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The *Arabidopsis* E3 SUMO Ligase SIZ1 Regulates Plant Growth and Drought Responses [W]

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Posttranslational modifications of proteins by small ubiquitin-like modifiers (SUMOs) regulate protein degradation and localization, protein–protein interaction, and transcriptional activity. SUMO E3 ligase functions are executed by SIZ1/SIZ2 and Mms21 in yeast, the PIAS family members RanBP2, and Pc2 in human. The *Arabidopsis thaliana* genome contains only one gene, *SIZ1*, that is orthologous to the yeast *SIZ1*/*SIZ2*. Here, we show that *Arabidopsis SIZ1* is expressed in all plant tissues. Compared with the wild type, the null mutant *siz1-3* is smaller in stature because of reduced expression of genes involved in brassinosteroid biosynthesis and signaling. Drought stress induces the accumulation of SUMO-protein conjugates, which is in part dependent on SIZ1 but not on abscisic acid (ABA). Mutant plants of *siz1-3* have significantly lower tolerance to drought stress. A genome-wide expression analysis identified ~1700 *Arabidopsis* genes that are induced by drought, with SIZ1 mediating the expression of 300 of them by a pathway independent of *DREB2A* and ABA. SIZ1-dependent, drought-responsive genes include those encoding enzymes of the anthocyanin synthesis pathway and jasmonate response. From these results, we conclude that SIZ1 regulates *Arabidopsis* growth and that this SUMO E3 ligase plays a role in drought stress response likely through the regulation of gene expression.

INTRODUCTION

Eukaryotic cells use a variety of small polypeptides for post-translational modification of proteins (Vierstra and Callis, 1999; Melchior, 2000; Hay, 2001; Pickart, 2001; Gill, 2004; Kerscher et al., 2006). In addition to ubiquitin, these small peptides include ubiquitin-like proteins, such as RUB1/Nedd8, SUMO, HUB, ISG15, and ATG (Hochstrasser, 2000; Dittmar et al., 2002). Whereas a major function of ubiquitin is to mark proteins for intracellular degradation, SUMO modification of proteins leads to a number of biological consequences from antagonism of ubiquitination to regulation of transcription factor activity, alteration of protein subcellular localization and changes in protein–protein interaction (Hochstrasser, 2000, 2001; Gill, 2003; Girdwood et al., 2004; Johnson, 2004; Watts, 2004).

The sumoylation pathway resembles the better-studied ubiquitination pathway. It requires the sequential action of three enzymes, E1, E2, and E3 (Kurepa et al., 2003; Colby et al., 2006). The sumoylation process begins with the activation of the SUMO C-terminal by an E1 activating enzyme, a subsequent transfer to an SUMO E2 conjugating enzyme, and then with the help of an E3 ligase, SUMO is finally conjugated to a substrate protein. Sumoylated proteins can be removed from conjugates by SUMO proteases that are responsible for SUMO recycling. Genes encoding all these components are present in the *Arabidopsis thaliana* genome (Vierstra and Callis, 1999; Kurepa et al., 2003; Lois et al., 2003; Murtas et al., 2003). Recent reports have revealed that the desumoylation system confers a high specificity, in contrast with the redundancy of the conjugating system (Chosed et al., 2006). Because of the emerging importance of sumoylation in plant development, some components of the sumoylation machinery, including SUMO (Lois et al., 2003; Colby et al., 2006), SCE1a (Lois et al., 2003), SIZ1 (Miura et al., 2005, 2007), and different SUMO proteases (Murtas et al., 2003; Chosed et al., 2006; Colby et al., 2006) have been investigated. Recent observations suggested that the *Arabidopsis* sumoylation system plays an important role in many aspects of plant developmental processes. SUMO conjugate levels increased when plants were subjected to a number of stresses, implicating sumoylation in plant stress responses (Kurepa et al., 2003; Miura et al., 2005, 2007; Yoo et al., 2006). Moreover, increased sumoylation levels have been shown to attenuate abscisic acid (ABA)-mediated growth inhibition and amplify the induction of ABA- and stress-responsive genes, e.g., *RD29A* (Lois et al., 2003).

So far, only two *Arabidopsis* mutants have been described as being altered in sumoylation (*esd4*, Murtas et al., 2003; *siz1*, Miura et al., 2005). A SUMO protease mutant, *esd4*, displays an early flowering phenotype and alterations in shoot development, implicating SUMO-protein conjugates in these developmental processes (Murtas et al., 2003). A key component of the sumoylation pathway is the E3 ligase, which confers substrate specificity. Therefore, it is reasonable to assume that changes in SUMO E3 ligase activity would have a significant impact on processes that are regulated by sumoylation. Yeast SIZ1 (for

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SAP and Miz domain) and human PIAS (for Protein Inhibitor of Activated STAT) proteins have been identified as SUMO E3 ligases that catalyze sumoylation of several proteins, including septins, LEF1, and STAT1 (Johnson and Gupta, 2001; Sachdev et al., 2001; Takahashi et al., 2001; Nishida and Yasuda, 2002; Ungureanu et al., 2003). In *Arabidopsis*, analysis of *siz1* mutants showed that SIZ1, a SUMO E3 ligase, regulates the expression of genes involved in phosphate starvation response and during the cold acclimation process (Miura et al., 2005, 2007). The transcription factors PHR1 and ICE1, which regulate part of the response of gene expression to phosphate starvation and cold stress, respectively, appear to be targets of SIZ1 (Miura et al., 2005, 2007). In addition, Yoo et al. (2006) reported that *siz1* mutants have reduced basal tolerance to heat shock, although no significant differences in gene expression were detected and Lee et al. (2006) found that SIZ1 regulates salicylate-dependent innate immunity in *Arabidopsis*.

Sumoylation has been implicated in the regulation of many aspects of eukaryotic development (Girdwood et al., 2004; Watts, 2004). To explore the role of the sumoylation process in plant development, we functionally characterized the *Arabidopsis* SUMO E3 ligase SIZ1. Here, we demonstrate that SIZ1 is expressed in almost all plant tissues. Detailed characterization of the knockout mutant *siz1-3* reveals that the E3 ligase is involved in the control of cell expansion and proliferation and in responses of plants to hormone and drought stress. We also performed a comparison between the expression profile of *siz1* with wild-type plants grown under control conditions and exposed to drought stress. Our results reveal that SIZ1 regulates the expression of an important set of genes under control conditions and in response to drought stress. These results indicate that SIZ1 regulates plant growth and the response to water deficit by changes in gene expression.

RESULTS

SIZ1 Is Expressed in All Plant Tissues

A major aim of our work is to study the role of SIZ1 and, therefore, sumoylation in *Arabidopsis* development and physiology. SIZ1 activity could be required in all plant cells or only in specific tissues/cell types. To investigate this issue, we generated transgenic plants expressing *SIZ1* promoter-β-glucuronidase (GUS) fusion genes to determine SIZ1 expression profile during plant development. Because no coding sequence can be detected in 3.65 kb upstream of the *SIZ1* coding sequence (from ATG of At5g60410 to ATG of At5g60400) (data obtained from The Institute for Genomic Research, http://www.tigr.org/tdb/e2k1/ath1/), we constructed three different promoter-GUS fusion genes containing 3133, 3535, and 4311 bp of 5′ sequences upstream of the *SIZ1* start codon (Figure 1A). Analysis of the GUS expression pattern in different transgenic lines showed no detectable differences among the three promoters. Figure 1 shows the representative expression profile of *pSIZ1-2:GUS* containing 3535 bp of 5′ upstream sequences.

In 3-d-old germinating seedlings, *SIZ1* was expressed in all organs except part of the hypocotyl and the basal region of the cotyledons (Figure 1B). A similar expression pattern in hypocotyl was observed in 3-week-old plants (Figure 1F). *SIZ1* was not expressed, at detectable levels, in juvenile leaves and in the basal region of developing young leaves (Figure 1F). However, in developing adult leaves, *SIZ1* expression was detected in leaf blades and petioles (Figures 1F and 1G). *SIZ1* was strongly expressed in the root system, especially in the primary root and lateral root tips (Figures 1C and 1D). Figure 1E shows strong expression of *SIZ1* in lateral root primordial, suggesting that this gene may play a role in lateral root development.

In flowers, *GUS* activity was observed in inflorescence stems, sepals, stamen filaments, and stigma (Figure 1H; data not shown), and only low expression levels were detected in anther and vascular tissues of petals. In young developing siliques, *SIZ1* expression was mainly found in stigma and pedicel (Figure 1I), while in adult siliques, expression was present all over the carpel (Figure 1J). No GUS staining was seen in developing seeds (data not shown).

The *siz1-3* Mutant Is Affected in Growth

The widespread expression of *SIZ1* in many different cell types suggested that the SUMO E3 ligase, and therefore the sumoylation system, may be involved in many different aspects of growth and development. To investigate this point further, we characterized the T-DNA knockout mutant *siz1-3* (Miura et al., 2005). As a control, we generated a complementation line by transforming *siz1-3* with a construct containing the *SIZ1* gene under the control of the constitutive promoter 35S (*siz1*+35S-*SIZ1*, named C-*siz1-3*). Analysis of mRNA and protein levels in this line revealed that plants of *C-siz1-3* expressed *SIZ1* mRNA and protein at a slightly lower level than wild-type plants (Figures 2A and 2B).

One week after germination on Murashige and Skoog (MS) plates, *siz1-3* plants showed no visible difference when compared with wild-type plants (data not shown). However, when germinated on soil, *siz1-3* was significantly smaller than both the wild type and C-*siz1-3* (Figure 2C). As juvenile leaves began to develop, *siz1-3* exhibited deficiencies in leaf elongation and enlargement (Figure 2C, panels a to c), and these differences were clearly manifested in rosette leaves of 5-week-old plants (Figure 2C, panel d). Similar differences could be seen in 25-d-old plants grown in vitro (see Supplemental Figure 1 online). In addition, 8-week-old *siz1-3* plants were significantly shorter compared with wild-type and C-*siz1-3* plants (Figure 2C, panel e). Compared with wild-type leaves, *siz1-3* leaves were reduced in length and width by approximately twofold (Figure 2D), and as a consequence, there was a 4.5-fold reduction in total leaf area (Figure 2D). Microscopy analysis revealed that *siz1-3* leaves contained smaller epidermal and mesophyll cells when compared with wild-type leaves (Figure 2E). In a 160,000-μm^2 area, the *siz1-3* leaf contained 114 ± 16.7 epidermal cells and 45 ± 5.6 stomata, whereas a wild-type leaf contained 47.9 ± 5.8 epidermal cells and 17.4 ± 2.8 stomata. Therefore, *siz1* leaves had ~2.3 times more cells per unit area than wild-type leaves. Wild-type leaf phenotype was recovered in C-*siz1-3*, attributing these mutant phenotypes to a deficiency in *SIZ1*. These results indicate that SIZ1, and therefore the sumoylation process, plays an important role in *Arabidopsis* cell expansion and proliferation.

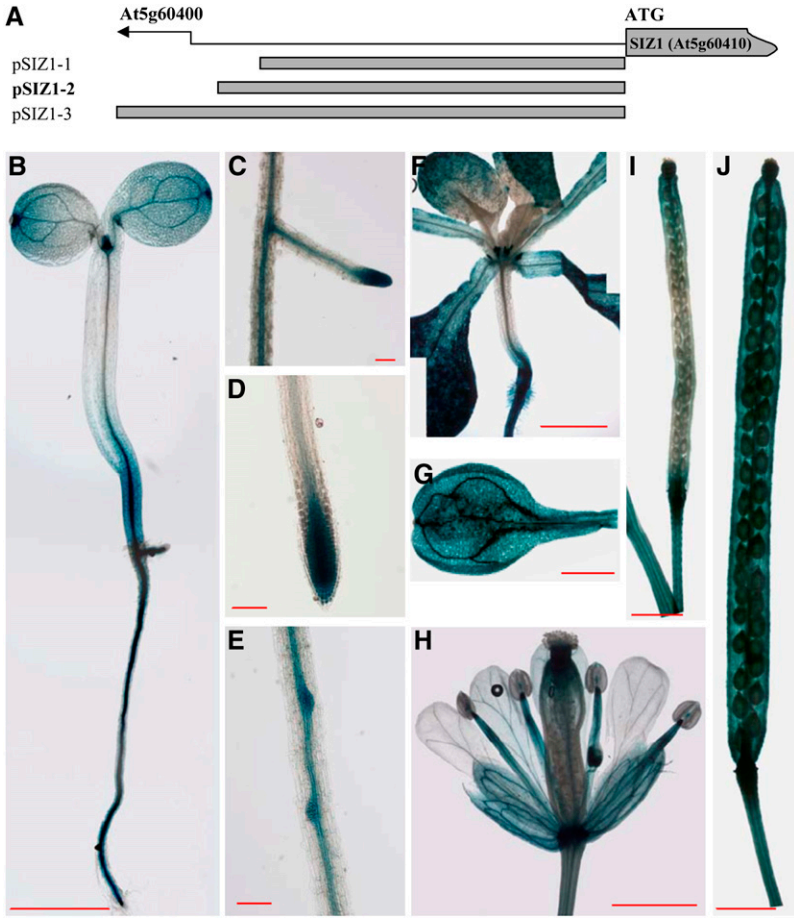

Figure 1. Expression Profile of *pSIZ1-GUS*.

(A) Schematic diagrams of *SIZ1* promoter fragments used for promoter-*GUS* fusions. *pSIZ1-1* (3133-bp 5′ sequence), *pSIZ1-2* (3535-bp 5′ sequence), and *pSIZ1-3* (4311-bp 5′ sequence) were fused to a *GUS* open reading frame.
(B) to **(J)** Expression patterns of *pSIZ1-2:GUS* in a 3-d-old seedlings **(B)**, lateral root **(C)**, primary root tip **(D)**, lateral root primordia **(E)**, 10-d-old seedlings **(F)**, adult leaf **(G)**, flower **(H)**, young silique **(I)**, and old silique **(J)** (see Results for details). Bars = 1 mm, except in **(C)** to **(E)**, where bars = 0.1 mm.

SIZ1 Regulates Anthocyanin Accumulation

Visual observation of *siz1-3* indicated reduced anthocyanin accumulation in petioles of adult plants when compared with the wild type and C-*siz1-3* (Figure 2C, panel d). We compared anthocyanin content of 5-week-old plants of the wild type, *siz1-3*, and C-*siz1-3* grown under control conditions. Figure 2F shows that *siz1-3* plants accumulated significantly lower anthocyanin levels than the wild type and C-*siz1-3*. The anthocyanin biosynthetic pathway is catalyzed by several enzymes, including PAL1, CHS1, and CHI (Solfanelli et al., 2006). RNA gel blot analysis revealed that, whereas the expression of *PAL1* and *CHI* was not significantly changed in *siz1-3*, the *CHS1* transcript accumulation was clearly lower in the mutant compared with the wild type (Figure 2G). Moreover, the reduced *CHS1* transcript in *siz1-3* was restored to wild-type levels in C-*siz1-3* plants. These results provide evidence that SIZ1 regulates the synthesis of anthocyanin by regulating *CHS1* expression.

Accumulation of SUMO-Protein Conjugates in Response to Drought Stress

Abiotic stresses, such as heat shock, low temperatures, ethanol, and H_2O_2 and phosphorus deficiency, have been reported to trigger a significant increase in SUMO-protein conjugate levels (Kurepa et al., 2003; Murtas et al., 2003; Yoo et al., 2006; Miura et al., 2007). Figure 3A shows that SUMO-protein conjugate levels were also elevated by drought treatment. This induction appeared to be ABA independent because no significant difference was observed between wild-type and the ABA-deficient mutant *aba2* (Figure 3A). To explore the possible role of SIZ1 in this process, we compared changes in SUMO-protein conjugate levels in the wild type, *siz1-3*, and C-*siz1-3*. Drought-induced accumulation of SUMO-protein conjugates was reduced in *siz1-3* but restored to near wild-type levels in C-*siz1-3* (Figure 3B), suggesting that SIZ1 mediates, in part, the increase of SUMO-protein conjugate levels in response to drought stress.

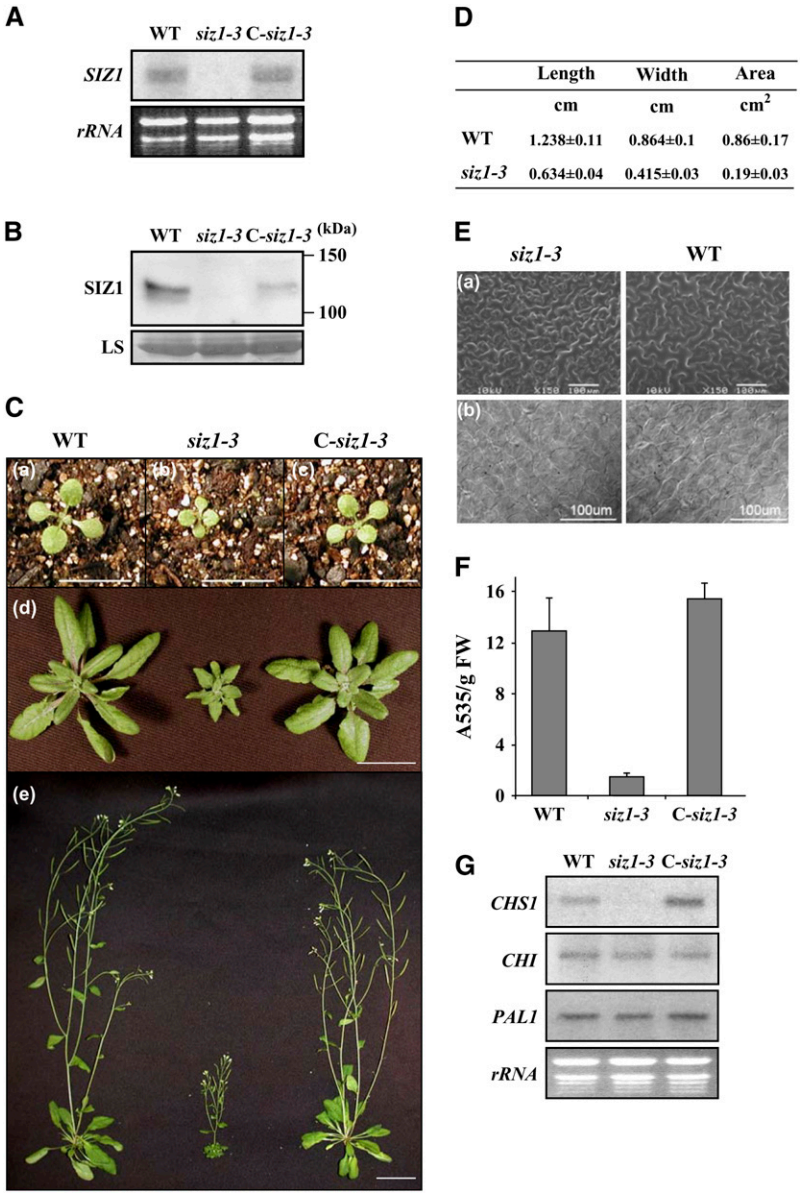

Figure 2. Functional Characterization of *SIZ1*.

(A) RNA gel blot analysis of *SIZ1*. Two-week-old wild-type, *siz1-3*, and *C-siz1-3* plants were used. rRNAs in the bottom panel were used as loading controls.

(B) Protein gel blot analysis of SIZ1 using affinity-purified anti-SIZ1 antibodies. Two week-old wild-type, *siz1-3*, and *C-siz1-3* plants were used. LS, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit.

(C) One-week-old (panels a to c), 5-week-old (d), and 8-week-old (e) wild-type, *siz1-3*, and *C-siz1-3* plants. Bars =10 mm in (a) to (c) and 3 cm in (d) and (e).

(D) Leaf lamina length, width, and area in 25-d-old wild-type and *siz1-3* mutant plants. Data are average values ± SD ($n = 12$).

(E) Analysis of epidermal (a) and mesophyll (b) cells of the 5th leaf of 25-d-old *siz1-3* and wild-type plants by scanning electron microscopy. In a 160,000-μm^2 area, a *siz1-3* leaf contains 114 ± 16.7 epidermal cells and 45 ± 5.6 stomata, whereas a wild-type leaf contains 47.9 ± 5.8 epidermal cells and 17.4 ± 2.8 stomata ($n = 6$). Note that the mesophyll cell size of *siz1-3* leaf is smaller than that of the wild type. Bars = 100 μm.

(F) Anthocyanin contents of wild-type, *siz1-3*, and *C-siz1-3* plants. Error bars represent SD ($n = 3$). Data are shown as relative units. FW, fresh weight.

(G) RNA gel blot analysis of total RNAs (10 μg) from 5-week-old wild-type, *siz1-3*, and *C-siz1-3* plants using specific probes for *CHS1*, *CHI*, and *PAL1*. rRNAs in the bottom panel were used as loading controls.

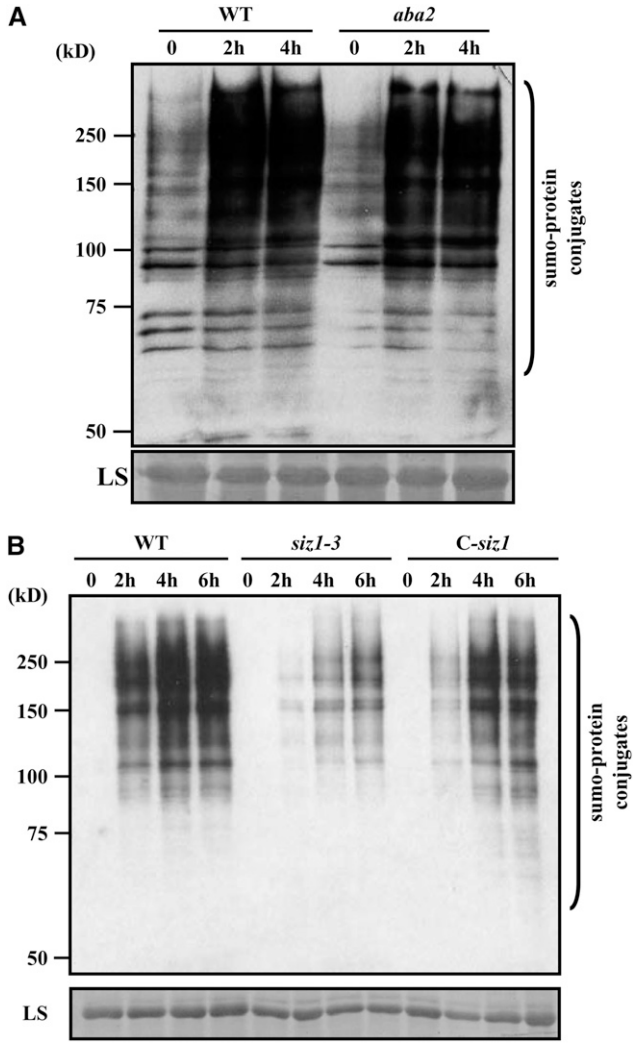

Figure 3. Accumulation of SUMO-Protein Conjugates Induced by Dehydration Is Partially Dependent on SIZ1.

Protein extracts were analyzed by protein gel blots using anti-SUM1 polyclonal antibodies to detect SUMO-protein conjugates. The large subunit (LS) of Rubisco (55 kD) was used as a loading control. Each lane contained 10 μg of protein.

(A) Three-week-old wild-type and *aba2* mutant plants exposed to drought for 0, 2, and 4 h.

(B) Three-week-old wild-type, *siz1-3*, and *C-siz1-3* plants exposed to drought for 0, 2, 4, and 6 h.

SIZ1 Is a Positive Regulator of Drought Stress Tolerance

The SIZ1-dependent increase in SUMO-protein conjugate levels in response to drought suggests a possible role of this E3 ligase in the stress response. To investigate this possibility, we quantified the tolerance to drought stress of wild-type, *siz1-3*, and C-*siz1-3* plants by measuring their loss of fresh weight and their capacity to survive 2 weeks after withholding water. Figure 4 shows that *siz1-3* was significantly more sensitive to drought stress than the wild type. In the first series of experiments, *siz1-3* lost 50% of fresh weight in 37 min compared with 49 min in the wild type (Figure 4A). This decreased capacity of *siz1-3* to retain water was more dramatic in experiments where water was withheld from soil-grown plants for 2 weeks. Upon resumption of watering, 89% ± 5.2% of wild-type plants survived, whereas none of the *siz1-3* plant recovered (Figure 4C). Moreover, plants of *C-siz1-3* displayed wild-type-level tolerance to this stress with a 93% ± 3.1% survival rate (Figure 4C). To rule out the possibility that these results were affected by plant size, we also measured the loss of fresh weight of wild-type, *siz1-3*, and C-*siz1-3* plants grown on plates. Note that in contrast with plants grown on soil, these plants have a similar size irrespective of the genotypes, and whole plants were used for these experiments. The results of these experiments confirmed that *siz1-3* was more sensitive to drought stress (see Supplemental Figure 2 online). Finally, SIZ1 protein levels showed a transient increase at 2 h after drought treatment (Figure 4D) consistent with its role in mediating synthesis of SUMO-protein conjugates.

Transcriptome Analysis of *siz1-3*

To study the molecular basis of *siz1-3* phenotypes, we performed a genome-wide expression analysis using samples from wild-type and *siz1-3* plants under normal growth conditions and after 2 h of drought stress. Under control conditions, the expression of ~1600 *Arabidopsis* genes (7% of the total) was deregulated in *siz1-3* (more than twofold difference relative to the wild type) (see Supplemental Table 1 online). Among them, we found 317 genes whose expression was lower in *siz1-3* than in wild-type plants (see Supplemental Table 2 online). Interestingly, 11 of these genes encode proteins involved in brassinosteroid biosynthesis and the signaling pathway and four in the auxin signaling pathway (Table 1). These results indicate that under normal growth conditions, SIZ1 regulates brassinosteroid and auxin biosynthesis and signaling pathways, and through these pathways, influences *Arabidopsis* development. In addition, we found 643 genes that showed an increased mRNA accumulation in *siz1-3* compared with wild-type plants (see Supplemental Table 3 online). This group of genes included *PR-1*, *PR-2*, and *PR-5*, which were previously reported to show increased expression in the mutant (Lee et al., 2006).

The responses and tolerance of *Arabidopsis* to drought stress are to a large extent underpinned by changes in gene expression (Shinozaki et al., 2003; Sakuma et al., 2006). Therefore, it is not surprising that after 2 h of drought treatment the expression of 25% of the *Arabidopsis* genes was significantly altered in the wild type (see Supplemental Table 4 online). Among them, ~1700 genes showed a significant increase in their expression levels (more than twofold induction; see Supplemental Table 5 online). In this category, we found many genes previously reported to be induced by ABA, pathogen attack, heat shock, jasmonic acid, or auxin (Table 2). In addition, drought-induced expression of ~1044 genes was significantly deregulated in *siz1-3* (see Supplemental Table 6 online). Moreover, *siz1-3* seemed to be needed for the appropriate drought induction of 262 of these genes (more than twofold expression decrease in *siz1-3*; see Supplemental Table 7 online). Included in this category (Tables 3 and 4) were genes previously implicated in drought stress

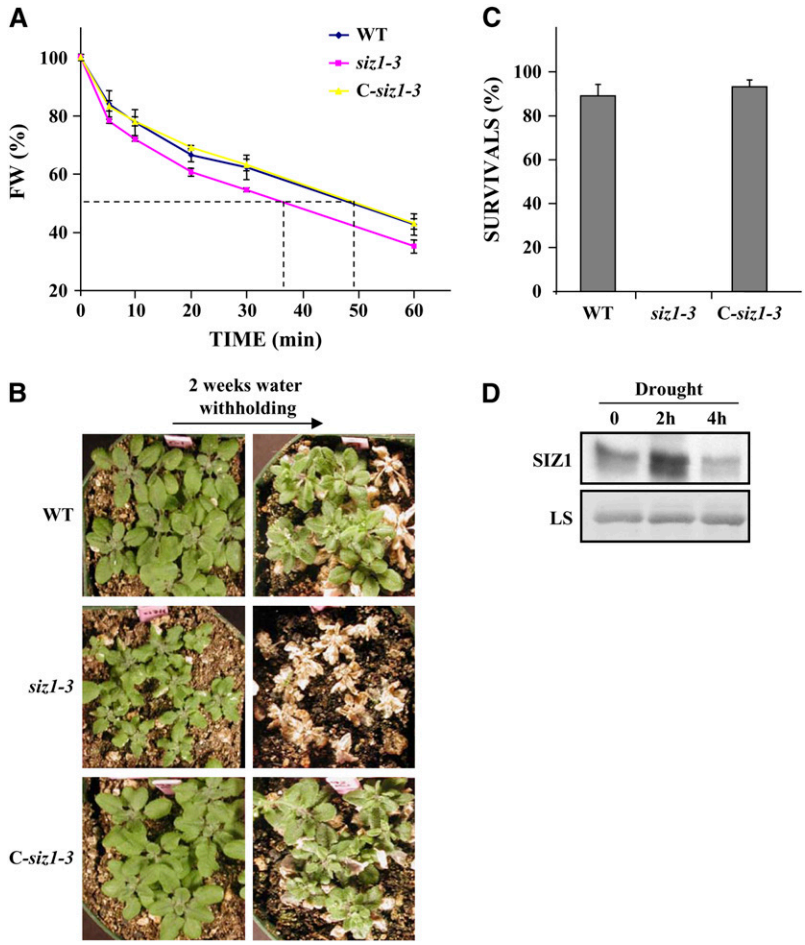

Figure 4. SIZ1 Plays an Important Role in Drought Tolerance.

(A) Water loss quantification in percentage of fresh weight lost (0, 5, 10, 20, 30, and 60 min) in 3-week-old wild-type, *siz1-3*, and *C-siz1-3* plants. The percentage of fresh weight (FW) remaining after the treatment was determined. Data represent average values ± SD ($n = 6$).

(B) Survival of plants subjected to water withholding for 3 weeks after resumption of watering for 1 week.

(C) Percentage of survival in **(B)**. Data represent average values ± SD ($n = 6$).

(D) Analysis of SIZ1 protein levels in 2-week-old wild-type plants exposed to drought for 0, 2, and 4 h. Protein extracts were analyzed by protein gel blots using affinity-purified anti-SIZ1 antibodies. LS, large subunit of Rubisco.

tolerance, such as *MYC2* (Abe et al., 2003), *ANNAT4* (Lee et al., 2004), *COR15A* (Li et al., 1993), *KIN1* (Gilmour et al., 1992), and *P5CS1* (Yoshiba et al., 1995). We found that the expression of genes coding for almost all of the anthocyanin biosynthetic enzymes were induced by drought in the wild type, and this induction was severely impaired in *siz1-3* (Table 4). The same situation applies to genes involved in brassinosteroid synthesis and in jasmonate responses (Tables 3 and 4). All these genes can be divided into two categories depending in how SIZ1 affects their expression. The first category included genes whose expression was lower in *siz1-3* compared with the wild type either under normal growth conditions or in response to drought stress, but these genes remained drought inducible in the mutant (Table 3). The second category included genes that were expressed at wild-type levels under normal conditions but did not respond significantly to drought stress in *siz1-3* (Table 4). Taken altogether, our results demonstrate that SIZ1, and therefore sumoylation of proteins, play an important role in the regulation of several hormone signaling pathways under normal growth conditions and during responses to drought stress.

The results obtained in the microarray experiments were validated by quantitative RT-PCR analysis, which uncovered four gene categories (Figure 5). In the first category (Figure 5, panels 1 to 8), basal but not drought-inducible expression was reduced by SIZ1 deficiency. For example, under control conditions, *SQS2* expression in the wild type was approximately threefold higher than in *siz1-3* (Figure 5, panel 2, column WT C/*siz1-3* C); however, the relative fold induction of this gene by drought was comparable in the mutant and the wild type (Figure 5, panel 2, columns WT D/C and *siz1-3* D/C). These results show

Table 1. Genes Underexpressed in *siz1-3* Plants under Normal Growth Conditions

Gene Name	AGI Code[a]	Ratio[b]	P Value	Description
Brassinosteroids				
CYP85A2	AT3G30180	4	1.52E-02	Cytochrome P450
BEE1	AT1G18400	4	8.45E-03	Helix-loop-helix DNA binding protein
SQP1	AT5G24150	4	2.48E-02	Squalene monooxygenase
LUP1	AT1G78970	4	4.43E-02	Lupeol synthase
DWF4	AT3G50660	3	1.01E-02	22α hydroxylase
SQS2	AT4G34650	3	4.70E-02	Squalene synthase
DET2	AT2G38050	2	7.00E-03	3-oxo-5-α-steroid 4-dehydrogenase
HMG1	AT1G76490	2	4.18E-02	3-hydroxy-3-methylglutaryl coenzyme A reductase
BIM1	AT5G08130	2	2.88E-02	Basic helix-loop-helix (bHLH) family protein
DWF1	AT3G19820	2	2.86E-02	Cell elongation protein
FK	AT3G52940	2	4.52E-02	Nuclear envelope membrane protein
Auxin				
ATR1	AT5G60890	4	2.13E-02	cyp450 reductase
IAA6	AT1G52830	4	2.99E-02	Auxin-responsive protein
PIN7	AT1G23080	3	4.76E-02	Auxin efflux carrier protein
AUX1	AT2G38120	2	4.56E-02	Putative transporter of amino acid–like molecules
ABA				
NCED3	AT3G14440	5	3.42E-02	9-*cis*-epoxycarotenoid dioxygenase
CIPK20	AT5G45820	5	9.17E-03	CBL-interacting Ser/Thr protein kinase
ABA1	AT5G67030	2	2.26E-02	Zeaxanthin epoxidase
Light				
PIF4	AT2G43010	3	2.40E-02	bHLH protein
PKS2	AT1G14280	3	4.45E-02	Phytochrome kinase substrate
PRR5	AT5G24470	2	2.13E-02	Pseudo-response regulator
PIL6	AT3G59060	2	4.07E-02	Myc-related bHLH transcription factor

[a] AGI, Arabidopsis Genome Initiative.
[b] Expression fold decrease in *siz1-3* relative to wild-type plants.

that under drought conditions, the expression of these groups of genes in *siz1-3* was still threefold lower than that in the wild type. In the second category, exemplified by *HMG1*, basal level expression was reduced in *siz1-3* compared with the wild type (Figure 5, panel 9, column WT C/*siz1-3* C). On the other hand, drought treatment had little effect on *HMG1* expression in the wild type but induced *HMG1* expression by threefold in *siz1-3* (Figure 5, panel 9, column *siz1-3* D/C). The third category of genes included those encoding enzymes of the anthocyanin biosynthetic pathway (Figure 5, panels 10 to 13). Drought-inducible but not basal (Figure 5, columns *siz1-3* D/C and WT C/*siz1-3* C) expression of these genes clearly required SIZ1. Finally, expression of genes in the fourth category appear to be insensitive to SIZ1 abundance under both control and drought conditions (Figure 5; panels 14 to 16).

DISCUSSION

Compared with a growing long list of SUMO-modified proteins identified in yeast and mammals, little is known about sumoylated proteins and their roles in plants. In *Arabidopsis*, sumoylation has been implicated in stress responses (Kurepa et al., 2003; Miura et al., 2005, 2007; Yoo et al., 2006), ABA signaling (Lois et al., 2003), flowering-time regulation (Murtas et al., 2003), phosphorus starvation (Miura et al., 2005), and innate immunity (Lee et al., 2006). These results indicate a central role for the sumoylation process in environmental responses and in different aspects of plant development. Here, we describe the role of SIZ1, an *Arabidopsis* SUMO E3 ligase (Miura et al., 2005), in *Arabidopsis* growth and drought stress tolerance.

SIZ1 Regulates *Arabidopsis* Growth

Because SIZ1 is expressed in almost all plant tissues (Figure 1), it is reasonable to assume that this E3 ligase plays a role in plant development. Morphological analysis showed that *siz1-3* has reduced plant height and leaf size (Figure 2). Interestingly, although *siz1* leaves show a decrease in cell size (2.3 times smaller than wild-type cells), this could not completely explain the reduction in leaf area (4.5 times smaller than the wild type) (Figure 2). These results suggest that SIZ1 is involved in cell expansion and cell proliferation. This phenotype can be attributed to a SIZ1 deficiency because wild-type morphology was restored in the complementation line, which displays comparable accumulation levels of SUMO proteins as wild-type plants (Figure 3).

Genome-wide expression analyses show that under normal growth conditions, SIZ1 regulates the expression of ~1600 genes in *Arabidopsis*. Among these, 11 genes encode important components of the brassinosteroid biosynthetic and signaling

Table 2. Drought-Inducible Genes

Gene Name	AGI Code	P Value	Description
Abiotic stress			
ABF3	AT4G34000	1.61E-02	ABA-responsive element binding factor
ADH1	AT1G77120	1.20E-02	Alcohol dehydrogenase
ANNAt4	AT2G38750	2.18E-02	Calcium-dependent membrane binding protein
At *MRP*	AT1G04120	1.89E-02	ABC transporter family protein
COR15A	AT2G42540	8.99E-03	Cold-regulated protein
COR47	AT4G38410	3.31E-03	Cold-regulated protein
ERD10	AT1G20450	2.54E-02	Dehydrin
ERD2	AT1G29330	3.37E-03	Endoplasmic reticulum retention signal receptor
MYB2	AT2G47190	1.79E-02	MYB transcription factor
MYC2	AT1G32640	8.82E-03	Transcription factor
P5CS1	AT2G39800	8.10E-03	δ 1-Pyrroline-5-carboxylate synthetase
RAB18	AT5G66400	9.22E-03	Dehydrin
RAP2.4	AT1G78080	8.18E-03	ERF/AP2 transcription factor family
RAP2.6	AT1G43160	1.32E-02	ERF/AP2 transcription factor
RCI2B	AT3G05890	1.90E-02	Low-temperature and salt-responsive protein
RD20	AT2G33380	1.60E-03	Calcium binding protein
RD29A	AT5G52310	1.74E-02	Low-temperature-responsive 78-kD protein
RD29B	AT5G52300	2.85E-03	Low-temperature-responsive 65-kD protein
SAL1	AT5G63980	4.34E-02	FIERY1 protein
SUS1	AT5G20830	1.56E-03	Sucrose synthase
Anthocyanin			
*CHS1**	AT5G13930	7.61E-03	Chalcone synthase
DFR	AT5G42800	2.57E-02	Dihydroflavonol reductase
F3H	AT3G51240	3.10E-02	Flavanone 3-hydroxylase
FLS	AT5G08640	2.85E-02	Flavonol synthase
PAP1	AT1G56650	2.76E-02	MYB domain
TT5	AT3G55120	5.03E-03	Chalcone-flavanone isomerase
TT7	AT5G07990	1.86E-02	Flavonoid 3′-monooxygenase/flavonoid 3′-hydroxylase (F3′H)
TT8	AT4G09820	7.86E-03	bHLH protein
UGT78D1	AT1G30530	1.94E-02	UDP glucose:flavonoid 3-*o*-glucosyltransferase
ABA			
*ABF3**	AT4G34000	1.61E-02	ABA-responsive element binding protein
*ABI1**	AT4G26080	1.08E-03	Protein phosphatase 2C
*ABI2**	AT5G57050	6.56E-03	Protein phosphatase 2C
AHG3	AT3G11410	9.43E-03	Protein phosphatase 2C
*AtHB12**	AT3G61890	1.51E-02	Homeodomain Leu zipper class I
*AtHB7**	AT2G46680	9.23E-04	Homeobox Leu zipper
*MYB102**	AT4G21440	1.00E-02	MYB transcription factor
*ATR1**	AT4G24520	1.42E-03	cyp450 reductase
*COR13**	AT4G23600	3.70E-02	Cys lyase
GBF3	AT2G46270	1.05E-02	bZIP G-box binding protein
*HAB1**	AT1G72770	6.15E-04	Protein phosphatase 2C
*MYB7**	AT2G16720	8.57E-03	Myb transcription factor
PRN	AT3G59220	5.00E-02	Cupin domain–containing protein
Jasmonic			
AOC3	AT3G25780	3.33E-02	Allene oxide cyclase
AOS	AT5G42650	5.66E-03	Allene oxide synthase
DAD1	AT2G44810	1.65E-03	Chloroplastic phospholipase A1
ESP	AT1G54040	3.85E-03	Epithiospecifier protein
JAR1	AT2G46370	2.44E-03	Auxin-responsive GH3 family protein
JMT	AT1G19640	2.61E-02	*S*-adenosyl-L-Met:jasmonic acid carboxyl methyltransferase
LOX3	AT1G17420	2.85E-02	Lipoxygenase
TAT3	AT2G24850	2.29E-02	Tyrosine aminotransferase
Auxin			
CYP79B2	AT4G39950	1.89E-02	Cytochrome P450
IAA5	AT1G15580	2.42E-02	Auxin-responsive protein
IAR3	AT1G51760	3.82E-02	IAA–amino acid conjugate hydrolase

(Continued)

Table 2. (continued).

Gene Name	AGI Code	P Value	Description
ILL6	AT1G44350	3.60E-02	IAA–amino acid hydrolase
ILR1	AT3G02875	2.71E-03	IAA–amino acid hydrolase
Ethylene			
*ACS2**	AT1G01480	2.49E-02	1-Aminocyclopropane-1-carboxylate
*ERS2**	AT1G04310	3.93E-03	Two-component His kinase
Brassinosteroid			
SQS2	AT4G34650	1.27E-02	Squalene synthase
SQP1	AT5G24150	2.03E-02	Squalene monooxygenase 1,1
Defense response			
ASA1	AT5G05730	1.14E-02	Anthranilate synthase
DHS1	AT4G39980	1.66E-02	1-Deoxy-D-arabino-heptulosonate 7-phosphate
EDS5	AT4G39030	1.95E-03	Orphan multidrug and toxin extrusion transporter
MLO11	AT5G53760	2.74E-03	Seven transmembrane MLO family protein
*PAL1**	AT2G37040	1.86E-02	Phe ammonia-lyase
PLP7	AT3G54950	3.73E-03	Patatin-related
WRKY18	AT4G31800	2.40E-02	WRKY family transcription factor
Heat shock			
HSFA2	AT2G26150	1.24E-02	Heat stress transcription factor
HSFA6B	AT3G22830	8.11E-03	Heat stress transcription factor
HSP17.6-C11	AT5G12020	4.71E-03	Heat shock protein
*HSP17.8**	AT1G07400	2.51E-03	Heat shock protein
HSP23.5-M	AT5G51440	3.91E-02	Mitochondrial small heat shock protein
*HSP70**	AT5G02500	2.65E-02	Heat shock protein
HSP81-1	AT5G52640	1.84E-02	Heat shock protein
Phosphate starvation			
PLDZ2	AT3G05630	1.83E-03	Phospholipase D protein
Light			
PIF3	AT1G09530	2.05E-03	Transcription factor
PTF1	AT3G02150	1.60E-03	TCP family transcription factor
Development			
BIGPETAL	AT1G59640	5.36E-03	bHLH encoding gene

The asterisk indicates genes previously described as being stress inducible. IAA, indole-3-acetic acid.

pathway (Table 1, Figure 5). Similar to *siz1-3*, knockout mutants of some of these genes display a dwarf phenotype due to a defect in cell elongation (*dwf1*, Takahashi et al., 1995; *det2*, Li et al., 1996; *dwf4*, Azpiroz et al., 1998; *hmg1*, Suzuki et al., 2004) and cell proliferation (*det2* and *dwf1*, Nakaya et al., 2002). Moreover, *siz1-3* also shows a decrease in the expression of genes implicated in auxin signaling (Table 1), another hormone that regulates plant development (Tanaka et al., 2006). There is evidence for an interaction between brassinosteroid and auxin signaling and that brassinosteroid may act through an auxin-mediated pathway (Arteca et al., 1988; Mandava, 1988; Sasse, 1999; Nakamura et al., 2006). Taken together, these results suggest that SIZ1 modulates brassinosteroid biosynthesis and signaling pathways and, through these processes, *Arabidopsis* growth. One reasonable hypothesis is that a regulator of the brassinosteroid pathway is either activated or inactivated via sumoylation by SIZ1. That SIZ1 regulates plant growth is not surprising since in other eukaryotes a large number of proteins involved in different aspects of development are known to be sumoylated (Girdwood et al., 2004; Watts, 2004). Moreover, analysis of mutants affected in the SUMO protease, ESD4, uncovers a role of sumoylation in the regulation of flowering time and the control of plant development (Murtas et al., 2003). Recently, Miura et al. (2005) reported that SIZ1 is involved in the regulation of root growth in response to phosphate starvation.

Under normal growth conditions *siz1-3* accumulated four times less anthocyanin than the wild type, indicating that SIZ1 positively regulates pigment accumulation in adult plants (5-week-old plants) (Figure 2C, panel d). Transcript analyses revealed that *CHS1* levels were significantly lower in *siz1-3*, which may account for its lower anthocyanin content. This is consistent with previous notion that *CHS1* plays a central role in flavonoid biosynthesis (Li et al., 1993; Saslowsky et al., 2000). The regulation by SIZ1 appears to be age dependent because *CHS1* expression is only affected in adult plants (5-week-old plants). Deficiencies in anthocyanin accumulation have also been reported in *dwf1*, a brassinosteroid-deficient mutant (Luccioni et al., 2002). These data, together with our results, suggest that brassinosteroid regulates anthocyanin accumulation in adult plants and that SIZ1, and therefore the sumoylation process, mediates this process.

SIZ1 Is a Positive Regulator of Drought Tolerance

Previous work has reported an increase in the accumulation of SUMO-protein conjugates in response to abiotic stresses, such as heat shock, cold, ethanol, or H_2O_2 (Kurepa et al., 2003; Murtas

Table 3. Drought-Inducible Genes Underexpressed in *siz1-3*

Gene Name	AGI Code	P Value	WT C[a]	WT D2 h[b]	Ratio[c]	*siz1*C[d]	*siz1* D2 h[e]	Ratio[f]	Description
Stress									
ATR1	AT5G60890	2.26E-03	38	1196	31	9	250	27	Myb-like transcription factor
ANNAT4	AT2G38750	6.09E-03	243	1323	5	37	186	5	Calcium-dependent membrane binding protein
RCI2B	AT3G05890	2.43E-02	227	943	4	82	355	4	Low-temperature and salt-responsive protein
P5CS1	AT2G39800	1.50E-02	1264	4680	4	37	401	11	δ 1-Pyrroline-5-carboxylate synthetase
MYC2	AT1G32640	7.69E-03	1080	3020	3	435	1486	4	Transcription factor
COR15a	AT2G42540	3.91E-02	10518	23450	2	964	3040	3	Cold-regulated protein
KIN1	AT5G15960	2.12E-02	21422	43230	2	1130	2912	3	Cold- and ABA-inducible protein
Jasmonic									
JMT	AT1G19640	7.85E-03	18	864	46	6	333	54	*S*-adenosyl-L-Met:jasmonic acid carboxyl methyltransferase
COI3	AT4G23600	7.72E-04	3031	18327	6	656	6640	10	Cys lyase
AOS	AT5G42650	1.32E-03	1711	7748	4	351	3304	9	Allene oxide synthase
ESP	AT1G54040	8.15E-04	664	1468	2	109	248	2	Epithiospecifier protein
LOX1	AT1G55020	1.83E-02	47	102	2	38	45	1	Lipoxygenase
Brassinosteroid									
SQS2	AT5G24150	1.68E-03	33	162	5	12	31	3	Squalene synthase
SQP1	AT4G34650	1.45E-02	107	410	4	30	76	2	Squalene monooxygenase 1,1

[a] Absolute expression value of wild-type plants under normal conditions.
[b] Absolute expression value of wild-type plants exposed for 2 h to drought stress.
[c] Expression fold increase in wild-type dehydrated plants relative to control plants.
[d] Absolute expression value of *siz1-3* plants under normal conditions.
[e] Absolute expression value of *siz1-3* plants exposed for 2 h to drought stress.
[f] Expression fold increase in *siz1-3* dehydrated plants relative to control plants.

et al., 2003; Yoo et al., 2006; Miura et al., 2007). Here, we show that *Arabidopsis* plants exposed to drought stress accumulate increased levels of sumoylated proteins by an ABA-independent pathway (Figure 3). Moreover, this increase is also highly dependent on SIZ1 activity since the accumulation of SUMO-protein conjugates is significantly lower in *siz1-3* compared with the wild type. This SIZ1-mediated sumoylation likely plays an important role in conferring drought stress tolerance because *siz1-3* plants were clearly more sensitive to drought stress compared with wild-type plants. In addition, this hypersensitive phenotype was

Table 4. Drought-Inducible Genes with Decreased Induction by Drought in *siz1-3*

Gene Name	AGI Code	P Value	WT C[a]	WT D2 h[b]	Ratio[c]	*siz1*C[d]	*siz1* D2 h[e]	Ratio[f]	Description
Stress									
RD29B	AT5G52300	4.47E-03	33	677	21	37	186	5	Low-temperature-responsive 65-kD protein
SAL1	AT5G63980	8.72E-04	243	1490	6	736	521	1	FIERY1 protein
Anthocyanin									
PAP1	AT1G56650	8.96E-03	56	1341	24	31	320	10	MYB domain–containing transcription factor
DFR	AT5G42800	1.60E-03	15	220	15	27	33	1	Dihydroflavonol reductase
CHS1	AT5G13930	3.96E-03	35	419	12	46	37	1	Chalcone synthase
TT8	AT4G09820	6.46E-03	7	69	10	7	8	1	bHLH protein
TT5	AT3G55120	5.06E-03	109	460	4	100	204	2	Chalcone-flavanone isomerase
F3H	AT3G51240	9.45E-03	34	126	4	54	31	1	Flavanone 3-hydroxylase
FLS	AT5G08640	3.82E-02	31	100	3	49	48	1	Flavonol synthase
TT7	AT5G07990	1.57E-03	14	44	3	13	11	1	Flavonoid 3′-monooxygenase/flavonoid 3′-hydroxylase (F3′H)
UGT78D1	AT1G30530	1.12E-02	326	736	2	361	229	1	UDP glucose:flavonoid 3-*o*-glucosyltransferase
Jasmonic									
ATTPSO3	AT4G16740	1.54E-03	6	511	79	4	43	10	Monoterpene synthase

[a] Absolute expression value of wild-type plants under normal conditions.
[b] Absolute expression value of wild-type plants exposed for 2 h to drought stress.
[c] Expression fold increase in wild-type dehydrated plants relative to control plants.
[d] Absolute expression value of *siz1-3* plants under normal conditions.
[e] Absolute expression value of *siz1-3* plants exposed for 2 h to drought stress.
[f] Expression fold increase in *siz1-3* dehydrated plants relative to control plants.

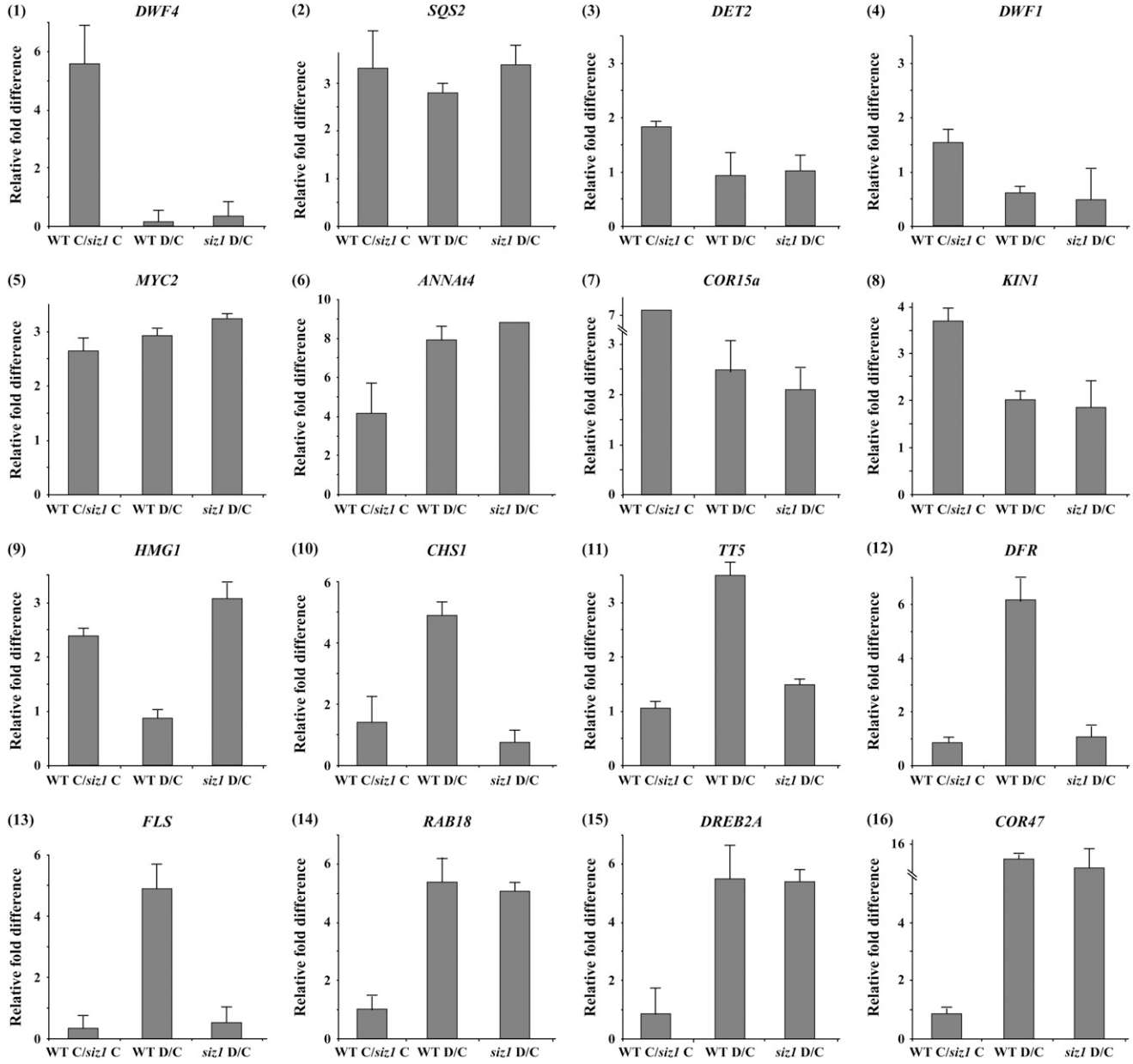

Figure 5. SIZ1 Regulates Gene Expression.

Relative expression levels (fold difference) of 16 genes in wild-type and *siz1-3* plants under control conditions (labeled as WT C/*siz1* C), in wild-type plants after 2 h of drought stress with respect to wild-type plants under control conditions (labeled as WT D/C), and in *siz1-3* plants after 2 h of drought stress with respect to *siz1-3* plants under control conditions (labeled as *siz1* D/C). The expression data were obtained by quantitative RT-PCR. Amplification of eIF4a mRNA within the same reactions was performed as a loading control. Error bars represent SD ($n = 3$). (1) *DWF4*, (2) *SQS2*, (3) *DET2*, (4) *DWF1*, (5) *MYC2*, (6) *ANNAt4*, (7) *COR15a*, (8) *KIN1*, (9) *HMG1*, (10) *CHS1*, (11) *TT5*, (12) *DFR*, (13) *FLS*, (14) *RAB18*, (15) *DREB2A*, and (16) *COR47*.

completely rescued in plants of the complementation line (Figure 4). Consistent with its role in stress tolerance, SIZ1 levels transiently increase in response to drought (Figure 4). Our results, along with previous reports that *siz1* mutants show a decrease in the accumulation of SUMO-protein conjugates under heat shock and cold (Miura et al., 2005, 2007; Yoo et al., 2006), suggest that SIZ1 plays a general role in abiotic stress responses.

We note that the accumulation of SUMO-protein conjugates in *siz1-3*, although significantly decreased compared with wild-type plants, remains inducible by drought stress, indicating the

existence and contributions of additional and yet unidentified SUMO E3 ligases or other proteins with SUMO E3 ligase activity.

Genome-Wide Transcriptome Analysis of *Arabidopsis* Response to Drought Stress

Previous work has shown that the tolerance to drought stress is mediated by changes in gene expression (Seki et al., 2002; Shinozaki et al., 2003; Sakuma et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2006). Here, we present results of a genome-wide expression analysis of *Arabidopsis* plants subjected to drought. We found that after 2 h of exposure to dehydration, the expression level of 1700 *Arabidopsis* genes is induced more than two times (Table 2; see Supplemental Table 5 online). Seki et al. (2002) previously analyzed 7000 *Arabidopsis* genes and reported that 280 genes are induced more than five times under drought stress. We have expanded this analysis to the entire *Arabidopsis* genome (22,500 genes) and found ~600 genes to be induced more than five times by this stress. Taken into consideration the differences in materials and experimental conditions between our experiments and those of Seki et al. (2002), we conclude that the results obtained are comparable. Several of these drought-inducible genes (e.g., *DREB2A*, *MYC2*, or *ABF3*) have previously been characterized as important regulators of this response (Shinozaki et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2006). Table 2 also includes genes that were previously reported as being involved in responses to other stresses but found to be drought inducible in our experiments. It is possible that these are general stress-responsive genes whose expression is induced by many stresses.

SIZ1 Regulates Both Basal and Drought-Inducible Gene Expression

Among the 1700 drought-inducible genes, the induction of 262 genes is positively regulated by SIZ1, demonstrating the important role of this SUMO E3 ligase in drought responses. Our analysis showed that SIZ1 could regulate either basal or drought-inducible gene expression (Figure 5). For example, basal but not drought-inducible expression of *MYC2* and *ANNAt4* is reduced by SIZ1 deficiency (Figure 5). Since optimal expression of these genes is needed for drought stress tolerance in *Arabidopsis* (Abe et al., 2003; Lee et al., 2004), their lower expression in *siz1-3* could account for the mutant's hypersensitivity to drought. By contrast, drought-inducible but not basal expression of four genes (*CHS1*, *TT5*, *DFR*, and *FLS*) implicated in the anthocyanin biosynthetic pathway (Figure 5, panels 10 to 13) is regulated by SIZ1, consistent with the role of this SUMO E3 ligase in regulating anthocyanin accumulation in adult plants. Anthocyanin accumulation is an important component of the reactive oxygen species detoxification system in *Arabidopsis* (Nagata et al., 2003; Filkowski et al., 2004), and its reduced levels in *siz1-3* may contribute to the mutant's hypersensitivity to drought stress and also suggest a role for anthocyanin in drought stress tolerance.

We found that basal expression of four genes involved in the brassinosteroid biosynthethic pathway (Figure 5, panels 1 to 4) is also reduced by SIZ1 deficiency, leading to an overall decrease in transcript levels of these genes even upon drought treatment. Kagale et al. (2006) have recently reported that treatment of *Arabidopsis* with brassinosteroids can increase its tolerance to drought stress. Therefore, it is likely that normal responses to drought may entail an increase in brassinosteroid accumulation. Future work should be directed toward the determination of a possible increase in brassinosteroid accumulation and increased flux through the brassinosteroid signaling pathway under drought and the possible role of brassinosteroid in conferring drought stress tolerance. Finally, drought induction of jasmonate-responsive genes has been previously reported (Fujita et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2006). Although these results suggest a role for jasmonate in drought stress response, the mechanism of action still remains unknown.

Results obtained with *RD29B*, *PAP1*, *TT5*, and *ATTPSO3* indicated that SIZ1-independent pathways are also involved in the regulation of these genes in response to drought stress. Therefore, the function for SIZ1 in the response of *Arabidopsis* to drought stress is complex, and it may regulate different signaling pathways at different levels.

The tolerance of *Arabidopsis* to dehydration is mediated mainly by three independent signaling pathways: the first one is dependent on ABA, the second one is regulated by the transcription factor DREB2A, and the last one regulates *ERD1* gene expression (Shinozaki et al., 2003; Sakuma et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2006). SIZ1 is not involved in the regulation of the two ABA-independent pathways because the expression of *ERD1* and *DREB2A*, and some of its target genes (i.e., *RD29A* [data not shown] and *COR47* [Figure 5]), is not affected in *siz1-3*. By contrast, SIZ1 is needed for the basal expression of some ABA-dependent genes (i.e., *MYC2*, *COR15A*, and *KIN1*; Figure 5) at wild-type levels, although ABA is not required for the accumulation of SUMO-protein conjugates and the drought induction of other ABA-dependent genes (i.e., *CBF4* and *RAB18*; Figure 5) is not affected in *siz1-3*. Therefore, our results suggest that SIZ1 regulates part of the ABA-dependent signaling pathway by an ABA-independent process and SIZ1 likely acts in a new independent signaling pathway in the response to dehydration stress.

In conclusion, our results show that SIZ1 is an important component not only in the control of plant growth but also in drought stress responses. With respect to the former, SIZ1 appears to execute its function by regulating brassinosteroid and auxin pathways. With respect to the latter, the absence of SIZ1 activity reduces the expression of several key genes (e.g., *MYC2* and *ANNAT4*) implicated in drought stress tolerance of *Arabidopsis*. Therefore, we can reasonably assume that the increased sensitivity of *siz1* to drought can be attributed to the role of SIZ1 in drought stress responses. Considering that SIZ1 is a SUMO E3 ligase that most probably targets multiple substrates, protein sumoylation likely features prominently in drought tolerance. Our analysis of SUMO-protein conjugate levels in *siz1-3* also indicates the existence of other proteins with SUMO E3 ligase activity in *Arabidopsis* (i.e., SUMO E2 conjugating enzymes and other SUMO E3 ligases). The identification of additional SUMO E3 ligases and the isolation and identification of sumoylated proteins will help to dissect the biological functions of the sumoylation machinery in plant growth and development.

METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) and *siz1-3* mutant plants were used. Seeds of the T-DNA insertion line *siz1-3* (SALK_034008) were obtained from the ABRC. Seeds were surface sterilized with 30% bleach containing 0.05% Triton X-100 for 15 min and rinsed five times with sterilized water. Unless specified, treated seeds were plated on MS medium with 1% sucrose (1× MS salt, pH 5.7, 1% sucrose, and 0.8% agar) and kept in darkness at 4°C for 4 d to break dormancy. Soil-grown plants were obtained by sowing seeds in pots containing a mixture of organic substrate and vermiculite (3:1 v/v). In all cases, plants were transferred to 16 h light/8 h dark at 22°C under white fluorescent light (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Three week-old plants were cut near the stem-root junction and the detached rosette place in a flow laminar hood for 2, 4, and 6 h. After treatments, plants were immediately frozen in liquid N_2 and stored at –80°C. Samples were used for RNA gel blot and protein gel blot experiments.

Water loss was investigated by two methods. Short-term assays were performed by allowing detached rosette of 4-week-old plants to dehydrate in a flow laminar hood, and the fresh weight was measured at different times after the treatment. Long-term experiments were performed by withholding water to 2-week-old plants grown on soil for 3 weeks, after which, the plants were watered again for 1 week. The number of plants that survived 1 week after resumption of watering was determined.

Plasmid Constructs

SIZ1 cDNA clone APZL63a07 (GenBank accession number AV530225) was obtained from the Kazusa DNA Research Institute. To facilitate subcloning of *SIZ1* into different vectors, appropriate restriction sites were introduced by PCR with Pfu Turbo polymerase (Stratagene), and PCR products were verified by DNA sequencing. The binary vector pBA002 containing a 35S promoter was used for plasmid constructs (Kost et al., 1998).

Plant Protein Extraction and Protein Gel Blot Analysis of SUMO Conjugates

Approximately 200 mg of 3-week-old *Arabidopsis* seedlings were homogenized in 200 μL 4× SDS protein sampler buffer containing 5 mM *N*-ethylmaleimide (Murtas et al., 2003; Peng et al., 2003). Protein samples were resolved in 7.5% SDS/polyacrylamide gels, analyzed by protein gel blots with affinity-purified anti-SUMO1 or anti-SIZ1 polyclonal antibodies polyclonal antibodies, and detected with the ECL plus detection kit (Amersham Biosciences). Polyclonal antibodies were raised in rabbit by Cocalico and immunopurified using recombinant 6His-SUM1 or 6His-SIZ1.

GUS Staining

GUS staining was performed according to Hu et al. (2003).

RNA Isolation and Gel Blot Analysis

Total RNA was isolated from 3-week-old *Arabidopsis* plants using Qiagen RNeasy Plant Mini kits. For RNA gel blot analysis, 10 μg of total RNA was separated under denaturing conditions in a 1.5% agarose gel. *SIZ1* mRNA was probed using a 493-bp fragment from the 15th exon of the *SIZ1* gene amplified with the primer pair miz-9-F (5′-CGAGAATGATTTAGTGATC-3′) and miz-9-R (5′-TTTAAAACCCGACTGAGC-3′). The specific probes for *KIN1* (Kurkela and Borg-Franck, 1992), *CHS1*, *CHI*, and *PAL1* (Solfanelli et al., 2006) were obtained by PCR from genomic DNA of the Columbia-0 ecotype. Probe was prepared using a Rediprime II random primer labeling system (Amersham Biosciences), and hybridization was performed according to the manufacturer's instructions. RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least three times.

Complementation Experiments

Seeds harvested from heterozygous *siz1-3* plants were germinated and grown on soil under short-day conditions (10 h light/14 h dark) for 5 weeks before being transferred to long-day conditions (16 h light/8 h dark) for 4 to 5 weeks. Flowering plants were transformed by vacuum infiltration via agrobacteria with *35S:HA-SIZ1* in a binary vector carrying Basta resistance. Basta-resistant plants were selected, and T-DNA insertion was determined by PCR screening to identify lines homozygous for insertion at the *siz1* locus.

Analysis of Anthocyanin Content

Relative anthocyanin levels were determined according to Solfanelli et al. (2006).

Morphometric Measurements

The analysis of leaf length, width, and area was performed with the 5th leaf of 25-d-old ($n = 12$) wild-type and *siz1-3* mutant plants using public domain image analysis software (ImageJ version 1.32; http://rbs.info.nih.gov/ij/).

Microarray Analysis

Genome-wide expression studies were performed with three biological replicates of wild-type and *siz1-3* plants treated with drought stress for 0 or 2 h. One microgram of total RNA was used for reverse transcription using the MessageAmp II aRNA kit (Ambion) and 15 μg of labeled cRNA for hybridization. GeneChip (Affymetrix ATH1) hybridization and scanning were performed at the Genomic Resource Center (The Rockefeller University; http://www.rockefeller.edu/genomics). All microarray data will be available in the public repository Gene Expression Omnibus upon publication (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE6583.

Statistical Analysis of the Microarray Analysis

Statistical analysis of microarray data was performed using Genespring GX7.3.1 software (Agilent Technologies). After normalization using gcRMA, an analysis of variance test was used for the statistical analysis. The Welch *t* test (variances not assumed equal) was used for the parametric test, and the Benjamini and Hochberg false discovery rate for multiple testing corrections was used with a P value ≤ 0.05. By this statistical analysis, we generated lists of the genes that present significant differences in their expression between wild-type control and *siz1-3* control, wild-type control and wild type exposed 2 h to drought stress, and wild type and *siz1-3* both exposed 2 h to drought stress. All genes that were considered to show significant expression differences by the previous tests were then filtered by a twofold change of expression level between samples from wild-type and *siz1-3* control plants, wild-type control and wild-type plants exposed 2 h to drought stress, and between wild-type and *siz1-3* plants exposed 2 h to drought stress.

Quantitative RT-PCR Analysis

Two micrograms of total RNA were used to reverse transcribe target sequences using oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was

performed in the presence of the double-stranded DNA-specific dye SYBR green PCR Master Mix (Applied Biosystems). Amplification was monitored in real time with the 7900 HT sequence detection system (Applied Biosystems). All reactions were performed in triplicate using three independent RNA samples. The sequences of the primers used are given in Supplemental Table 8 online.

Accession Number

The Arabidopsis Genome Initiative locus identifier and GenBank accession number for *SIZ1* are At5g60410 and AV530225, respectively.

Supplemental Data

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

Supplemental Figure 1. Phenotypes of *siz1-3*, the Wild Type, and C-*siz1-3* after 25 d of Growth in in Vitro Conditions.

Supplemental Figure 2. Loss of Fresh Weight of Wild-Type, *siz1-3*, and C-*siz1-3* Plants over Time.

Supplemental Table 1. Genes That Show Deregulated Expression in *siz1-3* Relative to the Wild Type.

Supplemental Table 2. Genes Underexpressed in *siz1-3* Plants (More Than Twofold Repression Relative to the Wild Type).

Supplemental Table 3. Genes Overexpressed in *siz1-3* Plants (More Than Twofold Induction Relative to the Wild Type).

Supplemental Table 4. Genes Whose Expression Is Regulated by Drought in Wild-Type Plants.

Supplemental Table 5. *Arabidopsis* Drought-Inducible Genes (More Than Twofold Induction).

Supplemental Table 6. Drought-Inducible Genes That Show Deregulated Expression in Response to Drought in *siz1-3* with Respect to the Wild Type.

Supplemental Table 7. Drought-Inducible Genes Downregulated in *siz1-3* Relative to Wild-Type Plants.

Supplemental Table 8. Primers Used in the RT-PCR Experiment (Figure 5).

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