) before (inserted) and after bolting. **B**, Quantification of the plant biomass of Col-0, *siz1-3*, *siz1-3 snc1-11*, and four lines of *siz1-3* harboring *p35S::CPR1* construct. Ten plants of each genotype were used for biomass quantification. **C** and **D**, Pathogen growth assay of Col-0, *siz1-3*, *snc1-11*, *siz1-3 snc1-11*, and four lines of *siz1-3* harboring *p35S::CPR1* construct by vacuum (C) and dipping (D) inoculation. For B, C, and D, letters indicate statistical differences ($P < 0.01$; one-way analysis of variance, Tukey's honestly significant difference test) of different genotypes.

modification of SNC1 protein expressed in *N. benthamiana*. The GFP-tagged wild-type SNC1 protein (SNC1:GFP) or SNC1-1 mutant protein (SNC1-1:GFP) were coexpressed with human influenza hemagglutinin (HA)-tagged SUMO1 (HA:SUMO1) or SUMO2 (HA:SUMO2) so that sumoylation could be monitored by the HA tag. The SNC1:GFP and SNC1-1:GFP proteins were immunoprecipitated (IPed) by anti-GFP antibody, and the proteins pulled down were separated on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyzed by anti-GFP and anti-HA antibodies, respectively. Compared with the plant tissues expressing only the SNC1:GFP or HA-SUMO1, tissues coexpressing SNC1:GFP and HA:SUMO1 showed additional bands with sizes larger than the SNC1:GFP fusion (Fig. 6B). These bands were present in both the wild-type SNC1 and SNC1-1 samples coinfiltrated with HA-SUMO1 or HA-SUMO2 (Fig. 6B). No significant differences in modification were observed between the wild type and the mutant SNC1 proteins (Fig. 6B). This indicates that both SNC1:GFP and SNC1-1:GFP proteins can be modified, and the modification can come from both SUMO1 and SUMO2.

DISCUSSION

In *Arabidopsis*, SUMO E3 SIZ1 was shown to negatively regulate plant disease resistance in an SA-dependent manner (Lee et al. 2007); however, the target of *SIZ1* in immunity was not known. Here, we provided genetic evidence that the plant immune receptor gene *SNC1* is negatively regulated by *SIZ1*. The growth defect of the *siz1* mutant is partially rescued by the loss-of-function *snc1-11* mutation and high temperature (Figs. 1 and 2), and *SIZ1* overexpression inhibited the dwarf phenotypes of *SNC1*-related autoimmune mutants (Fig. 3). These data indicate that *SIZ1* level is critical for *SNC1* activity. Moreover, *SNC1* is a major target in *SIZ1*-mediated defense response because the *snc1-11* mutation or overexpression of CPR1 that degrades SNC1 protein abolished the disease resistance defect of the *siz1* mutant (Figs. 2 and 4). In addition, our data suggest that resistance (*R*) genes other than *SNC1* may also be targeted by *SIZ1* in plant immunity control. The *siz1-3* growth phenotype was partially suppressed by the *snc1-11* mutation but was more completely suppressed by the SA-degrading coding gene *nahG*, the *pad4* mutation (Lee et al. 2007), and overexpression of *CPR1* that regulates multiple *R* genes (Fig. 4). Together, this study indicates that *SNC1* and perhaps other *R* genes are mediators of *SIZ1* function in immunity regulation.

SIZ1 may regulate *SNC1* at transcriptional or post-transcriptional levels. In this study, we did not find evidence for transcriptional regulation of *SNC1* as the primary mode for *SIZ1*. One known transcriptional regulator of *SNC1* is the H3K4 methyltransferase gene *ATXR7*, and it does not appear to be involved in the immunity function of *SIZ1* (Fig. 5C). A direct target of *SIZ1*, *FLD*, regulates the expression of *FLC* at the transcriptional level in the flowering time control, and it does not appear to be involved in *SNC1* regulation either (Fig. 5). In addition, *SIZ1* overexpression has a larger impact on the SNC1 protein level than the *SNC1* transcript level (Figs. 3C and D). These data suggest that *SNC1* might not be modulated by *SIZ1* primarily at the transcript level, and its transcript level change with altered *SIZ1* level could be due to a feedback regulation from the SNC1 activity (Li et al. 2007).

SIZ1 more likely affects SNC1 at a posttranscriptional level, which is indicated by the great reduction of the SNC1:GFP protein but mild effect on *SNC1* transcripts with *SIZ1* overexpression (Fig. 3). Because sumoylation can be a targeting signal for ubiquitination and ubiquitin-dependent protein degradation (Manente et al. 2011; Miteva et al. 2010; Praefcke et al.

2012; Prudden et al. 2007; Uzunova et al. 2007), we hypothesize that *SIZ1* can target the SNC1 protein for sumoylation. Consistent with this hypothesis, several putative sumoylation sites and SIM are found in the SNC1 protein sequences (Fig. 6A). Furthermore, the SNC1 protein is conjugated with SUMO1 and SUMO2 when transiently expressed in *N. benthamiana* leaves (Fig. 6B). This is the first time a plant NB-LRR protein has been found to be sumoylated, suggesting that these immune receptor proteins are subject to multiple posttranslational modifications in addition to ubiquitination.

However, it remains to be determined whether or not SNC1 is sumoylated by *SIZ1* in vivo and, if so, how sumoylation might affect SNC1 stability or activity. One potential effect of sumoylation of SNC1 is to promote subsequent ubiquitination of SNC1 by CPR1. This is suggested by the rescue of the growth and defense mutant phenotypes of *siz1-3* by overexpression of the F-box protein CPR1/CPR30 (Fig. 4). It will be interesting to test whether or not sumoylation of SNC1 is the targeting signal for ubiquitination of SNC1 by CPR1/CPR30. Future studies should further uncover the molecular links between sumoylation and plant immunity and reveal intricate regulations of plant immune receptor genes at multiple levels.

Fig. 5. Genetic interaction analyses of *SIZ1*, *ATXR7*, and *FLD*. **A**, Growth phenotypes of Col-0, *fld-3*, *siz1-3*, and *siz1-3 fld-3* double mutant. **B**, Growth phenotypes of Col-0, *siz1-3*, and *siz1-3* harboring *p35S::FLD* construct (*FLD-OE/siz1-3*). Images of one representative transgenic line is shown. **C**, Growth phenotypes of Col-0, *atxr7-1*, *siz1-3*, and *siz1-3 atxr7-1*. **D**, Growth phenotypes of Col-0, *atxr7-1*, *bon1-1*, and *bon1-1 atxr7-1*.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis seed were either sowed directly on soil or plated on half-strength Murashige and Skoog (MS) medium plate (Murashige and Skoog 1962; Tamada et al. 2009) containing 0.7% agar and 1% sucrose. For pathogen tests, seed were directly planted on soil. The growth chamber was set with light at either 24 h/day for growth phenotyping or 12 h/day for pathogen growth tests, with light intensity at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity at 50 to 70%.

Mutant characterization.

The previously published mutants *siz1-3* (SALK_034008) (Lee et al. 2007; Miura et al. 2005), *atxr7-1* (SALK_149692) (Tamada et al. 2009), and *fld-3* (SALK_075401) (He et al. 2003) were obtained from the *Arabidopsis* Biological Resource Center. Single mutants were crossed to generate double mutants *siz1-3 snc1-11*, *siz1-3 atxr7-1*, and *siz1-3 fld-3* by genotyping plants in the F2 population. Genotyping primers were salk_034008LP (TCCCTCGTAGACATCTGATGG) and salk_034008RP (AAAGAGAGAGTGAGCGAAGGG) for *siz1-3*, cs25760_LP (TCTTGTGACAGGTGCAACTTG) and cs25760_RP (AAACAAAGCTAGGCACAAGGC) for *atxr7-1*,

and SALK_075401_LP (TTCTCTCAGGTCAAGAGCCTG) and SALK_075401_RP (GTGGAGATCACTGCTTTCTGC) for *fld-3*.

Plant transformation, transgenic plant selection, and characterization.

The full-length cDNA of the *SIZ1* gene was amplified from the wild-type Col-0 using *SIZ1_F* (ATGGATTTGGAAGC TAATTGTAAG) and *SIZ1_R* (TTAAACTCCGGTGTCTTGTCT GAT) primers. The full-length cDNA of *FLD* was amplified from the wild-type Col-0 using *FLD_F* (ATGGTCTCATTCTCCG CACC) and *FLD_R* (TCATTGTTCAATCTTTTTCATCG) primers. Both *SIZ1* and *FLD* cDNA fragments were cloned into the Gateway entry vector pCR8 TOPO TA (Invitrogen) and, subsequently, into the destination vectors PGWB402 (Nakagawa et al. 2007) or PMDC32 (Curtis and Grossniklaus 2003) to generate the 35S promoter-driven overexpression constructs. *PGWB402::SIZ1* was transformed into *bon1-1* and the *pSNC1::SNC1::GFP/Col-0* homozygous line (Zhu et al. 2010) to generate the *SIZ1*-overexpression lines in each background, and *PGWB402::FLD* was transformed into *siz1-3* to generate *FLD*-overexpression lines in the *siz1-3* background. The transgenic plants were obtained using kanamycin at 50 $\mu\text{g/ml}$ for selection on MS plates. The *PMDC32::SIZ1* was transformed into *snc1-1* to

Fig. 6. Sumoylation of the SNC1 protein. **A**, Predicted small ubiquitin-like modifier interaction motifs (SIM) and sumoylation sites of SNC1 protein. Locations of the SIM and sumoylation sites (K residue) are indicated. A score higher than the cutoff is an indication of high confidence in predicted sites. **B**, Sumoylation of the wild-type SNC1:GFP and the mutant SNC1-1:GFP proteins detected by Western blot. Total proteins and immunoprecipitated (IPed) proteins (approximately 20-fold concentrated) were loaded as input and IP samples, respectively, and detected with anti-green fluorescent protein (GFP) and anti-human influenza hemagglutinin (HA) antibodies. Arrowhead indicates sumoylated SNC1 and SNC1-1 proteins.

generate *SIZ1*-overexpression lines in the *snc1-1* background, and transgenic plants were obtained using hygromycin at 50 µg/ml for selection on MS plates. A previously reported construct of *p35S::CPR30 (CPR1)* (Gou et al. 2009, 2012) was transformed into *siz1-3* to generate the *CPR1*-overexpression lines in the *siz1-3* background.

Bacterial growth assay.

The bacterial growth assay was carried out as previously described (Gou et al. 2015). Briefly, *P. syringae* pv. *tomato* strain DC3000 was grown on plates with King's B medium. The bacteria were resuspended and diluted with 10 mM MgCl₂ and 0.02% Silwet L-77 to an optical density at 600 nm (OD₆₀₀) = 0.05 for dipping inoculation and OD₆₀₀ = 0.002 for vacuum infiltration. Plants were immersed in inoculums for 10 s by dipping or 2 min by vacuum. They were then covered to keep humidity immediately after inoculation and uncovered 1 h later. Bacterial growth in plants was assayed at 1 h (day 0) and 3 days (day 3) after inoculation. Three whole seedlings were collected as one sample, weighed, ground in 1 ml of 10 mM MgCl₂ with 0.02% Silwet L-77, and shaken at room temperature for 1 h. Serial dilutions of the ground solution were spotted on King's B media, and the number of CFU per fresh weight was determined. Three samples were analyzed for each genotype and condition combination.

RNA extraction and qRT-PCR analysis.

Total RNA were extracted from 10-day-old plate-grown seedlings using the Trizol reagent (Invitrogen), as instructed by the manufacturer. RNA blot analysis of *PR1* expression was performed as described (Gou et al. 2009). For qRT-PCR, cDNA was synthesized from the mRNA using M-MuLV reverse transcription (NEB). *SNC1_F* (GAATCGAATGTCTCTATCTGC) and *SNC1_R* (CTGTAAAGTCGGCGAGCTCA) were used as primers for *SNC1* amplification, and the *PR1_F* (CTTTGTAGC TCTTGATAGTGCTCT) and *PR1_R* (TCCTGCATATGATGCT CTTATTGA) primers for *PR1* amplification. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for real-time PCR. The cycle threshold (Ct) value was calculated by the CFXManager Software v3.0 (Bio-Rad). The standard rules (Udvardi et al. 2008) were followed to quantify the relative gene expression using the comparative Ct method against the internal standard *UBQ10*.

Arabidopsis total protein extraction and Western blot analyses.

Arabidopsis seedlings grown on MS plate for 12 days were used for total protein extraction using the buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol (DTT), 0.25% Triton-X 100, and 1× complete protease inhibitor cocktail (Sigma-Aldrich). Western blot was performed using the polyclonal anti-GFP antibody (Invitrogen) or monoclonal anti-HA antibody (Convance).

Sumoylation site prediction and in vivo sumoylation assay.

The prediction of sumoylation sites was done using the GPS-SUMO 1.0 software (<http://sumosp.biocuckoo.org/>) with a medium threshold setting (Zhao et al. 2014). For in vivo sumoylation assay, the *Arabidopsis AtSUMO1* and *AtSUMO2* genes were tagged by 3xHA at the N terminus and expressed under the 35S promoter in pHPT vector. The previously used *35S::SNC1:GFP* and *35S::SNC1-1:GFP* *Agrobacterium* strains (Zhu et al. 2010) were singly infiltrated, or coinfiltrated with *35S::HA:SUMO1* and *35S::HA:SUMO2* *Agrobacterium* strains into *N. benthamiana* leaves, and leaf tissues were collected 3 days postinoculation. Total proteins were extracted using the buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM

EDTA, 150 mM NaCl, 10% glycerol, 10 mM DTT, 0.2% 2-mercaptoethanol, 1% Triton-X 100, 2% polyvinyl-pyrrolidone, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor cocktail (Sigma-Aldrich), and a small portion was used as an input. The SNC1:GFP or SNC1-1:GFP proteins were IPed using GFP-Trap magnetic agarose beads (chromotek). Both the input and IPed protein samples were resolved by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes for Western blotting. Western blot was performed using the monoclonal anti-GFP antibody (JL-8; Clontech) or monoclonal anti-HA antibody (Convance).

ACKNOWLEDGMENTS

We thank T. Nakagawa for the PGWB vectors and the *Arabidopsis* Biological Resource Center for the *Arabidopsis* mutants. This work was supported by grants from National Science Foundation of the United States (IOS-0919914 and IOS-1353738) to J. Hua and Xinjiang Key Laboratory Special Funds of China (2014KL008) to Q. Huang.

LITERATURE CITED

- Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. 1998. Structure determination of the small ubiquitin-related modifier SUMO-1. *J. Mol. Biol.* 280:275-286.
- Cheng, Y. T., Li, Y., Huang, S., Huang, Y., Dong, X., Zhang, Y., and Li, X. 2011. Stability of plant immune-receptor resistance proteins is controlled by SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* 108:14694-14699.
- Colby, T., Matthäi, A., Boeckelmann, A., and Stuibler, H. P. 2006. SUMO-conjugating and SUMO-deconjugating enzymes from *Arabidopsis*. *Plant Physiol.* 142:318-332.
- Curtis, M. D., and Grossniklaus, U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133:462-469.
- Desterro, J. M., Rodriguez, M. S., and Hay, R. T. 1998. SUMO-1 modification of IκBα inhibits NF-κB activation. *Mol. Cell* 2:233-239.
- Gou, M., and Hua, J. 2012. Complex regulation of an R gene SNC1 revealed by auto-immune mutants. *Plant Signal. Behav.* 7:213-216.
- Gou, M., Shi, Z., Zhu, Y., Bao, Z., Wang, G., and Hua, J. 2012. The F-box protein CPR1/CPR30 negatively regulates R protein SNC1 accumulation. *Plant J.* 69:411-420.
- Gou, M., Su, N., Zheng, J., Huai, J., Wu, G., Zhao, J., He, J., Tang, D., Yang, S., and Wang, G. 2009. An F-box gene, CPR30, functions as a negative regulator of the defense response in *Arabidopsis*. *Plant J.* 60:757-770.
- Gou, M., Zhang, Z., Zhang, N., Huang, Q., Monaghan, J., Yang, H., Shi, Z., Zipfel, C., and Hua, J. 2015. Opposing effects on two phases of defense responses from concerted actions of HSC70 and BON1 in *Arabidopsis*. *Plant Physiol.* 169:2304-2323.
- Hanania, U., Furman-Matarasso, N., Ron, M., and Avni, A. 1999. Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. *Plant J.* 19:533-541.
- Hay, R. T. 2005. SUMO: A history of modification. *Mol. Cell* 18:1-12.
- He, Y., Michaels, S. D., and Amasino, R. M. 2003. Regulation of flowering time by histone acetylation in *Arabidopsis*. *Science* 302:1751-1754.
- Hecker, C. M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. 2006. Specification of SUMO1- and SUMO2-interacting motifs. *J. Biol. Chem.* 281:16117-16127.
- Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* 419:135-141.
- Huang, Y., Minaker, S., Roth, C., Huang, S., Hieter, P., Lipka, V., Wiermer, M., and Li, X. 2014. An E4 ligase facilitates polyubiquitination of plant immune receptor resistance proteins in *Arabidopsis*. *Plant Cell* 26:485-496.
- Ishida, T., Fujiwara, S., Miura, K., Stacey, N., Yoshimura, M., Schneider, K., Adachi, S., Minamisawa, K., Umeda, M., and Sugimoto, K. 2009. SUMO E3 ligase HIGH PLOIDY2 regulates endocycle onset and meristem maintenance in *Arabidopsis*. *Plant Cell* 21:2284-2297.
- Ishida, T., Yoshimura, M., Miura, K., and Sugimoto, K. 2012. MMS21/HPY2 and SIZ1, two *Arabidopsis* SUMO E3 ligases, have distinct functions in development. *PLoS One* 7:e46897.
- Jin, J. B., Jin, Y. H., Lee, J., Miura, K., Yoo, C. Y., Kim, W. Y., Van Oosten, M., Hyun, Y., Somers, D. E., Lee, I., Yun, D. J., Bressan, R. A., and Hasegawa, P. M. 2008. The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion

- pathway and through affects on FLC chromatin structure. *Plant J.* 53: 530-540.
- Johnson, K. C., Dong, O. X., Huang, Y., and Li, X. 2012. A rolling stone gathers no moss, but resistant plants must gather their MOSES. *Cold Spring Harb. Symp. Quant. Biol.* 77:259-268.
- Kerscher, O. 2007. SUMO junction—What's your function? New insights through SUMO-interacting motifs. *EMBO Rep.* 8:550-555.
- Kim, D. Y., Han, Y. J., Kim, S. I., Song, J. T., and Seo, H. S. 2015a. *Arabidopsis* CMT3 activity is positively regulated by AtSIZ1-mediated sumoylation. *Plant Sci.* 239:209-215.
- Kim, S. I., Park, B. S., Kim, D. Y., Yeu, S. Y., Song, S. I., Song, J. T., and Seo, H. S. 2015b. E3 SUMO ligase AtSIZ1 positively regulates SLN1-mediated GA signaling and plant development. *Biochem. J.* 469:299-314.
- Kurepa, J., Walker, J. M., Smalle, J., Gosink, M. M., Davis, S. J., Durham, T. L., Sung, D. Y., and Vierstra, R. D. 2002. The small ubiquitin-like modifier (SUMO) protein modification system in *Arabidopsis*. Accumulation of SUMO1 and -2 conjugates is increased by stress. *J. Biol. Chem.* 278:6862-6872.
- Lee, J., Nam, J., Park, H. C., Na, G., Miura, K., Jin, J. B., Yoo, C. Y., Baek, D., Kim, D. H., Jeong, J. C., Kim, D., Lee, S. Y., Salt, D. E., Mengiste, T., Gong, Q., Ma, S., Bohnert, H. J., Kwak, S. S., Bressan, R. A., Hasegawa, P. M., and Yun, D. J. 2007. Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ1 SUMO E3 ligase. *Plant J.* 49:79-90.
- Li, Y., Yang, S., Yang, H., and Hua, J. 2007. The TIR-NB-LRR gene *SNC1* is regulated at the transcript level by multiple factors. *Mol. Plant-Microbe Interact.* 20:1449-1456.
- Manente, A. G., Pinton, G., Taviano, D., Lopez-Rodas, G., Brunelli, E., and Moro, L. 2011. Coordinated sumoylation and ubiquitination modulate EGF induced EGR1 expression and stability. *PLoS One* 6:e25676.
- Melchior, F. 2000. SUMO—Nonclassical ubiquitin. *Annu. Rev. Cell Dev. Biol.* 16:591-626.
- Miteva, M., Keusekotten, K., Hofmann, K., Praefcke, G. J., and Dohmen, R. J. 2010. Sumoylation as a signal for polyubiquitylation and proteasomal degradation. *Subcell. Biochem.* 54:195-214.
- Miura, K., Jin, J. B., Lee, J., Yoo, C. Y., Stirm, V., Miura, T., Ashworth, E. N., Bressan, R. A., Yun, D. J., and Hasegawa, P. M. 2007. SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*. *Plant Cell* 19:1403-1414.
- Miura, K., Lee, J., Jin, J. B., Yoo, C. Y., Miura, T., and Hasegawa, P. M. 2009. Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106:5418-5423.
- Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A. S., Raghothama, K. G., Baek, D., Koo, Y. D., Jin, J. B., Bressan, R. A., Yun, D. J., and Hasegawa, P. M. 2005. The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. U.S.A.* 102:7760-7765.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15:473-497.
- Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* 104:34-41.
- Orth, K., Xu, Z., Mudgett, M. B., Bao, Z. Q., Palmer, L. E., Bliska, J. B., Mangel, W. F., Staskawicz, B., and Dixon, J. E. 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. *Science* 290:1594-1597.
- Praefcke, G. J., Hofmann, K., and Dohmen, R. J. 2012. SUMO playing tag with ubiquitin. *Trends Biochem. Sci.* 37:23-31.
- Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J., Tainer, J. A., McGowan, C. H., and Boddy, M. N. 2007. SUMO-targeted ubiquitin ligases in genome stability. *EMBO J.* 26:4089-4101.
- Tamada, Y., Yun, J. Y., Woo, S. C., and Amasino, R. M. 2009. ARABIDOPSIS TRITHORAX-RELATED7 is required for methylation of lysine 4 of histone H3 and for transcriptional activation of FLOWERING LOCUS C. *Plant Cell* 21:3257-3269.
- Udvardi, M. K., Czechowski, T., and Scheible, W. R. 2008. Eleven golden rules of quantitative RT-PCR. *Plant Cell* 20:1736-1737.
- Uzunova, K., Götttsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E. S., Praefcke, G. J., and Dohmen, R. J. 2007. Ubiquitin-dependent proteolytic control of SUMO conjugates. *J. Biol. Chem.* 282:34167-34175.
- Verger, A., Perdomo, J., and Crossley, M. 2003. Modification with SUMO. A role in transcriptional regulation. *EMBO Rep.* 4:137-142.
- Xia, S., Cheng, Y. T., Huang, S., Win, J., Soards, A., Jinn, T. L., Jones, J. D., Kamoun, S., Chen, S., Zhang, Y., and Li, X. 2013. Regulation of transcription of nucleotide-binding leucine-rich repeat-encoding genes SNC1 and RPP4 via H3K4 trimethylation. *Plant Physiol.* 162:1694-1705.
- Yang, S., and Hua, J. 2004. A haplotype-specific Resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in *Arabidopsis*. *Plant Cell* 16:1060-1071.
- Zhang, Y., Goritschnig, S., Dong, X., and Li, X. 2003. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1, constitutive 1*. *Plant Cell* 15:2636-2646.
- Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y., and Ren, J. 2014. GPS-SUMO: A tool for the prediction of sumoylation sites and SUMO-interaction motifs. *Nucleic Acids Res.* 42: W325-W330.
- Zheng, Y., Schumaker, K. S., and Guo, Y. 2012. Sumoylation of transcription factor MYB30 by the small ubiquitin-like modifier E3 ligase SIZ1 mediates abscisic acid response in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 109:12822-12827.
- Zhu, Y., Qian, W., and Hua, J. 2010. Temperature modulates plant defense responses through NB-LRR proteins. *PLoS Pathog.* 6:e1000844.
- Zou, B., Yang, D. L., Shi, Z., Dong, H., and Hua, J. 2014. Monoubiquitination of histone 2B at the disease resistance gene locus regulates its expression and impacts immune responses in *Arabidopsis*. *Plant Physiol.* 165:309-318.