Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling

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SUMO (small ubiquitin-related modifier) conjugation (i.e., sumoylation) to protein substrates is a reversible posttranslational modification that regulates signaling by modulating transcription factor activity. This paper presents evidence that the SUMO E3 ligase SIZ1 negatively regulates abscisic acid (ABA) signaling, which is dependent on the bZIP transcription factor ABI5. Loss-of-function T-DNA insertion *siz1–2* and *siz1–3* mutations caused ABA hypersensitivity for seed germination arrest and seedling primary root growth inhibition. Furthermore, expression of genes that are ABA-responsive through ABI5-dependent signaling (e.g., *RD29A*, *Rd29B, AtEm6, RAB18, ADH1*) was hyperinduced by the hormone in *siz1* seedlings. *abi5–4* suppressed ABA hypersensitivity caused by *siz1* (*siz1–2 abi5–4*), demonstrating an epistatic genetic interaction between *SIZ1* and *ABI5*. A K391R substitution in ABI5 [ABI5(K391R)] blocked SIZ1-mediated sumoylation of the transcription factor in vitro and in *Arabidopsis* protoplasts, indicating that ABI5 is sumoylated through SIZ1 and that K391 is the principal site for SUMO conjugation. In *abi5–4* plants, *ABI5(K391R)* expression caused greater ABA hypersensitivity (gene expression, seed germination arrest and primary root growth inhibition) compared with *ABI5* expression. Together, these results establish that SIZ1-dependent sumoylation of ABI5 attenuates ABA signaling. The double mutant *siz1–2 afp-1* exhibited even greater ABA sensitivity than the single mutant *siz1*, suggesting that SIZ1 represses ABI5 signaling function independent of AFP1.

abscisic acid | signaling | SUMO | sumoylation

The phytohormone abscisic acid (ABA) regulates numerous processes, including many that are necessary for plant growth and development and environmental stress adaptation (1–3). ABA accumulates in developing embryos, where it regulates seed development and storage product accumulation and facilitates the initiation and maintenance of seed dormancy (2). The hormone prevents premature seed germination before embryos are developmentally and physiologically mature, which ensures seedling establishment in favorable environmental conditions. Furthermore, ABA enhances seed desiccation tolerance by inducing the expression of genes encoding effectors that provide hyperosmotic protection as embryos dehydrate in the later maturation stages. Water deficit induces ABA biosynthesis in stratified seed, arresting germination until the environment becomes more conducive for seedling development.

Effectors and mechanisms of ABA perception and signal transduction are the focus of intensive research efforts (2, 3). ABA receptors identified to date include FCA (flowering control locus A), CHLH (H subunit, magnesium-protoporphyrin-IX chelatase), GCR2 [G (guanine nucleotide-binding protein) protein–coupled receptor 2], and GTG1 and GTG2 [G protein–coupled receptor (GPCR)-like G proteins] (4–7). GTG1 and GTG2 are predicted to have GPCR topology and to exhibit GTP-binding and GTPase activities (7). These proteins are hypothesized to be membrane-localized ABA receptors involved in G protein–mediated transduction of the hormonal signal (7).

Among the numerous effectors of ABA signal transduction identified to date are the ABA-insensitive (ABI) determinants, detected by a genetic screen for mutations that rendered seeds less responsive to ABA-mediated inhibition of germination (3, 8). *ABI1* and *ABI2* encode protein phosphatase 2Cs, whereas *ABI3, ABI4,* and *ABI5* encode B3, APETALA2-like, and basic leucine zipper (bZIP) domains containing transcription factors, respectively (9–11).

ABI3 and ABI5 function as intermediates in ABA signaling to regulate seed maturation and germination, as well as expression of genes that facilitate desiccation tolerance as embryos dehydrate in later maturational stages (11–13). Furthermore, ABA or hyperosmotic stress of seeds during stratification induces biosynthesis of the hormone regulated by *ABI3* and *ABI5* (11, 12). *ABI5* expression suppresses ABA insensitivity of seed germination caused by *abi3* (12), and overexpression of *ABI3* enhances *ABI5* expression (14), indicating that *ABI5* is genetically epistatic to *ABI3*.

Recombinant ABI5 physically interacts with the ABRE (ABA-responsive element, ACGTGG/TC) *cis* regulatory promoter sequence in ABA-responsive genes, such as *AtEm6, RD29A/LTI78* (responsive to desiccation/low-temperature induced), *RD29B/LTI65, RAB18* (response to ABA), and *ADH* (alcohol dehydrogenase) (3, 13). Chromosomal immunoprecipitation analysis has revealed that ABA stimulates association of ABI5 with the *AtEm6* promoter, which presumably is necessary for transactivation (12). Recent evidence has established that HY5 binds to the promoter of *ABI5* to function as an integrator of light and ABA signaling during seed germination, early seedling growth, and root development (15).

ABA induces ABI5 accumulation through transcriptional activation and enhanced protein stability (11). ABI5 stabilization and activation is correlated with phosphorylation (11). AFP (an ABI5-binding protein) facilitates ABI5 proteasome degradation (16), which is linked to the RING-finger ubiquitin E3 ligase KEG (KEEP ON GOING) (17). An *abi5* mutation suppresses ABA-hypersensitivity of *afp-1* and *keg* mutants (16, 17). These findings indicate that AFP and KEG are negative

regulators of ABA signaling, acting through the degradation of ABI5 (16, 17).

Sumoylation regulates diverse biological processes, including cell cycle progression, DNA repair and transcription in yeast and metazoans (18). SUMO conjugation to protein substrates, similar to ubiquitination, occurs in a series of biochemical steps that are catalyzed sequentially by SUMO-activating, -conjugating, and -ligating enzymes (18). Proteases of the cysteine protease superfamily deconjugate SUMO from protein substrates (18). In plants, SUMO conjugation and deconjugation determinants and sumoylation have been linked functionally to ABA and Pi starvation signaling, growth and flowering, defense against phytopathogens, thermotolerance, and cold acclimation (summarized in ref. 19). SIZ1 (SAP and Miz), the principal SUMO E3 ligase in *Arabidopsis*, has been reported to function in all of these processes (20–26). *Arabidopsis* SIZ1 is the plant prototype of yeast SIZ and mammalian PIAS proteins that regulate gene expression through chromatin remodeling and nuclear body sequestration (18).

This study established that *siz1* mutations cause ABA hypersensitivity, resulting in inhibition of germination and seedling primary root growth, implicating sumoylation in the regulation of ABA signaling, as SUMO1/2 overexpression attenuates ABA-mediated growth inhibition (27). SIZ1 was found to modulate ABA signaling by facilitating sumoylation of ABI5 at K391. Wild-type *ABI5* expression suppressed the *abi5–4* mutation; however, this capacity was abrogated by a *K391R* substitution, which also prevented sumoylation of the protein. Together, these results indicate that SUMO modification negatively regulates ABI5 function in ABA signaling during seed germination and seedling growth.

Results

siz1 Enhances ABA Sensitivity of Seeds and Seedlings.
Exogenous application of ABA during or immediately after stratification results in seed germination and primary root growth inhibition in seedlings (8). We found that ABA (0.5 μM) inhibited *siz1* (*siz1–2* and *siz1–3*) seed germination, as well as cotyledonary and primary root expansion, relative to wild type (Fig. 1*A*). *siz1* seeds were hypersensitive to ABA at all concentrations evaluated (0.1–5 μM) [Fig. 1*A* and *B*; supporting information (SI) Fig. S1]. At the highest ABA levels evaluated ($\geq$ 2.5 μM), a percentage of *siz1* seeds failed to germinate during the experimental interval (Fig. S1), as well as for varying periods thereafter (data not shown). Wild-type seed germination, although delayed by ABA, was maximal (nearly 100%) within 6 days after sowing (Fig. S1).

ABA inhibited primary root growth of *siz1* seedlings relative to that of wild-type seedlings (Fig. 1*C* and *D*). Expression of the wild-type allele *ProCaMV35S::SIZ1:GFP* suppressed the ABA hypersensitivity of *siz1–2* seedlings (Fig. 1*D*), confirming that *SIZ1* contributes to ABA responsiveness of seedlings. *SIZ1* transcript abundance in these transgenic lines was comparable to that of wild type (Fig. S2). Together, these results implicate *SIZ1* as a negative regulator of ABA function in seeds and seedlings.

siz1 Enhances ABA-Induced Gene Expression.
ABA signaling results in activation of transcription factors that interact with ABRE *cis* elements and induce expression of genes associated with germination and dehydration responses to germination (13). Consequently, expression of ABA-responsive genes containing ABRE elements was evaluated in *siz1* and wild-type seedlings. ABA-induced *RD29A*, *RD29B*, *AtEm6*, *RAB18*, and *ADH1* expression was greater in *siz1* than in wild-type seedlings. The difference in relative expression level was greatest for *AtEm6* and *ADH* (Fig. 2). Interestingly, *ABI5* expression was similar in the *siz1* and wild-type seedlings (Fig. 2). These results directly implicate *SIZ1* as a negative regulator of ABA signaling, but through a mechanism that does not involve transcriptional regulation of *ABI5*.
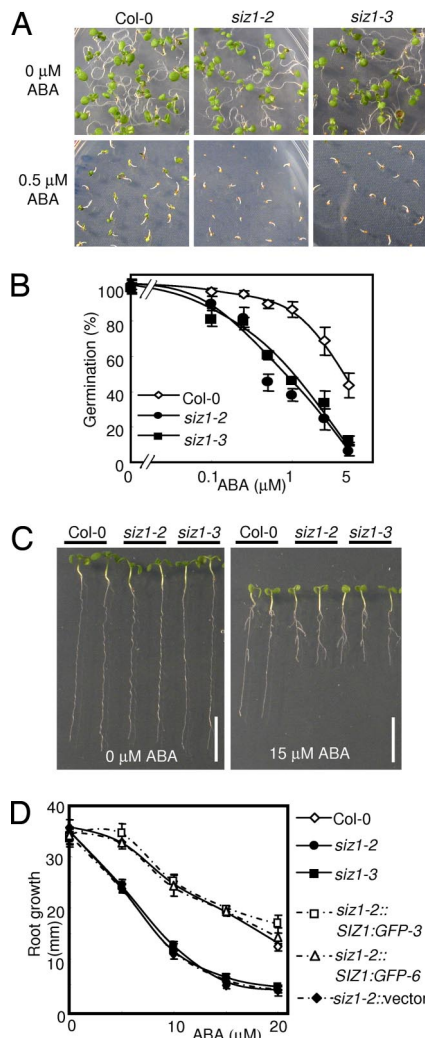

Fig. 1. *siz1* mutation increases ABA inhibition of seed germination and seedling primary root growth. Col-0 (wild type), *siz1–2*, and *siz1–3* seeds were sown, or seedlings were transferred onto medium without or supplemented with ABA. (*A*) A representative example of results 7 days after sowing. (*B*) Seed germination frequencies were determined 4 days after sowing for an average of 30–34 seeds from 5 independent experiments $\pm$ SE; a 100% germination frequency indicates that all seeds germinated. (*C* and *D*) Seedlings (3–1/2 days old) of equivalent size were transferred onto media, and primary root growth was determined after a 7-day growth period (root length at the end minus root length at the beginning of the growth period). (*C*) A representative example of the results. (Scale bar = 10 mm.) (*D*) Root growth values expressed as mean $\pm$ SE ($n \geq$ 15). *siz1–2::SIZ1:GFP-3* and *siz1–2::SIZ1:GFP-6* are representative lines illustrating that *SIZ1:GFP* was transformed into *siz1–2* and genetically complemented the mutation. The *siz1–2::vector* is the control line included for comparison.

Genetic Interaction Between *SIZ1* and *ABI5* or *AFP*.
Genetic interaction between *ABI5* and *SIZ1* was assessed by crossing *abi5–4* and *siz1–2* to produce the double mutant. F$_2$ progeny were genotyped for the presence of both *siz1–2* and *abi5–4*, and those homozygous at both loci were selected for evaluation. *abi5–4* suppressed ABA sensitivity of *siz1–2* for both seed germination (Fig. 3*A*) and seedling primary root growth (Fig. 3*B* and *C*). These results indicate that *ABI5* is genetically epistatic to *SIZ1*.

AFP negatively regulates ABA signaling by facilitating proteasome degradation of ABI5 (16). The *siz1–2 afp-1* double mutation caused additive ABA-hypersensitive seed germination and primary root growth phenotypes (Fig. 3). These results suggest that both SIZ1 and AFP are negative regulators of
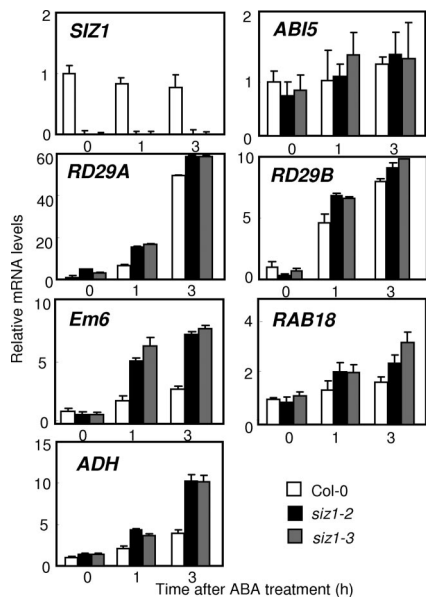
Fig. 2. *SIZ1* negatively regulates expression of ABA-responsive genes. Seven days after sowing seeds of wild type, *siz1–2*, and *siz1–3*, either water (0) or ABA (100 μM) was applied in a foliar spray. The seeds were then incubated for the times indicated. Relative mRNA levels were determined by quantitative RT-PCR analysis. Transcript levels of *SIZ1*, *ABI5*, *RD29A*, *RD29B*, *Em6*, *RAB18*, and *ADH* are illustrated. Data are mean $\pm$ SD (n = 3) from 1 representative experiment. Three independent experiments were performed; the results from each experiment exhibit similar relative trends. The gene expression was similar in the seedlings treated for 3 h with water and the seedlings without treatment (data not shown).

ABI5-dependent ABA signaling, but likely through independent mechanisms.

SIZ1 Mediates Sumoylation of ABI5. Because our results infer that *SIZ1* and *ABI5* are genetic interactors and that *ABI5* expression is not affected by *siz1*, we posited that SIZ1 negatively regulates ABA signaling through sumoylation of ABI5. SUMOplot (http://www.abgent.com/tool/sumoplot) predicted that ABI5 contains a sumoylation motif (ΨKXE) identifying K391 as the probable SUMO conjugation residue (28). SIZ1 facilitated SUMO1 conjugation to ABI5 in an in vitro assay (Fig. 4*A*); however, substitution of K391 by R blocked sumoylation, indicating that K391 is the residue to which SUMO1 is conjugated. SUMO1 and 2 are considered functionally redundant (29). *T7:SUMO1* and *HA:ABI5* or *HA:ABI5(K391R)* cDNAs were cotransformed into protoplasts isolated from wild-type or *siz1–2* plants. SUMO1 conjugation to ABI5 in wild-type protoplasts was unaffected by ABA (Fig. 4*B* and *C*). Neither ABI5-SUMO1 nor ABI5(K391R) conjugation product was detected in protein extracts isolated from *siz1–2* protoplasts, even though a substantial amount of protein was loaded onto the gel (Fig. 4*C*). Together, these results indicate that SIZ1 mediates sumoylation of ABI5 at residue K391. ABI5 was less abundant in the *siz1–2* seedlings than in the wild-type seedlings (Fig. 4*D*), supporting the notion that sumoylation of ABI5 may increase the stability of the protein; that is, the wild-type seedlings probably contain both sumoylated and unsumoylated ABI5.

Sumoylation of ABI5 Negatively Regulates ABA Responses. To investigate whether sumoylation of ABI5 affects plant responses to ABA, transgenic plants expressing $P_{CsV}:ABI5$ or $P_{CsV}:ABI5(K391R)$ in the *abi5–4* background were generated. Plants of independent T_4 and T_5 homozygous lines with equivalent expression of the respective transgenes were identified based on quantification of
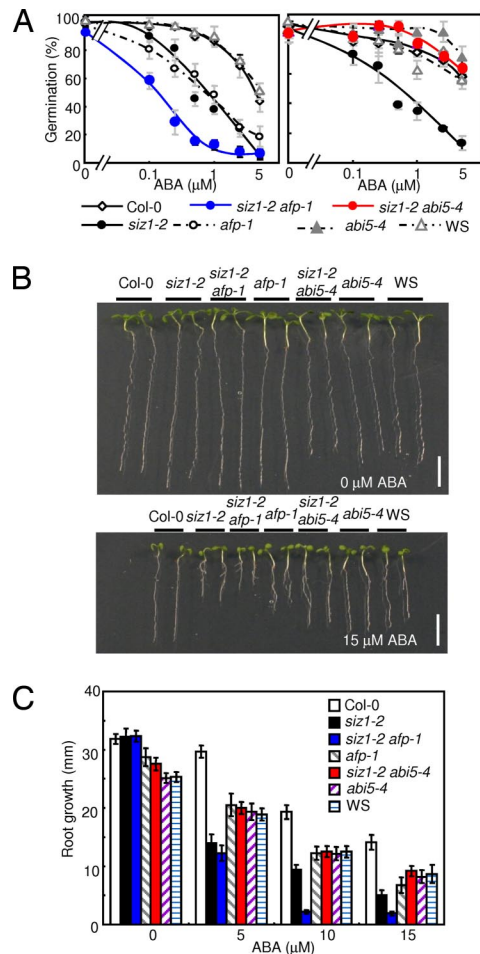

Fig. 3. ABA sensitivity caused by *siz1–2* is suppressed by *abi5–4* and enhanced by *afp-1*. (*A*) Germination frequencies of wild type (Col-0 and WS), *siz1–2 afp-1*, and *siz1–2 afp-1* seeds (*Left*) and wild type, *siz1–2*, *abi5–4*, and *siz1–2 abi5–4* seeds (*Right*) 4 days after sowing. Each value is the average of 30–34 seeds from 5 independent experiments ($\pm$ SE). (*B* and *C*) Primary root growth of 3-day-old seedlings that were transferred to medium without or with supplementation with 15 μM ABA. (*B*) Photographs showing representative results of seedlings 7 days after transfer. (Scale bar = 10 mm.) (*C*) Root growth values for seedlings 7 days after transfer (mean $\pm$ SE; $n \geq 12$) from 1 of 3 representative experiments. F_3 and F_4 seeds of *abi5–4 siz1–2* and *afp-1 siz1–2* were used in these experiments.

mRNA abundance (Fig. S3) and used for phenotypic evaluation of ABA responses. Expression of wild-type *ABI5* in *abi5–4* suppressed ABA insensitivity (Fig. 5*A* and *B*); that is, the seed germination and primary root growth responses to ABA of these plants were similar to those of wild-type plants. However, expression of *ABI5(K391R)* in *abi5–4* resulted in seed germination and primary root growth hypersensitivity to ABA (Fig. 5*A* and *B*). Expression of ABA-responsive genes (*RD29A*, *RD29B*, *AtEm6*, *RAB18*, and *ADH*) also was hypersensitive to ABA (Fig. 5*C*). The ABA sensitivity of these genes in *abi5–4* plants expressing *ABI5* was similar to that in wild-type (WS) plants (Fig. 5*C*). These results indicate that SUMO conjugation to ABI5 negatively regulates ABA signaling.

Discussion

Our results indicate that the *Arabidopsis* SUMO E3 ligase SIZ1 is a negative regulator of ABA signaling that inhibits germination, causes postgerminative primary root growth arrest (Fig. 1), and activates expression of ABA-responsive genes, such as *Em6*
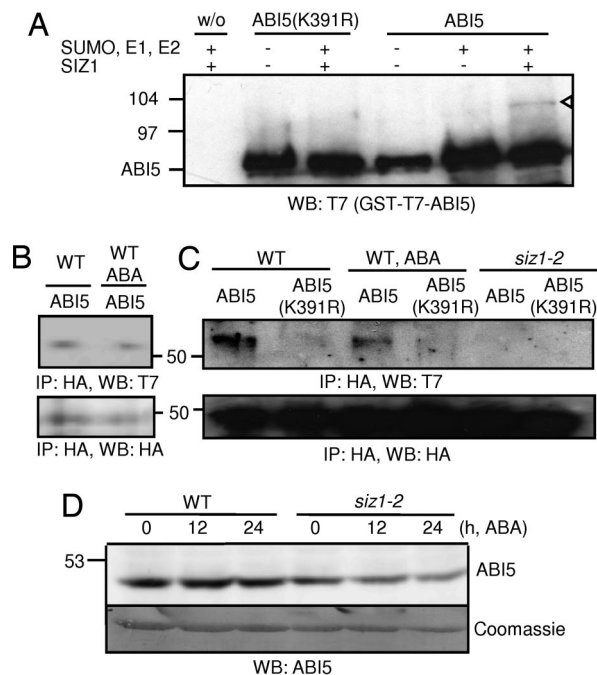
Fig. 4. SIZ1-mediated SUMO1 conjugation to ABI5 and ABI5 abundance in *siz1–2*. (*A*) In vitro sumoylation was performed using affinity-purified recombinant GST-T7-ABI5 or GST-T7-ABI5(K391R) as a substrate (21, 38). The reaction mixture contained *Arabidopsis* His-SAE1 (E1), His-SAE2 (E1), His-SCE1 (E2), GST-SIZ1 (E3), and His-SUMO1. ABI5 proteins were detected with anti-T7 (GST-T7-ABI5, ≈85 kDa). His-SUMO1 exhibited a ≈20-kDa protein (38); SUMO1-ABI5 conjugation was ≈105 kDa, as indicated by the arrowhead. No band was detected without substrates (w/o). (*B* and *C*) In vivo sumoylation was assessed after expression of T7-SUMO1 and HA-ABI5 or HA-ABI5(K391R) expression in *Arabidopsis* protoplasts (21). Soluble extracts from untreated or ABA-treated protoplasts (see *Materials and Methods*) were immunoprecipitated with anti-HA (IP: HA). Western blot analysis was performed with anti-T7 to detect T7-SUMO1-HA-ABI5 conjugates (WB: T7; *Top Panel*). To confirm the existence of equivalent levels of HA-ABI5 or HA-ABI5(K391R) in extracts, protein was detected using anti-HA (WB: HA; *Bottom Panel*). (*D*) ABI5 levels in wild-type or *siz1–2* plants as determined by Western blot analysis. Two-week-old seedlings were treated without (0, water) or with ABA for 12 or 24 h using a foliar spray application. ABI5 protein levels were analyzed with anti-ABI5. A nonspecific Coomassie blue–stained band is shown as a loading control.

and *ADH* (Fig. 2). Our genetic and biochemical data support the notion that SIZ1 regulates ABA signaling through sumoylation of the bZIP transcription factor ABI5 at K391 (Figs. 3 and 4). Expression of *ABI5(K391R)* in *abi5–4* plants enhances ABA signaling that inhibits seed germination, causes seedling primary root growth arrest, and transcriptionally activates ABA-responsive genes to a greater extent than expression of *ABI5* (Fig. 5). Thus, sumoylation of ABI5 at K391 is responsible for the negative regulation of ABA signaling. We posit that SIZ1-mediated sumoylation of ABI5 inactivates the transcription factor but protects the protein from proteasome degradation, which is facilitated by AFP and KEG (Fig. 6).

Sumoylation Protects ABI5 from Proteasome Degradation. Sumoylation and ubiquitination can interact competitively or cooperatively on the same substrate to regulate protein stability and function (30). SUMO conjugation to IκBα or NEMO competes for the same K residue, which, when ubiquitinated, results in proteasome degradation of the proteins (18, 30). The ABI5-binding protein AFP and the RING-type ubiquitin E3 ligase KEG act as negative regulators of ABA signaling, presumably through mechanisms resulting in ubiquitin-mediated degradation of ABI5 (16, 17). The 26S proteasome subunit RPN10 also is linked to degradation of ABI5 (31). It
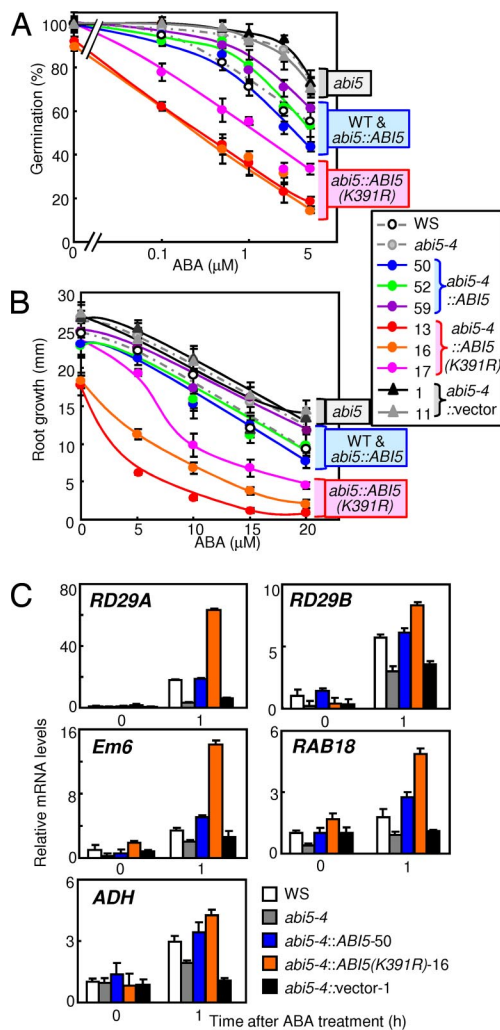

Fig. 5. *ABI5(K391R)* expression causes ABA hypersensitivity in *abi5–4* plants. Independent individual lines expressing *ABI5* or *ABI5(K391R)* in *abi5–4* were obtained. Plants of lines with similar transgene transcript abundances were identified (see Fig. S3). T$_4$ and T$_5$ progeny (homozygous) were used for phenotypic analyses. (*A* and *B*) Seed germination and primary root growth were measured as described in Fig. 1; 5 independent experiments ± SE (*A*) and the mean ± SE, $n \geq 15$ (*B*). (*C*) Transcript abundance of *RD29A*, *RD29B*, *Em6*, *RAB18*, and *ADH* was determined using quantitative PCR analysis of 1-week-old seedlings before (0 h) or 1 h after ABA treatment (100 μM by foliar spray). mRNA levels are expressed relative to transcript abundance in wild-type seedlings at 0 h. Data are mean ± SD (*n* = 3) from 1 representative data set of 3 individual experiments.

is feasible that SIZ1-mediated sumoylation of ABI5 protects the transcription factor from degradation, because less ABI5 was detected in the *siz1–2* plants (Fig. 4*D*).

The additive genetic interaction between *siz1–2* and *afp* (Fig. 3) suggests that SIZ1 and AFP regulate ABI5 through different mechanisms (Fig. 6). AFP and ABI5 colocalize to nuclear bodies, where the transcription factor is postulated to undergo proteasome degradation (16). Sumoylation of transcription factors facilitates recruitment into nuclear bodies (18), and SIZ1 is localized to nuclear foci (20). Thus, it is plausible that ABI5 may be compartmentalized into at least 2 different types of nuclear bodies, one of which is the site of AFP colocalization and ABI5 proteasome degradation. Compartmentalization of ABI5 into the alternative nuclear body is facilitated by SIZ1-mediated sumoylation and renders the transcription factor inactive, but not susceptible to proteolytic digestion.
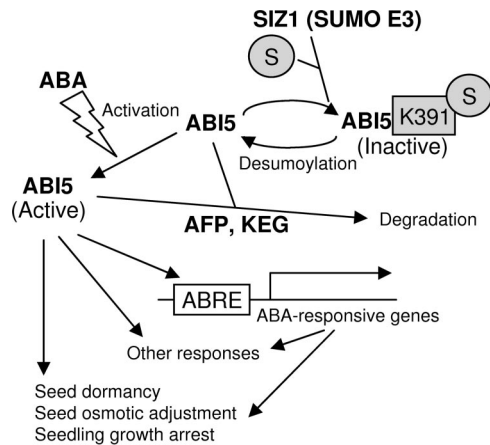
Fig. 6. A model illustrating how SIZ1 negatively regulates ABA signaling through sumoylation of ABI5. SIZ1-mediated sumoylation of ABI5 at K391 negatively regulates ABI5 activity and ABA signaling, which inhibits seed germination and seedling primary root growth (Figs. 1–5). Sumoylated ABI5 is inactive but presumably can be activated by desumoylation involving a SUMO protease. ABA activates desumoylated ABI5 by phosphorylation. AFP and KEG facilitate proteasome-dependent degradation of ABI5 (16, 17). It is posited that SIZ-mediated sumoylation regulates ABI5 activity in ABA signaling by facilitating the maintenance of an inactive form of the transcription factor that is not susceptible to proteolytic degradation.

Sumoylation and Phosphorylation in ABA Signaling. SUMO conjugation to substrate proteins regulates and is regulated by post-translational modifications that alter stability, localization, and activity of transcription factors (30). For instance, phosphorylation of c-Jun and p53 reduces sumoylation of these proteins (32), whereas phosphorylation of HSF1 and HSF4 enhances sumoylation (33). ABA induces phosphorylation of SnRK2 [SNF1 (sucrose nonfermenting 1)-related protein kinase 2] family members, activating the proteins (34). In turn, SnRK2.2 and SnRK2.3 phosphorylate ABI5 in response to ABA (35). The *snrk2.2 snrk2.3* double mutation makes seed germination and seedling primary root growth insensitive to ABA (35). These results indicate that ABI5 is phosphorylated as a response to ABA and infers that phosphorylation activates the transcription factor. SIZ1-mediated SUMO conjugation to ABI5 negatively affects ABA regulation of seed germination and seedling primary root growth. Consequently, it is plausible that sumoylation of ABI5 negatively affects activity by modulating protein phosphorylation.

Sumoylation/Desumoylation Plays an Important Role in Precise Regulation of ABI5 Activity by a Reversible Mechanism. Several reports have indicated that ABA signaling is tightly controlled and that ABI5 plays a central role in this signaling (9, 10). Based on our results, we hypothesize that ABI5 is sumoylated to make a pool of inactive ABI5, and that desumoylation is required to release ABI5 from inactive form (Fig. 6). This neutral ABI5 may be activated by phosphorylation after ABA treatment (16) to enhance the expression of ABA-responsive genes, which contain the ABRE *cis* element in their promoters. Such activation also may enhance seed dormancy, osmotic adjustment, and growth inhibition (Fig. 6). Without sumoylation, such as ABI5(K391R), cells cannot make an inactive pool. Because the ABI5 protein level was decreased in *siz1* (Fig. 4D), it is more likely that sumoylation protects ABI5 from degradation, thereby accelerating ABA-mediated inhibition of germination and postgermination growth (Fig. 5).

Because of the additive effects of *siz1–2 afp-1* (Fig. 3), sumoylation and AFP-facilitated degradation of ABI5 are differently, and perhaps competitively, regulated (Fig. 6). Unlike degradation, the sumoylation/desumoylation mechanism revers-

ibly regulates the transcription factor. SUMO modification may protect ABI5 from degradation and also may play a role in switching ABI5 activity to neutral (by desumoylation) or off (by sumoylation). This switching mechanism may be required for precise regulation of ABI5 activity (Fig. 6).

Materials and Methods

Plant Materials and ABA Treatment. The *Arabidopsis thaliana* genetic resources used in this study, *siz1–2*, *siz1–3* (Col-0 ecotype; 20), *abi5–4*, and *afp-1*, were kindly provided by Dr. N.H. Chua [Wassilewskija (WS) ecotype; see ref. 16]. F$_3$ and F$_4$ homozygous double mutants were obtained by crossing *siz1–2* (male) to *abi5–4* or *afp-1* mutants (female). Diagnostic PCR analyses were performed to identify the *siz1–2* mutation, as described previously (20), and to identify the *abi5–4* and *afp-1* mutations, as described previously (11, 16). T5 homozygous *siz1–2::SIZ1:GFP* transgenic plants were used for the experiments.

ABI5- or *ABI5(K391R)*-expressing transgenic plants in the *abi5–4* background were obtained by *Agrobacterium*-mediated floral transformation, as described previously (21). The *ABI5* or *ABI5(K391R)* coding region, amplified with primers ABI5-BinaF and ABI5-EGR (Table S1), was inserted into the binary vector pCsV1300, the expression of which is driven by the cassava vein mosaic virus promoter (CsV) (36). The abundance of transgene was detected by RT-PCR using the primers ABI5K391RF and NOS-transR (Fig. S3).

The seeds were surface-sterilized and then kept in the dark for 3 days at 4 °C to break dormancy. The seeds were sowed onto Murashige and Skoog (MS) medium containing 0.8% agar. Germination frequencies (i.e., percentage of seeds sown) were obtained by scoring radicle emergence (*n* = 5; 30–34 seeds per plates; 3 times). Note that 100% germination means that all seeds germinated for all genotypes. To investigate the inhibition of root growth by ABA, 3.5-day-old seedlings were transferred onto plates supplemented with ABA (Sigma). Root growth is measured as the difference in root length between the beginning and the end of the growth evaluation period.

Quantitative RT-PCR. ABA was applied as an aqueous foliar spray (100 μM ABA in water) onto seedlings grown on agar medium for 7 days after sowing (37). About 3 mL of ABA solution was sprayed onto each plate, after which the seedlings were incubated for 1 h or 3 h. Seedlings were harvested before and 1 h and 3 h after ABA application. Total RNA from 1-week-old plants was isolated using TRIzol reagents (Invitrogen) according to the manufacturer's protocol. A 3-μg RNA template was used for first-strand cDNA synthesis performed using SuperScript II reverse transcriptase (Invitrogen) with an oligo(dT$_{21}$) primer. Primer pairs for quantitative PCR (Table S2) were designed, and PCR was performed as described previously (20, 21).

Purification of Recombinant Proteins and in Vitro and in Vivo SUMO Conjugation Assays. The *ABI5* ORF was amplified from the cDNA clone (U85657, obtained from the Arabidopsis Biological Resource Center) with primers ABI5-T7F and ABI5-expR (Table S1). The PCR product encoding wild-type *ABI5* (pGST-T7-ABI5) or a mutated *ABI5(K391R)* [AA$_{1172}$A to AGA by site-directed mutagenesis with primers *ABI5K391RF* and *ABI5K391RR*; pGST-T7-ABI5(K391R)] was inserted into pGEX-5X-T7 (21). Recombinant proteins from pGST-T7-ABI5, pGST-T7-ABI5(K391R), pGST-SIZ1 (22), or other expression vectors (kindly provided by Dr. H.-P. Stuible) were prepared as described previously (21, 38). In vitro sumoylation assays were performed as described previously (38), but modified by adding 2 μg of GST-SIZ1 and 0.1 μg of E2 enzyme (instead of 2 μg of E2) to the assay mixture. Immunoblot analyses with anti-T7 antibody (Novagen) were performed to detect GST-T7-ABI5 or GST-T7-ABI5(K391R).

For transient expression in *Arabidopsis* protoplasts, the *ABI5* or *ABI5(K391R)* coding region, amplified with primers ABI5-HAF and ABI5-expR (Table S2), was inserted into the plasmid p326-HAN (21) to produce the HA-ABI5 or HA-ABI5(K391R) fusion protein driven by the 35S promoter. *T7:SUMO1* (21) and *HA:ABI5* or *HA:ABI5(K391R)* were coexpressed in wild-type or *siz1–2* protoplasts (21). After incubation at 23 °C for 42 h, the protoplasts were incubated with or without ABA (at a final concentration of 40 μM) at 23 °C for 1 h. Soluble extracts were immunoprecipitated with agarose-immobilized goat anti-HA (QED Bioscience). Immunoblot analyses were performed with anti-HA (Santa Cruz Biotechnology) or anti-T7 (Novagen).

Immunoblot Analysis. Two-week-old seedlings were treated with ABA by a foliar spray and incubated for 12 and 24 h. Samples were prepared as described previously (20). After 30 μg of protein was loaded onto an SDS/PAGE gel, immunoblot analysis with anti-ABI5 antibody (kindly provided by Dr. L. Lopez-Molina; see ref. 39) was performed.

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