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SIZ1 Controls Cell Growth and Plant Development in Arabidopsis Through Salicylic Acid

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Regular Paper

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The post-translational conjugation of small ubiquitin-related modifiers (SUMOs) to other proteins is involved in regulation of many processes in eukaryotic development; although its role in plant development is beginning to be dissected. Previously, we demonstrated that the *siz1* mutant, which is impaired in SUMO E3 ligase, showed a dwarf-like shoot phenotype with accumulation of salicylic acid (SA), and the expression of *nahG*, a bacterial salicylate hydroxylase that catabolizes SA, in *siz1* reduced the SA level and suppressed dwarfism. Herein, we provide evidence that the *SIZ1* gene controls cell division and elongation through regulation of the SA level. Mature *siz1-2* and *siz1-3* plants exhibited a dwarf-like shoot phenotype that is attributable to decreased leaf cell volume and number relative to the wild type. Cell division and expansion defects caused by *siz1* were also suppressed by the expression of *nahG*. Expression of *XTH8* and *XTH31*, encoding xyloglucan endotransglycosylase/hydrolase, which are thought to facilitate leaf cell expansion, was down-regulated in *siz1* leaves. However, reduced *XTH8* and *XTH31* expression in *siz1* plants was restored in *nahG siz1-2* plants. These results indicate that *SIZ1* regulates cell growth and plant development with regulation of SA accumulation. Also, *XTH8* and *XTH31* genes may be responsible for reduced leaf cell expansion.

Keywords: *Arabidopsis thaliana* • Cell division and expansion • Post-translational modification • Salicylic acid • SUMO • Sumoylation.

Abbreviations: BR, brassinolide; GA, gibberellic acid; HPY2, high ploidy 2; IAA, indole-3-acetic acid; MMS21, methyl methansulfonate sensitive 21; PIAS, protein inhibitor of activated STAT; RT–PCR, reverse transcription-PCR; SA, salicylic acid; SAE, SUMO activation enzyme; SCE, SUMO conjugating enzyme; SIZ1, SAP and MIZ domain protein; SUMO, small ubiquitin-related modifier; XTH, xyloglucan endotransglycosylase/hydrolase.

Introduction

SUMO (small ubiquitin-related modifier) conjugation to a protein substrate (sumoylation) in plants and other organisms requires the sequential function of the E1 activation enzyme (SAE), the E2 conjugation enzyme (SCE) and the E3 ligase (Kurepa et al. 2003, Colby et al. 2006, Miura et al. 2007a). Deconjugation of SUMO from target proteins is carried out by ubiquitin-like cysteine proteases (Miura et al. 2007a). In Arabidopsis, it appears that *SUMO1* and *SUMO2* are functionally redundant genes, *SAE2* and *SCE* are essential, and SUMO proteases are encoded by multiple genes (Miura et al. 2007a). Two functional SUMO E3 ligases, SIZ1 and HPY2/MMS21, have been identified (Miura et al. 2005, Ishida et al. 2009, Huang et al. 2009). SIZ1 is an ortholog of SIZ/PIAS-type (SAP and MIZ/protein inhibitor of activated STAT) E3 ligases (Miura et al. 2005) and HPY2 (high ploidy 2) is an ortholog of MMS21/NSE2-type (methyl methanesulfonate-sensitive 21/non-SMC-element 2) SUMO E3 ligases (Huang et al. 2009, Ishida et al. 2009). RanBP2 (Ran-binding protein 2), Pc2 (polycomb 2), the PHD domain of the KAP1 co-repressor, Topors and HDAC4 (histone deacetylase 4) SUMO E3 ligases (Geiss-Friedlander and Melchior 2007) have yet to be identified in plants.

Studies indicate that sumoylation functions in plant development (Murtas et al. 2003, Lee et al. 2007, Saracco et al. 2007, Jin et al. 2008) and in stress responses such as those that occur because of phosphate deficiency, salt, high and low temperature, and drought stresses (Kurepa et al. 2003, Lois et al. 2003, Miura et al. 2005, Yoo et al. 2006, Catala et al. 2007, Miura et al. 2007b, Conti et al. 2008; Miura and Hasegawa 2008). Many of these processes were linked to sumoylation directly through the SIZ1 SUMO E3 ligase (Miura et al. 2007a). In addition, SIZ1 regulates abscisic acid and salicylic acid (SA) signaling (Lois et al. 2003, Lee et al. 2007, Miura and Ohta 2009, Miura and Hasegawa 2009; Miura et al. 2009). *siz1* resulted in constitutive plant defense against plant pathogens through an SA-mediated signaling pathway (Lee et al. 2007).

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SA plays a crucial role in plant defense and is generally involved in the activation of defense responses against biotrophic pathogens as well as the establishment of systemic acquired resistance (Grant and Lamb 2006). Plants treated with exogenous SA or mutants accumulating more SA are more resistant to viral and fungal infection compared with wild-type plants without SA treatment (Bari and Jones 2009). Interestingly, accumulation of SA also causes plant morphological defects. Arabidopsis mutants which are constitutively resistant to pathogens due to SA accumulation exhibit altered leaf shape, reduced height and/or spontaneous cell death (Rate et al. 1999, Shah et al. 1999, Rate and Greenberg 2001, Vanacker et al. 2001, Suarez-Rodriguez et al. 2007, Zhang et al. 2007). *agd2-1* (for aberrant growth and death2) that accumulated SA showed resistance to *Pseudomonas syringae* and dwarfism (Rate and Greenberg 2001). *syp121-1 syp122-1* (syntaxin) double mutation increases SA, resulting in dwarfism, to defend against *P. syringae* (Zhang et al. 2007). The constitutive pathogen response 5, *cpr5*, mutant also exhibits SA accumulation, resistance to pathogen and plant dwarfism caused by reduction of cell size (Kirik et al. 2001). These findings suggest that plant innate immunity is linked to regulation of cellular growth and plant development.

Sumoylation has been implicated in cell cycle regulation that affects eukaryotic development (Watts 2004). *Drosophila* PIAS protein is essential for viability, and is required for proper chromosome structure and chromosome inheritance (Hari et al. 2001). Mutations to *SAE2*, *SCE1* or both *SUMO1* and *SUMO2* are lethal, causing growth arrest early in Arabidopsis embryogenesis (Saracco et al. 2007). Arabidopsis *SIZ1* is expressed in leaf blades, petioles, primary and lateral root tips, inflorescence stems, sepals, stamen filaments and stigma, but not in part of the hypocotyls and basal region of developing young leaves (Catala et al. 2007). *SIZ1* is also necessary for vegetative growth and development (Catala et al. 2007), although the mechanisms have not been elucidated. Here we present evidence that establishes SIZ1 function in cell division and expansion through regulation of SA accumulation. *siz1-2* and *siz1-3* mutations caused defects in leaf cell division and expansion, resulting in formation of smaller leaves and shoot dwarfism. As described previously (Lee et al. 2007), the *siz1* mutant showed a dwarf-like phenotype with accumulation of SA. In addition, expression of the bacterial *nahG* gene, encoding a salicylate hydroxylase that catalyzes the conversion of SA to catechol (Yamamoto et al. 1965), reduced SA accumulation (Lee et al. 2007). Here, we demonstrate that *nahG* also suppressed the cell division and expansion defects caused by *siz1* and resulted in normal plant growth and development. *nahG* expression in *siz1-2* plants enhanced, to wild-type levels, expression of *XTH8* and *XTH31* encoding xyloglucan endotransglycosylase/hydrolases (XTHs) that are thought to be involved in promoting leaf cell elongation. This study establishes that SIZ1 functions in vegetative growth and development by regulating leaf cell division and expansion through SA signaling that is associated with expression of *XTH* genes.

Results

Growth and vegetative development is reduced by siz1 mutations

Eight-week-old *siz1-2* and *siz1-3* plants exhibited substantially shorter stems (internodal elongation), reduced leaf area (**Fig. 1A**) and reduced floral organ size (**Fig. 1B–G**) than wild-type plants. However, floral organ composition and organization of *siz1* plants were similar to those of the wild type (**Fig. 1B–E**), although these organs were smaller. No embryo abortion or abnormality was detected (not shown). These dwarf phenotypes of *siz1* plants were suppressed by introduction of Pro_{SIZ1}:*SIZ1:GFP* (**Fig. 1H**). Plants of a transgenic line that expressed *SIZ1* mRNA abundance (**Fig. 1I**) similar to that of the wild type exhibited a similar morphological phenocopy (**Fig. 1H**). Interestingly, vegetative and reproductive development times of *siz1* and wild-type plants were similar under long day conditions (Jin et al. 2008). Floral initiation occurred in *siz1* and wild-type plants at the same chronological age and with the same number of rosette leaves under long day conditions (Jin et al. 2008).

To quantify the effects of *siz1-2* mutations on leaf area, the fifth rosette leaves from 4-week-old *siz1* and wild-type plants were compared (**Fig. 2A, B**). *siz1* plants exhibited reduced leaf blade width and length relative to the wild type (**Fig. 3A**). As a consequence, the total leaf area (fifth rosette leaf) of *siz1-2* and *siz1-3* plants was 24±1.7 and 27±1.7 mm^2, respectively, whereas that of wild-type plants was 201±15 mm^2 (**Fig. 3B**). Reduction of leaf width, length and area was suppressed by expression of Pro_{SIZ1}:*SIZ1:GFP* in *siz1*.

SIZ1 regulates cell division and expansion

Microscopic analysis revealed that *siz1-2* leaves contained smaller palisade and parenchyma mesophyll cells than did wild-type leaves (**Figs. 4A, 5A**). The *siz1* mutation also reduced the biomass of roots (**Fig. 6A**) and cell volume in roots (**Fig. 6B**). These results suggest that SIZ1 regulates cell expansion as well as plant development in both shoot and root. Because of smaller cells, *siz1-2* and *siz1-3* leaves had 550±32 and 530±27 palisade cells in the first layer in a 0.25 mm^2 area, respectively, whereas wild-type leaves had 160±26 cells in a 0.25 mm^2 area (**Fig. 4B**). Leaves of *siz1* plants contained about half the number of palisade mesophyll cells in the first layer as wild-type and *siz1-2::SIZ1:GFP* plants (**Fig. 4C**). These values were extrapolated to estimate the total cells of the first layer of palisade mesophyll cells in *siz1* and wild-type plants. Transverse sections of the fifth rosette leaves of 4-week-old plants were observed by microscopy (**Fig. 5A**). The numbers of palisade and parenchymatous cells in the fifth rosette leaf of the *siz1* mutant in the leaf width direction were decreased (**Fig. 5B**). The reduction of cell numbers (**Figs. 4C, 5B**) suggests that SIZ1 is involved in controlling cell division.

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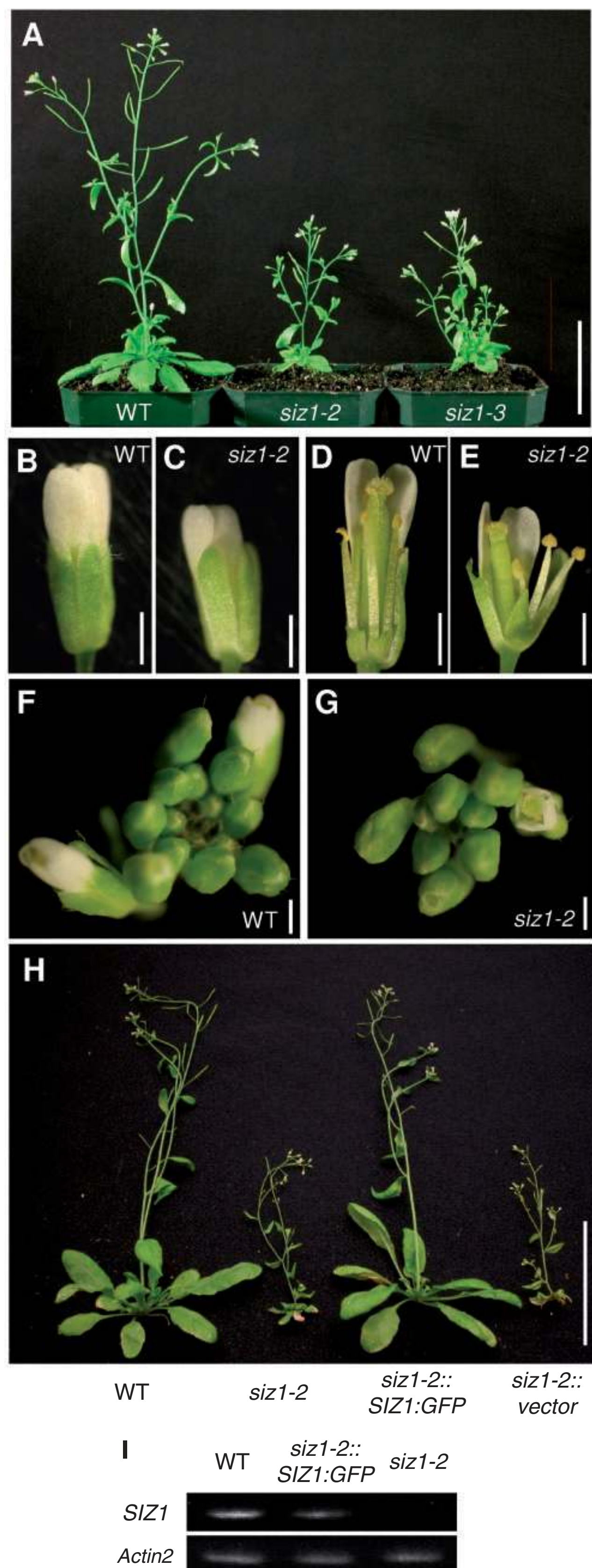

Fig. 1 *siz1* mutation drastically reduces plant growth at maturity. (A) Photographs are of representative 8-week-old wild-type (Col-0), *siz1-2* and *siz1-3* plants grown under long day conditions (16 h light/8 h dark) at 22°C. Flowers and those without two sepals and two petals of a wild-type (B, D) and *siz1-2* plant (C, E). Inflorescences of the wild type (F) and *siz1-2* mutant (G). (H) The wild-type *SIZ1* allele complemented the growth defect caused by the *siz1-2* mutation. Photographs are of representative wild-type, *siz1-2*, *siz1-2* harboring Pro_{SIZ1}:*SIZ1:GFP* and a vector control plant. Bar = 5 cm (A, H) or 1 mm (B–G). (I) *SIZ1* transcript abundance in wild type, *siz1-2* and *siz1-2* transformed with Pro_{SIZ1}:*SIZ1:GFP*. Expression levels of *SIZ1* in a transgenic line expressing the wild-type allele Pro_{SIZ1}:*SIZ1:GFP* in *siz1-2* were similar to those of the wild type.

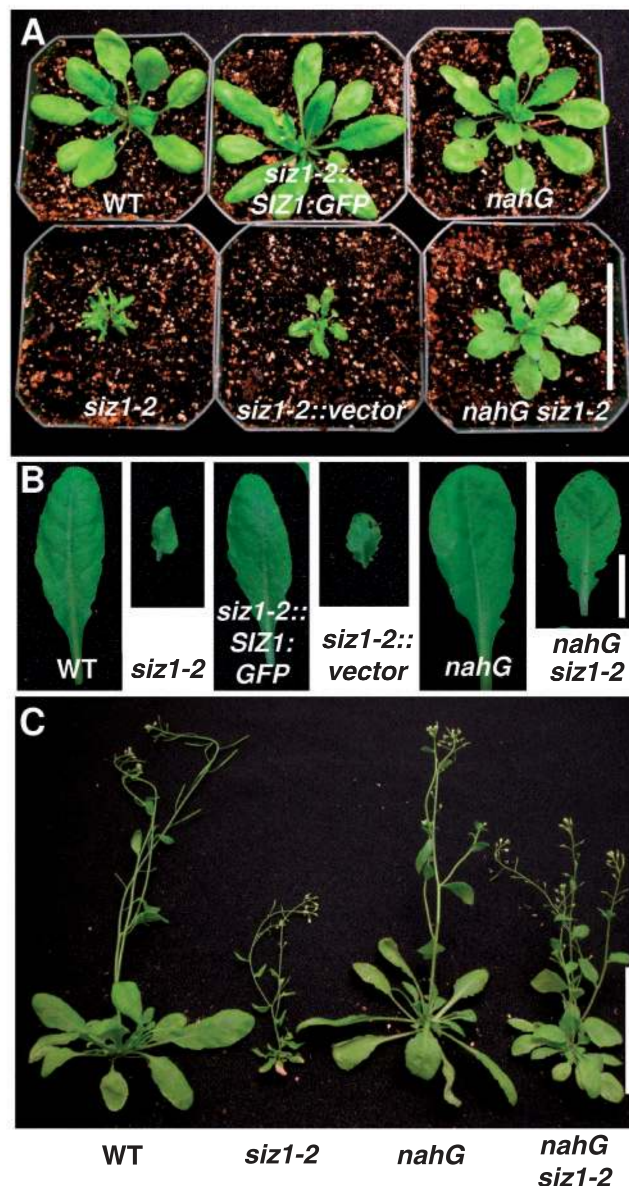

Fig. 2 The *siz1-2* mutation decreases leaf size, which are suppressed by *nahG*. Photographs are of representative 4-week-old wild-type, *siz1-2*, *siz1-2* harboring *SIZ1:GFP* or a vector, *nahG* and *nahG siz1-2* plants (A) and the fifth rosette leaves of these genotypes (B). (C) Eight-week-old wild-type, *siz1-2*, *nahG* and *nahG siz1-2* plants. Bars = 5 cm (A, C) or 1 cm (B).

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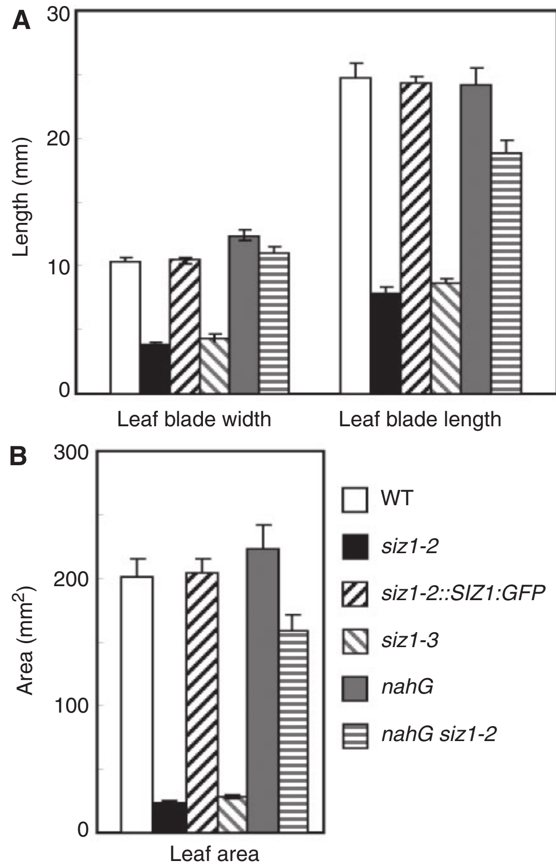

Fig. 3 Decrease in leaf blade width and length is caused by the *siz1* mutation. The leaf blade width and length (A) or leaf area (B) were measured by using the fifth rosette leaves of wild-type, *siz1-2*, *siz1-2* containing *SIZ1:GFP*, *siz1-3* and *nahG siz1-2* plants. Values are the mean ± SE, n = 10–14.

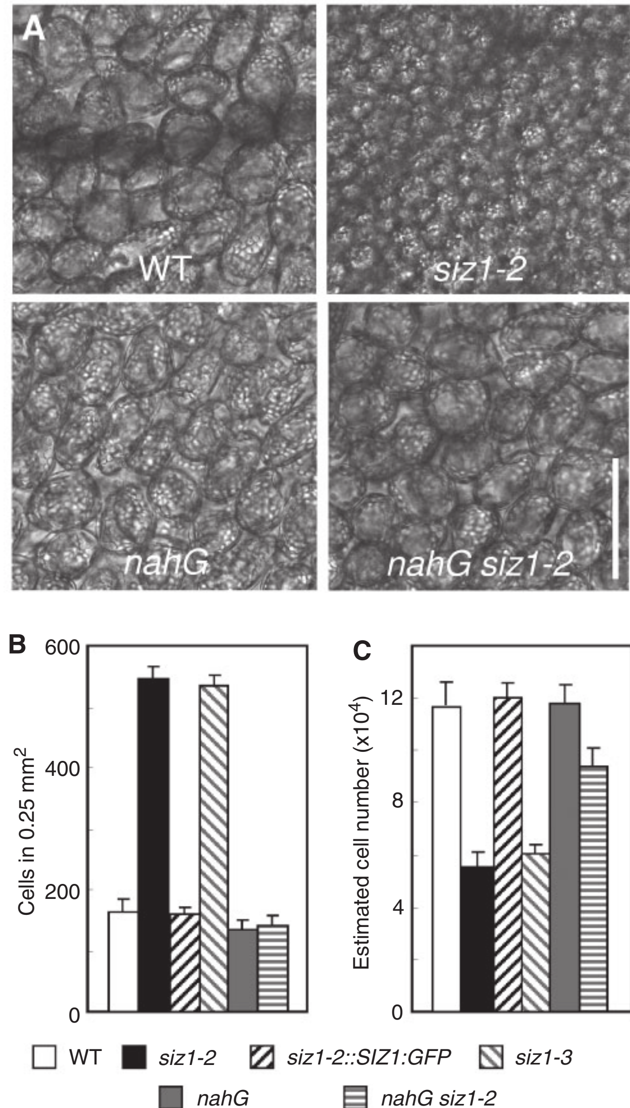

Fig. 4 The *siz1* mutation decreases the cell volume and cell proliferation and *nahG* recovers these defects. (A) Palisade mesophyll cells of the fifth rosette leaves of 4-week-old wild-type, *siz1-2*, *nahG* and *nahG siz1-2* plants were observed by microscopy. Bar = 100 µm. (B) The average cell number in a 0.25 mm² area was counted [n = 10 leaves (10 areas per each leaf)]. Based on average cell number per 0.25 mm² area (B) and leaf area (**Fig. 3B**), the total number of the first layer of palisade mesophyll cells was estimated (C, n = 10).

nahG recovers the defect in cell expansion and cell division caused by the siz1-2 mutation

Our previous results indicated that *SIZ1* regulates SA-dependent innate immunity (Lee et al. 2007). We investigated the role of *nahG* in regulation of plant development and cell division and expansion. Wild-type and *nahG*-expressing plants exhibited similar leaf size and plant development (**Figs. 2, 3**), because SA is accumulated only a little in wild-type and *nahG* plants under normal conditions (Lee et al. 2007). The fifth rosette leaf area of 4-week-old *nahG* plants was 220 ± 18 mm², while that of the wild type was 201 ± 15 mm². Introduction of *nahG* into *siz1-2* recovered plant growth and development (**Fig. 2**). It recovered leaf blade width more than leaf blade length (**Fig. 3A**). Therefore, the leaf area of *nahG siz1-2* was 160 ± 12 mm² (about 80% area compared with the wild type), whereas that of *siz1-2* was 24 ± 1.7 mm² (about 10% area compared with the wild type) (**Fig. 3B**).

Microscopic analyses revealed that the cell size of *nahG siz1-2* plants was similar to that of wild-type and *nahG* plants while cells of *siz1* plants were smaller (**Figs. 4A, 5A**). In a 0.25 mm² area, *nahG* and *nahG siz1-2* contained 140 ± 27 and 140 ± 28 cells, respectively, whereas wild-type and *siz1-2* contained 160 ± 26 and 550 ± 32 in a 0.25 mm² area, respectively (**Fig. 4B**). These means that the average cell volume of the wild type, *siz1-2*, *nahG* and *nahG siz1-2* is about 1.6, 0.45, 1.8 and 1.8 (×10³) µm², respectively, indicating that the cell volume of the wild type, *nahG* and *nahG siz1-2* is similar, but that of *siz1-2* is substantially reduced.

Calculation revealed that wild-type and *nahG* plants had 12 ± 1.2 and 12 ± 0.7 (×10⁴) palisade mesophyll cells in the first

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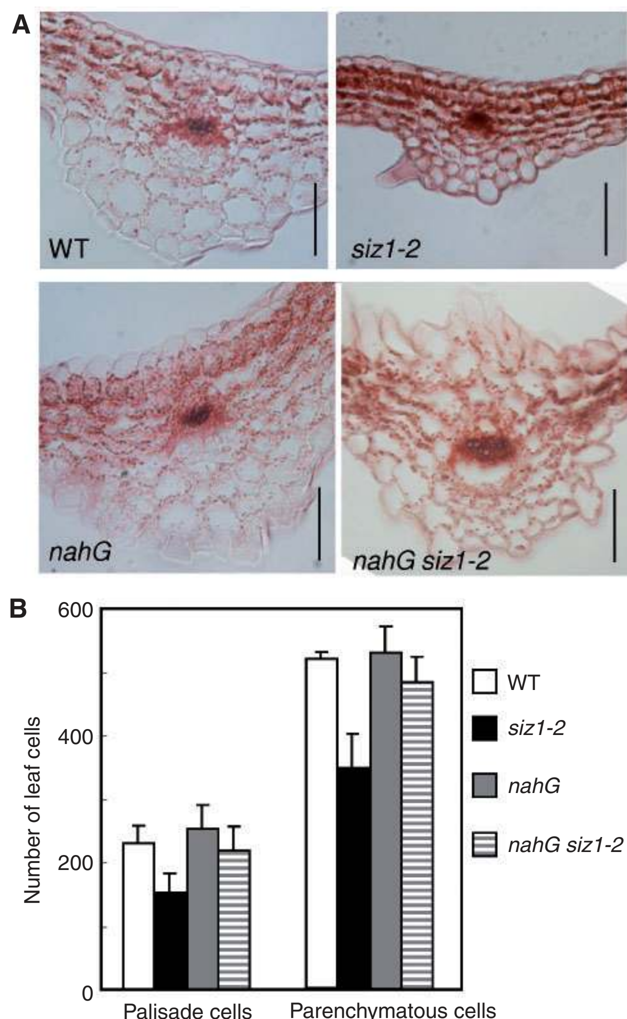

Fig. 5 Palisade and parenchymatous cells were smaller in *siz1-2* than in the wild type. (A) Transverse section of the fifth rosette leaves of 4-week-old wild-type, *siz1-2*, *nahG* and *nahG siz1-2* plants. Bars = 0.5 mm. (B) The number of cells aligned in the leaf width direction was counted. Values are the mean ± SE, *n* = 4.

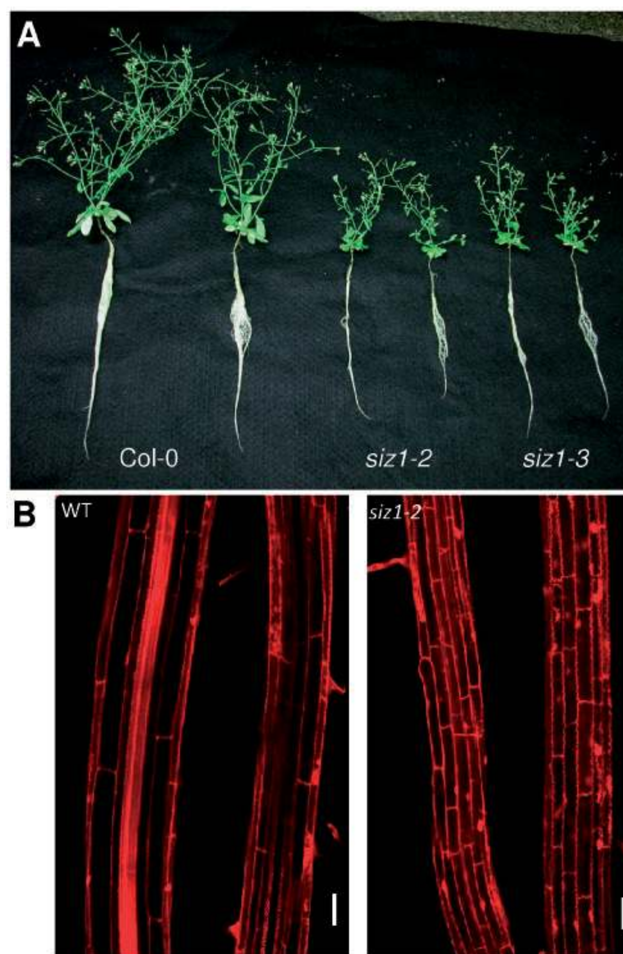

Fig. 6 The *siz1* mutation reduces biomass and cell elongation in roots. (A) Eight-week-old wild-type, *siz1-2* and *siz1-3* plants grown in hydroponic culture. (B) Photographs are of representative wild-type and *siz1-2* roots (two of each) stained with propidium iodide. Bars = 100 μm.

layer, whereas *siz1-2*, *siz1-3* and *nahG siz1-2* contained 5.5 ± 0.9, 6.0 ± 0.4 and 9.1 ± 0.6 ($\times 10^4$) cells, respectively, in the first layer (**Fig. 4C**). The number of palisade and parenchymatous cells of *nahG siz1-2* was also recovered (**Fig. 5B**), suggesting that introduction of *nahG* into *siz1-2* partially suppressed reduction of cell numbers in *siz1-2*. These results indicate that *nahG* recovered cell division and expansion defects caused by the *siz1-2* mutation.

Treatment with gibberellic acid (GA) or brassinolide (BR), but not indole-3-acetic acid (IAA), slightly enhanced leaf and plant development of wild-type and *siz1-2* plants in our conditions (**Fig. 7**). The leaf area of *siz1-2* to which GA (30 ± 1.8 mm^2) or BR (37 ± 2.7 mm^2) had been applied was about 1.7 or 2.0 times larger than that of *siz1-2* (18 ± 0.6 mm^2) (**Fig. 7C–F**). However, suppression of the dwarf-like phenotype of *siz1-2* by introduction of *nahG* was more substantial than that caused by GA or BR treatment. As flowering was enhanced (**Fig. 7E**), *siz1-2* plants were able to respond to gibberellic acid. These results suggest that dwarfism caused by the *siz1* mutation is not due to reduction of GA or BR biosynthesis or an abnormal response to GA or BR.

Leaves of SA-accumulating mutants such as *cpr5* and *mekk1* display hypersensitive cell death that is associated with constitutive defense in their leaves (Kirik et al. 2001, Ichimura et al. 2006). Four-week-old wild-type, *siz1-2*, *nahG* and *nahG siz1-2* plants were treated with trypan blue (**Fig. 9**), a common dye for visualizing dead cells (Shirasu et al. 1999), to ascertain if the cell death which occurs is associated with SA overaccumulation in *siz1* plants (Lee et al. 2007). Trypan blue-stained cells were observed in leaves of *siz1-2* leaves, but not in leaves of other genotypes, including *nahG siz1-2* (**Fig. 8**). These results suggest that *SIZ1* loss of function leads to cell death (**Fig. 8**) and activation of constitutive defense responses (Lee et al. 2007).

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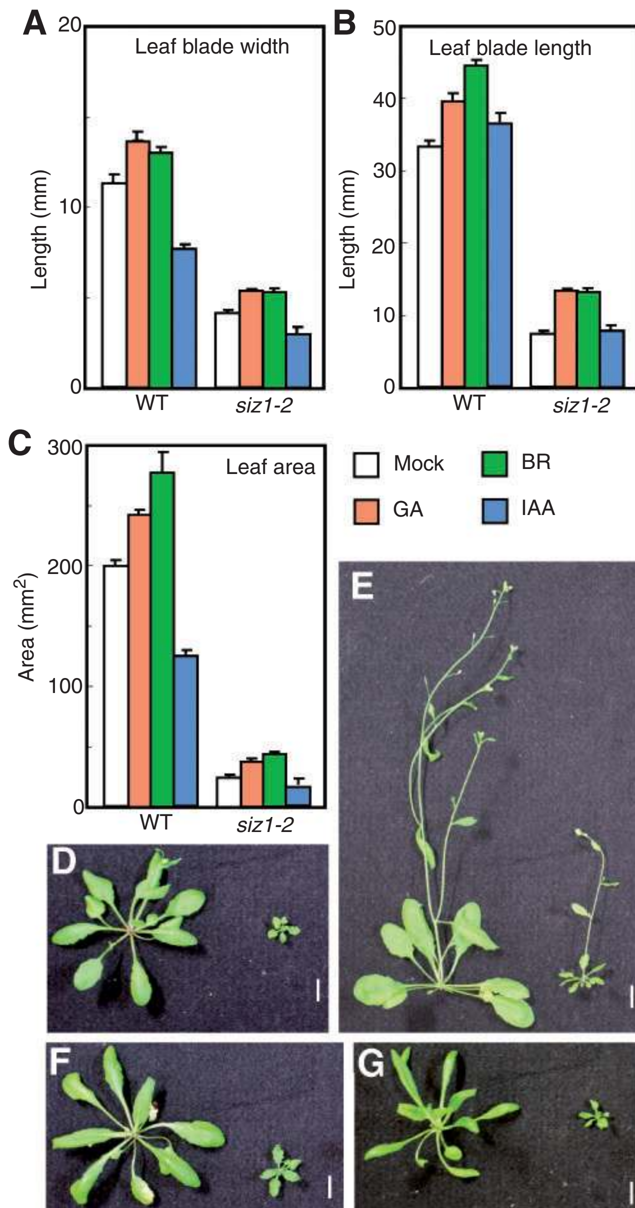

Fig. 7 Application of gibberellic acid, brassinosteroid or auxin did not recover *siz1* dwarfism as introduction of *nahG* did. Three-week-old plants were treated with 50 μM gibberellin A3 (GA), 0.1 p.p.m. brassinolide (BR) or 25 p.p.m. IAA by a foliar spray twice a week. Two weeks after treatment, the leaf blade width (A), length (B) and leaf area (C) were measured using the fifth rosette leaves of wild-type and *siz1-2* plants. Values are the mean ± SE, $n = 10–12$. Photographs are of representative wild-type (left) and *siz1-2* plants (right) treated with water (D), GA (E), BR (F) or IAA (G).

Expression of XTH8 and XTH31 is regulated by SIZ1 though control of SA accumulation

XTH genes encode enzymes that are implicated in cell wall loosening and cell expansion (Rose et al. 2002). XTHs endolytically cleave xyloglucan polymers and join the newly generated ends to other xyloglucan chains (Rose et al. 2002). Arabidopsis contains 33 *XTH* genes in the genome (Yokoyama and Nishitani 2001). To investigate which *XTH* genes are more likely to be involved in regulation of SA-dependent dwarfism, expression patterns of *XTH* genes with SA, *P. syringae* treatment, or in mutants with increased (*cpr5*, *mpk4*; Bowling et al. 1997, Petersen et al. 2000) or reduced (*nahG*) SA levels were extracted from the Genevestigator database (**Fig. 9A**; Zimmermann et al. 2004, Zimmermann et al. 2005) (http://www.genevestigator.ethz.ch). Among 33 *XTH* genes, expression levels of *XTH8*, *XTH17* and *XTH31* were strongly down-regulated in both *cpr5* and *mpk4* and not changed in *nahG* (**Fig. 9A**). Reverse transcription–PCR (RT–PCR) results indicated that *XTH8* and *XTH31*, but not *XTH17*, were down-regulated in *siz1* and expression of *XTH8* and *XTH31* was recovered in *nahG siz1-2* (**Fig. 9B**).

We also checked the expression level of *XTH24* (*MERI5*), a potential target for *AN* (*ANGUSTIFOLIA*), which regulates the width of leaves (Kim et al. 2002) and may play a role in leaf morphogenesis at the early stage (Verica and Medford 1997). The transcript levels of *XTH24* and *AN* were similar in all genotypes (**Fig. 9B**). Thus, *SIZ1* regulates SA-dependent *XTH8* and *XTH31* expression, but may not be involved in *AN*-dependent regulation of cell elongation.

Discussion

Here we establish that Arabidopsis *SIZ1* functions in cell growth and plant development through SA accumulation. *siz1* mutations caused dwarfism (**Fig. 1**), and reduced leaf size (**Figs. 2, 3**), cell size and cell number (**Figs. 4–6**). Further, expression of *XTH8* and *XTH31* was down-regulated in *siz1* plants (**Fig. 9**). All of the phenotypes were, to some extent, recovered by *nahG* expression in *siz1-2* (**Figs. 2–9**). Because *nahG* reduced accumulation of SA caused by the *siz1-2* mutation (Yoo et al. 2006, Lee et al. 2007), the results suggest that accumulation of SA is likely to inhibit cell growth, plant development and expression of *XTH8* and *XTH31*.

Development and viability regulated by sumoylation

The sumoylation system is essential for viability. In Arabidopsis, mutation in *SAE2* or *SCE1*, and double mutations in SUMO1 and 2 cause lethality (Saracco et al. 2007), similar to observations in yeast (Johnson and Blobel 1997, Johnson et al. 1997). Because SUMO1/2 conjugation was substantially decreased in the *siz1* mutant (Miura et al. 2005, Yoo et al. 2005, Saracco et al. 2007), SIZ1 appears to be responsible for most of the sumoylation. Though the *siz1* mutant exhibited a dwarf-like phenotype (**Fig. 1**), it is not lethal. One possible explanation is that other SUMO E3 ligases may overlap the SIZ1-dependent sumoylation system. Recently, another SUMO E3 ligase, HPY2, which shows similarity to yeast and human MMS21, has been identified (Huang et al. 2009, Ishida et al. 2009). HPY2 regulates endocycle onset, meristem maintenance and plant development in Arabidopsis (Ishida et al. 2009). The Arabidopsis SUMO protease, ESD4, also controls plant development, as well as regulation of flowering time (Murtas et al. 2003).

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Like the Arabidopsis mutants, loss of function in sumoylation mechanism causes several defects in development and cell growth in yeast and mammals. SUMO1 knock-out mice exhibited embryonic demise and immediate postnatal death (Alkuraya et al. 2006). In *Saccharomyces cerevisiae*, mutations in the single genes encoding SUMOs, SAE1, SAE2 or SCE1, cause cell cycle defects and arrest at the G_2/M transition (Dohmen et al. 1995, Johnson and Blobel 1997, Johnson et al. 1997). The SUMO E2 enzyme SCE1 plays an essential role in early embryonic development and this role is evolutionarily conserved. As the Arabidopsis *sce1* mutant shows embryonic lethality (Saracco et al. 2007), the *sce1* mutations lead to embryonic death in mouse and *Caenorhabditis elegans* (Jones et al. 2002). Loss of PIAS function in *Drosophila melanogaster* and *C. elegans* also leads to embryonic lethality with abnormal body morphology (Hari et al. 2001, Holway et al. 2006). In *S. cerevisiae*, the mutant cells with deletion of both *Siz1* and *Siz2* genes exhibit poor growth at low temperature (Johnson and Gupta 2001), and elimination of all three E3 activities (Siz1, Siz2 and Mms21) is synthetically lethal (Reindle et al. 2006). Taken together,

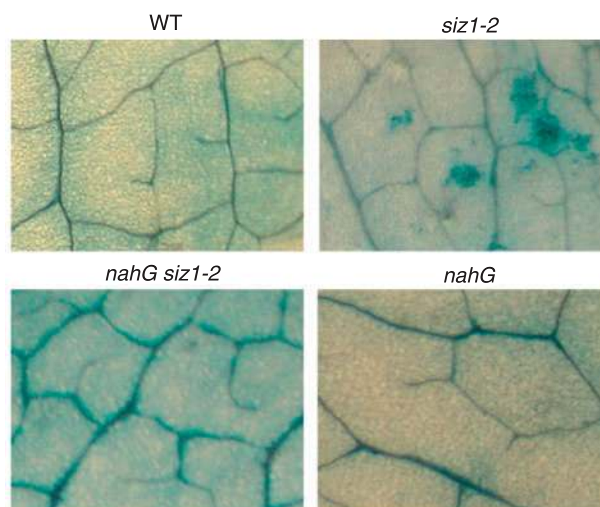

Fig. 8 The *siz1* mutation resulted in cell death. Leaves of 4-week-old wild-type, *siz1-2*, *nahG* and *nahG siz1-2* plants were stained with trypan blue.

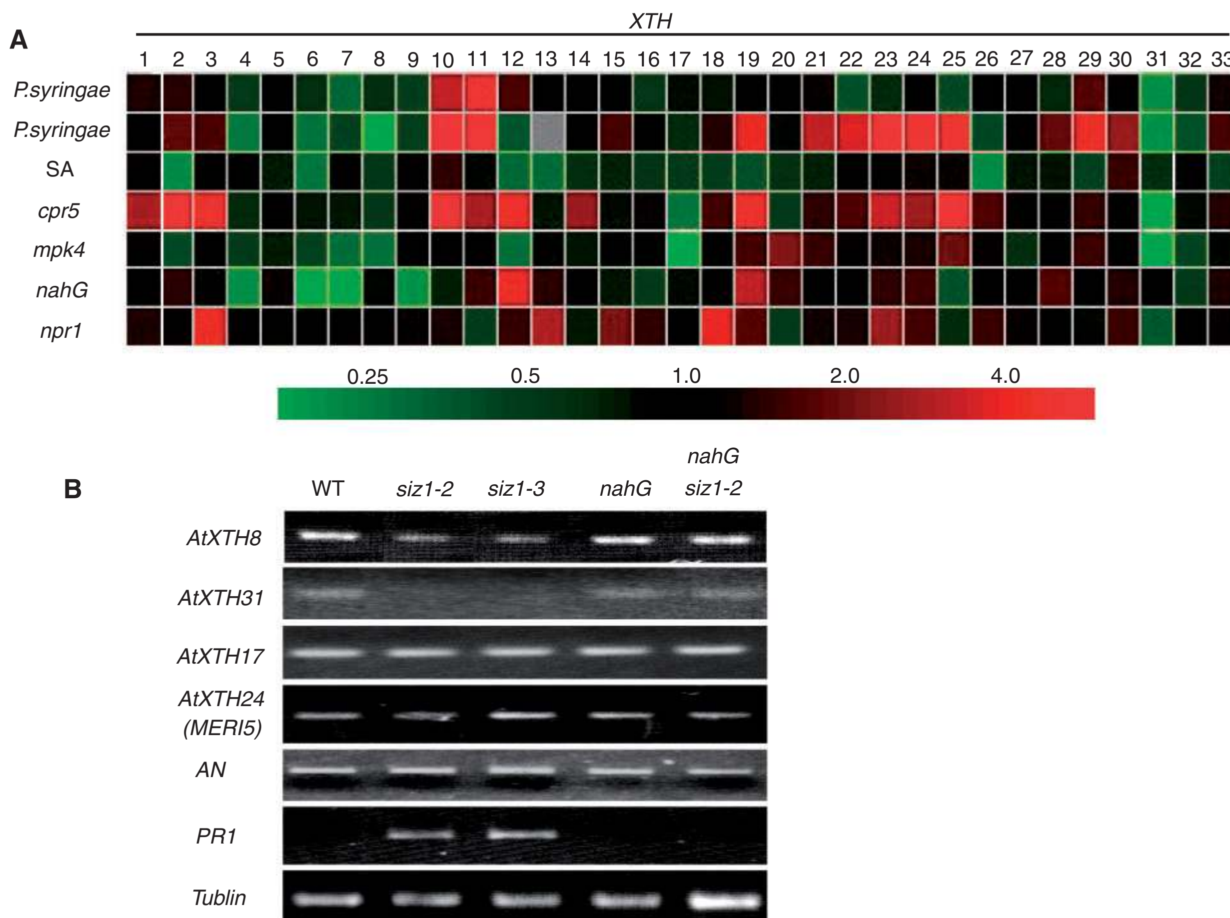

Fig. 9 *XTH* expression. (A) Genevestigator Arabidopsis *XTH* expression with treatment by *Pseudomonas syringae* and SA and in *cpr5*, *mpk* and *nahG* plants. (B) *XTH* expression in wild-type, *siz1*, *nahG* and *nahG siz1-2* plants. Total RNA was prepared from leaves of 4-week-old plants grown at 23°C, and semi-quantitative RT–PCR was performed. *PR1* expression was used to monitor SA accumulation in *siz1* mutants (Lee et al. 2007).

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SUMO pathway proteins have critical functions at both cellular and organismal levels.

SIZ1 controls cell growth by regulating SA

SIZ1 regulates cell proliferation (**Figs. 4** and **5**). Control of cell growth by SIZ1 is due to negative regulation of SA-mediated inhibition of cell division and elongation. SIZ1 appears to control both cell division and expansion, with the latter affected to a greater degree than the former. Interestingly, in vascular smooth muscle cells, SA or aspirin (acetyl-SA) inhibit cell proliferation (Marra and Liao 2001). SA and aspirin block IKK (IκB kinase), leading to inhibition of NF-κB activation (Yin et al. 1998), which is necessary for cell survival, proliferation and differentiation (Hayden and Ghosh, 2008).

XTHs catalyze the cleavage and molecular grafting of xyloglucan chains that are necessary for cell wall loosening and reorganization (Rose et al. 2002). Expression of the *Brassica campestris XTH1* in Arabidopsis enhanced stem elongation, which is caused by enhanced directional cell expansion (Shin et al. 2006). *acl* (*acaulis*) and *tfl* (*terminal flower*) mutations cause a reduction in cell size (Tsukaya et al. 1993, Tsukaya et al. 1995) and in height (Alvarez et al. 1992), respectively, which is associated with down-regulated expression of Arabidopsis *XTH9*, the ortholog of *BcXTH1* (Hyodo et al. 2003). The *xth21* mutation causes a dwarf phenotype compared with the wild type that was associated with altered deposition of cellulose in the wall and cell wall elongation (Liu et al. 2007). Our results implicate *XTH8* and *XTH31* as potential effectors of cell elongation that are negatively regulated by SA (**Fig. 9**). *XTH8* and *XTH31* are strongly expressed in early stages of leaf development and are presumably necessary for proper development (Becnel et al. 2006). Since *siz1* causes constitutive high levels of SA, we posit that the hormone imbalance disturbs appropriate *XTH8* and *XTH31* expression, leading to affects on cell development that result in dwarfism.

Because little SA is accumulated in wild-type plants under normal conditions (Lee et al. 2007), no significant difference in leaf size between wild-type and *nahG* plants was observed (**Fig. 2**). Chilling temperature enhances accumulation of SA in wild-type plants (Scott et al. 2004). Thus, *nahG* plants grow bigger than wild-type plants, and the cell size of *nahG* leaves was larger than that of wild-type leaves at 5°C (Scott et al. 2004). Furthermore, the *cpr5* mutant, which accumulates SA, is impaired in cell size and proliferation (Kirik et al. 2001). Taken together with the fact that the *siz1* mutation increased endogenous SA, leading to a decrease in cell division and elongation (**Figs. 4–6**), it is suggested that accumulation of endogenous SA inhibits cell expansion and proliferation.

This report provides another link between SIZ1 and SA accumulation that is connected to plant defense and development (Lee et al. 2007). Identification of specific SUMO conjugates that are involved in SIZ1-mediated regulation of the SA-dependent innate immunity response may provide a model for how SA integrates plant defense and developmental signaling.

Materials and Methods

Plant materials and growth conditions

The Arabidopsis T-DNA insertion mutants, *siz1-2* and *siz1-3* (Miura et al. 2005), and *nahG* plants (van Wees and Glazebrook, 2003) were in the *Arabidopsis thaliana* Col-0 background. *nahG siz1-2* plants were identified by diagnostic PCR (Lee et al. 2007). Arabidopsis plants were grown on soil in a growth room with a 16 h photoperiod and light intensity of 100–120 $\mu Em^{-2} s^{-1}$ at 22°C. To break seed dormancy, seeds were incubated at 4°C for at least 2 d. For complementation, Pro_{SIZ1}:*SIZ1:GFP* or Pro_{SIZ1}:*GUS:GFP* (a negative control) was transformed into *siz1-2* (Jin et al. 2008).

Three-week-old plants were treated with 50 μM gibberellin A3 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 0.1 p.p.m. brassinolide (Brassino Co., Ltd., Imizu, Japan) or 25 p.p.m. indole-3-acetic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) by a foliar spray twice a week. After 2 weeks of treatment, leaf blade length, width and area of fifth rosette leaves were measured.

Morphometric analysis

Leaf blade width, length and area were measured for the fifth rosette leaves of 4-week-old plants by using ImageJ version 1.36b (http://rsb.info.nih.gov/ij).

Microscopic analysis

Leaves were incubated with a fixing solution containing 90% ethanol and 10% acetate overnight. Then leaves were washed with 90, 70, 50 and 30% ethanol for 20 min at each step. Leaves were incubated in 80% chloral hydrate and 10% glycerol and observed using a Nikon E800 microscope with differential interference contrast (Nikon, Tokyo, Japan). Images were acquired using a SPOT RT-slider digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

To make sections, leaves from 4-week-old plants were fixed in a 1 : 1 : 18 solution of formaldehyde, acetic acid and 50% ethanol (FAA) overnight. These leaves were stained with 1% safranine in 50% ethanol for 1 h. After washing in 50% ethanol, leaves were incubated in 50 mM phosphate buffer (pH 7.2), 4% paraformaldehyde and 0.25% glutaraldehyde for 2 h. The samples were washed three times with phosphate-buffered saline, then were embedded in 5% agar. Leaf slices were cut on a vibrating microtome (30 μm thick) (Leica VT1200S, Nussloch, Germany). Tissue sections were observed under a microscope (DM RXA-6, Leica, Nussloch, Germany).

Roots from 4-week-old plants were visualized with 200 $\mu g\,ml^{-1}$ propidium iodide solution for 60 min. Roots were imaged with a Leica TCS-SP2 AOBS confocal microscope.

Leaves from 4-week-old WT, *siz1-2*, *nahG* and *nahG siz1-2* plants were submerged in lactic acid–glycerol–phenol–trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol and 20 mg of trypan blue, dissolved in 10 ml of distilled water) (Koch and Slusarenko 1990) and incubated

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at 95°C for 3 min. Leaves were incubated overnight, then transferred into chloral hydrate (2.5 mg ml^{-1} H_2O) and kept overnight. Chloral hydrate was removed and 70% glycerol was added. Plant cell death was observed using a Nikon E800 microscope.

RNA isolation and semi-quantitative RT–PCR

Total RNA from leaves of 4-week-old plants was extracted (Miura et al. 2007b). A 2.50 μg aliquot of RNA was used as template to synthesize first-strand cDNA with M-MLV Reverse Transcriptase (Promega , Madison, WI, USA) and random primers p(dN)$_6$ (Roche). Primer pairs for RT–PCR are as follows; *XTH8*, *XTH17*, *XTH24* and *XTH31* (Yokoyama and Nishitani 2001), *PR1* (Lee et al. 2007), *ANGUSTIFOLIA* (5′-TGAGACGGTGCCGTGGTATGG-3′ and 5′-GTTGCCTACTGGTGGATTCC-3′) and tubulin (5′-CGTGGATCACAGCAATACAGAGCC-3′ and 5′-CCTCCTGCACTTCCACTTCGTCTTC-3′).

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