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# SUMO E3 Ligases GmSIZ1a and GmSIZ1b regulate vegetative growth in soybean[OO]

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Research Article

**Abstract** SIZ1 is a small ubiquitin-related modifier (SUMO) E3 ligase that mediates post-translational SUMO modification of target proteins and thereby regulates developmental processes and hormonal and environmental stress responses in *Arabidopsis*. However, the role of SUMO E3 ligases in crop plants is largely unknown. Here, we identified and characterized two *Glycine max* (soybean) SUMO E3 ligases, *GmSIZ1a* and *GmSIZ1b*. Expression of *GmSIZ1a* and *GmSIZ1b* was induced in response to salicylic acid (SA), heat, and dehydration treatment, but not in response to cold, abscisic acid (ABA), and NaCl treatment. Although *GmSIZ1a* was expressed at higher levels than *GmSIZ1b*, both genes encoded proteins with SUMO E3 ligase activity *in vivo*. Heterologous expression of *GmSIZ1a* or *GmSIZ1b* rescued the mutant phenotype of *Arabidopsis siz1-2*, including dwarfism, constitutively activated expression of pathogen-related genes, and ABA-sensitive seed germination. Simultaneous downregulation of *GmSIZ1a* and *GmSIZ1b* (*GmSIZ1a/b*) using RNA interference (RNAi)-mediated gene silencing decreased heat shock-induced SUMO conjugation in soybean. Moreover, *GmSIZ1RNAi* plants exhibited reduced plant height and leaf size. However, unlike *Arabidopsis siz1-2* mutant plants, flowering time and SA levels were not significantly altered in *GmSIZ1RNAi* plants. Taken together, our results indicate that *GmSIZ1a* and *GmSIZ1b* mediate SUMO modification and positively regulate vegetative growth in soybean.

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## INTRODUCTION

Since the discovery of the SUMO peptide, researchers have established that SUMO conjugation to proteins (SUMOylation) profoundly influences biological processes including innate immunity, stress responses, DNA repair and transcriptional regulation (Enserink 2015). SUMOylation is a rapid and reversible post-translational modification that affects protein–protein interactions, protein targeting, enzymatic activity, and protein stability (Cubeñas-Potts and Matunis 2013). Although SUMOylation is widely known as a regulator of nuclear processes, growing evidence indicates that it also regulates non-nuclear processes, such as channel activity, receptor and cytoskeletal functions, autophagy, and exocytosis (Gill 2004; Wasik and Filipek 2014).

SUMOs have a similar three-dimensional structure to ubiquitin, but the amino acid sequences of these proteins share only approximately 20% similarity and the surface topology of SUMO is substantially different

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from that of ubiquitin (Müller et al. 2001). SUMO is translated as a pre-protein (known as pre-SUMO) and a SUMO-specific cysteine protease (SUMO protease) deletes a short C-terminal fragment (immediately downstream of a C-terminal GG motif) to produce active mature SUMO (also known as free SUMO). SUMO conjugates to its target substrate in a stepwise manner via activation (E1), conjugation (E2), and ligation (E3) (Müller et al. 2001). SUMO is often conjugated to a ΨKXE/D motif (where Ψ is a large hydrophobic residue; K is lysine; X is any residue; and E/D is glutamic acid or aspartic acid) in substrates, resulting in an isopeptide bond between the C-terminal G residue in SUMO and the K residue in the substrate (Johnson 2004). SUMO proteases cleave the isopeptide bond between SUMO and its substrate (Kim and Baek 2009).

SUMOylation has been implicated in the regulation of developmental, hormonal, and environmental responses in *Arabidopsis*, such as gametophyte development (Ling et al. 2012; Liu et al. 2014), embryogenesis (Saracco et al. 2007), photomorphogenesis (Sadanandom et al. 2015; Lin et al. 2016), flowering time (Murtas et al. 2003; Jin et al. 2008), cell proliferation (Huang et al. 2009; Ishida et al. 2009), abscisic acid (ABA) signaling (Miura et al. 2009; Zheng et al. 2012), gibberellic acid (GA) signaling (Kim et al. 2015), the salt stress response (Conti et al. 2008), thermal adaptation (Yoo et al. 2006; Miura et al. 2007), the drought stress response (Catala et al. 2007; Zhang et al. 2013), immune responses (Lee et al. 2007; Saleh et al. 2015), and nutrient (phosphate and nitrogen) starvation signaling (Miura et al. 2005; Park et al. 2011). The SUMO regulatory mechanism is conserved in *Oryza sativa* (rice), *Zea mays* (maize), Dendrobium (orchids), and *Malus domestica* (apple; Park et al. 2010; Liu et al. 2015; Augustine et al. 2016; Zhang et al. 2016). The SUMO E3 ligase OsSIZ1 regulates phosphate- and nitrogen-dependent responses, spikelet fertility, and plant development in rice (Thangasamy et al. 2011; Wang et al. 2011, 2015). Moreover, the OsOTS1 SUMO protease positively regulates salt stress responses in rice (Srivastava et al. 2016). Mutations in an *Arabidopsis* SUMO E3 ligase, *AtSIZ1*, increase salicylic acid (SA) levels, resulting in reduced plant stature, constitutively activated immune responses, and early flowering (Lee et al. 2007; Jin et al. 2008; Miura et al. 2010). However, whether this regulatory mechanism is conserved in other plant species remains to be determined.

The function of SUMOylation in soybean, an important crop plant, is unknown. In this study, we identified and characterized two soybean SUMO E3 ligases, *GmSIZ1a* and *GmSIZ1b*. We demonstrated that both are bona fide SUMO E3 ligases that positively regulate vegetative growth in soybean. *GmSIZ1a* and *GmSIZ1b* are required for heat shock-induced GmSUMO1 conjugation, implying that *GmSIZ1a*/*b*-mediated SUMO modifications regulate heat stress responses in soybean as they do in *Arabidopsis* (Yoo et al. 2006). However, in contrast to *Arabidopsis* plants harboring a mutation in *AtSIZ1*, downregulation of *GmSIZ1a*/*b* did not affect flowering time and SA production. Thus, SUMO E3 ligases may have distinct regulatory roles in soybean.

## RESULTS

### Identification of genes encoding putative SUMO E3 ligases in soybean

To investigate the role of soybean SUMO E3 ligase, we searched the soybean genome database (http://www.phytozome.net) for SIZ1 homologs. We identified *GmSIZ1a* (Glyma12g07590, 880 aa) and *GmSIZ1b* (Glyma11g15880, 879 aa) (Figure S1A). The primary amino acid sequences of these proteins showed 96.2% identity to each other, but approximately 64% identity to *AtSIZ1* (Figure S1B). Phylogenetic analysis of putative SIZ1/PIAS-type SUMO E3 ligases from various plant species showed that *GmSIZ1a* and *GmSIZ1b* were closely related to *AtSIZ1* in the dicot sub-class of SUMO E3 ligases (Figure S1C). The gene structure of *GmSIZ1a* and *GmSIZ1b* was similar to that of *AtSIZ1*, although *GmSIZ1a* and *GmSIZ1b* had one fewer exon (17 *vs.* 18 in *AtSIZ1*) and different intron sizes (Figure S1D). *GmSIZ1a* and *GmSIZ1b* contained all five conserved domains found in *AtSIZ1*, including the N-terminal scaffold attachment factor A/B/acinus/PIAS (SAP) domain; the plant homeodomain (PHD); the putative PINIT core domain; the SIZ/PIAS-RING (SP-RING) domain; and the SUMO-interacting motif (SIM) (Figure 1A) (Miura et al. 2005), suggesting that *GmSIZ1a* and *GmSIZ1b* may have SUMO E3 ligase activity.

### *GmSIZ1a* and *GmSIZ1b* are nuclear proteins

To determine the subcellular localization of *GmSIZ1a* and *GmSIZ1b*, we transiently expressed C-terminal green fluorescent protein (GFP) fusions of these proteins in *Arabidopsis* protoplasts (Figure 1B). While GFP alone was localized to both the nucleus and cytosol (Figure 1B,

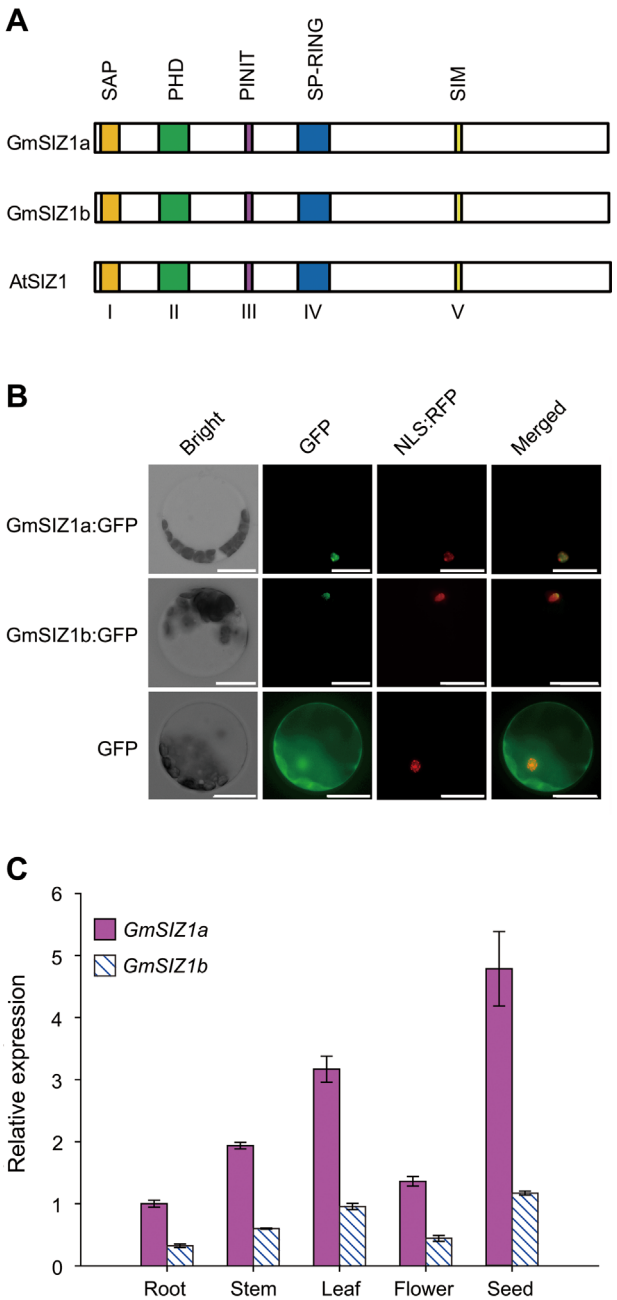


**Figure 1. Expression pattern of *GmSIZ1a* and *GmSIZ1b* and protein subcellular localization**

(**A**) Schematic representation of *GmSIZ1a*, *GmSIZ1b*, and *AtSIZ1*, showing conserved domains in color. (**B**) Subcellular localization. GmSIZ1a:GFP or GmSIZ1b:GFP was transiently co-expressed with NLS:RFP (nuclear localization signal:RFP; a nuclear marker) in *Arabidopsis* protoplasts. Green fluorescent protein (GFP) was used as a control. Bars = 20 μm. (**C**) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *GmSIZ1a* and *GmSIZ1b* expression in different tissues. Relative expression was normalized to that of *GmUBI3*, and data represent the mean ± *SD*, $n = 3$.

bottom panel), GmSIZ1a:GFP and GmSIZ1b:GFP were exclusively localized to the nucleus (Figure 1B, upper and middle panel, respectively), suggesting that *GmSIZ1a* and *GmSIZ1b* are nuclear proteins.

### Expression patterns of *GmSIZ1a* and *GmSIZ1b*

To characterize the expression profiles of *GmSIZ1a* and *GmSIZ1b* in different tissues of soybean, we analyzed *GmSIZ1a* and *GmSIZ1b* expression levels by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Figure 1C). Expression of both genes was higher in seeds and leaves than in other tissues examined. Next, we analyzed the promoter regions (1,500 bp upstream of the ATG translation start codon) of *GmSIZ1a* and *GmSIZ1b* using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html/), and identified several putative abiotic stress, biotic stress, and hormone response *cis*-acting regulatory elements, including those related to heat, low-temperature, defense, ABA, SA, jasmonic acid, and light (Table S1). This observation prompted us to determine the expression levels of *GmSIZ1a* and *GmSIZ1b* in response to SA, ABA, heat, dehydration, cold, and salt stress treatments (Figure 2). Consistent with previous reports, the controls *GmPR1*, *GmHSF12*, and *GmDREB2* were induced by SA, heat, and dehydration treatment, respectively (Figure 2A–C; Chen et al. 2007; Sandhu et al. 2009; Chung et al. 2013). Under these conditions, transcript accumulation of *GmSIZ1a* and *GmSIZ1b* also increased significantly (Figure 2A–C). By contrast, cold, ABA, and NaCl treatment activated the expression of the control gene *GmDREB2*, but did not significantly affect transcript accumulation of *GmSIZ1a* and *GmSIZ1b* (Figure 2D–F). These results suggest that *GmSIZ1a* and *GmSIZ1b* function in certain biotic/abiotic stress responses.

### Both *GmSIZ1a* and *GmSIZ1b* are functional SUMO E3 ligases

To explore the biochemical activity of *GmSIZ1a* and *GmSIZ1b*, we transformed *Arabidopsis siz1-2* plants with constructs expressing C-terminal GFP fusions of *GmSIZ1a* or *GmSIZ1b* under the *AtSIZ1* promoter (ProAtSIZ1:GmSIZ1a:GFP or ProAtSIZ1:GmSIZ1b:GFP, respectively), and three independent transgenic lines were used for further analysis (Figure S2A, B). Both *GmSIZ1a* and *GmSIZ1b* partially suppressed the

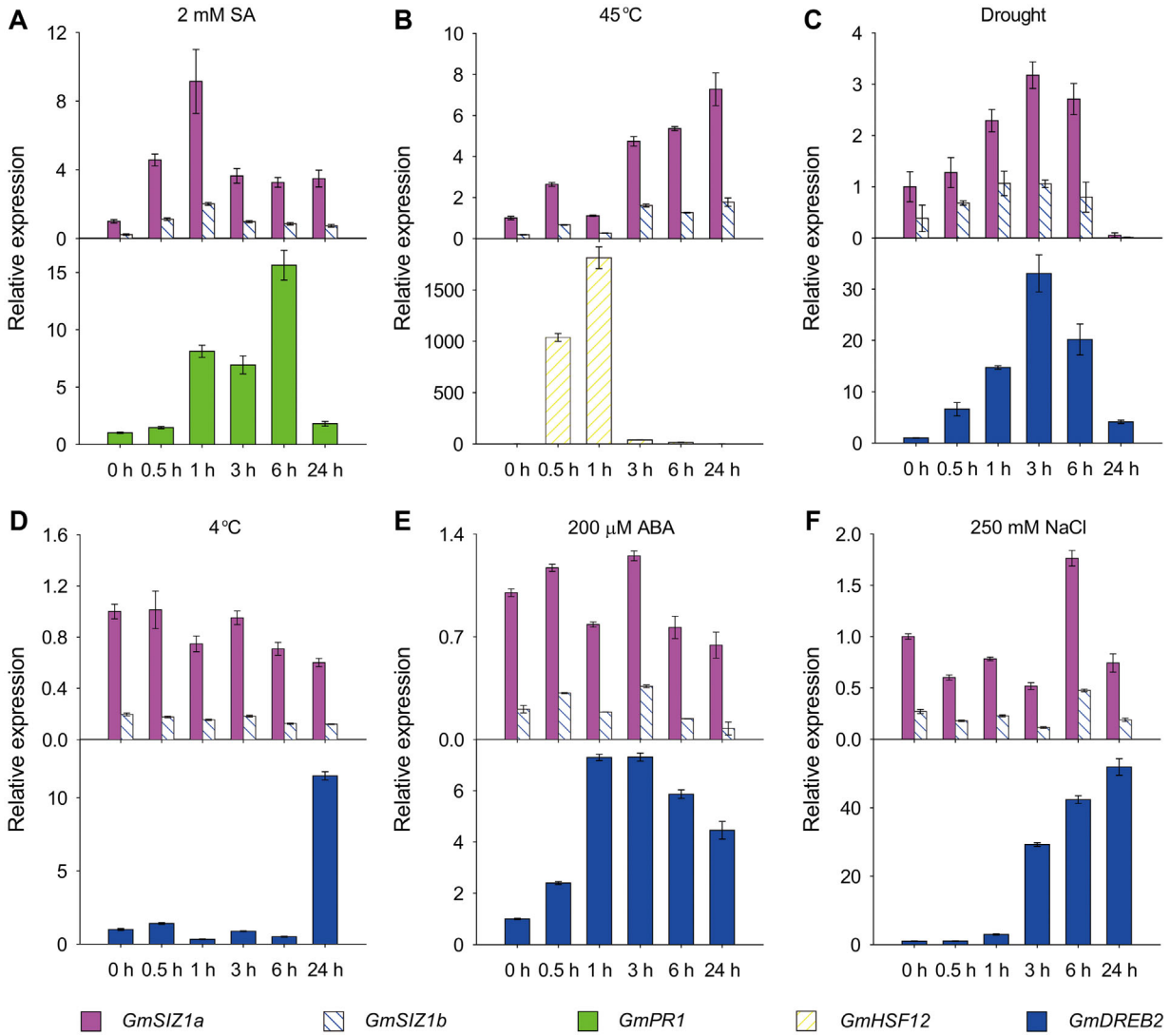


**Figure 2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *GmSIZ1a* and *GmSIZ1b* expression level in response to salicylic acid (SA), heat, drought, cold, abscisic acid (ABA) and NaCl treatments**

Fourteen-day-old soybean seedlings were treated with 2 mM SA (**A**), 45 °C heat stress (**B**), drought (**C**), 4 °C cold stress (**D**), 200 μM ABA (**E**), and 250 mM NaCl (**F**) for the indicated periods. *GmPR1*, *GmDREB2*, and *GmHSF12* were used as stress- or hormone-responsive positive controls. Relative expression was normalized to that of *GmUBI3*, and data represent the mean ± *SD*, $n = 3$.

dwarf phenotype of the *Arabidopsis siz1-2* plants (Figures 3A, B, S2C). Due to increased levels of SA in the *siz1-2* plants, the expression of *PR* genes and *ISOCHORISMATE SYNTHASE1* (*ICS1*) is increased (Lee et al. 2007). The elevated *PR* and *ICS1* gene expression level was almost completely rescued by the heterologous expression of *GmSIZ1a* or *GmSIZ1b* in *siz1-2* (Figure 3C). Moreover, the ABA-sensitive seed germination phenotype of *siz1-2* (Miura et al. 2009) was suppressed by the expression of *GmSIZ1a* or *GmSIZ1b* (Figure 3D, E). These results indicate that both *GmSIZ1a* and *GmSIZ1b* are biologically functional proteins that may have SUMO E3 ligase activity.

The reduced SUMO E3 ligase activity in *siz1* attenuates heat shock-induced SUMO conjugation (Miura et al. 2005). This activity was restored by expression of *GmSIZ1a* or *GmSIZ1b* in *siz1* (Figure 4A). We further analyzed the SUMO E3 ligase activity of *GmSIZ1a* and *GmSIZ1b in vivo* by transiently co-expressing GmSIZ1a:GFP, GmSIZ1b:GFP, or AtSIZ1:GFP with FLAG:AtSUMO1 in

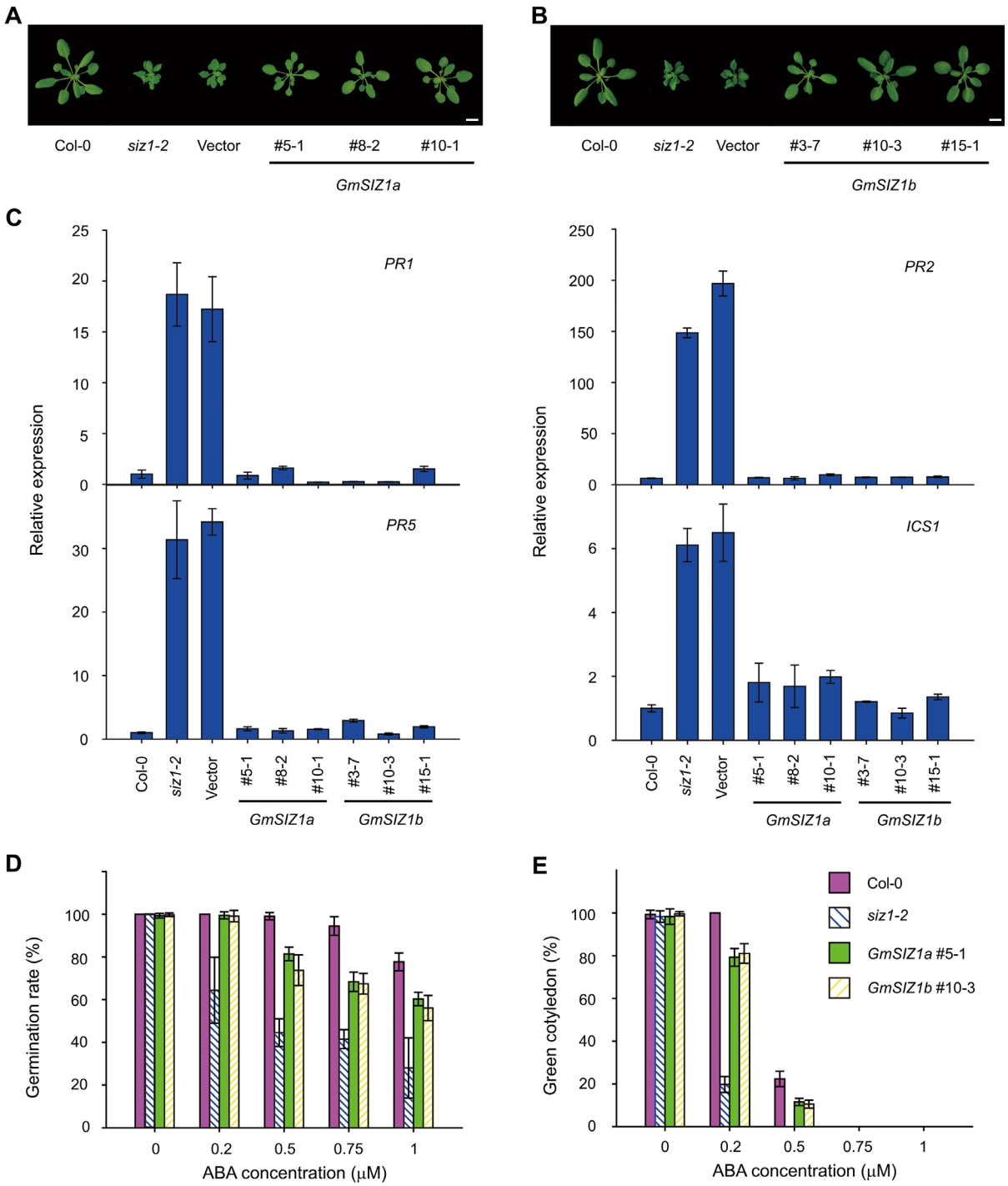


**Figure 3. *GmSIZ1a* and *GmSIZ1b* rescue the *Arabidopsis siz1-2* mutant phenotype**

(**A**) and (**B**) Morphological phenotype of wild-type, *siz1-2*, and complementation plants. *GmSIZ1a* (#5-1, #8-2 and #10-1) and *GmSIZ1b* (#3-7, #10-3 and #15-1) indicate lines expressing GmSIZ1a:GFP or GmSIZ1b:GFP driven by the *Arabidopsis* SIZ1 promoter in the *siz1-2* background, respectively. Vector indicates empty vector transformed into *siz1-2* plants. Bars = 1 cm. (**C**) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *Arabidopsis PR1*, *PR2*, *PR5*, and *ICS1* expression in Col-0, *siz1-2*, and complementation lines under normal conditions. Relative expression was normalized to that of *AtUBC* and data represent the mean ± *SD*, $n = 3$. (**D**) Seed germination rate of Col-0, *siz1-2*, *GmSIZ1a* #5-1, and *GmSIZ1b* #10-3 in the presence of the indicated concentrations of abscisic acid (ABA) at 4 days after planting. Data are the mean ± *SD*, $n = 3$. (**E**) Percentage of green cotyledons in Col-0, *siz1-2*, *GmSIZ1a* #5-1, and *GmSIZ1b* #10-3 seedlings at 7 days after planting in the presence of ABA. Data are the mean ± *SD*. Three biological replicates were performed.

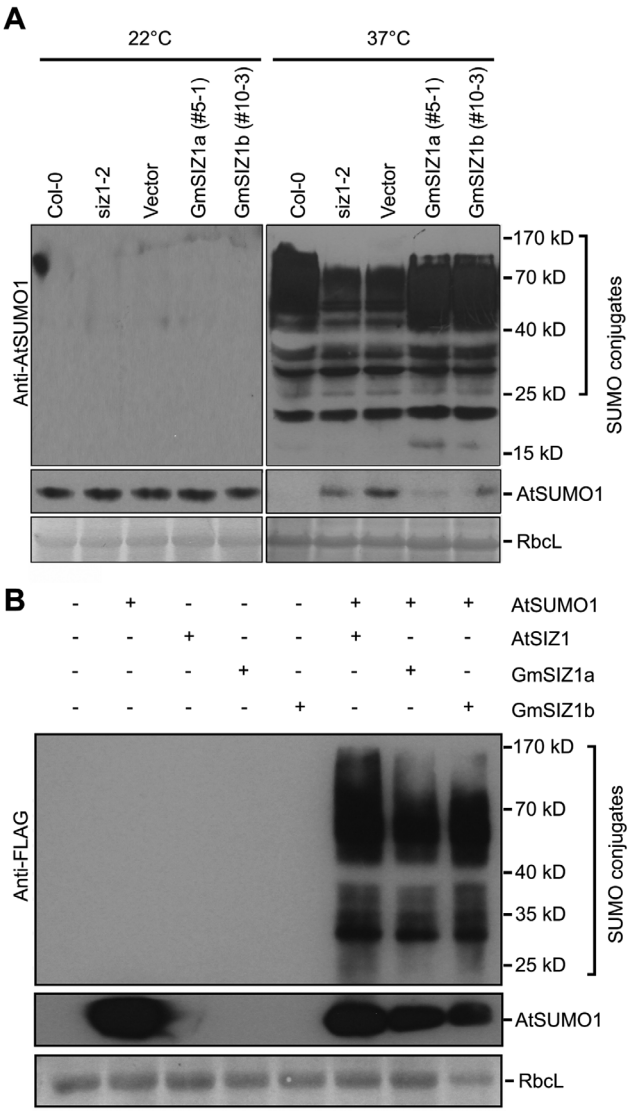


**Figure 4. *GmSIZ1a* and *GmSIZ1b* exhibit SUMO E3 ligase activity**

(**A**) Expression of *GmSIZ1a* or *GmSIZ1b* suppresses impaired heat shock-induced AtSUMO1 conjugation in *siz1-2*. Fourteen-day-old long-day-grown seedlings were subjected to heat shock treatment (37°C) for 30 min, and the heat shock-induced SUMO conjugates and free SUMO (AtSUMO1/2) were detected with anti-AtSUMO1 antibody. 22 °C indicates the non-stressed condition. Coomassie blue-stained Rubisco large subunit (RbcL) was used as the loading control. (**B**) *GmSIZ1a* and *GmSIZ1b* induce the accumulation of AtSUMO1 conjugates in *Nicotiana benthamiana*. AtSIZ1:GFP, GmSIZ1a:GFP, or GmSIZ1b:GFP was transiently expressed in *N. benthamiana* with or without FLAG:AtSUMO1. Proteins were extracted from the leaves three days after infiltration, and the SUMO1 conjugates and free SUMO (AtSUMO1) were detected with anti-FLAG antibody. Coomassie blue-stained RbcL was used as a loading control.

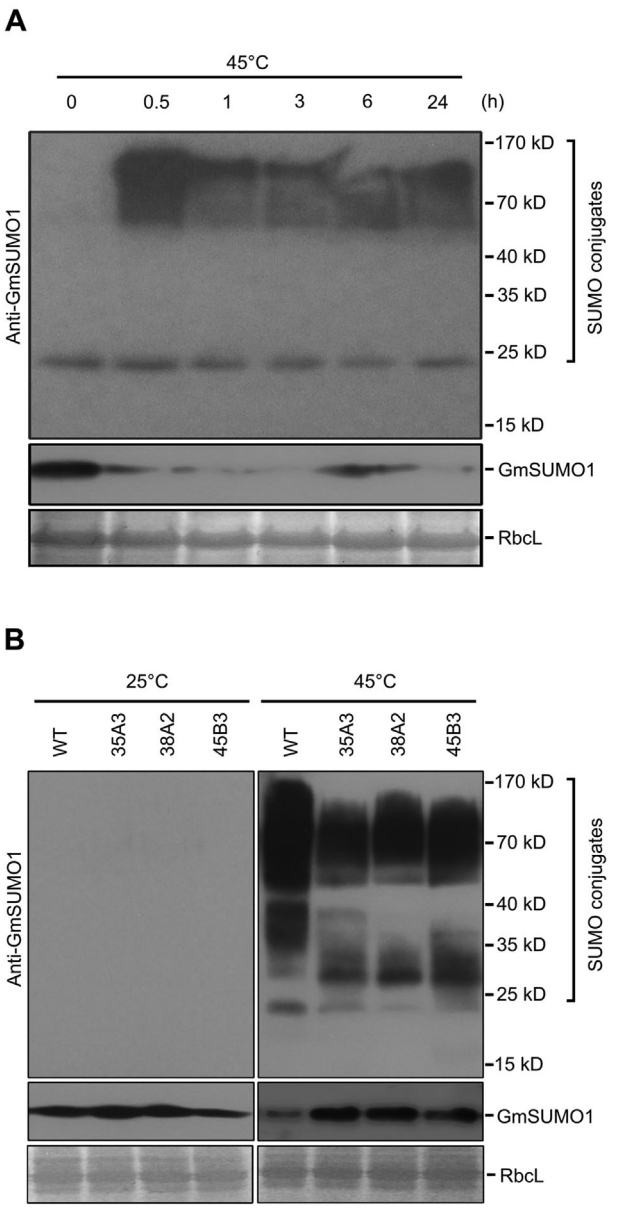


**Figure 5. Downregulation of *GmSIZ1a* and *GmSIZ1b* causes reduced heat shock-induced GmSUMO1 conjugation in soybean**

(**A**) Heat shock-induced GmSUMO1 conjugates in soybean. Fourteen-day-old Zhongdou 32 (wild type) soybean seedlings were treated with heat shock (45 °C) for the indicated periods, and GmSUMO1 conjugates and free SUMO (GmSUMO1) were detected using anti-GmSUMO1 antibody. Coomassie blue-stained RbcL was used as a loading control. (**B**) GmSUMO1 conjugates and free SUMO (GmSUMO1) were detected with anti-GmSUMO1 antibody under normal (25 °C) and heat shock (45 °C for 30 min) conditions. Proteins were extracted from 14-day-old wild-type (WT) soybean seedlings and three independent *GmSIZ1RNAi* lines (35A3, 38A2 and 45B3). RbcL was used as the loading control.

*Nicotiana benthamiana* leaves as described previously (Park et al. 2010). Under non-stress conditions, transient expression of AtSUMO1 alone did not promote SUMO conjugation, but co-expression of *AtSIZ1* with AtSUMO1 greatly induced SUMO conjugation. Similar to *AtSIZ1*, co-expression of *GmSIZ1a* or *GmSIZ1b* with AtSUMO1 strongly promoted SUMO conjugation (Figure 4B). These results indicate that both *GmSIZ1a* and *GmSIZ1b* possess SUMO E3 ligase activity.

### *GmSIZ1a* and *GmSIZ1b* function as SUMO E3 ligases in soybean

To characterize the function of *GmSIZ1a* and *GmSIZ1b* in soybean, we simultaneously downregulated both *GmSIZ1a* and *GmSIZ1b* by RNA interference-mediated gene silencing. We generated the *GmSIZ1RNAi* vector using shared sequences expected to silence both *GmSIZ1a* and *GmSIZ1b* simultaneously (Figures S1D, S3A). Three independent T3 *GmSIZ1RNAi* transgenic lines were obtained in the soybean cultivar Zhongdou 32 (35A3, 38A2 and 45B3) (Figure S3B–D). The expression of both *GmSIZ1a* and *GmSIZ1b* was significantly reduced in the *GmSIZ1RNAi* lines (Figure S3E).

To investigate whether the SUMO E3 ligase activity was reduced in the *GmSIZ1RNAi* plants, we analyzed heat shock-induced SUMO conjugation. We attempted to use commercial anti-SUMO1 antibody (Abcam, ab5316; a rabbit polyclonal antibody that reacts with *Arabidopsis* SUMO1/2) to detect the heat shock-induced SUMO conjugation in Zhongdou 32, but we did not observe substantial levels of SUMO conjugates. The Soybean genome contains three identical SUMO isoforms, GmSUMO1, 2 and 3, which are closely related to AtSUMO2 (Figure S4A, B). A predicted 3D structure comparison suggested that AtSUMO1 and GmSUMO1 have similar structures, but may have different epitopes (Figure S4C). Therefore, we generated anti-GmSUMO1 polyclonal antibody, and monitored SUMO conjugates under heat shock conditions (Figure 5A). Heat shock-induced GmSUMO1 conjugates were substantially increased at 0.5 h after heat shock treatment, but gradually decreased at later time points (Figure 5A). However, anti-GmSUMO1 antibody did not detect SUMO conjugates in *Arabidopsis* (Figure S4D).

In the absence of heat shock treatment, SUMO conjugates were hardly detected in *GmSIZ1RNAi* plants and the level of free SUMO was similar in wild-type and

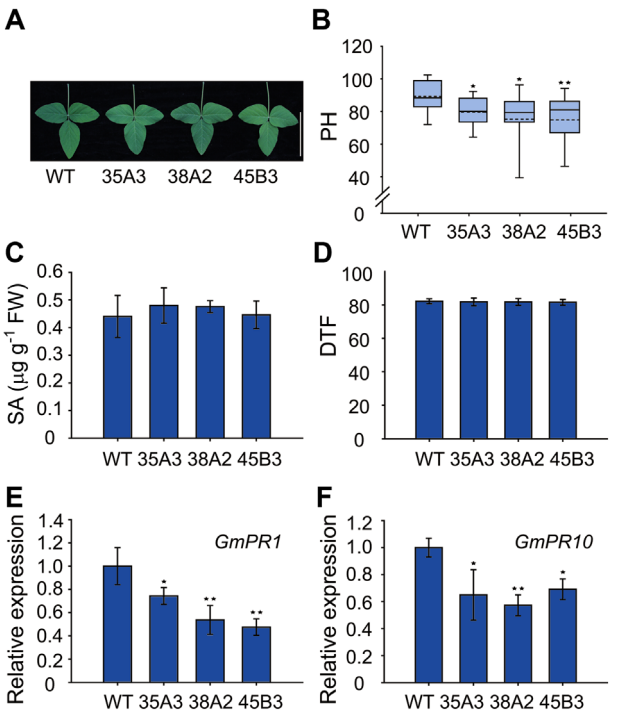


**Figure 6. Downregulation of *GmSIZ1a* and *GmSIZ1b* causes reduced plant height and leaf size, but does not affect salicylic acid (SA) levels or flowering time in soybean**

(**A**) First trifoliate leaf of the wild type and three independent *GmSIZ1RNAi* lines (35A3, 38A2, and 45B3). Bar = 10 cm. (**B**) Plant height (PH) of 5-week-old soybean plants. (**C**) SA levels in 14-day-old wild-type and *GmSIZ1RNAi* plants. Data represent the mean ± *SD*. (**D**) Flowering time (days to flower, DTF) of soybean plants. (**E**) and (**F**) quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *GmPR1* and *GmPR10* in 14-day-old WT and *GmSIZ1RNAi* leaves. Relative expression was normalized to that of *GmUBI3*, and data represent the mean ± *SD*. Twenty soybean plants (Zhongdou 32 cultivars; WT) and each *GmSIZ1RNAi* transgenic line in the Zhongdou 32 background (35A3, 38A2 and 45B3) were grown in a greenhouse, and plant height and flowering time were recorded. (One-way ANOVA test, $^{**}P < 0.01$, $^{*}P < 0.05$).

*GmSIZ1RNAi* plants (Figure 5B, left panel). Under heat-shock conditions, the SUMO conjugates were greatly increased in the wild type, but were increased to a lesser extent in *GmSIZ1RNAi* plants. In agreement with increased levels of SUMO conjugates, the level of free SUMO was lower in wild-type plants than in *GmSIZ1RNAi* plants under heat-shock conditions (Figure 5B). These results indicate that *GmSIZ1a* and *GmSIZ1b* facilitate SUMO conjugation in soybean.

### *GmSIZ1a* and *GmSIZ1b* regulate vegetative growth in soybean

Mutations in *Arabidopsis SIZ1* cause elevated SA levels, which results in dwarfism and early flowering (Lee et al. 2007; Jin et al. 2008). Similar to the *Arabidopsis siz1-2* plants, the *GmSIZ1RNAi* plants showed slightly reduced leaf size and plant height compared to those of the wild type (Figure 6A, B). However, downregulation of *GmSIZ1a/b* did not alter the flowering time (Figure 6C). To test if the *GmSIZ1RNAi* plants accumulated higher levels of SA, as did *Arabidopsis siz1*, we examined the SA levels in wild-type and *GmSIZ1RNAi* leaves under normal conditions (Figure 6D). Surprisingly, wild-type and *GmSIZ1RNAi* plants accumulated similar basal levels of SA. Moreover, in contrast to the constitutive activation of *PR* gene expression observed in *Arabidopsis siz1-2* pants (Lee et al. 2007), *GmPR1* and *GmPR10* expression was not induced in *GmSIZ1RNAi* plants, suggesting that downregulation of *GmSIZ1a* and *GmSIZ1b* expression does not cause a constitutive immune response (Figure 6E, F). Overall, our results indicate that *GmSIZ1a* and *GmSIZ1b* positively regulate vegetative growth.

## DISCUSSION

The functions of SUMO E3 ligases in the regulation of environmental stress responses and developmental processes have been extensively characterized in *Arabidopsis* (Park and Yun 2013). However, it was hitherto unknown whether SUMO E3 ligases have conserved functions in soybean. In this study, we identified two identical SIZ1 homologs in soybean, *GmSIZ1a* and *GmSIZ1b*, which localized to the nucleus (Figure S1A, B). Both proteins exhibited SUMO E3 ligase activity (Figures 3, 4) and promoted GmSUMO1 conjugation in soybean (Figure 5). Interestingly, downregulation of *GmSIZ1a/b* in soybean resulted in plants with reduced height and leaf size (Figure 6A, B). Thus, our study established that the SUMO E3 ligases *GmSIZ1a* and *GmSIZ1b* mediate SUMO modification of nuclear proteins and regulate vegetative growth in soybean.

In *Arabidopsis*, mutation of *AtSIZ1* causes reduced plant stature (Catala et al. 2007). The dwarfism of *siz1* plants is due to defects in cell division and expansion, which are caused by hyper-accumulation of SA in the mutant (Miura et al. 2010). The rice *Ossiz1* loss-of-function mutant also exhibits reduced plant height and shorter leaves, which are also likely attributable to defects in cell proliferation and expansion (Wang et al. 2011). Thus, it is possible that the reduced plant stature of *GmSIZ1RNAi* plants is also due to defects in cell division and expansion. In contrast to the *Arabidopsis siz1* mutant, *GmSIZ1RNAi* plants did not accumulate a higher level of SA than the wild type (Figure 6D; Lee et al. 2007). Moreover, exogenous SA treatment stimulates shoot and root growth in soybean (Rivas-San Vicente and Plasenciaa 2011). These results suggest that *GmSIZ1a* and *GmSIZ1b* regulate vegetative growth through an SA-independent mechanism in soybean. It has been shown that *AtSIZ1* is required for nitrogen assimilation and that application of exogenous ammonium rescues the dwarf phenotype of *siz1-2* plants (Park et al. 2011). By contrast, *Ossiz1* rice mutant plants accumulate higher levels of nitrogen, suggesting that OsSIZ1 negatively regulates nitrogen assimilation in rice (Wang et al. 2015). Whether GmSIZ1a/b affects vegetative growth by regulating nitrogen assimilation remains to be determined.

SUMO modification also regulates flowering time in *Arabidopsis*. Mutations in SUMO E3 ligases (i.e., *SIZ1* and HPY2/*MMS21*) or SUMO proteases (i.e., *ESD4* and *OTS1/2*) cause early flowering in *Arabidopsis* (Murtas et al. 2003; Conti et al. 2008; Jin et al. 2008; Kwak et al. 2016). The early flowering phenotype of *siz1* and *esd4* plants under short-day conditions is mainly due to hyper-accumulation of SA (Jin et al. 2008; Villajuana-Bonequi et al. 2014). In addition to *Arabidopsis*, SA promotes flowering in various plant species, such as *Nicotiana tabacum* (tobacco), members of the Lemnaceae family, and *Helianthus annuus* (sunflower). However, in the short-day species *Pharbitis nil*, exogenous SA application promotes flowering only under nutrient deprivation conditions (Rivas-San Vicente and Plasenciaa 2011). We found that downregulation of *GmSIZ1a/b* did not affect flowering time or SA levels in soybean (Figure 6). Thus, we speculate that *GmSIZ1a/b* modifies the nuclear localization of SUMO substrates that are not involved in the regulation of flowering time and SA accumulation. However, we cannot exclude the possibility that complete loss-of-function mutations of *GmSIZ1a/b* would alter flowering time and SA accumulation in soybean. Furthermore, *GmSIZ1a/b* may function redundantly with other SUMO E3 ligases to regulate flowering time and SA accumulation in soybean.

As plants are sessile organisms and are often exposed to abiotic stresses and pathogen attacks, they have evolved multi-layered defense systems. SUMOylation plays a pivotal role in the regulation of abiotic stress responses (i.e., ABA signaling and salt, cold, heat and drought stress responses) and immune responses (Yoo et al. 2006; Catala et al. 2007; Lee et al. 2007; Miura et al. 2007, 2009; Conti et al. 2008; Zheng et al. 2012; Zhang et al. 2013). *GmSIZ1a* and *GmSIZ1b* expression was induced by SA, heat, and dehydration treatments (Figure 2A–C). Moreover, *GmSIZ1a* and *GmSIZ1b* were required for heat shock-induced SUMO conjugation in soybean (Figure 5B). Downregulation of *GmSIZ1a* and *GmSIZ1b* did not cause constitutively elevated SA levels and *PR* gene expression in the absence of pathogen infection (Figure 6D–F). However, we cannot exclude the possibility that mutations in *GmSIZ1a* and *GmSIZ1b* cause higher levels of SA and *PR* gene expression under pathogen infection conditions than those observed in the wild type. Similar to SIZ1 in *Arabidopsis*, GmSIZ1a/b may also regulate heat, drought, and biotic stress responses in soybean. Although cold, ABA, and NaCl treatments did not affect the expression level of *GmSIZ1a* and *GmSIZ1b*, we cannot exclude the possibility that *GmSIZ1a/b* also regulate cold, ABA, and salt stress responses (Figure 2D–F), since SUMO modification regulates these stress responses at the post-translational level (Miura et al. 2007, 2009; Conti et al. 2014). Given that SUMO modification is a key regulatory mechanism in biotic/abiotic stress responses in *Arabidopsis*, it would be of great interest to determine if *GmSIZ1a/b* regulate environmental stress responses in soybean.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Soybean cultivar *Glycine max* L. Merr. cv. Zhongdou 32 was used in this study. Soybean seeds were germinated on filter paper moistened with deionized water for 1 d and then transferred to soil for further growth in the greenhouse (in the spring, under natural light plus incandescent light, at 25 °C to 30 °C) or in a plant growth room (16 h light (100 $\mu$mol m$^{-2}$ s$^{-1}$)/8 h darkness, at 25 °C). The *Arabidopsis* wild type and *siz1-2* (Salk_065397) plants used in this work were in the Columbia (Col-0) background. The *Arabidopsis* plants were grown in a growth room under long-day conditions (16 h light (100 $\mu$mol m$^{-2}$ s$^{-1}$ fluorescent lights)/8 h darkness) at 22 °C.

### Bioinformatics analysis

Amino acid sequences of plant SIZ1 homologs were collected from the National Center for Biotechnology Information (NCBI) and Phytozome databases. ClustalW2 software was used to align these sequences, and the percent identity was determined and the divergence matrices were constructed using DNASTAR software (DNAStar Inc., Madison, WI, USA). The phylogenetic tree was constructed using the neighbor-joining algorithm as instructed in MEGA 5.1 (Kumar et al. 2008). The homology model of the three-dimensional (3D) structure was constructed using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and the figures were generated using PyMOL (http://pymol.org/).

### RNA isolation and real-time reverse transcription-PCR

RNA was isolated and qRT-PCR was performed as described previously (Zhou et al. 2013). Briefly, total RNAs were isolated from *Arabidopsis* or soybean seedlings using TRIZOL reagents (RNAiso Plus, Code D9108B; TaKaRa). qRT-PCR analyses were performed on an MX3000P QPCR system. *Arabidopsis UBIQUITIN-CONJUGATING ENZYME* (*UBC*) or soybean *UBIQUITIN-LIKE PROTEIN3* (*GmUBI3*) was used as the internal control. The primers used for qRT-PCR are listed in Table S2.

### Plasmid construction

To generate p326-GmSIZ1a/b:GFP, full-length *GmSIZ1a* or *GmSIZ1b* cDNA without the termination codon was amplified using the gene-specific primers GmSIZ1a/b-F-*BamH*I and GmSIZ1a/b-R-*BamH*I. The resulting products were inserted in frame at the *BamH*I site of the p326-GFP vector (Zhou et al. 2013).

To generate pCambia1302-ProAtSIZ1:GmSIZ1a/b:GFP, full-length *GmSIZ1a* or *GmSIZ1b* cDNA without the termination codon was amplified using the gene-specific primers GmSIZ1a/b-F-*Hind*III and GmSIZ1a/b-R-*Nco*I, and ligated into the pCambia1302 vector. pCambia1302-GmSIZ1a/b:GFP plasmids were digested with *Xma*I, and the *AtSIZ1* promoter (Jin et al. 2008) was inserted into the *Xma*I site of pCambia1302-GmSIZ1a/b:GFP.

To generate pCambia3300-GmSIZ1a/b:GFP, 326-GmSIZ1a/b:GFP plasmids were digested with *BamH*I and the insert was ligated into the *BamH*I site of the pCambia3300-GFP vector.

To generate pCambia3300-GmSIZ1RNAi, pFGC1008 was digested with *Sac*I/*Pme*I, and the insert was ligated into the *Sac*I/*Pme*I sites of pCambia3300. A *GmSIZ1a* fragment was amplified with the GmSIZ1RNAi-F and GmSIZ1RNAi-R primers, and ligated into the *Asc*I/*Swa*I and *BamH*I/*Spe*I sites of the pCambia3300-RNAi vector.

To generate pET30a-His:GmSUMO1, *GmSUMO1* cDNA was amplified with the gene-specific primers SUMO-F-*BamH*I and SUMO-R-*Xho*I, and inserted into the *BamH*I and *Xho*I sites of the pET30a vector. All primer sequences are listed in Table S2.

### Subcellular localization

Plasmids were transformed into *Arabidopsis* protoplasts using polyethylene glycol (PEG)-mediated DNA transfection (Jin et al. 2001). The fluorescence images were captured using a fluorescence microscope (Olympus BX53).

### Antibody preparation

pET30a-His:GmSUMO1 plasmid was transformed into the *Escherichia coli* BL21 (DE3) strain, and the expressed His-GmSUMO1 fusion protein was purified with Ni Sepharose 6 Fast Flow resin (GE Healthcare). The purified full-length soybean SUMO1 protein was used to generate anti-GmSUMO1 rabbit polyclonal antibody.

### Analysis of SUMO conjugation

Total proteins were extracted from *Arabidopsis*, *N. benthamiana* or soybean leaves using protein extraction buffer containing 150 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5% (w/v) sodium dodecyl sulfate (SDS), 0.5% (v/v) NP40, 6 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 30% (v/v) glycerol. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and detected with anti-AtSUMO1 (Abcam, ab5316), anti-FLAG (Sigma, F3165), or anti-GmSUMO1 antibody.

### Generation of transgenic plants

The pCambia3300-GmSIZ1RNAi plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method. Soybean transformation was carried out using the *Agrobacterium*-mediated cotyledonary-node method as described previously (Paz et al. 2004). Expression and integration of the *bar* marker gene in the *GmSIZ1RNAi* lines were confirmed based on leaf Glufosinate resistance, using *bar* gene detection strips (QuickStix for LibertyLink/bar, Envirologix, USA) and genomic DNA PCR. pCambia1302-ProAtSIZ1:GmSUMO1a/b:GFP were introduced into *A. tumefaciens* strain GV3101, and transformed into *Arabidopsis siz1-2* using the floral-dip method as previously described (Clough and Bent 1998).

### SA measurement

Salicylic acid was extracted from 14-day-old long-day-grown soybean seedlings and quantified using a LECO Pegasus IV gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) system as previously described (Duan et al. 2012).

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## AUTHOR CONTRIBUTIONS

B.C. performed most of the research. X.K. generated the transgenic *Arabidopsis* plants and C.Z., S.S., Z.Z., Y.W. and X.L. performed some of the phenotypic analyses and designed the experiments. X.F.Z. and Y.H.J cloned the genes and constructed the plasmids. J.B.J. designed the research, supervised the study, and wrote the manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12504/suppinfo

**Figure S1**. Amino acid sequence analysis of soybean *GmSIZ1a* and *GmSIZ1b*, and their gene structures

(**A**) Amino acid sequence alignment of *GmSIZ1a*, *GmSIZ1b*,and *AtSIZ1*. Identical and similar amino acid residues are highlighted with black and gray boxes, respectively. (**B**) Percentage of identity and divergence among *GmSIZ1a*, *GmSIZ1b*, and *AtSIZ1* amino acid sequences. (**C**) Phylogenetic tree of SIZ1 homologs in various plant species. A neighbor-joining tree was constructed based on an alignment of the complete protein sequences. The numbers on the side of each branch indicate bootstrap values from 1,000 replicates. The scale bar indicates the substitution rate per site. (**D**) Schematic representation of the *GmSIZ1a*, *GmSIZ1b*, and *AtSIZ1* gene structures. The blue boxes represent exons and the green lines represent introns. The gray boxes represent UTR regions. PF1/PR1 and PF2/PR2 primer pairs were used for qRT-PCRanalysis of *GmSIZ1a* and

*GmSIZ1b* expression, respectively. Black line indicates the region used to generate *GmSIZ1RNAi* constructs.

**Figure S2.** Expression of *GmSIZ1a* or *GmSIZ1b* rescues the dwarf phenotype of *Arabidopsis siz1-2*

(**A**) Presence of the *siz1-2* mutation was analyzed in complementation lines. *GmSIZ1a* (#5-1, #8-2, and #10-1) and *GmSIZ1b* (#3-7, #10-3, and #15-1) indicate the expression of GmSIZ1a:GFP or GmSIZ1b:GFP driven by the *Arabidopsis SIZ1* promoter in *siz1-2*, respectively. Vector indicates empty vector transformed into *siz1-2*. Primer pair LB_a1 (LB)/RP was used to determine the presence of a T-DNA insertion in the *siz1* genetic background, and primer pair LP/RP was used to confirm homozygosity. (**B**) qRT-PCR analysis of transgene expression in Col-0, *siz1-2*, the vector control, and *GmSIZ1a/b* transgenic plants.Relative expression was normalized to that of *AtUBC*, and data represent the mean ±*SD*, n=3. (**C**) Leaf length and rosette diameter of five-week-old long-day-grown plants. The lengths of leaves from 5 plants were measured.

**Figure S3.** Generation of *GmSIZ1RNAi* soybean transgenic plants

(**A**) Schematic representation of the *GmSIZ1RNAi* construct and nucleotide sequence alignment of the *GmSIZ1a/b* cDNA fragment, which was used to generate the *GmSIZ1RNAi* construct. (**B**) Glufosinate resistance of *GmSIZ1RNAi* (35A3, 38A2 and 45B3) leaves. The non-transgenic soybean cultivar Zhoungdou32 (WT) was used as a negative control. Dashed white box indicates Glufosinate (200 $gL^{-1}$ glufosinate ammonium, BAYER, Germany) painted leaf area. (**C**) A *bar* gene strip (QuickStix for LibertyLink/*bar*, Envirologix USA) was used to detect the presence of the *bar* gene in the transgenic plants. Upper and lower arrowheads indicate the control and positive line, respectively. (**D**) PCR analysis confirmed the presence of the *bar* gene in the transgenic plants.The Bar-F and Bar-R primers were used for the PCR analysis. (**E**) qRT-PCR analysis of *GmSIZ1a* and *GmSIZ1b* levels in WT and *GmSIZ1RNAi* soybean transgenic lines. Relative expression was normalized to that of *GmUBI3*, and data represent the mean ± *SD*, n = 3. The numbers above the columns indicate the relative transcription level of *GmSIZ1a* or *GmSIZ1b* in the transgenic lines compared to in the wild type.

**Figure S4.** Amino acid sequence analysis and 3D structures of SUMO homologs

(**A**) Amino acid sequence alignment of SUMO homologs in *Arabidopsis*, soybean, and rice. Identical and similar amino acid residues are indicated with black and gray boxes, respectively. (**B**) Phylogenetic analysis of SUMO homologs in *Arabidopsis*, soybean, and rice. The neighbor-joining tree was constructed based on an alignment of the complete protein sequences. The numbers on the side of each branch indicate bootstrap values from 1,000 replicates, and the scale bar indicates the substitution rate per site. (**C**) Three-dimensional structure modeling of AtSUMO1 and GmSUMO1. (**D**) Detection of heat shock-induced SUMO conjugates and free SUMO in soybean and *Arabidopsis* using anti-GmSUMO1 antibody. Coomassie blue-stained Rubisco large subunit (RbcL) was used as the loading control.

**Table S1.** Putative cis-acting elements in the *GmSIZ1a* and *GmSIZ1b* promoters

**Table S2.** Primers used in this study