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

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

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Paul M. Hasegawa
Abstract

SUMO (small ubiquitin-related modifier) conjugation (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 that 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. Further, expression of genes that are ABA-responsive through ABI5-dependent signaling (e.g. RD29A, Rd29B, AtEm6, RAB18 and ADH1) was hyper-induced by the hormone in siz1 seedlings. abi5-4 suppressed ABA hypersensitivity caused by siz1 (siz1-2 abi5-4), revealing 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. ABI5(K391R) expression in abi5-4 plants caused greater ABA hypersensitivity (gene expression, seed germination arrest and primary root growth inhibition) than ABI5 expression in abi5-4. 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.

Introduction

The phytohormone abscisic acid (ABA) regulates numerous processes including those 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 (2), 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. Further, ABA enhances seed desiccation tolerance by inducing expression of genes encoding effectors that provide hyper-osmotic protection as embryos dehydrate in the later maturation stages. Water deficit induces ABA biosynthesis in stratified seed leads to germination arrest 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 receptor2) and GTG1 and GTG2 (GPCR-like G proteins) (4-7). GTG1 and GTG2 are predicted to have GPCR topology and exhibited GTP-binding and GTPase activities (7). These proteins are hypothesized to be membrane-localized ABA receptors that are posited to be involved in G protein-mediated transduction of the hormonal signal (7).

Among the numerous effectors of ABA signal transduction identified to date are the ABI (ABA-insensitive) determinants 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, while ABI3, ABI4 and ABI5 encode B3, APETALA2-like, and basic leucine zipper (bZIP) domain containing transcription factors, respectively (9-11).

ABI3 and ABI5 function as intermediates in ABA signaling that regulate seed maturation and germination, and expression of genes that facilitate desiccation tolerance as embryos dehydrate during later stages of maturation (11-13). Further, ABA or hyper-osmotic stress of seeds during stratification induces biosynthesis of the hormone that is regulated by ABI3 and ABI5 (11, 12). ABI5 expression suppressed ABA insensitivity of seed germination caused by abi3 (12) and overexpression of ABI3 enhanced 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) and RD29B/LTI65, RAB18 (response to ABA), and ADH (alcohol dehydrogenase) (3, 13). Chromosomal immunoprecipitation analysis revealed that ABA stimulates association of ABI5 with the AtEm6
promoter, which is presumably necessary for transactivation (12). Recent evidence 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 (ABI five binding protein) facilitated ABI5 proteasome degradation (16) that was linked with the RING finger ubiquitin E3 ligase KEG (Keep On Going) (17). An abi5 mutation suppressed ABA-hypersensitivity of afp-1 and keg mutants (16, 17). These results indicate that AFP and KEG are negative regulators of ABA signaling, acting through the degradation of ABI5 (16, 17).

Sumoylation regulates diverse biological processes such as 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 super family 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 19). SIZ1 (SAP and Miz) is the principal SUMO E3 ligase in Arabidopsis and 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 that resulted 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 modulated 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 prevents 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 seedling primary root growth inhibition (8). ABA (0.5 µM) inhibited siz1 (siz1-2 and siz1-3) seed germination, and cotyledonary and primary root expansion relative to wild-type (Fig. 1A). siz1 seeds were hypersensitive to ABA at all concentrations evaluated (0.1 to 5 µM) (Fig. 1A, 1B, and Supplemental Fig. S1). At the highest ABA levels evaluated (≥2.5 µM), a percentage of siz1 seeds failed to germinate during the experimental interval (Supplemental Fig. S1) and for periods thereafter (not shown). Wild-type seed germination, although delayed by ABA, was maximal (nearly 100%) within six days after sowing (Supplemental Fig. S1).

ABA inhibited primary root growth of siz1 seedlings relative to that of wild-type seedlings (Fig. 1C and D). Expression of the wild-type allele ProCaMV35S:SIZ1:GFP suppressed ABA hypersensitivity of siz1-2 seedlings (Fig. 1D), confirming that SIZ1 contributes to ABA responsiveness of seedlings. SIZ1 transcript abundance in these transgenic lines was
comparable to that of wild type (Supplemental 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 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 relative expression level difference was greatest for AtEm6 and ADH (Fig. 2). Interestingly, ABI5 expression was similar in 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.

**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. F2 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. 3A) and seedling primary root growth (Fig. 3B and C). These results indicate that ABI5 is genetically epistatic to SIZ1.

AFP negatively regulates ABA signaling by facilitating proteasome degradation of ABI5 (16). 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 ABI5-dependent ABA signaling but likely through independent mechanisms.
**SIZ1 mediates sumoylation of ABI5**

Since the results inferred 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 one sumoylation motif (ΨKXE; 28) identifying K391 as the probable SUMO conjugation residue. SIZ1 facilitated SUMO1 conjugation to ABI5 in an *in vitro* assay (Fig. 4A). However, substitution of K391 by R blocked sumoylation (Fig. 4A), indicating that K391 is the residue to which SUMO1 is conjugated. SUMO1 and 2 are considered to be 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. 4B and 4C). 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. 4C). Together, these results indicate that SIZ1 mediates sumoylation of ABI5 at residue K391. ABI5 was less abundant in *siz1-2* than in wild-type seedlings (Fig. 4D), supporting the notion that sumoylation of ABI5 may increase stability of the protein, i.e. wild type probably contains 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_{Cry}:ABI5* or *P_{Cry}:ABI5(K391R)* in the *abi5-4* background were generated. Plants of independent T₄ and T₅ homozygous lines were identified with equivalent expression of the respective transgene based on quantification of mRNA abundance (Supplemental Fig. S3). These plants were used for phenotypic evaluation of ABA responses. Expression of wild type
ABI5 in abi5-4 suppressed ABA insensitivity (Fig. 5A and 5B). That is, the seed germination and primary root growth responses to ABA of these plants were the same as those of wild-type plants (Fig. 5A and 5B). However, expression of ABI5(K391R) in abi5-4 resulted in seed germination and primary root growth hypersensitivity to ABA (Fig. 5A and 5B). Expression of ABA-responsive genes (RD29A, RD29B, AtEm6, RAB18, and ADH) was also hypersensitive to ABA (Fig. 5C). ABA sensitivity of these genes in abi5-4 plants expressing ABI5 was similar to that in wild-type (WS) plants (Fig. 5C). These results indicate that SUMO conjugation to ABI5 negatively regulates ABA signaling.

Discussion

A conclusion from the presented evidence is that the Arabidopsis SUMO E3 ligase SIZ1 is a negative regulator of ABA signaling that inhibits germination, causes post-germinative primary root growth arrest (Fig. 1), and activates expression of ABA responsive genes such as Em6 and ADH (Fig. 2). Genetic and biochemical data support the notion that SIZ1 regulates ABA signaling through sumoylation of the bZIP transcription factor ABI5 at K391 (Fig. 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 negative regulation of ABA signaling. We posit that SIZ1-mediated sumoylation of ABI5 inactivates the transcription factor but protects the protein from proteasome degradation that 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 that when ubiquitinated results in proteasome degradation of the proteins (18, 30). AFP, ABI5 binding protein, and KEG, RING-type ubiquitin E3 ligase, are 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 is feasible that SIZ1-mediated sumoylation of ABI5 protects the transcription factor from degradation, as a lower abundance of ABI5 was detected in siz1-2 plants (Fig. 4D).

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 co-localize to nuclear bodies, where it is postulated that the transcription factor undergoes proteasome degradation (16). Sumoylation of transcription factors facilitates recruitment into nuclear bodies (18) and SIZ1 is localized to nuclear foci (20). It is thus plausible that ABI5 is compartmentalized into, at least, two different types of nuclear bodies. One nuclear body type is where AFP co-localizes and in which proteasome degradation of ABI5 occurs. 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.

**Sumoylation and Phosphorylation in ABA Signaling**

SUMO conjugation to substrate proteins regulates and is regulated by posttranslational 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 that
activates 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 phosphorylation of the protein.

**Sumoylation/Desumoylation Plays an Important Role in Precise Regulation of ABI5 Activity by a Reversible Mechanism**

Several reports demonstrate 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 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 expression of ABA-responsive genes, which contain the ABRE *cis*-element in their promoter. Such activation may also enhance seed dormancy, osmotic adjustment, and growth inhibition (Fig. 6). Without sumoylation, such as ABI5(K391R), cells could not make an inactive pool. As ABI5 protein level was decreased in *siz1* (Fig. 4D), it is more likely that sumoylation protects ABI5 from degradation. Therefore, ABA-mediated inhibition of germination and postgerminative growth was accelerated (Fig. 5).

Because *siz1-2 afp-1* showed additive effects (Fig. 3), sumoylation and AFP-facilitated degradation of ABI5 are differently, and perhaps competitively, regulated (Fig. 6). Unlike degradation, the sumoylation/desumoylation mechanism reversibly regulates the transcription
factor. SUMO modification may protect ABI5 from degradation and play a role in switching ABI5 activity 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. Arabidopsis thaliana genetic resources for this research were siz1-2, siz1-3 (Col-0 ecotype; 20), and abi5-4 and afp-1, which were kindly provided by Dr. NH Chua (Wassilewskija (WS) ecotype; 16). F3 and F4 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 (20), and to identify abi5-4 and afp-1 mutations as described previously (11, 16). T5 homozygous siz1-2::SIZ1:GFP transgenic plants were used for experiments.

ABI5 or ABI5(K391R)-expressing transgenic plants in the abi5-4 background were obtained by Agrobacterium-mediated floral transformation as described (21). The ABI5 or ABI5(K391R) coding region, amplified with primers ABI5-BinaF and ABI5-EGR (Supplemental Table 2), was inserted into the binary vector pCsV1300 (expression is driven by cassava vein mosaic virus promoter, CsV) (36). The abundance of transgene was detected by RT-PCR with the primers ABI5K391RF and NOS-transR (Supplemental Fig. S3).

Seeds were surface sterilized and then kept for 3 days in the dark at 4°C to break dormancy. Thereafter, seeds were sowed onto Murashige and Skoog (MS) medium containing 0.8% agar. Germination (% of seeds sown) frequencies were obtained by scoring radicle emergence (n = 5, 30-34 seeds per plates, three times). Note that 100% germination means that all seeds germinated for all genotypes. To investigate inhibition of root growth by ABA,
3.5-day-old seedlings were transferred onto plates supplemented with ABA (Sigma). Root growth is the root length difference at the beginning and 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, and then seedlings were incubated for 1 or 3 h. Seedlings were harvested prior to and one and three hours after ABA application. Total RNA from one-week-old plants was isolated using TRIZOL reagents (Invitrogen) according to the manufacture’s protocol. Three micrograms of RNA were used as a template for first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) with an oligo(dT21) primer. Primer pairs for quantitative PCR (Supplemental Table 1) were designed and PCR was performed as described (20, 21).

**Purification of Recombinant Proteins and In Vitro and In Vivo SUMO Conjugation Assays.**

The *ABI5* open reading frame was amplified from the cDNA clone (U85657 obtained from Arabidopsis Biological Resource Center) with primers ABI5-T7F and ABI5-expR (Supplemental Table 2). The PCR product encoding wild-type *ABI5* (pGST-T7-ABI5) or a mutated *ABI5(K391R)* [AA1172A 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 (21, 38). In vitro sumoylation assays were performed as described (38) with the modifications that 2 μg of GST-SIZ1 and 0.1 μg of E2 enzyme (instead of 2 μg of E2) were added to the assay mixture. Immunoblot analysis with anti-T7 antibody (Novagen) was 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 (Supplemental Table 2), 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 co-expressed in wild-type or *siz1-2* protoplasts (21). After incubation at 23°C for 42 h, then protoplasts were incubated with or without ABA (40 μM final concentration) at 23°C for 1 h. Soluble extracts were immunoprecipitated with Agarose Immobilized Goat anti-HA (QED Bioscience Inc). Immunoblot analysis was performed with anti-HA (Santa Cruz Biotechnology, Inc) 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 (20). Thirty micrograms of protein was loaded onto an SDS-PAGE gel, and immunoblot analysis with anti-ABI5 antibody (kindly provided by Dr. L Lopez-Molina; 39) was performed.

**Acknowledgements**

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**References**


**Figure Legends**

Fig. 1. *siz1* mutation increases ABA inhibition of seed germination and seedling primary root growth. Col-0 (wild type), *siz1*-2, or *siz1*-3 seeds were sown, or seedlings transferred onto medium without or supplemented with ABA. (A) The illustration is a representative example of results seven days after sowing. (B) Seed germination frequencies were determined four days after sowing for an average of 30-34 seeds from five independent experiments ± standard error
(SE). A 100% germination frequency is indicative that all seeds germinated. (C, D) Three and half-day-old seedlings of equivalent size were transferred onto media and primary root growth was determined after a seven-day growth period (root length at the end minus root length at the beginning of the growth period). (C) A representative example of results is illustrated, bars = 10 mm. (D) Each root growth value is the mean ± 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; $siz1-2::$vector is the control line for comparison.

Fig. 2. $SIZ1$ negatively regulates expression of ABA-responsive genes. Seven days after sowing seeds of wild type, $siz1-2$, or $siz1-3$; water (0) or ABA (100 μM) was applied as foliar spray application. Seeds were then incubated for the time 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 ± SD ($n = 3$) from one representative experiment. Three independent experiments were performed and results from each exhibited similar relative trends. Expression of genes in seedlings treated for 3 h with water was similar to that from seedlings without treatment (data not shown).

Fig. 3. ABA sensitivity caused by $siz1-2$ is suppressed by $abi5-4$ and enhanced by $afp-1$. (A) Illustrated are germination frequencies of wild-type (Col-0 and WS), $siz1-2$, $afp-1$ or $siz1-2 afp-1$ seeds (left), or wild-type, $siz1-2$, $abi5-4$ or $siz1-2 abi5-4$ seeds (right) four days after sowing. Each value is the average of 30-34 seeds from five independent experiments, ± SE. (B, C) Primary root growth of three-day-old seedlings that were transferred to medium without or
supplemented with 15 μM ABA. (B) Photographs illustrate representative results of seedlings seven days after transfer, bars = 10 mm. (C) Each root growth value is for seedlings seven days after transfer (mean ± SE), n ≥ 12 from one of three representative experiments. F_{3} and F_{4} seeds of abi5-4 siz1-2 and afp-1 siz1-2 were used in these experiments.

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, 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 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 there were 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 determined by western blot analysis. Two-week-old seedlings were treated without (0, water) or with ABA for 12 or 24 h by 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.

Fig. 5. ABI5(K391R) expression causes ABA hyper-sensitivity in abi5-4 plants. Independent
individual lines expressing \textit{ABI5} or \textit{ABI5(K391R)} in \textit{abi5-4} were obtained. Plants of lines with similar transgene transcript abundances were identified (Supplemental Fig. S3). \textsc{T}_4 and \textsc{T}_5 progeny (homozygous) were used for phenotypic analyses. (A, B) Seed germination and primary root growth were measured as described in Fig. 1; five independent experiments ± SE (A) and the mean ± SE, \(n \geq 15\) (B). (C) Transcript abundance of \textit{RD29A}, \textit{RD29B}, \textit{Em6}, \textit{RAB18}, and \textit{ADH} was determined using quantitative PCR analysis of one-week-old seedlings prior to (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 one representative data of three individual experiments.

Fig. 6. The model illustrates how \textit{SIZ1} negatively regulates ABA signaling through sumoylation of \textit{ABI5}. \textit{SIZ1}-mediated sumoylation of \textit{ABI5} at K391 negatively regulates \textit{ABI5} activity and ABA signaling that inhibits seed germination and seedling primary root growth (Fig. 1-5). Sumoylated \textit{ABI5} that is inactive but is presumed to be activated by desumoylation involving a SUMO protease. ABA activates desumoylated \textit{ABI5} by phosphorylation. AFP and KEG facilitate proteasome-dependent degradation of \textit{ABI5} (16, 17). It is posited that \textit{SIZ}-mediated sumoylation regulates \textit{ABI5} activity in ABA signaling by facilitating the maintenance of an inactive form of the transcription factor that is not susceptible to proteolytic degradation.
Fig. 1. Miura et al.

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Fig. 2. Miura et al.
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Fig. 3. Miura et al. 
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Fig. 4. Miura et al.
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Fig. 5. Miura et al.
(1 column x 18cm)
Fig. 6. Miura et al.
(1 column x 7cm)
Supplemental Fig. S1. *siz1* mutation causes hypersensitivity to ABA-mediated inhibition of seed germination. Germination frequency of wild type (Col-0), *siz1-2*, and *siz1-3* seeds was determined on medium without (0) or supplemented with 0.5, or 2.5 mM ABA. Each value is the average of 30-34 seeds from 5 independent experiments, ± SE.
Supplemental Fig. S2. Illustrated is SIZ1 transcript abundance in Col-0 (wild type), siz1-2, two independent siz1-2 lines transformed with SIZ1:GFP. Expression levels of SIZ1 in transgenic lines expressing the wild-type allele ProCaMV35S:SIZ1:GFP in siz1-2 were similar to that of wild type.
Supplemental Fig. S3. Illustrated is *ABI5* or *ABI5(K391R)* transcript abundance in independently transformed *abi5-4* lines (T4 generation). (A) Semi-quantitative RT-PCR analysis was performed using primers ABI5K391RF and NOS-transR that detects mRNA produced by the transgene but not native *ABI5* (top panel); compare with lanes indicating vector in *abi5-4*, WS, and *abi5-4*. Numbers above columns indicate the independent transgenic line number; those circled identify lines that were used for phenotypic and gene expression analyses. (B) Illustrated is the diagram of the binary vector for expression of *ABI5* or *ABI5(K391R)*. *ABI5* or *ABI5(K391R)* cDNA expression, which was driven by the CsV (cassava vein mosaic virus) promoter. Arrowheads indicate the locations at which primers (ABI5K391RF and NOS-transR) annealed for PCR amplification.
Table 1. Primer Sequences Used to Detect SIZ1 or Genes Involved in ABA Signaling by the Quantitative PCR Method

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<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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<tr>
<td>SIZ1</td>
<td>5'-ATAGCGGCCCTGGAATCAT-3'</td>
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<td>5'-GCCCTTGCTTTGTACTGTCATTTCATAC-3'</td>
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<td>ABI5</td>
<td>5'-GAGAATGCGCAGCTAAAACA-3'</td>
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<td>5'-GTGGACAACTCGGGTTTCTC-3'</td>
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<td>RD29A</td>
<td>5'-CTTGATGTTCAACGGAAAGGT-3'</td>
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<td>5'-CAATCTCCGGTACTCTCTCCA-3'</td>
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<td>Em6</td>
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<td>5'-TTAAGTGCTTGGGCTCTGAAATTG-3'</td>
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<td>RAB18</td>
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<td>5'-ACGGGAAAGCTTTCTCTGATC-3'</td>
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<td>ADH</td>
<td>5'-ATCAATCCGAGCTCCT-3'</td>
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<td>5'-GCCACACGGATTCTAGCACC-3'</td>
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Table 2. Primer Sequences Used for Construction of Vectors.

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<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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<tr>
<td>ABI5-T7F</td>
<td>5'-CGGAATTCATGGTAACTAGAGAAACGAA-3'</td>
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<td>ABI5-expR</td>
<td>5'-ATTCTCGAGTTAGAGGGTGGACAGTACCGGG-3'</td>
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<td>ABI5K391RF</td>
<td>5'-GTGGAGAAGAAGAATGCAGC-3'</td>
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<td>ABI5K391RR</td>
<td>5'-CGGGATCCCTTACCTCTGA-3'</td>
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<td>ABI5-HAF</td>
<td>5'-CGGGATCCCATGATGGTAACTAGAGAAGA-3'</td>
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<td>ABI5-BinaF</td>
<td>5'-CGGGATACGTGTTAAGTAGAGAAGA-3'</td>
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<td>ABI5-EGR</td>
<td>5'-CGGGATCTGTTAGGTGACAACCTC-3'</td>
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<tr>
<td>NOS-transR</td>
<td>5'-GCCAATGTTGGAACGATCGGGA-3'</td>
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