# Specific Domain Structures Control Abscisic Acid-, Salicylic Acid-, and Stress-Mediated SIZ1 Phenotypes[1][W]

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SIZ1 (for yeast SAP and MIZ1) encodes the sole ortholog of mammalian PIAS (for protein inhibitor of activated STAT) and yeast SIZ SUMO (for small ubiquitin-related modifier) E3 ligases in Arabidopsis (*Arabidopsis thaliana*). Four conserved motifs in SIZ1 include SAP (for scaffold attachment factor A/B/acinus/PIAS domain), PINIT (for proline-isoleucine-asparagine-isoleucine-threonine), SP-RING (for SIZ/PIAS-RING), and SXS (for serine-X-serine, where X is any amino acid) motifs. SIZ1 contains, in addition, a PHD (for plant homeodomain) typical of plant PIAS proteins. We determined phenotypes of *siz1-2* knockout mutants transformed with *SIZ1* alleles carrying point mutations in the predicted domains. Domain SP-RING is required for SUMO conjugation activity and nuclear localization of SIZ1. Salicylic acid (SA) accumulation and SA-dependent phenotypes of *siz1-2*, such as diminished plant size, heightened innate immunity, and abscisic acid inhibition of cotyledon greening, as well as SA-independent basal thermotolerance were not complemented by the altered SP-RING allele of *SIZ1*. The SXS domain also controlled SA accumulation and was involved in greening and expansion of cotyledons of seedlings germinated in the presence of abscisic acid. Mutations of the PHD zinc finger domain and the PINIT motif affected in vivo SUMOylation. Expression of the PHD and/or PINIT domain mutant alleles of *SIZ1* in *siz1-2* promoted hypocotyl elongation in response to sugar and light. The various domains of SIZ1 make unique contributions to the plant's ability to cope with its environment.

Posttranslational protein modifications regulate the function of proteins, affecting activity, stability, interaction type, and interaction strength with partners as well as subcellular localization. The known protein modifications that elicit such changes are phosphorylation, prenylation, myristoylation, methylation, ubiquitination, and SUMO (for small ubiquitin-related modifier) conjugation. Although ubiquitination and SUMOylation of target proteins occur by similar mechanisms, both have vastly different functions. Ubiquitination is used to label proteins destined for degradation, whereas SUMOylation is a transient and reversible process that elicits long-term metabolic and developmental effects that are largely antagonistic to ubiquitination (Hay, 2005). In animals and yeast, SUMOylation has been extensively studied. SUMO modifications control cell cycle progression, DNA repair, and subcellular localization and are involved in transcriptional regulation (Matunis et al., 1996; Johnson and Blobel, 1999; Hardeland et al., 2002; Gill, 2005; Geiss-Friedlander and Melchior, 2007; Martin et al., 2007). Impairing SUMO modification capability results in pathological conditions such as neurodegenerative diseases and cancer in humans and loss of viability in yeast (Li and Hochstrasser, 2003; Dorval and Fraser, 2007; Kim and Baek, 2009). In plants, perturbation of the SUMO modification capability is reported to affect abscisic acid (ABA) responses, flowering time, phosphate starvation responses, cold tolerance, basal thermotolerance, and salicylic acid (SA)-dependent defense responses (Lois et al., 2003; Novatchkova et al., 2004; Miura et al., 2005, 2007a, 2007b, 2009; Catala et al., 2007; Jin et al., 2008).

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The process of SUMOylation/deSUMOylation is conserved across species (Miura et al., 2007a). SUMOylation is mechanistically related to ubiquitination. SUMO proteins derive from precursor proteins that are cleaved by a SUMO-specific peptidase/isopeptidase to expose a conserved C-terminal di-Gly (GG) motif. SUMOylation begins with a SUMO-activating enzyme (E1), which catalyzes ATP-dependent adenylation of the SUMO C terminus. Subsequent transfer of the SUMO to an internal Cys residue forms a high-energy thioester bond. The activated SUMO is then transferred to the conjugating enzyme (E2), resulting in the formation of a thioester between the C-terminal Gly of SUMO and a Cys residue on the E2 protein. The conjugating enzyme (E2), in cooperation with SUMO E3 ligase (E3), then catalyzes the transfer and ligation of SUMO to the target protein. SUMO forms an isopeptide bond with the ε-amino group of the target protein at a conserved ΨKXE/D sequence (where Ψ is a large hydrophobic amino acid, K is Lys, X is any amino acid, and E/D are Glu/Asp). Deconjugation of SUMO is catalyzed by a SUMO-specific peptidase/isopeptidase (Hay, 2005; Kerscher et al., 2006). At the molecular level, SUMOylation affects protein-protein interactions by masking interfaces or inducing conformational changes on the target protein (Geiss-Friedlander and Melchior, 2007). The conjugated SUMO can itself create novel interactions. A large number of SUMO targets have been discovered, and SUMOylated proteins are found in most cellular compartments (Geiss-Friedlander and Melchior, 2007). Thus, selection of an improper SUMO isoform, mutations affecting the function of individual SUMO-conjugating and -deconjugating enzymes, or mislocalization of any these components of the SUMOylation machinery can have profound effects.

Several types of SUMO E3 ligases have been identified across species. They are named for vertebrate-specific nuclear pore protein RanBP2 (for RanGAP1-binding protein 2; NUP358), human Polycomb group member Pc2 (for Polycomb group 2), class II histone deacetylases, and proteins containing an SP-RING (for SIZ/PIAS-RING) domain. The largest group of SUMO E3 ligases is characterized by the essential SP-RING domain. This group can be subdivided into two distinct subgroups: one is the NES2/MMS21 (for non-SMC element 2/methyl methanesulfonate sensitive 1) group, which contains only the SP-RING domain, and the other is the SIZ/PIAS (for yeast SAP and MIZ/protein inhibitor of activated STAT) group, which shares a conserved N-terminal domain in addition to the SP-RING. The only PIAS-type SUMO E3 ligase identified in Arabidopsis (*Arabidopsis thaliana*) is SIZ1 (At4g60410; Miura et al., 2005, 2007a). Orthologs of the NSE2/MMS1-type SUMO E3 ligases are also predicted to occur in Arabidopsis based on sequence annotation, and one member, HIGH PLOIDY2/AtMMS21 (At3g15150), has been characterized (Miura et al., 2007a; Huang et al., 2009; Ishida et al., 2009).

All PIAS proteins share high sequence identity (Hay, 2005; Miura et al., 2007a; Palvimo, 2007). They are characterized by four structural motifs: an N-terminal SAP (for scaffold attachment factor A/B/acinus/PIAS) motif, a PINIT (for Pro-Ile-Asn-Ile-Thr) motif, an SP-RING zinc finger domain, and a SUMO-interacting motif (Sharrocks, 2006; Miura et al., 2007a; Palvimo 2007). SIZ1 and PIAS proteins of other plants also contain a special zinc finger domain called PHD (for plant homeodomain; Miura et al., 2007a; Garcia-Dominguez et al., 2008). The SP-RING domain is required for SUMO E3 ligase activity of several PIAS proteins (Johnson and Gupta, 2001; Takahashi and Kikuchi, 2005; Reindle et al., 2006; Garcia-Dominguez et al., 2008). PHD domains of non-PIAS proteins have ubiquitin E3 ligase or SUMO E3 ligase activity (Lu et al., 2002; Coscoy and Ganem, 2003; Ivanov et al., 2007). The PHD domain of SIZ1 functions as a SUMO E3 ligase for selected targets such as bromodomain-containing proteins (Garcia-Dominguez et al., 2008). The PINIT motif of *Saccharomyces cerevisiae* SIZ1 appears to cooperate with the SP-RING domain to form a module with E3 ligase activity toward selected targets (Reindle et al., 2006). The SP-RING domain, SAP domain, and PINIT domains are all known to regulate subcellular localization of ScSIZ1 (Takahashi and Kikuchi, 2005; Reindle et al., 2006). The SAP domain binds nucleic acids and is predicted to be involved in chromatin biology, chromatin-nuclear matrix attachment, and transcriptional regulation (Liu et al., 2001; Gross et al., 2004; Okubo et al., 2004; Garcia-Dominguez et al., 2008; Suzuki et al., 2009). SUMO-interacting motifs contribute to SUMO selection or selection of downstream effectors of the target (Minty et al., 2000; Geiss-Friedlander and Melchior, 2007).

At least five mammalian and two *S. cerevisiae* PIAS family SUMO E3 ligases have so far been identified (Hay, 2005; Geiss-Friedlander and Melchior, 2007). Because of functional redundancy, it is not possible to assign an in vivo function to the various domains on mammalian and yeast PIAS family SUMO E3 ligases. In Arabidopsis, however, the sole PIAS protein that is known is SIZ1. Thus, SIZ1 affords a unique opportunity to study whether the four domains can be associated with defined specific phenotypes in a mutational analysis. Our study revealed several stress-responsive phenotypes that were associated with the SP-RING domain of SIZ1. Mutations in the PHD or PINIT domain perturbed sugar and light sensing, and an SXS (Ser-X-Ser; where X is any amino acid) domain mutation affected ABA sensitivity of the cotyledons.

## RESULTS

### Properties of SIZ1 Affected by SP-RING, PHD, and PINIT Domain Mutations

In schematic form, Figure 1A depicts the conserved structural motifs of SIZ1 as they have been identified in PIAS proteins. The N-terminal DNA-binding SAP

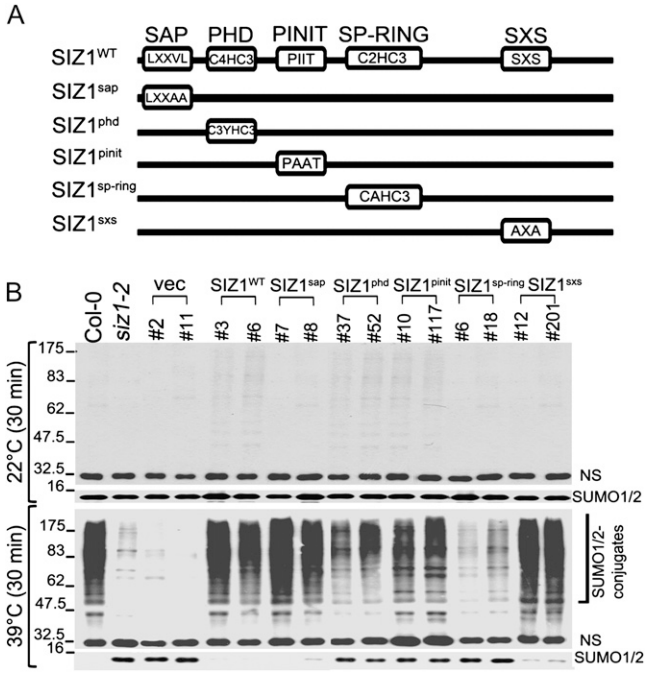


**Figure 1.** Point mutations of SIZ1 and their effects on the pattern of heat shock-induced SUMO conjugation. A, Schematic representation of SIZ1 ($SIZ1^{WT}$) and derived mutants. The location of SIZ1 conserved domains is indicated on $SIZ1^{WT}$, and critical amino acid sequences within these domains are shown within the boxes. The *siz1* mutants are named for their altered domain, and the nature and location of the amino acid substitutions in these mutants are indicated within the respective boxes. B, Immunoblot analysis of the effect of the mutations on SUMO1/2 conjugation activity in vivo. Total proteins were extracted from unstressed (22°C, 30 min) or heat-shocked (39°C, 30 min) 10-d-old seedlings of the wild type (Col-0), *siz1-2* mutant (*siz1-2*), *siz1-2* mutant transformed with empty vector (vec), or *siz1-2* transformant expressing the indicated cDNAs from the *SIZ1* promoter. Two independent lines of each transformant were analyzed. Shown are immunoblots of SDS-PAGE gels of these protein extracts (10 $\mu$g per lane) probed with anti-SUMO1, which detects both SUMO1 and SUMO2. NS, Nonspecific signal.

domain of PIAS proteins consists of a conserved 35-amino acid stretch carrying an LXXΨL (where X is any amino acid and Ψ is a hydrophobic amino acid) signature that has been shown to be important in trans-repression activity and the assembly of nuclear receptor coactivator complexes. The corresponding LXXVL sequence in SIZ1 was mutated to LXXAA by simultaneously introducing V22A and L23A mutations, yielding the $SIZ1^{sap}$ allele (Liu et al., 2001; Gross et al., 2004). The PHD domain, a hallmark of plant PIAS proteins (Miura et al., 2005, 2007a), has been shown to function in SUMO conjugation by deletion analysis (Garcia-Dominguez et al., 2008). It consists of a C4HC3-type zinc finger motif that was changed to C3YHC3 by the C134Y mutation, to yield the $SIZ1^{phd}$ allele. The PINIT motif of some mammalian PIAS proteins regulates their subcellular localization and affects substrate selectivity (Duval et al., 2003; Wong et al., 2004). PIIT (for Pro-Ile-Ile-Thr) is the modified form of the PINIT motif that occurs in SIZ1. Simultaneous introduction of I274A and I275A mutations in SIZ1 converted this motif to PAAT (for Pro-Ala-Ala-Thr), to yield the $SIZ1^{pinit}$ allele. The SP-RING domain of PIAS proteins from all sources functions as an E3 ligase for the SUMO modification pathway (Sachdev et al., 2001; Kotaja et al., 2002; Takahashi and Kikuchi, 2005; Garcia-Dominguez et al., 2008). It is characterized by a C2HC3 zinc finger, and alterations of any Cys in this motif abrogate substrate recognition, E2 recognition, and ligase activity of yeast SIZ1 (Takahashi and Kikuchi, 2005). A C379A mutation was created in SIZ1 to alter the C2HC3 zinc finger to CAHC3, yielding the $SIZ1^{sp\text{-}ring}$ allele. The SXS motif is located in a region bearing homology to a SUMO-interacting domain of mammalian PIASx$\alpha$. This domain was established by deletion analysis as important for noncovalent interaction of mammalian PIASx$\alpha$ with SUMO1 (Minty et al., 2000). The $SIZ1^{sxs}$ allele was created by simultaneously introducing S604A and S606A mutations in order to convert the SXS motif to AXA (for Ala-X-Ala).

Several homozygous lines of *siz1-2* transformants expressing GFP fusions of wild-type *SIZ1* ($SIZ1^{WT}$) or the altered *SIZ1* alleles were generated (Fig. 1A) to elucidate biochemical and physiological functions of the targeted domains. Even though we attempted immunoblotting with anti-GFP and anti-$His_6$ based on SIZ1 constructs (see "Materials and Methods") to investigate SIZ1 protein abundance, we failed to detect SIZ1 proteins, owing to the fact that expression of the protein from its native promoter is low. Alternatively, transgenic plants expressing $SIZ1^{mutant}$ transcript at levels approximately equal to the *SIZ1* transcript level of wild-type ecotype Columbia-0 (Col-0) were chosen for further analysis (Supplemental Fig. S1). Even though we could not detect the protein in leaf extracts by immunoblotting, SIZ1 protein was expressed in vivo in all transformants. This is based on the detection by fluorescence microscopy of all transiently expressed $SIZ1^{mutant}$-GFP fusion proteins in protoplasts (Fig. 2; data not shown).

The effect of each mutation on SUMO conjugation activity was tested by an immunoblot analysis of crude protein extracts of heat-shocked seedlings with an anti-SUMO1 antiserum that detects both SUMO1 and SUMO2 (Fig. 1B; Kurepa et al., 2003). As expected, heat shock increased the concentration of SUMO-conjugated proteins (proteins of size greater than 50 kD) and reduced the amount of free SUMO1/2 (10 kD) in wild-type Col-0 seedlings (positive control) but not in the *siz1-2* seedlings (negative control; Fig. 1B; Yoo et al., 2006). Expression of $SIZ1^{WT}$, $SIZ1^{sap}$, and $SIZ1^{sxs}$ in the *siz1-2* background restored heat-induced SUMO conjugation to the level found in Col-0 seedlings (Fig. 1B). Expression of $SIZ1^{phd}$ and $SIZ1^{pinit}$ in the *siz1-2* background partly restored SUMO conjugation levels, whereas expression of $SIZ1^{sp\text{-}ring}$ or an empty vector in the *siz1-2* background failed to restore significant SUMO conjugation capability in heat-shocked seed-

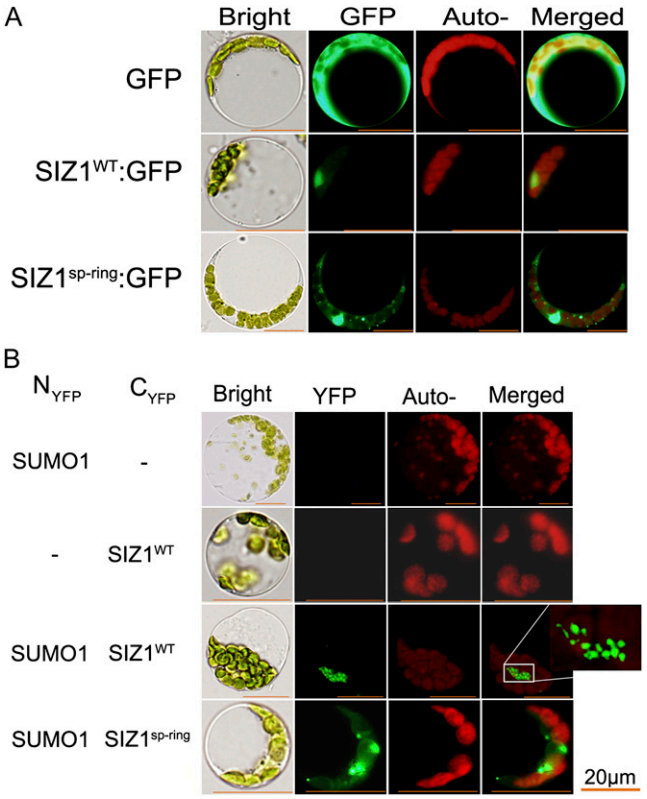


**Figure 2.** Transient expression analysis of SP-RING domain function in leaf mesophyll cell protoplasts. A, Mutation of the SP-RING domain disrupts nuclear localization of SIZ1. GFP, $SIZ1^{WT}$:GFP, and $SIZ1^{sp\text{-}ring}$:GFP fusions were transiently expressed in Col-0 protoplasts from the constitutive CaMV 35S promoter. The GFP signals were examined 16 h after transformation. Chlorophyll autofluorescence (Auto-) was used to identify chloroplasts. B, Mutation of the SP-RING domain does not disrupt interaction with SUMO. $Prom_{CsV}$::$N_{YFP}$:*SUMO1*, $Prom_{CaMV35S}$::$C_{YFP}$, $Prom_{CsV}$::$N_{YFP}$, $Prom_{CaMV35S}$::$SIZ1^{WT}$:$C_{YFP}$, and $Prom_{CaMV35S}$::$SIZ1^{sp\text{-}ring}$:$C_{YFP}$ constructs were introduced into Col-0 protoplasts in the indicated combinations, and YFP fluorescence signals, indicative of positive interaction, were examined 16 h after transformation. The YFP signal obtained in the interaction of SUMO with $SIZ1^{WT}$ is magnified to reveal the punctate nature within the nucleus. Chlorophyll autofluorescence was used to identify chloroplasts. Bar = 20.0 $\mu$m.

lings. Thus, the SP-RING domain is of importance for heat shock-induced SUMO conjugation in Arabidopsis seedlings, while the PHD and PINIT domains are required for full SUMO conjugation activity.

Three domains, SP-RING, SAP, and PINIT, control nuclear targeting of PIAS proteins (Duval et al., 2003; Reindle et al., 2006). GFP-fused $SIZ1^{WT}$, $SIZ1^{sap}$, $SIZ1^{phd}$, $SIZ1^{pinit}$, $SIZ1^{sp\text{-}ring}$, and $SIZ1^{sxs}$ proteins were transiently expressed in protoplasts obtained from leaf mesophyll cells of the Col-0 line, and the GFP signals were detected by florescence microscopy as described (Jin et al., 2001). Upon transformation with native GFP, a diffuse fluorescence signal was detected (Fig. 2A). Compartmentalized GFP signals were observed upon expression of $SIZ1^{WT}$ (Fig. 2A), $SIZ1^{sap}$, $SIZ1^{phd}$, $SIZ1^{pinit}$, and $SIZ1^{sxs}$ fusion proteins (data not shown). Similar localization data were obtained using the same GFP-fused $SIZ1^{WT}$ construct (Miura et al., 2005), suggesting that the GFP fusion proteins of $SIZ1^{WT}$, $SIZ1^{sap}$, $SIZ1^{phd}$, $SIZ1^{pinit}$, and $SIZ1^{sxs}$ are nucleus localized. Transformation with the $SIZ1^{sp\text{-}ring}$:GFP fusion construct yielded signals both within and outside the nucleus (Fig. 2A), suggesting that the SP-RING domain of SIZ1 functions in nuclear localization. A similar distribution was observed for an SP-RING mutant of PIAS3L (Duval et al., 2003).

To test whether the SP-RING domain mutation affected interaction with SUMO, bimolecular fluorescence complementation was carried out in leaf mesophyll protoplasts obtained from the Col-0 line (Fig. 2B). As expected, no yellow fluorescent protein (YFP) signal was observed in protoplasts transformed with $Prom_{CsV}$::$N_{YFP}$:*SUMO1* or $Prom_{CaMV35S}$::$SIZ1^{WT}$:$C_{YFP}$ alone. Fluorescence signals indicating interaction were observed in protoplasts coexpressing $Prom_{CsV}$::$N_{YFP}$:*SUMO1* and $Prom_{CaMV35S}$::$SIZ1^{WT}$:$C_{YFP}$. The signal was compartmentalized as shown by the nuclear localization of $SIZ1^{WT}$ (Fig. 2A; Miura et al., 2005). $SIZ1^{sp\text{-}ring}$:$C_{YFP}$ was expected to localize in nuclei and in the cytosol (Fig. 2A). Indeed, fluorescence signals were observed both in nucleus and cytosol of protoplasts coexpressing $Prom_{CsV}$::$N_{YFP}$:*SUMO1* and $Prom_{CaMV35S}$::$SIZ1^{sp\text{-}ring}$:$C_{YFP}$ and show that the C379A mutation in the SP-RING domain did not affect the interaction of SIZ1 with SUMO1 in either compartment. The SP-RING domain is known as a requirement for localization of mammalian PIASy to nuclear bodies (Jackson, 2001). The punctate nature of the signal observed in protoplasts cotransformed with $Prom_{CsV}$::$N_{YFP}$:*SUMO1* and $Prom_{CaMV35S}$::$SIZ1^{WT}$:$C_{YFP}$ but not in protoplasts coexpressing $Prom_{CsV}$::$N_{YFP}$:*SUMO1* and $Prom_{CaMV35S}$::$SIZ1^{sp\text{-}ring}$:$C_{YFP}$ suggests that SIZ1 localizes to discrete subnuclear structures by a process requiring the SP-RING domain.

The SXS motif interacted strongly with SUMO in a yeast two-hybrid assay (Minty et al., 2000). This motif is typically flanked by a hydrophobic core on one side and acidic amino acids on the other. The two-hybrid assay established that the two Ser residues in the SXS motif and the juxtaposed hydrophobic core were essential for the interaction of a mammalian PIAS protein with SUMO. SUMO1ΔGG lacks the Gly residues that form a covalent bond with the Lys residue in the SUMOylation motif of target proteins and therefore participates only in noncovalent interactions between SUMO and PIAS proteins (Ihara et al., 2005). We used a split ubiquitin assay system to record noncovalent interactions between SUMO1ΔGG and the SIZ1 mutants described (Fig. 1A) and were unable to observe any differences between the wild type and mutant *SIZ1* alleles (Supplemental Fig. S2). Considering recent studies that established the juxtaposed hydrophobic aliphatic amino acid core as essential for SUMO binding, and not the SXS motif (Minty et al., 2000), this result can be understood. Using the spilt ubiquitin assay (Supplemental Fig. S2), we confirmed that in-

teraction between SIZ1 and intact SUMO1 is unaffected by a C379A mutation in the SP-RING domain of SIZ1 (Fig. 2B).

### The SP-RING Domain Plays an Important Role in the Regulation of SA Accumulation and SA-Dependent Phenotypes

The null *siz1-2* mutation led to elevated SA in planta that then generated a dwarf phenotype (Fig. 3A; Lee et al., 2007). The null *siz1-2* mutation also enhanced innate immunity as a result of SA accumulation, as measured by the accumulation of PR1 protein and reduced growth of the bacterial pathogen *Pseudomonas syringae* pv *tomato* on inoculated leaves (Fig. 3, C and D; Lee et al., 2007).

Expression of $SIZ^{WT}$, $SIZ1^{sap}$, $SIZ1^{phd}$, or $SIZ1^{pinit}$ from the *SIZ1* promoter in the *siz1-2* mutant restored size (Fig. 3A; Supplemental Table S1), SA levels, PR1 protein abundance, and pathogen susceptibility (Fig. 3, B–D) to those of wild-type plants. Compared with Col-0 control plants, empty vector transformants of *siz1-2* and transformants of *siz1-2* expressing $SIZ1^{sp\text{-}ring}$ from the *SIZ1* promoter had elevated levels of SA and PR1 protein in leaves (approximately 15- and 10-fold higher SA levels, respectively; Fig. 3, B and C). The SA levels in leaves of the $SIZ1^{sp\text{-}ring}$ transformants of *siz1-2* ($1.59 \pm 0.13$ or $1.71 \pm 0.16$ $\mu$g SA $g^{-1}$ fresh weight) were significantly lower than those in the *siz1-2* mutant ($2.10 \pm 0.06$ $\mu$g SA $g^{-1}$ fresh weight) or empty vector transformants of *siz1-2* ($2.32 \pm 0.062$ $\mu$g SA $g^{-1}$ fresh weight) but were significantly higher than the level in Col-0 ($0.16 \pm 0.06$ $\mu$g SA $g^{-1}$ fresh weight). These data suggest that the SP-RING domain makes a major contribution to the regulation of SA levels by SIZ1. PR1 transcript and protein levels in leaves mirrored the SA levels (Fig. 3, B and C; Supplemental Fig. S4). PR1 transcript and protein were detected in leaf extracts of only the *siz1-2* mutant, empty vector, and $SIZ1^{sp\text{-}ring}$ transformants. The levels of PR1 transcript and protein were similar in leaf extracts of the *siz1-2* mutant and empty vector transformant but slightly lower in $SIZ1^{sp\text{-}ring}$ transformants. The elevated SA and PR1 levels in the $SIZ1^{sp\text{-}ring}$ transformant of *siz1-2* correlated with a strong dwarf phenotype (Fig. 3A; Supplemental Table S1) and pathogen resistance (Fig. 3D). The PR1 protein was slightly decreased in *siz1-2* by transformation with $SIZ1^{sp\text{-}ring}$, confirming that although the SP-RING domain is a major contributor to the regulation of SA accumulation by SIZ1, it is not the sole contributor. Transformants of *siz1-2* expressing $SIZ1^{sxs}$ from the *SIZ1* promoter accumulated an intermediate level of SA ($0.62 \pm 0.08$ $\mu$g SA $g^{-1}$ fresh weight) that was significantly greater than the SA content of Col-0. Compared with wild-type Col-0, the SA levels in leaves of *siz1-2* transformants expressing $SIZ1^{sp\text{-}ring}$ and $SIZ1^{sxs}$ were 10- and 4-fold higher, respectively (Fig. 3B). Accordingly, PR1 transcript was detected in $SIZ1^{sxs}$ transformants of *siz1-2* but transcript levels were reduced in comparison with empty vector and $SIZ1^{sp\text{-}ring}$ transformants (Supplemental Fig. S4). Interestingly, PR1 protein abundance, pathogen susceptibility, and size of $SIZ1^{sxs}$ transformants were not significantly different from those of the wild type or $SIZ1^{WT}$ transformants of *siz1-2* (Fig. 3; Supplemental Table S1). It is speculated that the SXS motif also contributes to SIZ1-mediated SA accumulation and signaling.

### The SP-RING Domain Is Involved in Regulation of Basal Thermotolerance and the SA-Independent Phenotype

The null *siz1-2* mutation renders seed germination hypersensitive to heat shock (Yoo et al., 2006). A *NahG siz1-2* transgenic that expresses *Pseudomonas putida* salicylate hydroxylase and has very low levels of endogenous SA is also hypersensitive to heat shock. The heat shock sensitivity of seed germination or basal thermotolerance of the *siz1-2* mutant, therefore, is independent of SA content (Yoo et al., 2006). The domain of SIZ1 important for an SA-independent phenotype was ascertained by testing heat shock sensitivity of seed germination (Fig. 4). In the absence of heat shock, radicle emergence (germination) was observed within 2 d in all seeds for Col-0, *siz1-2*, and *siz1-2* transformants (Fig. 4; data not shown). Germination was delayed by heat shock in all lines. Four days after heat shock, between 78% and 88% of seeds of Col-0 and *siz1-2* transformants expressing $SIZ1^{WT}$, $SIZ1^{sap}$, $SIZ1^{phd}$, $SIZ1^{pinit}$, or $SIZ1^{sxs}$ had visible radicles (Fig. 4). However, for the *siz1-2* mutant and *siz1-2* transformants that carried either the empty vector or expressing $SIZ1^{sp\text{-}ring}$, seed germination was substantially repressed by heat shock. Only 15% to 25% of seeds had germinated in these lines 4 d after the heat shock (Fig. 4). The difference between Col-0 and *siz1-2* or empty vector as well as $SIZ1^{sp\text{-}ring}$ transformants of *siz1-2* was evident from 3 to 6 d after heat shock (Supplemental Fig. S3). However, no significant difference ($P < 0.05$) was observed in the percentage of germinated seeds for *siz1-2*, *siz1-2* transformants carrying empty vector, or *siz1-2* transformants expressing $SIZ1^{sp\text{-}ring}$ through the 7-d period. Thus, the SP-RING domain is essential for regulation of the SA-independent phenotype of thermotolerance at seed germination.

### The SXS Domain Modulates the Inhibitory Effect of ABA on Cotyledon Greening

ABA inhibits seed germination and induces chlorosis in leaves and cotyledons of Arabidopsis seedlings (Tuteja, 2007). Overexpression of AtSUMO1/2 increased the level of SUMOylation in planta and reduced the effect of ABA, while cosuppression of *AtSCE1a* decreased the level of SUMOylation in plants and accentuated the effect of ABA on seedlings (Lois et al., 2003; Saracco et al., 2007; Miura et al., 2009). Accordingly, when seeds of the *siz1-2* mutant and Col-0 were germinated in the presence of 0.2 $\mu$M ABA, expansion of the cotyledon of *siz1-2* seedlings was

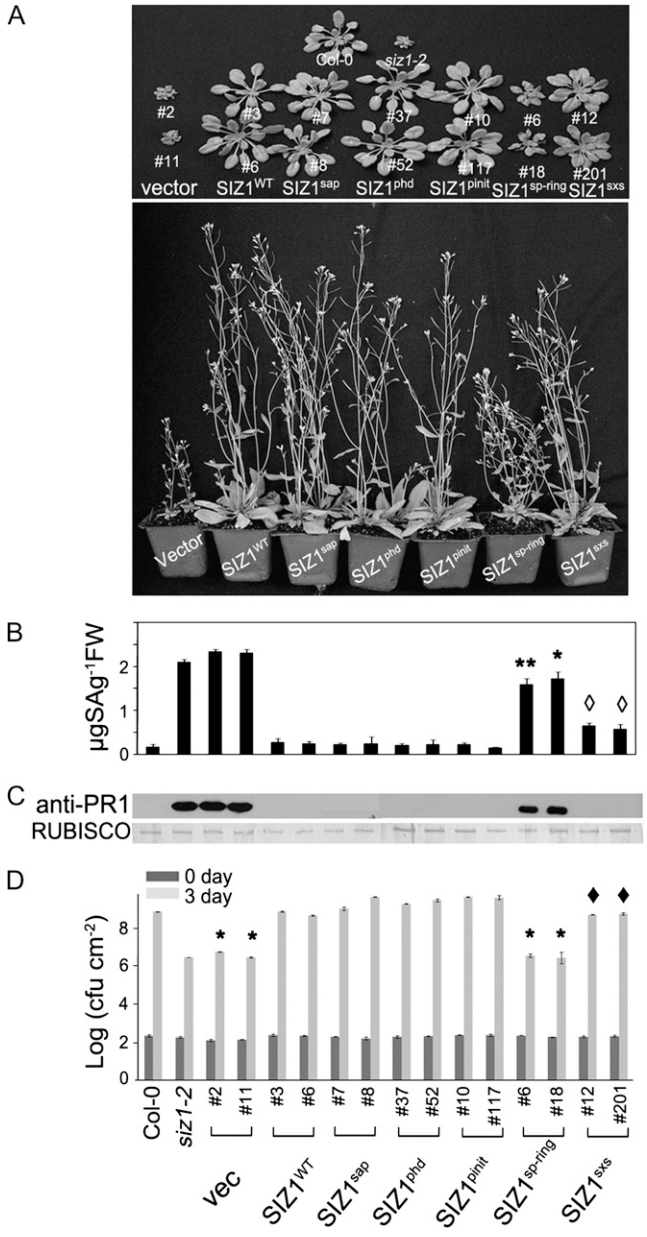


**Figure 3.** Complementation of SA-dependent phenotypes of the *siz1-2* mutant. A, Morphology of Col-0, *siz1-2*, and the indicated *siz1-2* transformants. The transformants are described in Figure 1. The T3 generation plants were grown in soil for 4 weeks (top) or 7 weeks (bottom) under a 16-h-light/8-h-dark photoperiod at 22°C. Two different lines of each transformant are shown. B, SA levels in rosette leaves of Col-0, *siz1-2*, and the indicated *siz1-2* transformants. Leaf tissues were collected from 4-week-old soil-grown plants and SA was quantified by HPLC. Values represent means ± SD obtained from three replicates of each sample. For *siz1-2* transformants expressing the $SIZ1^{sp\text{-}ring}$ mutant allele, significant differences from Col-0 and *siz1-2* by Student's *t* test at $P < 0.05$ (asterisk) or $P < 0.01$ (double asterisk) are indicated. For *siz1-2* transformants expressing the $SIZ1^{sxs}$ mutant allele, significant differences from Col-0 and *siz1-2* at $P < 0.05$ (diamond) are indicated. FW, Fresh weight. C, Accumulation of PR1 protein was monitored in these leaves. Shown are immunoblots of total leaf protein (2 $\mu$g) that were separated by SDS-PAGE and probed with anti-PR1. Rubisco content visualized by Coomassie Brilliant Blue staining is shown as a loading control. D, Growth of *P. syringae* pv *tomato* DC3000 in Col-0, *siz1-2*, and the indicated *siz1-2* transformants. Leaves of 4-week-old plants were inoculated with $1 \times 10^5$ colony-forming units (cfu) $mL^{-1}$ *P. syringae* pv *tomato* DC3000, and bacterial growth ($log_{10}$ cfu $cm^{-2}$ leaf) was monitored on 0 and 3 d postinoculation. Values represent means ± SD obtained from three independent experiments. Symbols indicate no significant differences from *siz1-2* (asterisks) and from Col-0 (diamonds) at $P < 0.05$. vec, Vector.

inhibited (Fig. 5A). Cotyledon expansion reverted to wild-type levels in transformants of *siz1-2* expressing $SIZ1^{WT}$ from the *SIZ1* promoter but not in empty vector transformants of *siz1-2* mutants (Fig. 5A). Also, at the time of cotyledon opening and epicotyl exposure, cotyledon greening was strongly inhibited by 0.2 $\mu$M ABA (about 75%) in empty vector transformants of *siz1-2* but not in transformants of *siz1-2* expressing $SIZ1^{WT}$ (about 5%; Fig. 5, B and C).

The inhibition of cotyledon greening in transformants expressing $SIZ1^{sap}$, $SIZ1^{phd}$, or $SIZ1^{pinit}$ (3% to 10%) was similar to that observed in transformants expressing $SIZ1^{WT}$ (7%; Fig. 5, B and C). However, 0.2 $\mu$M ABA had a strong inhibitory effect on cotyledon expansion (Fig. 5) and cotyledon greening (Fig. 5, B and C) in transformants expressing $SIZ1^{sp\text{-}ring}$ or $SIZ1^{sxs}$ from the *SIZ1* promoter. Inhibition of cotyledon greening was about 75% for empty vector transformants of *siz1-2*, about 66% for the $SIZ1^{sp\text{-}ring}$ transformants, and about 35% for $SIZ1^{sxs}$ transformants (Fig. 5, B and C). Comparison of the data in Figure 5C with data on SA levels in leaves of all transformants of *siz1-2* mutant (Fig. 3B) showed a direct relationship between leaf SA content and the inhibitory effect of 0.2 $\mu$M ABA on cotyledon greening. SA is known to enhance ABA sensitivity in seed germination (Nishimura et al., 2005; Xie et al., 2007). This raised the possibility that the inhibitory effect of ABA on cotyledon greening was mediated by SA. Accordingly, seedlings of *NahG* and *NahG siz1-2* were germinated in the absence or presence of 0.2 $\mu$M ABA, and the percentage of seedlings with green cotyledons was estimated at the stage of epicotyl exposure, as for the *siz1-2* transformants (Fig. 5, B and C). The data showed that the average inhibition of cotyledon greening in *NahG* (5%; Fig. 5B) and *NahG siz1-2* (10%; Fig. 5B) seedlings in medium containing 0.2 $\mu$M ABA was very similar to that observed for the $SIZ1^{WT}$, $SIZ1^{sap}$, $SIZ1^{phd}$, or $SIZ1^{pinit}$ transformants of *siz1-2* (7%–17%; Fig. 5, B and C). Since transformation of *NahG* into SA-accumulating *siz1-2* plants greatly depletes endogenous SA levels in leaves (Lee et al., 2007) and abolishes ABA inhibition of cotyledon greening (Fig. 5, B and C), the influence of ABA on cotyledon greening could be mediated by SA level.

### PHD and PINIT Domains Control Hypocotyl Length in Response to Sugar and Light

The plant-specific PHD domain in PIAS-type E3 ligases (Miura et al., 2007a) could be responsible for

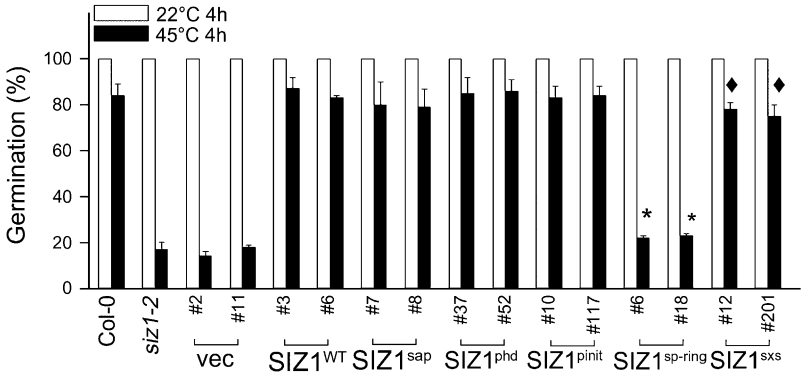


**Figure 4.** Complementation of the thermosensitive seed germination phenotype of the *siz1-2* mutant. Stratified seeds of Col-0, *siz1-2*, and the indicated *siz1-2* transformants were subjected to heat shock (45°C, 4 h) or incubated at ambient temperature (22°C, 4 h; controls) and immediately sown on plates. The plates were maintained at 16 h of light/8 h of dark at 22°C. Germination data, expressed as the percentage of seeds that had visible radicles, were obtained on day 4 after heat shock. The data are means ± SD from three independent experiments ($n = 50$). Symbols indicate no significant difference from *siz1-2* (asterisks) and Col-0 (diamonds) at $P < 0.05$. vec, Vector.

generating the light-related phenotype of altered hypocotyl length. When seeds were germinated under normal growth conditions, hypocotyl lengths of Col-0, the *siz1-2* mutant, and transformants of *siz1-2* expressing empty vector, $SIZ1^{WT}$, $SIZ1^{sap}$, $SIZ1^{sp\text{-}ring}$, or $SIZ1^{sxs}$ from the *SIZ1* promoter were comparable (Fig. 6A). On the other hand, the hypocotyls of transformants of *siz1-2* expressing $SIZ1^{phd}$ and $SIZ1^{pinit}$ were significantly longer (Fig. 6A). The long-hypocotyl phenotype was stronger in transformants of *siz1-2* expressing $SIZ1^{phd}$ than in $SIZ1^{pinit}$ transformants. It was not affected by the substitution of Glc for Suc (Fig. 6, C and D) but was abrogated either by omission of sugar in the growth medium (Fig. 6B) or in continuous darkness (data not shown). The PHD domain of SIZ1 has SUMO E3 ligase activity (Garcia-Dominguez et al., 2008). The PINIT domain of mammalian PIAS1 and PIAS3 has been shown to be involved in SUMO and in target selection (Wong et al., 2004). Our data (Fig. 1B) indicated that both the C134Y mutation in the PHD domain and the PIIT-to-PAAT mutation in the PINIT domain impaired the formation of SUMO conjugates. In addition, Figure 6 shows that $SIZ1^{phd}$ and $SIZ1^{pinit}$ alleles create a sugar- and light-dependent long-hypocotyl phenotype in seedlings of $SIZ1^{phd}$ and $SIZ1^{pinit}$ transformants that is not evident in wild-type or *siz1-2* mutant seedlings. In conclusion, the sugar- and light-dependent long-hypocotyl phenotype in seedlings of $SIZ1^{phd}$ and $SIZ1^{pinit}$ transformants appears to be based on mutations that either alter SUMO or target selectivity or on alterations in the relative abundance of conjugates with different SUMO isoforms.

## DISCUSSION

SUMOylation regulates development and is involved in biotic and abiotic stress responses in Arabidopsis. The E3 ligase, SIZ1, plays a pivotal role in controlling SUMOylation and, hence, significantly affects stress responses, growth, and development in plants (Lois et al., 2003; Miura et al., 2007a). Compared with the list of SUMO-modified proteins and their roles in yeast and mammals (Reindle et al., 2006; Vertegaal et al., 2006), fewer plant SUMO targets have been identified and their functions analyzed at the molecular level. The transcription factors PHR1, ICE1, ABI5, and FLD have been identified as targets of SUMOylation through SIZ1, explaining the biological effects of SUMOylation on responses to low phosphate, low temperature, ABA, and the regulation of flowering time (Miura et al., 2005, 2007b, 2009; Jin et al., 2008). The transcription factors Global Transcription Factor3 (GTE3) and AtMYB30 have been identified for targets of SUMO in in vitro assays (Garcia-Dominguez et al., 2008; Okada et al., 2009). As a step toward uncovering SUMOylation targets based on their interaction with SIZ1, the objective of our study was to associate phenotypes with mutations in the SIZ1 E3 ligase. Point mutations based on studies of yeast and mammalian PIAS-type SUMO E3 ligases were introduced into the known domains of SIZ1. Following the characterization of effects exerted by these mutations, we expect that domain-specific SIZ1 interactors may eventually be identified. Indeed, various phenotypes associated with null mutation of SIZ1 can be associated with specific domains, providing an indication about the multiple effects that SIZ1 can orchestrate.

Our results indicate that an intact zinc finger motif in the SP-RING domain is a major contributor to SUMOylation capability of SIZ1, proper nuclear localization of SIZ1, and control of SA level (Figs. 1B, 2, and 3). Mutational analyses of PIAS/SIZ-type proteins coupled with in vitro tests have established that the Cys residues in the C2HC3 zinc finger motif of the SP-RING domain are required for E3 ligase activity, substrate selectivity, or interaction with E2 (Takahashi and Kikuchi, 2005; Reindle et al., 2006; Garcia-Dominguez et al., 2008). In fact, deletion of Cys-379 along with amino acids 380 to 384 of SIZ1 strongly impaired its in vitro SUMO E3 ligase activity with all tested substrates (Garcia-Dominguez et al., 2008). SUMOylation of two SIZ1-interacting proteins, namely AtSCE1 (the E2 enzyme) and GTE3, and autoSUMOylation of SIZ1 were impaired by this six-amino acid deletion. Therefore, it is not surprising that heat shock-induced SUMO conjugation in Arabidopsis seedlings was almost completely abolished by the mutation of only one Cys (Cys-379) in zinc finger of the

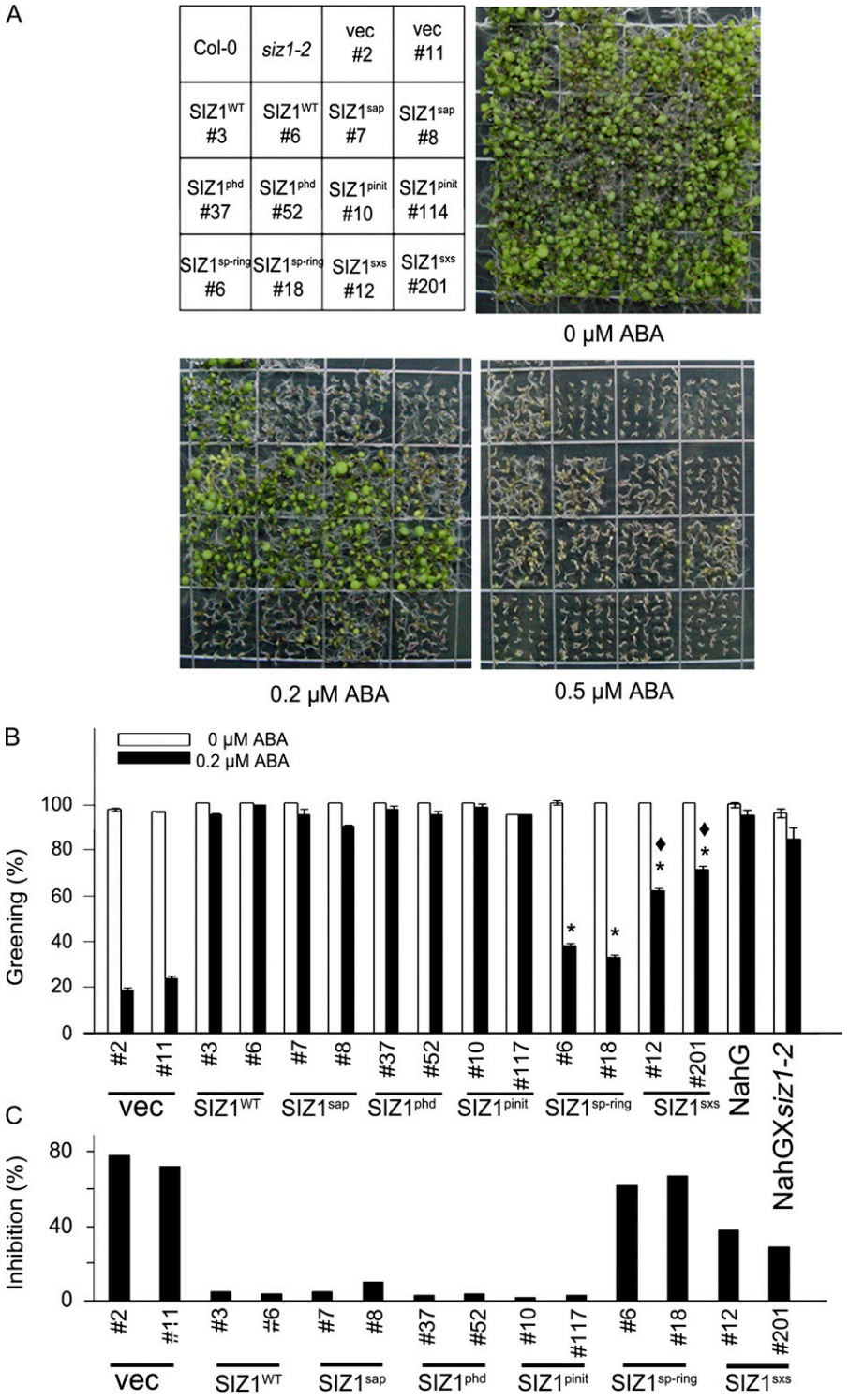


**Figure 5.** Complementation of ABA-induced inhibition of cotyledon expansion and cotyledon greening in *siz1-2* mutants. A, Cotyledon expansion of transgenic plants in the presence of ABA. Stratified seeds ($n = 25$) of Col-0, *siz1-2* mutant, and the indicated *siz1-2* transformants were spread in the illustrated pattern on 0.5× Murashige and Skoog medium containing 0, 0.2, and 0.5 $\mu$M ABA and maintained under a 16-h-light/8-h-dark daily photoperiod at 22°C. Photographs were taken after 7 d. B, Stratified seeds ($n = 50$) were spread on 0.5× Murashige and Skoog medium containing 0 and 0.2 $\mu$M ABA and maintained under a 16-h-light/8-h-dark daily photoperiod at 22°C. On day 6 of growth, the cotyledons spread apart to expose the epicotyls, and the number of seedlings with green cotyledons and the total number of seedlings on each plate were counted. The data are averages from three independent experiments and indicate the percentage (±SE) of seedlings with green cotyledons in each population. Significant differences from the vector (vec) transformant (asterisks) and SIZ$^{WT}$ transformant (diamonds) at $P < 0.05$ are indicated. C, Percentage inhibition of cotyledon greening was calculated from the data shown in B as 100× change in percentage greening by 0.2 $\mu$M ABA/percentage greening in the absence of ABA.

SP-RING domain of SIZ1 (Fig. 1B). SUMO conjugation under many other conditions would be expected to be severely impaired by this mutation. In keeping with this prediction, all tested phenotypes of *siz1-2* (stunted growth, SA accumulation, pathogen resistance, ABA sensitivity of seed germination and cotyledon greening, root growth inhibition under phosphate limitation, and abrogation of basal thermotolerance; Table I; Figs. 3–6; Supplemental Figs. S3 and S5) were associated with the SIZ1$^{sp\text{-}ring}$ mutation. The phenotype of *SIZ1$^{sp\text{-}ring}$* transformants of *siz1-2* closely resembled that of *siz1-2* mutants in most instances, indicating the importance of Cys-379 and the SP-RING domain for these phenotypes.

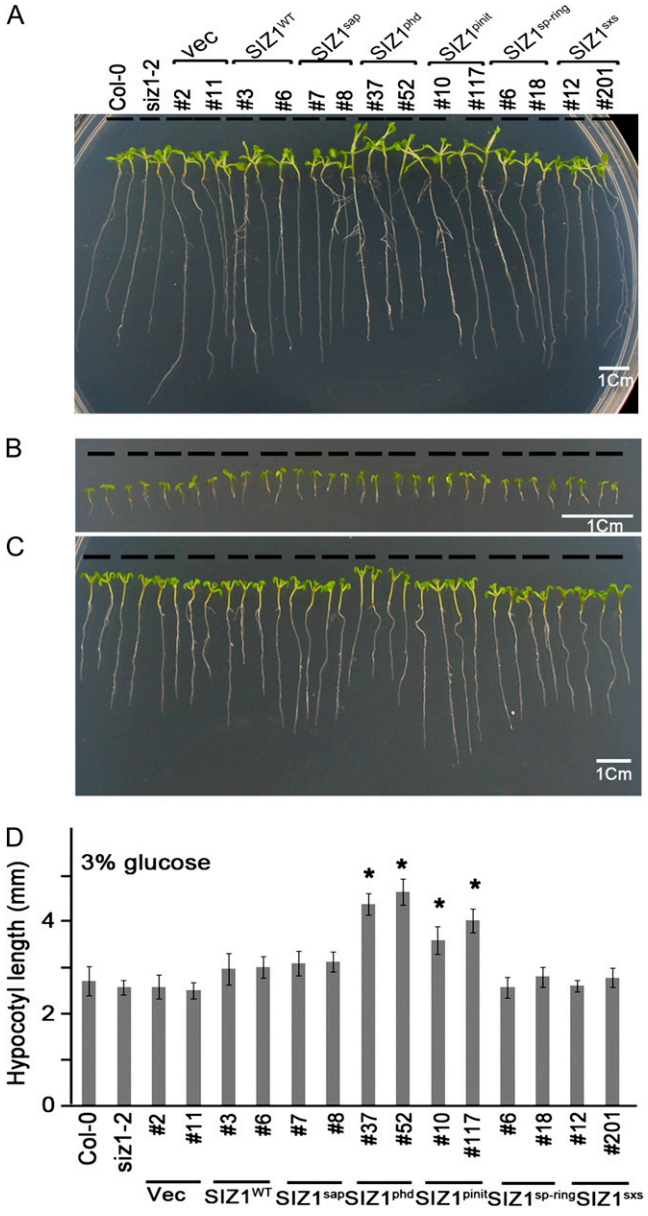


**Figure 6.** Effects of *SIZ1* mutations on hypocotyl length. Stratified seeds of Col-0, *siz1-2* mutant, and the indicated *siz1-2* transformants were spread on 0.5× Murashige and Skoog medium containing 2% Suc (A), no sugar (B), and 3% D-Glc (C) and maintained under a 16-h-light/8-h-dark daily photoperiod at 22°C. Photographs were taken 7 d later. D, Average hypocotyl lengths (±SD) of seedlings grown on plates containing 3% Glc ($n = 30$). Asterisks indicate significant differences from the $SIZ1^{WT}$ transformant at $P < 0.05$. Data are representative of three independent repeats. vec, Vector.

In addition to the SP-RING domain, there is evidence that the PHD and PINIT domains of PIAS-type SUMO E3 ligases participate in SUMO conjugation (Wong et al., 2004; Ivanov et al., 2007; Garcia-Dominguez et al., 2008). The PHD domain of SIZ1 has SUMO ligase activity (Fig. 1B; Garcia-Dominguez et al., 2008). The PHD domain of SIZ1 was not required for autoSUMOylation of SIZ1, but disruption of the C4HC3 zinc finger of this domain by deletion of amino acids 134 to 142 abolished the ability of SIZ1 to cooperate with the SP-RING domain for efficiently SUMOylating AtSCE1 in vitro (Garcia-Dominguez et al., 2008). An intact PHD domain was essential for SUMO conjugation to GTE3 in vitro, suggesting that the PHD domain functions as an E3 ligase but its target range is limited compared with that of the SP-RING domain. The mammalian transcriptional regulator KAP1 does not contain an SP-RING but utilizes a PHD domain for interaction with E2 enzyme and ligation of SUMO (Ivanov et al., 2007). Thus, the available evidence suggests that the PHD domain functions as a SUMO E3 ligase for a limited class of substrates. The PINIT domain of mammalian and *S. cerevisiae* PIAS/SIZ proteins is involved in target and SUMO selection (Wong et al., 2004; Takahashi and Kikuchi, 2005; Reindle et al., 2006). Accordingly, the C134Y point mutation that disturbs the PHD domain of SIZ1 and the PIIT-to-PAAT mutation that modifies the PINIT-like motif were found to partially impair SUMO conjugation in heat-shocked Arabidopsis seedlings (Fig. 1B). However, the PHD and PINIT domain mutations of SIZ1 were not associated with any of the tested phenotypes of the *siz1-2* mutant. Surprisingly, expression of $SIZ1^{phd}$ or $SIZ1^{pinit}$ but not empty vector, $SIZ1^{WT}$, or $SIZ1^{sp\text{-}ring}$ in the *siz1-2* mutant or Col-0 Arabidopsis (Fig. 6; data not shown) resulted in a dominant long-hypocotyl phenotype that was dependent on sugar and light. There was no evidence for the participation of the PHD or PINIT domain in subcellular localization of SIZ1 or interaction of SIZ1 with AtSUMO1 (Fig. 2; Supplemental Fig. S2). Thus, a possible explanation of the dominant sugar- and light-dependent long-hypocotyl phenotype of $SIZ1^{phd}$ and $SIZ1^{pinit}$ transformants is that the mutations allow selection of unusual SIZ1 targets that may or may not need to be SUMOylated in order to elicit the phenotype. Sugar and light signaling pathways are known to control hypocotyl elongation (Solfanelli et al., 2006; Catala et al., 2007; Shin et al., 2007; Buer and Djordjevic, 2009). Thus, the $SIZ1^{phd}$ and $SIZ1^{pinit}$ transformants could be valuable tools for the genetic interrogation of sugar and light signaling pathways in Arabidopsis.

SUMOylation targets are found in most subcellular compartments (Geiss-Friedlander and Melchior, 2007). Therefore, subcellular localization of SIZ1 can be important for regulating its function. PIAS-type SUMO E3 ligases and their chief targets are localized mostly in the nucleus, often in nuclear bodies (Sachdev et al., 2001; de Cristofaro et al., 2009). Mutation of the SP-RING domain Cys residues, the Ile residues of PINIT motif, or the terminal Leu residues of the SAP domain LXXLL motif in mammalian PIAS3L protein results in both nuclear and cytoplasmic distribution (Duval et al., 2003; Reindle et al., 2006). Nuclear retention of *S. cerevisiae* SIZ1 requires the SAP domain (Takahashi and Kikuchi, 2005). We found that SIZ1 was also nucleus localized and associated with subnuclear

**Table I.** *Summary of the phenotypes of siz1-2 transformants*

| Phenotype | Col-0 | *siz1-2* | Vector | $SIZ1^{WT}$ | $SIZ1^{sap}$ | $SIZ1^{phd}$ | $SIZ1^{print}$ | $SIZ1^{sp\text{-}ring}$ | $SIZ1^{sxs}$ |
|---|---|---|---|---|---|---|---|---|---|
| SUMO1/2 conjugates (SUMO1/2) by 39°C, 30 min | ++++ (−) | + (+++) | + (+++) | ++++ (−) | ++++ (−) | ++ (++) | ++ (++) | + (+++) | ++++ (−) |
| SA level | − | ++++ | ++++ | − | − | − | − | +++ | + |
| Disease resistance (bacterial pathogen) | − | +++ | +++ | − | − | − | − | ++ | − |
| Heat shock sensitivity (germination) | − | +++ | +++ | − | − | − | − | +++ | − |
| ABA sensitivity (germination) | − | +++ | +++ | − | − | − | − | +++ | + |
| ABA sensitivity (greening) | − | +++ | +++ | − | − | − | − | +++ | ++ |
| Hypocotyl length | − | − | − | − | − | +++ | ++ | − | − |
| Phosphate limitation (root growth inhibition) | − | +++ | +++ | − | − | − | − | +++ | − |

structures (Fig. 2). The nuclear and subnuclear localization of SIZ1 was abrogated by mutation of Cys-379 of the SP-RING. It is reported that SIZ1 SUMOylates itself on Lys-100 and Lys-488 under in vitro conditions, and deletion of the SP-RING zinc finger motif in SIZ1 abrogates self-SUMOylation (Garcia-Dominguez et al., 2008). This suggests that the nuclear speckles (subnuclear structure; Fig. 2; Miura et al., 2005) may result from a self-SUMOylation defect in the $SIZ1^{sp\text{-}ring}$ mutant. These data suggest that some of the phenotypes associated with the SP-RING mutation may be attributed to mislocalization of SIZ1 within the cell. In yeast, the local concentration of SIZ proteins is a major factor in substrate selectivity (Takahashi and Kikuchi, 2005; Reindle et al., 2006). Based on SUMO ligase activity associated with PHD and PINIT domains (Fig. 1B; Takahashi and Kikuchi, 2005; Reindle et al., 2006; Ivanov et al., 2007; Garcia-Dominguez et al., 2008), it is possible that mislocalization of SIZ1 in the $SIZ1^{sp\text{-}ring}$ transformants results in altered substrate selectivity. None of the other mutations that we tested had any effect on the subcellular location of SIZ1. Since the effects of point mutations were studied here, these studies do not in any way exclude the possibility that amino acids or regions outside the SP-RING domain of the protein participate in subcellular localization of SIZ1.

The ABA sensitivity of cotyledons of the *siz1-2* mutant and *siz1-2* transformants expressing $SIZ1^{sp\text{-}ring}$ or $SIZ1^{sxs}$ is a strong phenotype (Fig. 5). The SA level in green portions of 10-d-old *siz1-2* seedlings was found to be almost 2-fold higher than in Col-0 (Yoo et al., 2006). SA contents of leaves of 4-week-old *siz1-2* plants and *siz1-2* transformants expressing $SIZ1^{sp\text{-}ring}$ or $SIZ1^{sxs}$ also parallel the ABA sensitivities of these lines (Figs. 3B and 5). The ABA sensitivity of Arabidopsis cotyledons is greatly reduced in *NahG* transformants (Fig. 5). Agonistic and antagonistic effects of SA and ABA are well documented (Nishimura et al., 2005; Loake and Grant, 2007; Xie et al., 2007). These data strongly suggest that SA may be elevated in cotyledons of the 6-d-old *siz1-2* seedlings used to test the ABA effect (Fig. 5). We were unable to discover a molecular explanation for this phenotype. No change in SUMO1/2 conjugation pattern was observed in $SIZ1^{sxs}$ transformants (Fig. 1B). Neither noncovalent interaction of SIZ1 with SUMO1 nor subcellular localization of SIZ1 was affected by the SXS domain mutation (Supplemental Fig S2; data not shown). However, our tests were not designed to conclusively rule out participation of the SXS motif in SUMO selection. For example, it is possible that the SXS domain interacts with a different SUMO isoform than the one used in our test and affects conjugation of SUMO3 rather than SUMO1/2 (Fig. 1B; Supplemental Fig. S2).

Major effects of *siz1* null mutation on plant growth, development, and stress response are mediated via SA accumulation. The association of SA with plant response to biotic and abiotic stresses, and SA signaling, have been studied widely (Loake and Grant, 2007). However, regulation of SA biosynthesis, degradation, and modification, all of which could regulate the level of bioactive SA in plants, is not well understood. The phenotypic consequences of the SP-RING domain mutation suggest that SA levels are strongly regulated by SUMOylation. A RING-type ubiquitin E3 ligase

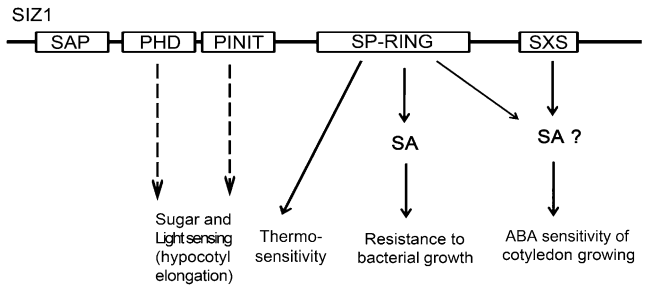


**Figure 7.** A model linking function to individual SIZ1 domains. The locations of conserved domains on SIZ1 ($SIZ1^{WT}$) protein are shown by the boxes. Phenotypes of *siz1-2* that could not be complemented fully by a mutant *SIZ1* allele, and the domain carrying that mutation, are shown connected by solid arrows. Resistance to bacterial growth is an SA-dependent phenotype, and our data suggest that sensitivity of cotyledons to ABA may also be SA dependent. Phenotypes that were observed only in *siz1-2* transformants carrying a mutant *SIZ1* allele, but not in wild-type or *siz1-2* null lines, are shown connected to the domain carrying that mutation by dashed arrows. We were unable to associate any phenotype with the combined V22A and L23A mutations in the SAP domain.

was recently shown to control SA accumulation and immune response in Arabidopsis (Yaeno and Iba, 2008). SUMOylation and ubiquitination have antagonistic effects on protein stability (Wilson and Heaton, 2008; de Cristofaro et al., 2009). Perhaps the stability of some component of the SA biosynthetic pathway remains compromised in *siz1-2* transformants expressing $SIZ1^{sp\text{-}ring}$. The ability of PIAS proteins to recruit and remove proteins from transcriptional complexes is an important function that does not always require SUMO ligase activity but is often dependent on SUMO interaction motifs containing an SXS signature (Sharrocks, 2006; Palvimo, 2007). The ABA sensitivity of seed germination and cotyledon greening of *siz1-2* transformants expressing $SIZ1^{sxs}$ suggests that SIZ1 also affects SA level independent of ligase activity. The SP-RING and SXS domain mutants of SIZ1 could be useful tools to clarify details of SA regulation in plants, which is crucial for coping with various biotic and abiotic stresses.

In summary, diverse phenotypes characteristic of SIZ1 are associated with specific domains and can be separated (Fig. 7). Although regulatory mechanism that govern responses by distinct structural domains and stress responsiveness still remain to be identified, our results demonstrate contributions of individual SIZ1 structural domains to the sum of functions that are orchestrated by the SIZ1 holoenzyme.

## MATERIALS AND METHODS

### Plant Material, Growth Conditions, and Transformation

The wild type and the *siz1-2* mutant (SALK_065397) of Arabidopsis (*Arabidopsis thaliana* Col-0) were genetic resources for this research (Miura et al., 2005). Plants were grown in soil at 22°C in a growth chamber at 120 $\mu$mol $m^{-2}$ $s^{-1}$ light intensity under a 16-h-light/8-h-dark daily photoperiod. All seeds were stratified for 3 d at 4°C before transfer to growth conditions. Unless mentioned otherwise, for experiments involving seedlings, stratified seeds were spread on a basal medium containing 1× Murashige and Skoog basal salt mixture, 2% Suc, 2.5 mM MES, pH 5.7, and 0.25% phytagel. Plates were incubated under a 16-h-light/8-h-dark daily photoperiod at 22°C/18°C.

Methods for *Agrobacterium tumefaciens*-mediated transformation have been described (Clough and Bent, 1998). Hygromycin-resistant transformants were screened for the presence of a T-DNA insert as well as the *siz1-2* mutation by diagnostic PCR. The PCR products corresponding to inserted *SIZ1* mutant domains were sequenced to confirm the mutation in planta. All transgenic lines were created in the siz1-2 genetic background and tested for the presence of the *siz1-2* allele by PCR using the primers listed in Supplemental Table 3A and the B primer, which corresponds to the $LBa_1$ primer described by the Salk Institute Genome Analysis Laboratory database (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/index.html). Homozygous T3 or T4 generation transgenic lines were used for phenotype and other analyses.

### Phenotype Analyses

Bacterial growth assays were performed exactly as described before (Lee et al., 2007) by infiltration of leaves of 4-week-old soil-grown plants with *Pseudomonas syringae* pv *tomato* DC 3000. The experiment was repeated three times with at least four replicates in each experiment.

Basal thermotolerance assays were performed essentially as described (Yoo et al., 2006). Eppendorf tubes containing stratified seeds (500 $\mu$L of water containing 50–100 $\mu$L of seeds) were subjected to heat shock treatment (4 h, 45°C) in a constant-temperature water bath (ISOTEMP 210; Fisher Scientific) and then sown on growth basal medium. Germination, quantified as number of seeds with visible radicles, was monitored every 24 h.

For measuring ABA sensitivity, seeds were germinated on basal medium without or with ABA (0.2 and 0.5 $\mu$M) supplement. Seedlings were photographed every 24 h. Greening was quantified at the time of cotyledon opening and epicotyl exposure as the percentage of seedlings with green cotyledons.

For the hypocotyl elongation assay, stratified seeds were plated on basal medium, medium containing no sugar, or 3% D-Glc, and hypocotyl length was measured after 7 d.

### DNA Methods

For site-directed mutagenesis, the $Prom_{CaMV35S}$::*SIZ1*:*GFP* construct (p326-sGFP vector; Miura et al., 2005) was used as template and substitution of single amino acids was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The primers used for mutagenesis are listed in Supplemental Table 2B. The mutations were confirmed by sequencing.

The $Prom_{SIZ1}$::$SIZ1^{WT}$:*GFP* construct used for plant transformation has been described earlier and shown to be functionally equivalent to the native *SIZ1* gene (Jin et al., 2008). For the mutant *SIZ1* alleles, the $SIZ1^{mutant}$ open reading frames from the above GFP constructs were isolated by PCR and cloned into the binary vector pCAMBIA 1302. The cauliflower mosaic virus 35S promoter (CaMV 35S) was then replaced with $Prom_{SIZ1}$, which is sequence 3.65 kb upstream of the *SIZ1* coding sequence. All $Prom_{SIZ1}$::$SIZ1^{variant}$:*GFP* plasmids were transformed into *A. tumefaciens* strain GV3101 and used to generate transgenic plants. A $Prom_{SIZ1}$::*GFP* construct was used as empty vector control.

The $Prom_{CaMV35S}$::$SIZ1^{variant}$:*GFP* constructs (p326-sGFP vector) were transiently expressed in protoplasts isolated from 14-d-old seedlings by polyethylene glycol-mediated transformation for determination of subcellular localization (Jin et al., 2008).

The spilt YFP vector YN/YC used for bimolecular fluorescence complementation was a kind gift of Dr. J.C. Koo (Chonbuk National University, JeonJu, Korea). This is a bidirectional expression plasmid with the $YFP_{N\ terminus}$ driven by the cassava vein mosaic virus promoter (CsV) and $YFP_{C\ terminus}$ driven by the CaMV 35S. The *SUMO1* (At4g26840) coding region was inserted in YN, resulting in the $Prom_{CsV}$::$YFP_{N\ terminus}$:*SUMO1* construct. The *SIZ1* coding region was inserted in YC, resulting in the $Prom_{CaMV35S}$:*SIZ1*:$YFP_{C\ terminus}$ construct. The constructs were cotransformed into leaf mesophyll protoplasts and observed with a fluorescent microscope (Olympus) after 24-h incubation at 22°C in the dark.

### Analysis of SUMOylation Profiles and PR1 Expression in Protein Extracts

For *PR1* gene expression, leaves of 4-week-old soil-grown plants were ground in liquid nitrogen and suspended in a buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 50 mM KCl, 1 mM EDTA, 10% Suc, 5% $\beta$-mercaptoethanol, and 4% SDS. The supernatant was collected by centrifugation at 12,000 rpm for 5 min at 4°C, and protein concentration was determined using Bradford reagent (Bio-Rad). PR1 was detected in the protein extracts exactly as described (Lee et al., 2007). For analysis of SUMOylation, proteins were extracted from seedlings as above and separated by SDS-PAGE, and the SUMOylation pattern was detected on immunoblots by probing with anti-SUMO1 antibody (ab5316; Abcam) exactly as described earlier (Yoo et al., 2006). The anti-SUMO antibody detects both SUMO1 and SUMO 2.

### SA Measurement

Leaves of 4-week-old soil-grown plants were harvested and frozen in liquid nitrogen for SA measurement. Extraction of leaf tissue and SA quantification by HPLC were performed as described earlier (Lee et al., 2007), except that a 5 mM solution of 3,4,5-trimethoxy-trans-cinnamic acid (60 $\mu$L) was used as an internal standard.

### Statistical Analysis

All data were analyzed for significant differences by Student's *t* test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_125434 (At5g60410).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Characterization of *siz1-2* transformants expressing in-frame *GFP* fusions to cDNAs of wild-type and mutant *SIZ1* from the *SIZ1* promoter.

**Supplemental Figure S2.** Analysis of the effect of mutations on the interaction between SIZ1 and SUMO1 using the yeast split ubiquitin assay system.

**Supplemental Figure S3.** Complementation of the thermosensitive seed germination phenotype of the *siz1-2* mutant.

**Supplemental Figure S4.** Analysis of *PR1* transcript in $SIZ1^{sp\text{-}ring}$ and $SIZ1^{sxs}$ transformants of *siz1-2*.

**Supplemental Figure S5.** Root growth phenotypes of *siz1-2* transformants under phosphate starvation.

**Supplemental Table S1.** Height comparison of *siz1-2* transformants.

**Supplemental Table S2.** List of PCR primers used in this study.

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