SUMO E3 Ligases GmSIZ1a and GmSIZ1b regulate vegetative growth in soybean

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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.

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.
Functional characterization of soybean SUMO E3 ligase

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 (Sadana 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,
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
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 a 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

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 plants (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, down-regulation 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). 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 Plasencia 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 OTSi/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 Plasencia 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. 2006; Catala et al. 2007; Lee et al. 2007; Miura et al. 2007, 2009; Conti et al. 2014). 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 µmol m⁻² s⁻¹) fluorescent lights)/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 µmol 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-BamHI and GmSIZ1a/b-R-BamHI. The resulting products were inserted in frame at the BamHI 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-HindIII and GmSIZ1a/b-R-NcoI, and ligated into the pCambia1302 vector. pCambia1302-GmSIZ1a/b:GFP plasmids were digested with Xmal, and the AtSIZ1 promoter (Jin et al. 2008) was inserted into the Xmal site of pCambia1302-GmSIZ1a/b:GFP.
To generate pCambia3300-GmSIZ1a/b:GFP, 326-GmSIZ1a/b:GFP plasmids were digested with BamHI and the insert was ligated into the BamHI site of the pCambia3300-GFP vector.

To generate pCambia3300-GmSIZ1RNAi, pFGC1008 was digested with SacI/Pmel, and the insert was ligated into the SacI/Pmel sites of pCambia3300. A GmSIZ1a fragment was amplified with the GmSIZ1RNAi-F and GmSIZ1RNAi-R primers, and ligated into the AscI/SwaI and BamHI/SpeI sites of the pCambia3300-RNAi vector.

To generate pET30a-His:GmSUMO1, GmSUMO1 cDNA was amplified with the gene-specific primers SUMO-F-BamHI and SUMO-R-XhoI, and inserted into the BamHI and XhoI 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 BX55).

**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, Envirowogix, USA) and genomic DNA PCR. pCambia3102-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

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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-PCR analysis 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. 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 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 g L⁻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 RbcL 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