

***SIZ1* controls cell growth and plant development in *Arabidopsis* through salicylic acid**

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Abbreviations:

Abbreviation: PIAS, protein inhibitor of activated STAT; SA, salicylic acid; SAE, SUMO activation enzyme; SCE, SUMO conjugating enzyme; SIZ1, SAP and MIZ domain protein; SUMO, small ubiquitin-related modifier

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Abstract

The posttranslational 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. Herein, we provide evidence that the *Arabidopsis* SUMO E3 ligase *SIZ1* controls cell division and elongation, and plant development. 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 wild type. Cell division and expansion defects caused by *siz1* were suppressed by the expression of *nahG*, a bacterial salicylate hydroxylase that catabolizes SA (salicylic acid). 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. Together, these results indicate that *SIZ1* controls cell growth and plant development through regulation of SA accumulation. And *XTH8* and *XTH31* genes may be responsible for reduced leaf cell expansion.

Keywords:

Arabidopsis thaliana, posttranslational modification, salicylic acid, SUMO, sumoylation, cell division and expansion

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., 2007a; 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 (Ishida et al., 2009; Huang et al., 2009). RanBP2 (Ran binding protein 2), Pc2 (polycomb 2), PHD domain of the KAP1 corepressor, 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 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; 2007b; Yoo et al., 2006; Catala et al., 2007; Conti et al., 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 (ABA) and salicylic acid (SA) signaling (Lois et al., 2003; Lee et al., 2007; Miura et al., 2009). *siz1* resulted in constitutive plant defense against plant pathogens through a SA-mediated signaling pathway (Lee et al., 2006).

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 to 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 pathogen due to SA accumulation, exhibit altered leaf shape, reduced height, and/or spontaneous cell death (Rate et al., 1999; Shah et al., 1999; Vanacker et al., 2001; Rate and Greenberg, 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 *Pseudomonas syringae* (Zhang et al., 2007). The constitutive pathogene 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, except part of the hypocotyls and basal region of developing young leaves (Catala et al., 2007). And *SIZ1* is necessary for vegetative growth and development (Catala et al., 2007), although the mechanisms have not been elucidated. Here is evidence that establishes SIZ1 function in cell division and expansion. *siz1-2* and *siz1-3* mutations caused defects in leaf cell division and expansion, resulting in formation of smaller leaves and shoot dwarfism. 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), 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 (XTH) 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), and reduced leaf area (Fig. 1A), and floral organ size (Fig. 1B-1G) than wild-type plants. However, floral organ composition and organization of *siz1* plants was similar to wild type (Figure 1B-1E), 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 similar *SIZ1* mRNA abundance (Fig. 1I) as 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 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 5th 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 wild type (Fig. 3A). As a consequence, total leaf area (5th rosette leaf) of *siz1-2* and *siz1-3* plants was 24 ± 1.7 and 27 ± 1.7 mm², respectively, whereas that of wild-type plants was 201 ± 15 mm² (Fig. 3B). Reduced leaf width, length and area were 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 (Fig. 4A, 5A). The *siz1* mutation also reduced 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 at 1st layer in a 0.25 mm² area, respectively, whereas wild-type leaves had 160 ± 26 cells/0.25 mm² (Fig. 4B). Leaves of *siz1*

plants contained about half the number of palisade mesophyll cells at 1st layer as wild-type and *siz1-2::SIZ1:GFP* plants (Fig. 4C). These values were extrapolated to estimate the total cells of 1st layer of palisade mesophyll cells in *siz1* and wild-type plants. Transverse section of the fifth rosette leaves of 4-week-old plants were observed by microscope (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 (Fig. 4C, 5B) suggests that SIZ1 is involved in controlling cell division.

nahG recovers 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 (Fig. 2 and 3), because SA is little accumulated in wild type and *nahG* plants under normal condition (Lee et al., 2007). The 5th rosette leaf area of 4-week-old *nahG* plants was $220 \pm 18 \text{ mm}^2$, while wild type was $201 \pm 15 \text{ mm}^2$. Introduction of *nahG* into *siz1-2* recovered plant growth and development (Fig. 2). It recovered leaf blade width than leaf blade length (Fig. 3A). Therefore leaf area of *nahG siz1-2* was $160 \pm 12 \text{ mm}^2$ (about 80% compared to wild type), whereas that of *siz1-2* was $24 \pm 1.7 \text{ mm}^2$ (about 10% area compared to wild type) (Fig. 3B).

Microscopic analyses revealed that cell size of *nahG siz1-2* plants was similar to that of wild-type and *nahG* plants while those of *siz1* plants were (Fig. 4A, 5A). In 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 0.25 mm² area, respectively (Fig. 4B). These means that average cell volume of wild type, *siz1-2*, *nahG*, and *nahG siz1-2* is about 1.6, 0.45, 1.8, and 1.8 ($\times 10^3$) μm^2 , respectively, indicating that cell volume of 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 ($\times 10^4$) palisade mesophyll cells at 1st 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 at 1st layer, respectively (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 defected 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* applied by GA ($30 \pm 1.8 \text{ mm}^2$) or BR ($37 \pm 2.7 \text{ mm}^2$) was about 1.7 or 2.0 times bigger than that of *siz1-2* ($18 \pm 0.6 \text{ mm}^2$) (Fig. 7C-F). However, suppression of the dwarf-like phenotype of *siz1-2* by introduction of *nahG* was more substantial than GA or BR treatment. As flowering was enhanced (Fig. 7E), *siz1-2* plants were able to respond to GA. These results suggest that dwarfism caused by the *siz1* mutation is not due to reduction of GA or BR biosynthesis or abnormal response to GA or BR.

Leaf 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 with trypan blue (Fig. 9), a common dye for visualizing dead cells (Shirasu et al., 1999), to ascertain if cell death occurs is associated with SA over-accumulation 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).

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

XTH genes encode enzymes that are implicated for 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, *Pseudomonas syringae* treatment, or in mutants, which increase (*cpr5*, *mpk4*; Bowling et al., 1997; Petersen et al., 2000) or reduces (*nahG*) SA level, were extracted from Genevestigator database (Fig. 9A; Zimmermann et al., 2004; 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). RT-PCR results indicated that *XTH8* and *XTH31*, not *XTH17*, was down-regulated in *siz1* and expression of *XTH8* and *XTH31* was recovered in *nahG siz1-2* (Fig. 9B).

We also checked expression level of *XTH24* (*MER15*), 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. *siz1* mutations caused dwarfism (Fig. 1), and reduced leaf size (Fig. 2 and 3), cell size and cell number (Fig. 4-6). Further, expression of *XTH8* and *XTH31* was downregulated in *siz1* plants (Fig. 9). All of the phenotypes were, to some extent, recovered by *nahG* expression in *siz1-2* (Fig. 2-9). Because *nahG* reduced accumulation of SA caused by the *siz1-2* mutation (Yoo et al., 2006; Lee et al., 2007), the results indicate *SIZ1* controls cell growth, plant development, and expression of *XTH8* and *XTH31* through regulation of SA accumulation.

Development and viability regulated by sumoylation

Sumoylation system is essential for viability. In *Arabidopsis*, the mutation in *SAE2* or *SCE1*, and the double mutations in *SUMO1* and 2 causes lethality (Saracco et al., 2007), similar to observation in yeast (Johnson and Blobel, 1997; Johnson et al., 1997). Because *SUMO1/2* conjugation were substantially decreased in the *siz1* mutant (Miura et al., 2005; Yoo et al., 2005; Saracco et al., 2007), *SIZ1* appears responsible for most of sumoylation. Though the *siz1* mutant

exhibited dwarf-like phenotype (Fig. 1), it is not lethal. One of possible explanation is that other SUMO E3 ligases may overlap SIZ1-dependent sumoylation system. Recently, another SUMO E3 ligase HPY2 (high ploidy 2), which shows similarity with yeast and human MMS21 (methyl methansulfonate sensitive 21), has been identified (Ishida et al., 2009; Huang et al., 2009). HPY2 regulates endocycle onset, meristem maintenance, and plant development in *Arabidopsis* (Ishida et al., 2009). The *Arabidopsis* SUMO protease, ESD4, also control plant development, as well as regulation of flowering time (Murtas et al., 2003).

Like the *Arabidopsis* mutants, loss-of-function in sumoylation mechanism causes several defects in development and cell growth in yeast and mammalian. The SUMO-1 knockout mice exhibited the embryonic demise and immediate postnatal death (Alkuraya et al., 2006). In *Saccharomyces cerevisiae*, mutations in the single genes encoding SUMO, SAE1, SAE2, or SCE1 cause cell cycle defects and arrest at the G2/M transition (Dohmen et al., 1995; Johnson et al., 1997; Johnson and Blobel, 1997). The SUMO E2 enzyme SCE1 plays an essential role in early embryonic development and this role is evolutionally 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, SUMO pathway proteins have critical functions at both cellular and organismic levels.

SIZ1 controls cell growth by regulating SA

SIZ1 regulates cell proliferation (Fig. 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) inhibits cell proliferation (Marra and Liao, 2001). SA and aspirin block IKK (I κ B kinase), leading to inhibition of NF- κ B 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 is necessary for cell wall loosening and reorganization (Rose et al., 2002). Expression of the *Brassica campestris XTH1* in *Arabidopsis* enhanced in 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; 1995) and in height (Alvarez et al., 1992), respectively, which is associated with downregulated expression of *Arabidopsis XTH9*, which is the ortholog of *BcXTH1* (Hyodo et al., 2003). The *xth21* mutation causes a dwarf phenotype compared to 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 of 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 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 decrease in cell division and elongation (Fig. 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 linked to plant defense and development (Lee et al., 2007). Identification of specific SUMO conjugates that are involved in SIZ1-mediated with regulation of 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 growth room with a 16-h photoperiod and light intensity of 100-120 $\mu\text{Em}^{-2}\text{sec}^{-1}$ at 22°C. To break seed dormancy, seeds were incubated at 4°C for at least 2 days. 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 applied with 50 μ M gibberellin A3, 0.1 ppm brassinolide, or 25 ppm indole-3-acetic acid by a foliar spray twice a week. After 2-week treatment, leaf blade length, width, and area of 5th rosette leaves were measured.

Morphometric analysis

Leaf blade width, length, and area were measured with the 5th rosette leaves of 4-week-old plants by using ImageJ version 1.36b (<http://rbs.info.nih.gov/ij>).

Microscopic analysis

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

To make section, leaves from 4-week-old plants were fixed in 1:1:18 solution of formaldehyde, acetic acid, and 50% ethanol (FAA) overnight. These leaves were stained with 1% safranin 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 with phosphate buffered saline three times, then were embedded into 5% agar. Leaf slices were cut on vibrating microtome (30 μ m 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 μ g/ml propidium iodide solution for 60 minutes. Roots were imaged with 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- phenol-trypan blue solution (10 μ l of lactic acid, 10 μ l of glycerol, 10 g of phenol, 20 mg of trypan blue, dissolved in 10ml of distilled water) (Koch et al., 1990) and incubated at 95°C for 3 min. Leaves were incubated overnight, then transferred into chloral hydrate (2.5mg/ml H₂O) and kept overnight. Chloral hydrate was removed and 70% glycerol was added. Plant cell death were observed using Nikon E800 microscope (Nikon, Tokyo, Japan).

RNA isolation and semi-quantitative RT-PCR

Total RNA from leaves of 4-week-old plants was extracted (Miura et al., 2007b). Two and half micrograms of RNA were used as template to synthesize first-strand cDNA with M-MLV

Reverse Transcriptase (Promega) and random primers p(dN)₆ (Roche). Primer pairs for RT-PCR are following; *XTH8*, *XTH17*, *XTH24*, and *XTH31* (Yokoyama and Nishitani, 2001), *PRI* (Lee et al., 2007), *ANGUSTIFOLIA* (5'-TGAGACGGTGCCGTGGTATGG-3' and 5'-GTTGCCTACTGGTGGATTCC-3'), and Tublin (5'-CGTGGATCACAGCAATACAGAGCC-3' and 5'-CCTCCTGCACTTCCACTTCGTCTTC-3').

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Figure legends

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 wild-type (B, D) and *siz1-2* plant (C, E). Inflorescences of wild type (F) and *siz1-2* (G). (H) The wild-type *SIZ1* allele complemented 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 plants. 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 transgenic line expressing the wild-type allele *Pro_{SIZ1}:SIZ1:GFP* in *siz1-2* was similar to that to wild type.

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

Fig. 3. Decrease in leaf blade width and length is caused by the *siz1-2* mutation. Length of leaf blade width and length (A) or leaf area (B) were measured by using the 5th rosette leaves of wild-type, *siz1-2*, *siz1-2* containing *SIZ1:GFP*, *siz1-3*, and *nahG siz1-2*. Values are the mean \pm SE, $n = 10-14$.

Fig. 4. The *siz1* mutation decreases in cell volume and cell proliferation and *nahG* recovers these defects. (A) Palisade mesophyll cells of the 5th rosette leaves of 4-week-old wild-type,

siz1-2, *nahG*, and *nahG siz1-2* plants were observed by microscopy. Bar = 100 μ m. (B)

Average cell number in 0.25-mm² area was counted ($n = 10$ leaves (10 areas per each leaf)).

Based on average cell number per 0.25mm² area (B) and leaf area (Fig. 4B), total number of 1st layer of palisade mesophyll cells were estimated (C, $n = 10$).

Fig. 5. Palisade and parenchymatous cells were smaller in *siz1-2* than that in 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 indicate 0.5-mm length. (B) The number of cells aligned in leaf-width

direction was counted. Values are the mean \pm 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 are

100- μ m length.

Fig. 7. Application of gibberellic acid, brassinosteroid, or auxin did not recover *siz1* dwarfism as

introduction of *nahG* did. Three-week-old plants were applied with 50 μ M gibberellin A3 (GA),

0.1 ppm brassinolide (BR), or 25 ppm indole-3-acetic acid (IAA) by a foliar spray twice a week.

Two weeks after treatment, length of leaf blade width (A), length (B), or leaf area (C) were measured by using the 5th rosette leaves of wild-type and *siz1-2* plants. Values are the mean \pm 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).

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 of *Pseudomonas syringae* and SA and in *cpr5*, *mpk4*, 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).

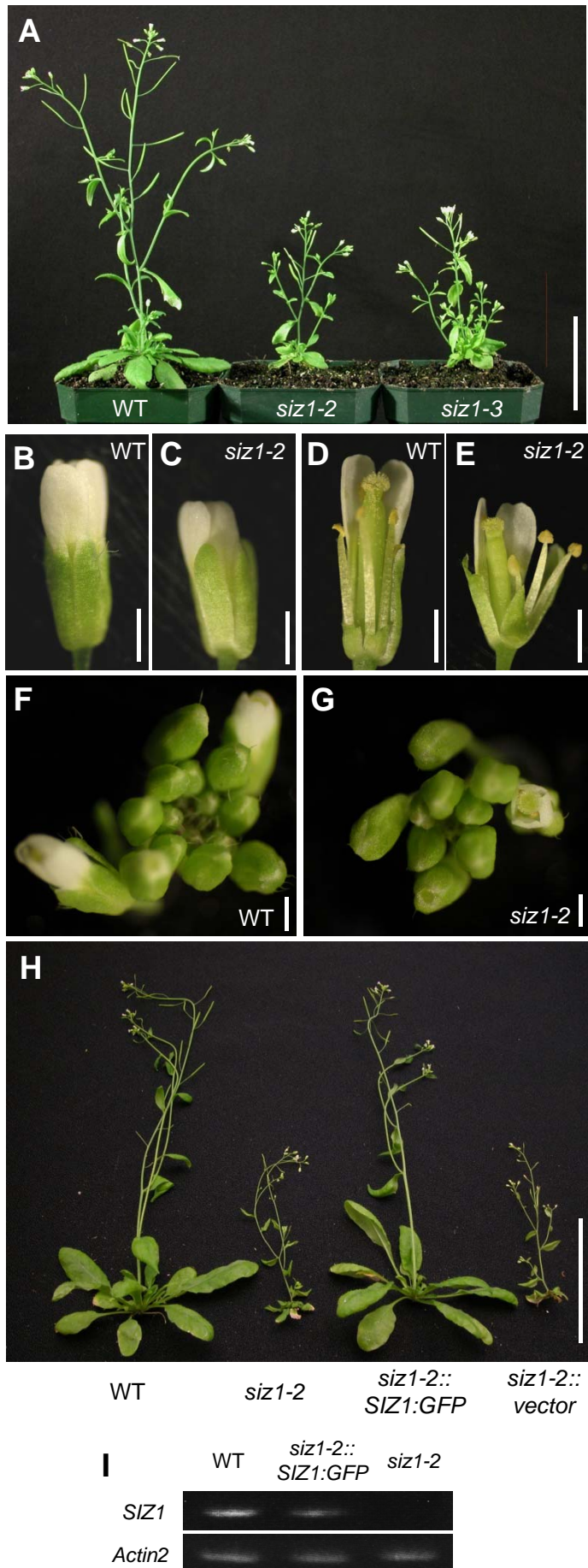


Figure 1. Miura et al.

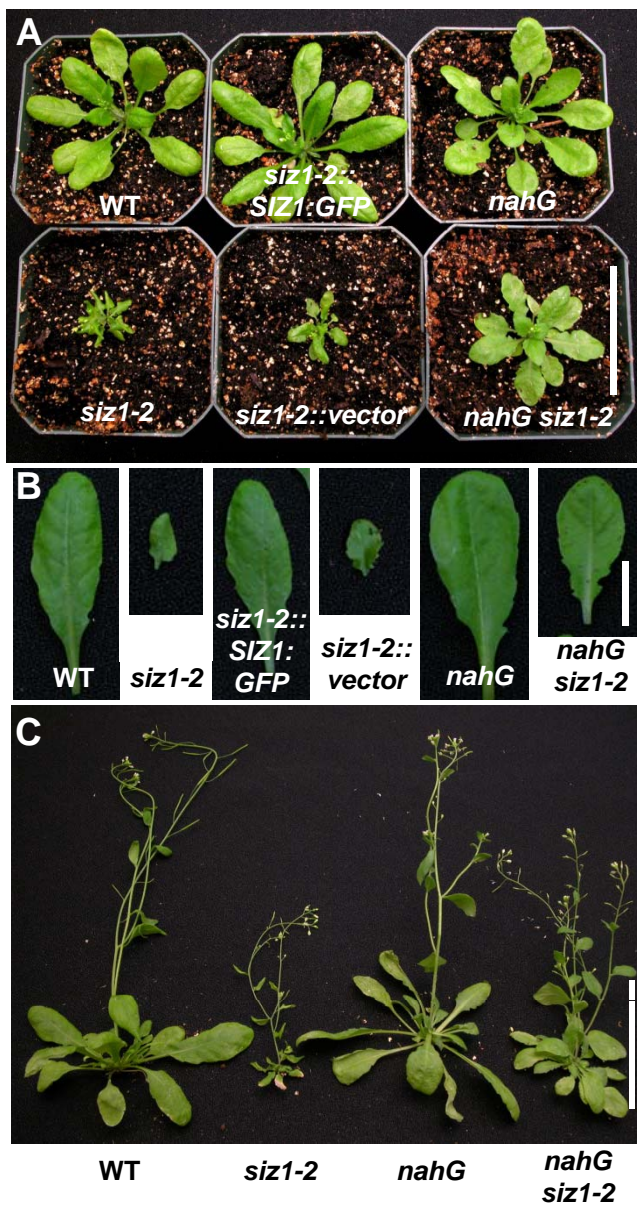


Figure 2. Miura et al.

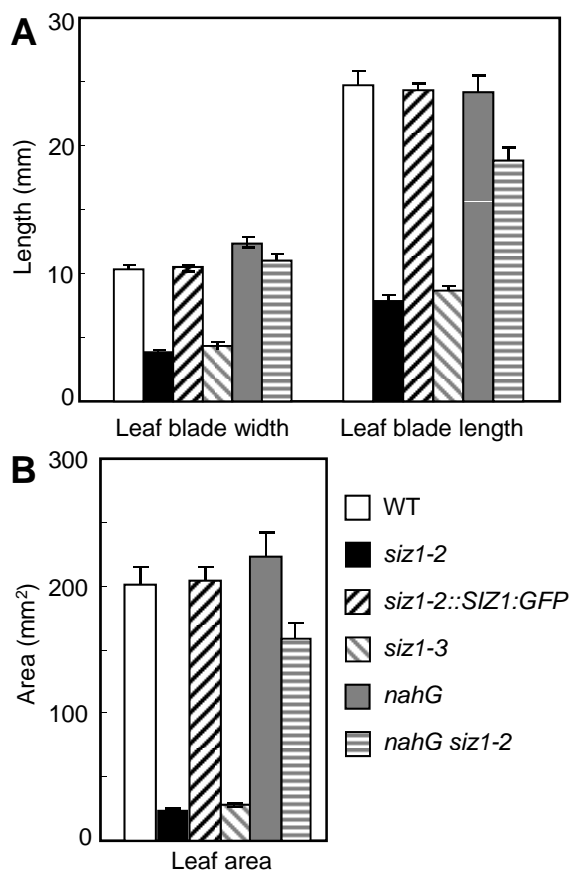


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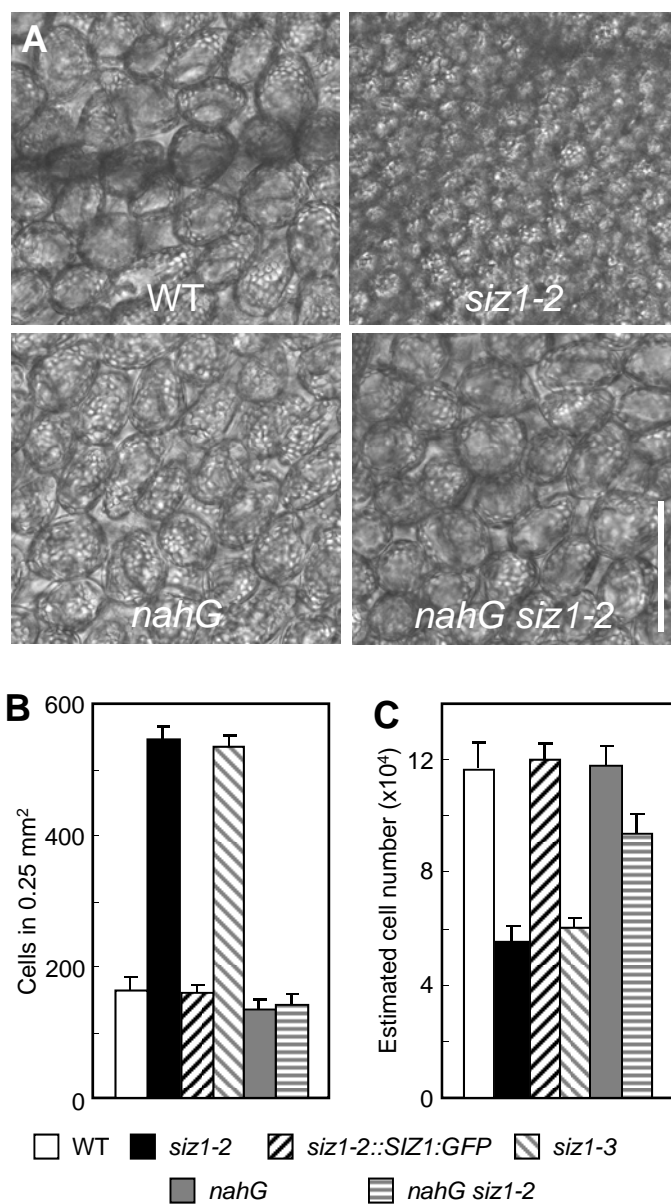


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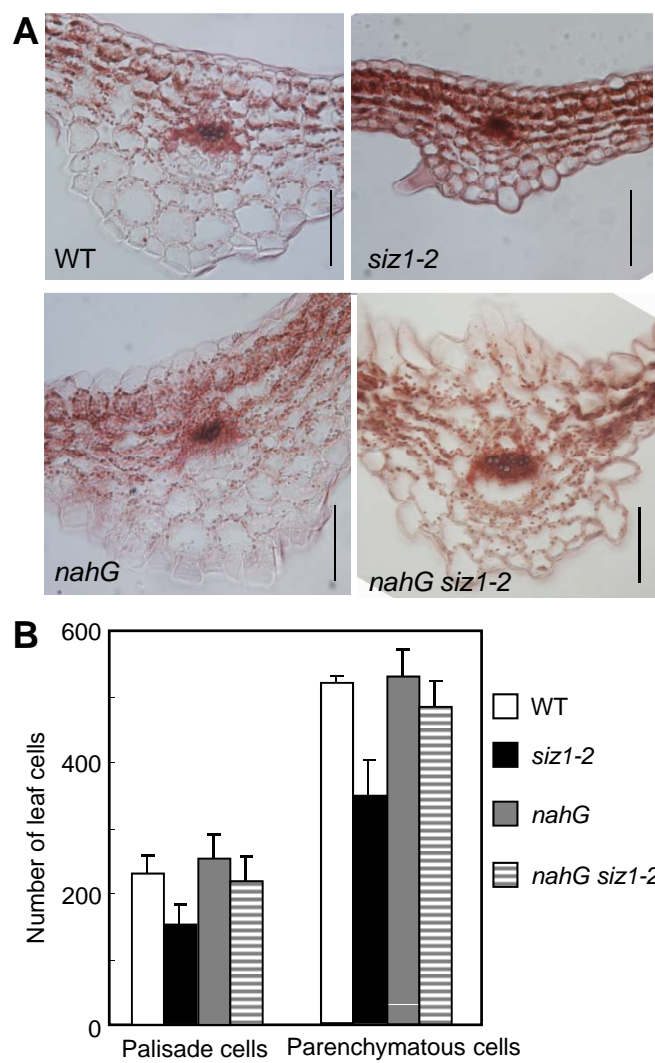


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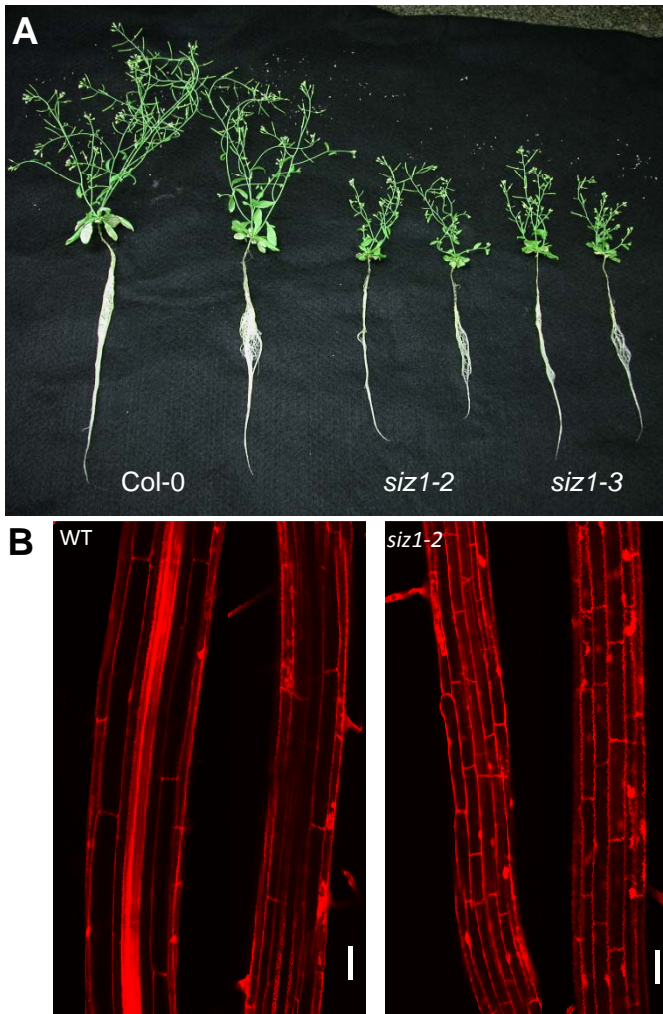


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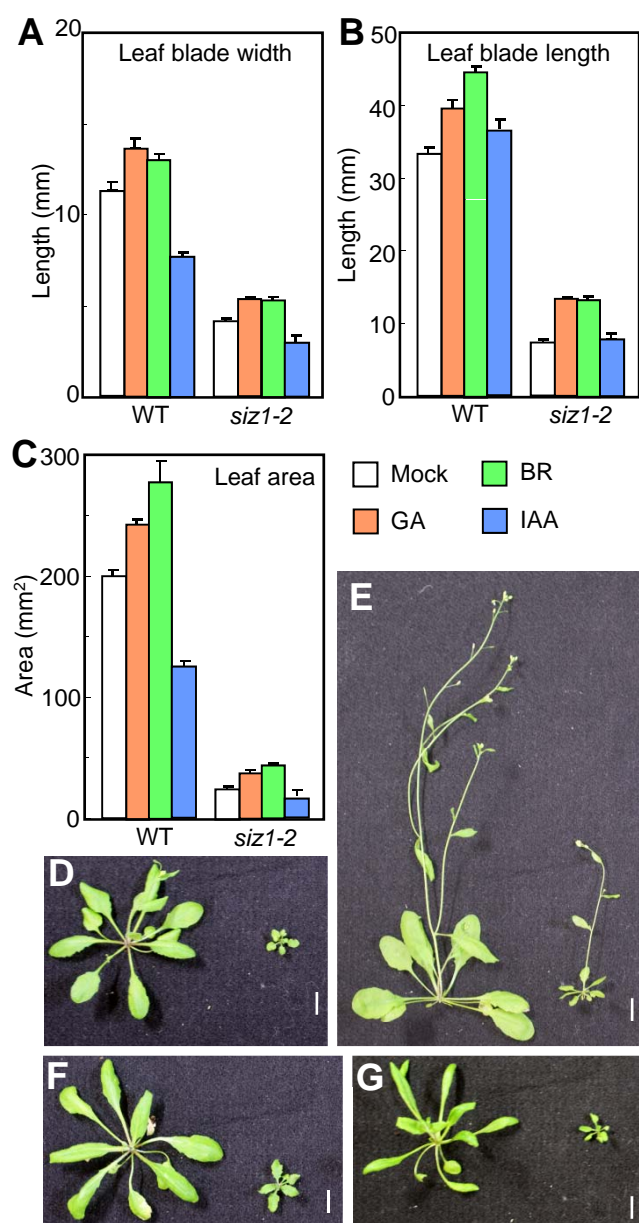


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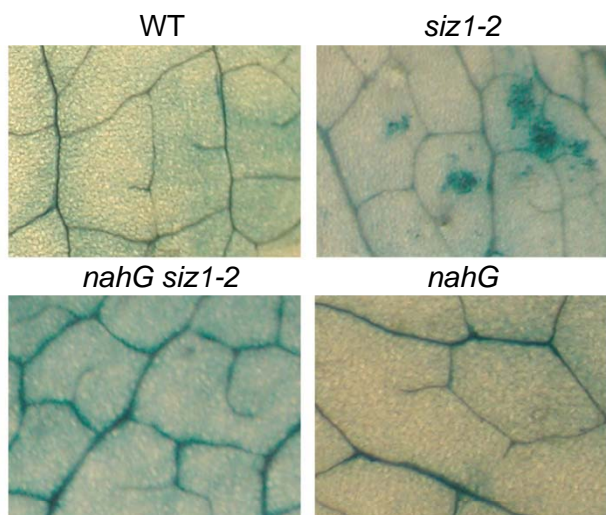


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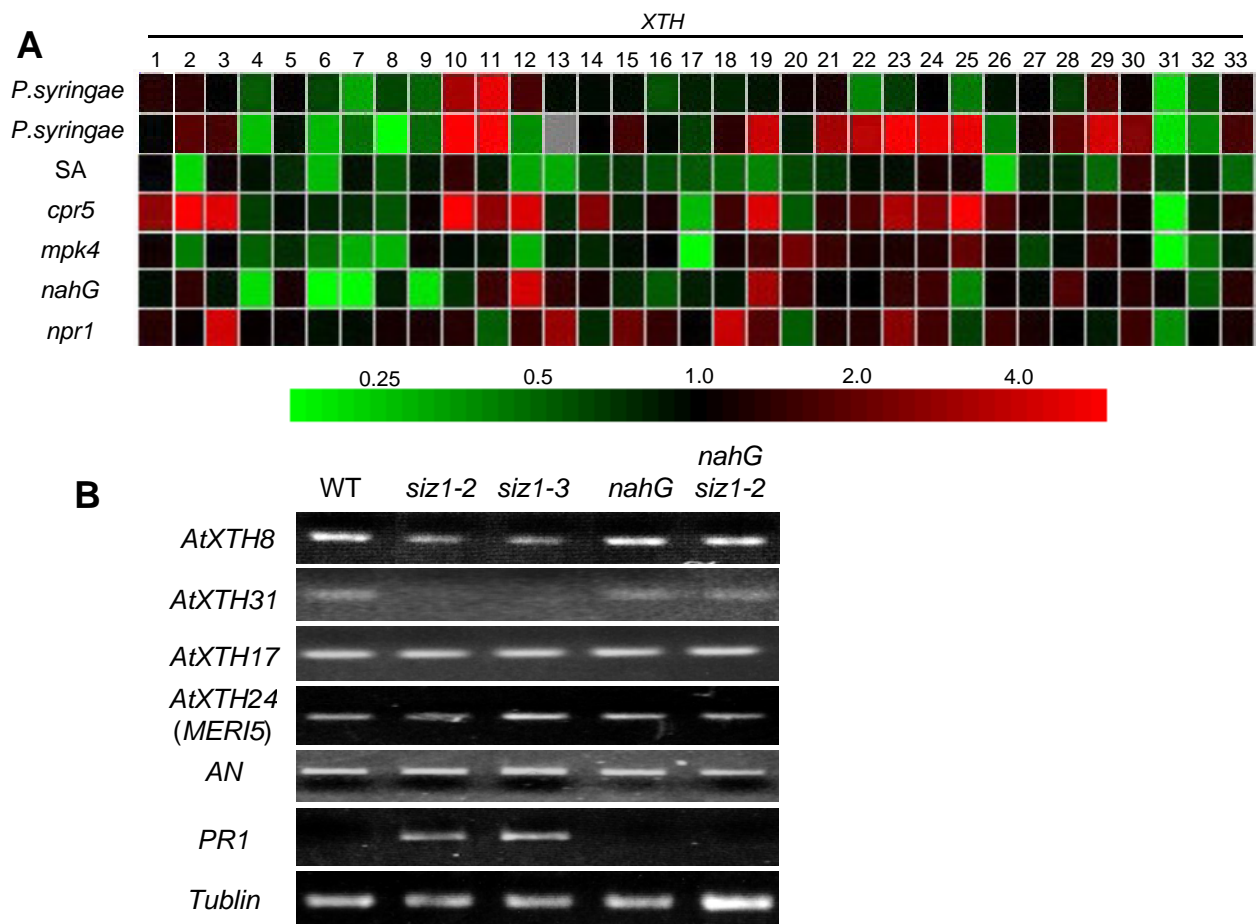


Figure 9. Miura et al.