Arabidopsis SUMO E3 Ligase SIZ1 Is Involved in Excess Copper Tolerance[1][W][OA]

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The reversible conjugation of the small ubiquitin-like modifier (SUMO) to protein substrates occurs as a posttranslational regulatory process in eukaryotic organisms. In Arabidopsis (*Arabidopsis thaliana*), several stress-responsive SUMO conjugations are mediated mainly by the SUMO E3 ligase SIZ1. In this study, we observed a phenotype of hypersensitivity to excess copper in the *siz1-2* and *siz1-3* mutants. Excess copper can stimulate the accumulation of SUMO1 conjugates in wild-type plants but not in the *siz1* mutant. Copper accumulated to a higher level in the aerial parts of soil-grown plants in the *siz1* mutant than in the wild type. A dramatic difference in copper distribution was also observed between *siz1* and wild-type Arabidopsis treated with excess copper. As a result, the shoot-to-root ratio of copper concentration in *siz1* is nearly twice as high as that in the wild type. We have found that copper-induced Sumoylation is involved in the gene regulation of metal transporters *YELLOW STRIPE-LIKE 1* (*YSL1*) and *YSL3*, as the *siz1* mutant is unable to down-regulate the expression of *YSL1* and *YSL3* under excess copper stress. The hypersensitivity to excess copper and anomalous distribution of copper observed in the *siz1* mutant are greatly diminished in the *siz1ysl3* double mutant and slightly in the *siz1ysl1* double mutant. These data suggest that SIZ1-mediated sumoylation is involved specifically in copper homeostasis and tolerance in planta.

Copper (Cu) is an essential metal for normal plant growth and development. It is also an important cofactor for many metalloproteins such as plastocyanin, Cu/zinc (Zn) superoxide dismutase, cytochrome *c* oxidase, laccase, amino oxidase, and polyphenol oxidase in plants (Clarkson and Hanson, 1980; Yruela, 2005, 2009; Pilon et al., 2006; Burkhead et al., 2009). Furthermore, Cu participates in numerous physiological processes, including photosynthesis, respiration, antioxidant activity, cell wall metabolism and lignification, and ethylene perception (Himelblau and Amasino, 2000; Burkhead et al., 2009). Because Cu has a high redox activity, excess Cu in the plant is toxic and easily interferes with numerous biochemical and physiological processes (Luna et al., 1994; Shen et al., 1998; Nielsen et al., 2003; Demirevska-Kepova et al., 2004). Consequently, plants have evolved different strategies and mechanisms to avoid such toxicity, which include the regulation of Cu uptake, chelation, efflux, sequestration, and storage to tightly regulate Cu homeostasis (Clemens, 2001; Puig and Thiele, 2002; Wintz and Vulpe, 2002; Meharg, 2005; Sharma and Dietz, 2006; Palmer and Guerinot, 2009; Pilon et al., 2009; Puig and Peñarrubia, 2009; Yruela, 2009). To date, several mechanisms for Cu tolerance have been identified. Excess Cu can be entrapped by cell wall binding in the apoplastic space (Nishizono et al., 1987; Konno et al., 2005). In the cytosol, Cu can be chelated by small cellular molecules like amino acids, Cu-binding proteins, or phenolic compounds to reduce the toxicity of the free forms of Cu. Sequestration into vacuolar compartments can also block the contact between free Cu ions and cellular components (Palma et al., 1990; Backor et al., 2004; Sharma and Dietz, 2006; Kovacik and Backor, 2007). In addition, Cys-rich metallothionein proteins and glutathione-derived phytochelatins can also chelate Cu to buffer the cellular Cu concentration (Lolkema et al., 1984; Zhou and Goldsbrough, 1994; Cobbett and Goldsbrough, 2002; Guo et al., 2003, 2008).

SUMO (for small ubiquitin-related modifier) proteins, small proteins with a molecular mass of about 12 kD, are ubiquitously expressed throughout the eukaryotic kingdom. Although the overall amino acid sequence identity between SUMO and ubiquitin is less than 18% in Arabidopsis (*Arabidopsis thaliana*; Kurepa et al., 2003), both proteins share a similar three-dimensional globular structure called the Ub fold that consists of an α-helix and four β-strands (Hay, 2001; Miura and Hasegawa, 2010). A cascade of enzymatic steps is required for sumoylation that is similar to that for ubiquitination. These steps require the participation of an E1-activating enzyme, an E2-conjugating

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enzyme (UBC9), and an E3 SUMO ligase to facilitate the transfer of SUMO from UBC9 to the acceptor Lys residue(s) in target proteins. Based on annotated databases and recent genetic and biochemical analyses, the components of sumoylation systems are also present in plants including algae (*Chlamydomonas reinhardtii*), dicots (Arabidopsis), and monocots (rice [*Oryza sativa*]; Colby et al., 2006; Miura et al., 2007; Nigam et al., 2008; Wang et al., 2008; Park et al., 2010; Shin et al., 2010). Sumoylation is involved in controlling cell growth and development (Miura and Hasegawa, 2010), embryogenesis (Colby et al., 2006; Saracco et al., 2007), and regulation of flowering time (Jin et al., 2008). In addition, sumoylation is involved in actions of both biotic and abiotic stresses (Kurepa et al., 2003) including salicylic acid-dependent pathogen defense (Lee et al., 2007), phosphate starvation responses (Miura et al., 2005), cold tolerance (Agarwal et al., 2006), drought response (Catala et al., 2007), and basal thermotolerance (Yoo et al., 2006).

A previous screening experiment did not detect changes in SUMO conjugation in Arabidopsis seedlings subjected to heavy metal stress (Kurepa et al., 2003). However, recent reports indicated that reactive oxygen species may function as key regulators of the sumoylation-desumoylation equilibrium by influencing the redox states of SUMO cascade enzymes and SUMO protease (Bossis and Melchior, 2006; Xu et al., 2007). Excess Cu can induce reactive oxygen species production through Haber-Weiss and Fenton reactions (Stadtman and Oliver, 1991; Maksymiec et al., 1994; Schützendübel and Polle, 2002). Therefore, we were interested to find out whether excess Cu can induce sumoylation responses and, if yes, whether such responses play a role in the tolerance of excess Cu stress.

Although several SUMO E3 ligases exist in plants, stress-responsive SUMO conjugation is mainly mediated by the SUMO E3 ligase SIZ1 (Ishida et al., 2009; Lois, 2010). In this study, we examined the role of sumoylation in Cu homeostasis and tolerance using the *siz1* mutant and an anti-SUMO1 antibody. In summary, we detected Cu hypersensitivity in the *siz1* mutant and a SIZ1-dependent accumulation of SUMO1 conjugates under excess Cu treatment. Interestingly, we observed an elevated shoot-to-root Cu concentration ratio in the *siz1* mutant. By examining the gene expression patterns and through functional studies of mutants, the involvement of *YSL1* and *YSL3* regulation in the control of Cu translocation is proposed.

RESULTS

The *siz1* Mutant Is Sensitive to Excess Cu

Stress-responsive SUMO conjugation is mediated mainly by the SIZ1 SUMO E3 ligase in Arabidopsis. To investigate whether sumoylation is involved in plant responses to excess Cu, we first examined the phenotype of the *siz1-2* mutant at exposure to a wide range of $CuSO_4$ concentrations (0.05–100 μM). We found that the *siz1-2* mutants were more sensitive to excess Cu than the wild type within a window of Cu concentration between 25 and 50 μM (Fig. 1A). Root growth experiments showed that the relative growth rate of primary root in the *siz1* mutant is slightly higher than that in the wild type in response to 5 to 50 μM Cu treatments (Fig. 1B). To confirm that the Cu-induced effect is specific to *siz1*, we further examined the phenotype of another mutant, *siz1-3*, which contains a different mutated allele, upon exposure of seedlings to excess Cu. As shown in Supplemental Figure S1, the sensitivity of both mutants to excess Cu is indistinguishable. The phenotype of *siz1* demonstrates the involvement of SIZ1 in the tolerance to Cu stress. Notably, the growth inhibition only occurred in shoots but not in roots (Supplemental Fig. S1).

Excess Cu Induces SIZ1-Dependent SUMO1 Sumoylation

Based on these observations, we hypothesized that sumoylation is involved in the tolerance to excess

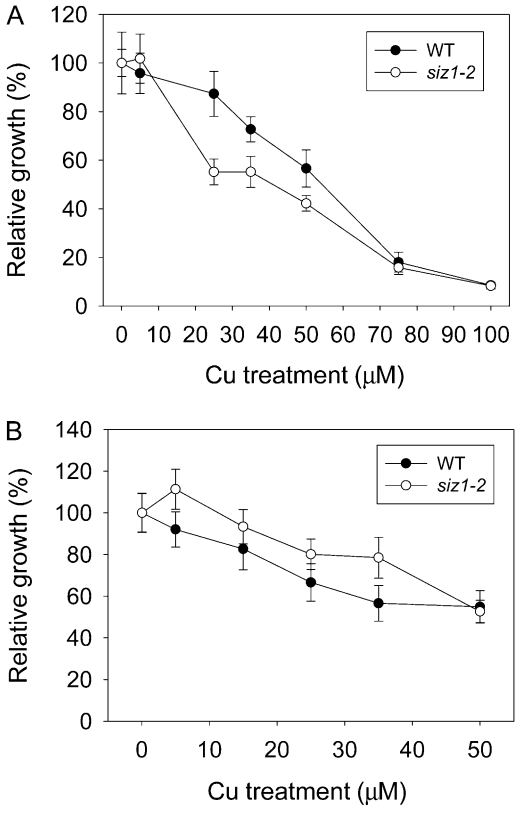

Figure 1. Growth of wild-type (WT) and *siz1* mutant seedlings under Cu stress. One-week-old wild-type (black circles) and *siz1-2* (white circles) seedlings were treated with various Cu concentrations, as indicated, for 10 d before fresh weights (A) or for 3 d (vertical growth) before root lengths (B) were measured. The normalized value against the growth in half-strength MS medium is presented. Means and error bars were calculated from six repeats with 10 plants each.

Cu in Arabidopsis. To investigate whether this post-translational modification of proteins occurs in plants encountering Cu stress, we performed immunoblotting analyses with anti-SUMO1 antibody to detect sumoylation. The antibody was prepared by immunizing a rabbit with purified recombinant SUMO1 (Supplemental Fig. S2). Before use, the antibody was affinity purified with recombinant SUMO1 protein immobilized on a polyvinylidene difluoride (PVDF) membrane. Furthermore, we used media-grown plant materials in this experiment instead of liquid-cultured seedlings that could be experiencing leaf-submerging stresses.

Considering that the root tissue is the uptake organ and could elicit a first response to excess Cu stress, we focused at first on protein sumoylation in roots. Immunoblot analysis with anti-SUMO1-specific antibodies resulted in the detection of SUMO1-protein conjugates in crude extracts of roots. The conjugates were induced by excess Cu in the wild type, while no conjugate formed in *siz1* mutants under Cu treatment (Fig. 2A). In the wild type, the induction is dose dependent in the range of tested Cu concentrations (10-50 μM). Compared with heat shock-induced sumoylation, Cu-induced sumoylation gives rise to a different pattern and is completely SIZ1 dependent (Fig. 2A). The extent of sumoylation is much less (about one-fifth) under Cu stress than under heat shock conditions. Time-course analysis revealed that the formation of SUMO1-protein conjugates as well as free SUMO1 increases rapidly upon exposure to excess Cu for about 2 h and then rapidly decreases after treatment for about 3 h (Fig. 2B; Supplemental Fig. S3). Cu-induced SUMO1 sumoylation was also observed at delayed time points in the shoot tissue (Supplemental Fig. S3). These data clearly demonstrate that sumoylation can be induced under excess Cu in Arabidopsis.

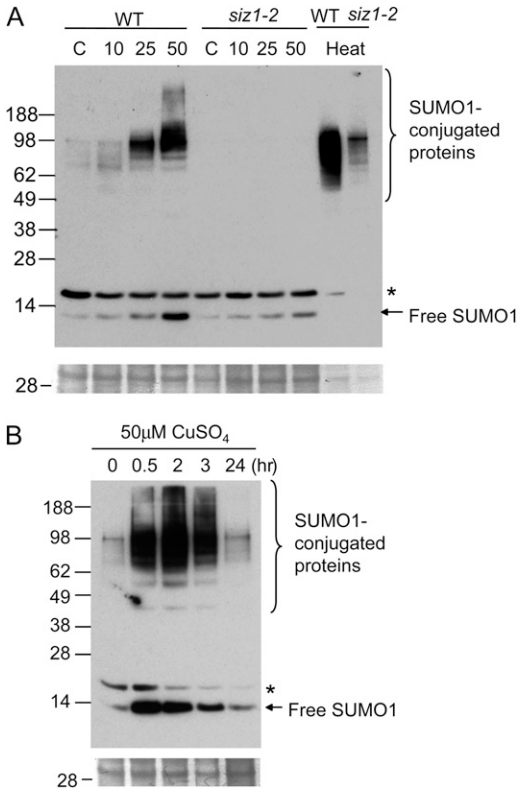

Figure 2. Sumoylation in roots under conditions of excess Cu. Western-blot analysis was performed with homemade anti-SUMO1 antibody. A, Sumoylation in roots of 12-d-old wild-type (WT) and *siz1-2* seedlings treated for 1 h with half-strength MS solution (control [C]) or half-strength MS solution containing 10, 25, or 50 μM $CuSO_4$ or subjected to heat shock at 37°C for 1 h. Loadings of heat shock samples are one-fifth of control and excess Cu-treated samples. B, Sumoylation of root proteins isolated from plants treated with 50 μM $CuSO_4$ for the indicated time periods. Asterisks indicate unidentified protein. Bottom panels show a gel portion stained with Coomassie blue.

Cu Distribution Is Abnormal in *siz1*

To examine whether *siz1* mutants accumulate more Cu than wild-type seedlings, we measured the Cu content in shoots and roots of plants after treatment with excess Cu. Results obtained at treatment of seedlings with 25 or 35 μM $CuSO_4$ were very similar. Interestingly, we found that shoots of *siz1* mutant seedlings accumulated higher concentrations of Cu as compared with the wild type. By contrast, the Cu concentration was more elevated in roots of wild-type seedlings. As a result, the *siz1* mutant possesses a shoot-to-root ratio for Cu that is about twice as high as that of the wild type (Fig. 3). In order to gain more insight into the accumulation of metals, the elemental profile of shoots of wild-type and *siz1* mutant plants, which were grown in soil for 3 weeks under normal conditions, was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Fig. 4). Among all metals inspected, Cu showed the most dramatic difference, with a concentration twice as high in the *siz1* mutant as compared with the wild type; by comparison, accumulation of other metals exhibited a small difference (manganese [Mn], Zn, and potassium [K]) or no difference (iron [Fe], aluminum, molybdenum, chromium, magnesium [Mg], calcium, and sodium). These results suggest that the Cu sensitivity of the *siz1* shoot is associated with Cu overaccumulation and that the Cu translocation from root to shoot is aberrant in *siz1*.

Expression of Cu-Related Transporter Genes in the *siz1* Mutant

Owing to the atypical distribution of Cu in the *siz1* mutant, we hypothesized that sumoylation contributes to the control of certain Cu-related transporters, including members of the ZIP, COPT, P-1b ATPase, and YSL families (Colangelo and Guerinot, 2006; Puig et al., 2007; Yruela, 2009; del Pozo et al., 2010). Thus, we examined the gene expression of these transporters in both wild-type and *siz1* plants under excess Cu

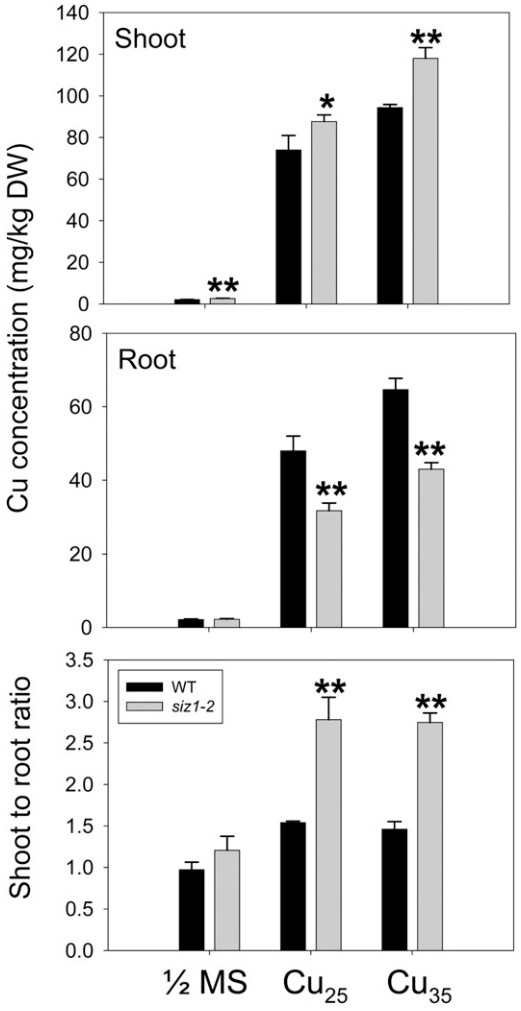

Figure 3. Cu content in shoot and root tissues. One-week-old wild-type (WT) and *siz1-2* seedlings were treated with excess Cu (25 or 35 μM $CuSO_4$, Cu_{25}, and Cu_{35}) for 10 d. Cu concentrations in the shoot (top panel) and root (middle panel) were determined by ICP-OES. In the bottom panel, calculated shoot-to-root ratios of Cu concentration are shown. Means and error bars were calculated from three repeats (* $P < 0.05$, ** $P < 0.01$). DW, Dry weight.

treatment by quantitative real-time reverse transcription (RT)-PCR (qPCR). The gene expression patterns of some transporters, including *ZIP4*, *COPT2*, *YSL1*, and *YSL3*, are different between the wild type and the *siz1* mutant (Fig. 5), while others are similar (Supplemental Fig. S4). Among these transporters, root *COPT2* expression is down-regulated after treatment with excess Cu. *COPT2* transcript levels in root were significantly lower in the *siz1* mutant than in the wild type under both normal and excess Cu conditions. The opposite trend was observed for the expression of root *ZIP4*. In addition, we found that *ZIP4* is up-regulated in the shoot of *siz1* but not in the wild type under excess Cu. Indeed, down-regulation of *COPT2* and up-regulation of *ZIP4* under excess Cu conditions were also reported previously (Sancenón et al., 2003; Wintz et al., 2003; del Pozo et al., 2010). The trends of regulation are comparable in the *siz1* mutant and the wild type. These observations may reflect the regulation by endogenous Cu status. On the other hand, we found that the expression of *YSL1* and *YSL3* in the shoot of *siz1* is roughly 2- to 3-fold higher than that in the wild type under both normal and excess Cu conditions. In the presence of excess Cu, the shoot expression of *YSL1* and *YSL3* was down-regulated in the wild type but not significantly regulated in the *siz1* mutant. This expression pattern was confirmed in a time-course experiment. The high expression of *YSL1* and *YSL3* in the

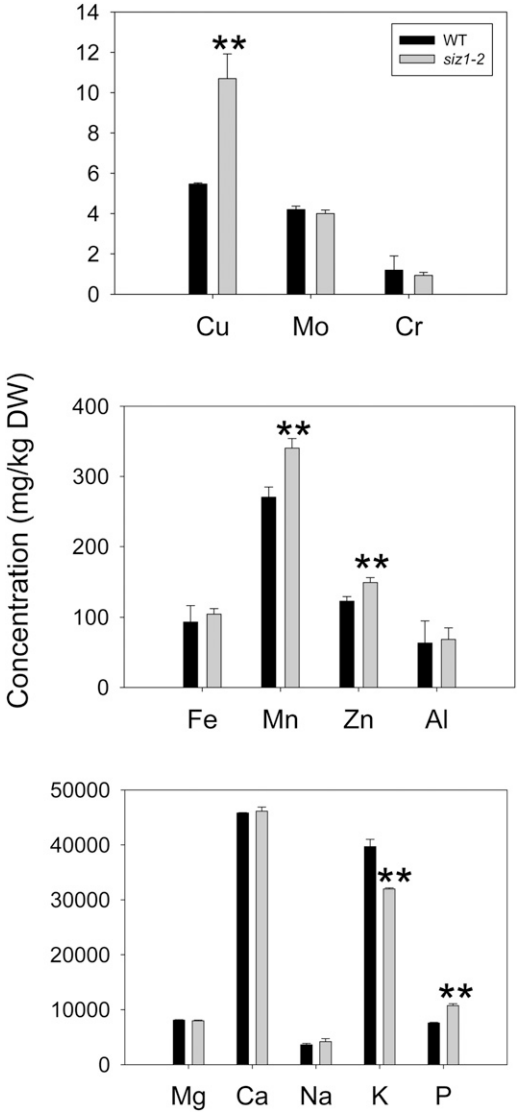

Figure 4. Elemental analysis of soil-grown wild-type (WT) and *siz1-2* plants. Determination of metal elements was done in the leaves of 3-week-old, soil-grown wild-type and *siz1-2* plants by ICP-OES. Means and error bars were calculated from three repeats (** $P < 0.01$). Al, Aluminum; Ca, calcium; Cr, chromium; DW, dry weight; Mo, molybdenum; Na, sodium; P, phosphorus.

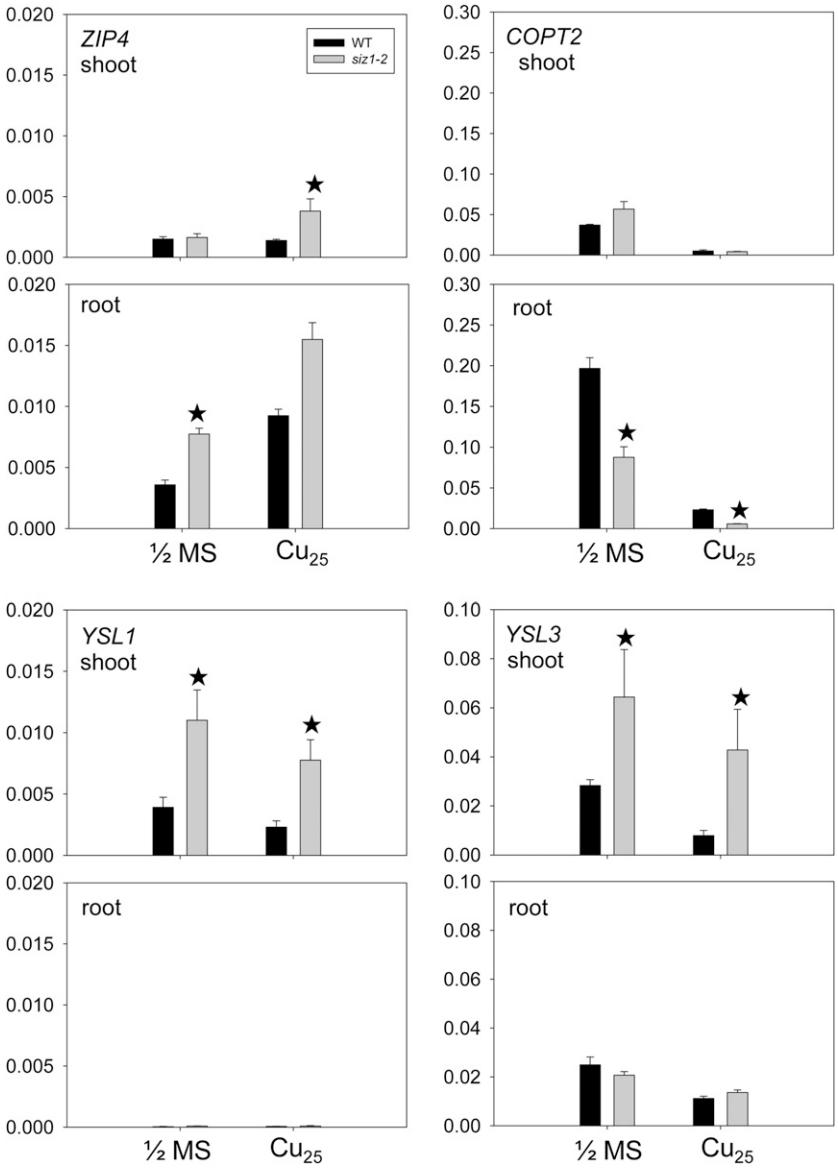

Figure 5. Differential expression of Cu-related transporter genes in *siz1*. Twelve-day-old wild-type (WT) and *siz1-2* plants were treated with half-strength MS medium or half-strength MS medium with 25 μM $CuSO_4$ (Cu_{25}) for 1 d. Gene expression of Cu homeostasis-related transporters in the shoot and root tissues was measured by qPCR. The *y* axis values represent expression relative to *ACT2*. Means and error bars were calculated from six samples of two biological repeats. Asterisks indicate genes with an expression ratio greater than 2 or lower than 0.5 for *siz1*: the wild type).

shoot lasted for the time examined (Fig. 6). These data imply that the action of sumoylation directly or indirectly regulates the mRNA levels of *YSL1* and *YSL3* through either transcription or mRNA stability under excess Cu conditions and that *SIZ1* is required for the control of basal transcription levels of *YSL1* and *YSL3* under normal conditions.

YSL1 and *YSL3* Are Downstream of Excess Cu-Induced SIZ1-Dependent Sumoylation and Involved in Cu Translocation

YSL1 and *YSL3* were previously reported to possess a similar function with regard to Fe loading into seeds (Chu et al., 2010). In order to examine whether *YSL1* and *YSL3* are responsible for the high accumulation of Cu in shoot and for the hypersensitivity of the *siz1* mutant to Cu, we created *siz1-2ysl1-2* and *siz1-2ysl3-1* double mutants for further tests. With regard to the overall phenotype, both *siz1-2ysl1-2* and *siz1-2ysl3-1* double mutants are similar to the *siz1* mutant grown on medium or soil (data not shown). However, a major recovery effect of *ysl3* on *siz1* was associated with Cu tolerance, suggesting that *YSL3* is situated downstream of sumoylation in the regulatory network (Fig. 7A). ICP-OES analysis further revealed that the *siz1ysl3* double mutant accumulated less Cu in shoot than the *siz1* mutant under excess Cu treatment. The reduction of Cu accumulation in the shoot and of the shoot-to-root Cu concentration ratio is smaller in *siz1ysl1* than in *siz1ysl3* but still statistically significant (Fig. 7, B and C). This result may reflect the lower relative expression of *YSL1* as compared with *YSL3* (Waters et al., 2006).

To confirm the Cu phenotype of *siz1-2ysl3-1*, experiments in two double mutants with different *ysl3* alleles, *siz1-2ysl3-1* and *siz1-2ysl3-2*, were conducted.

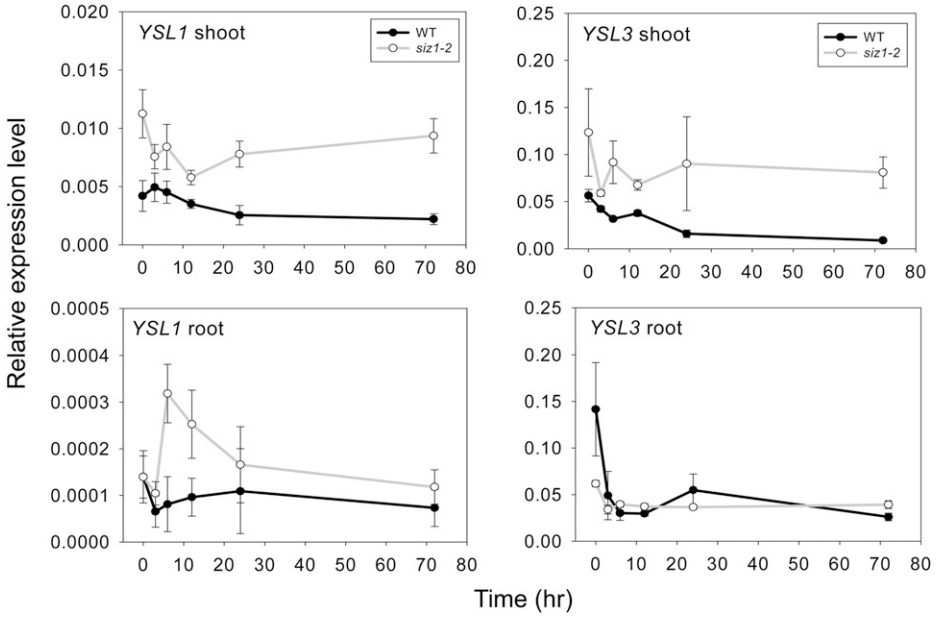

Figure 6. Time-course expression profiles of *YSL1* and *YSL3* in shoot and root tissues. Twelve-day-old wild-type (WT) and *siz1-2* plants were treated with half-strength MS medium or half-strength MS medium containing 25 μM $CuSO_4$ for 3, 6, 12, 24, and 74 h. Transcript levels of *YSL1* and *YSL3* were determined by qPCR analysis. The *y* axis values represent expression relative to *ACT2*. Means and error bars were calculated from six samples of two biological repeats.

Their reductions of Cu sensitivity, Cu accumulation, and shoot-to-root Cu concentration ratio are nearly identical (Fig. 7). These data support the downstream role of *YSL3* in the Cu-induced SIZ1-dependent sumoylation and the involvement of YSL3 in the Cu translocation from roots to shoots.

DISCUSSION

In this study, we found that *siz1* mutants clearly display a shoot growth-retarded phenotype under conditions of excess Cu (Fig. 1; Supplemental Fig. S1). Dose-response experiments were also performed at excess Zn, Fe, or cadmium levels; however, no obvious phenotype different from that of the wild type was observed, with the exception of a slightly more yellowish color of *siz1* as compared with wild-type plants under conditions of excess Zn (data not shown). In addition, excess Cu-induced sumoylation was demonstrated to occur in a SIZ1-dependent manner (Fig. 2). These data strongly suggest that sumoylation is involved in the mechanism of Cu tolerance. Although other SUMO E3 ligases have been found (i.e. MMS21 and HIGH PLOIDY2) in Arabidopsis (Ishida et al., 2009; Zhang et al., 2010), Cu-induced sumoylation is SIZ1 dependent. By contrast, SUMO1-conjugated complexes were detected in the *siz1* mutant under heat treatment (Fig. 2). Therefore, SUMO E3 ligases other than SIZ1 are involved in the process of heat stress but do not participate in Cu homeostasis and tolerance. These data indicate that the sumoylation response induced by excess Cu is not the same as that induced by heat shock in Arabidopsis. At this point, it remains to be investigated if other SUMO E3 ligases or SUMO paralogues are also involved in regulating plant responses to excess Cu.

The elemental profile of shoots of soil-grown plants revealed that the major difference of metal accumulation in *siz1* is Cu (Fig. 4). Moreover, a dramatic difference in the shoot-to-root ratio of Cu concentration between wild-type and *siz1* plants was observed under excess Cu treatment (Fig. 3). These data suggest that the mechanism controlling the partition of Cu between root and shoot is dysfunctional in *siz1*, causing a higher transport of Cu ions to the shoot, and indicate that sumoylation is involved in the regulation of Cu translocation from roots to shoots. In addition, we observed minor but significant increases in the accumulation of Zn and Mn and a decrease in the accumulation of K in the *siz1* mutant (Fig. 4). In the Cu-tolerant plant *Commelina communis*, Cu, Zn, and Mn have similar uptake patterns and are distributed at high levels in the vascular cylinder. This distribution pattern is different from that of Fe, which is located in the epidermis and endodermis (Shi et al., 2011). SIZ1-dependent sumoylation seems to be involved in the regulation of one or more common component(s) related to Cu, Zn, and Mn increases. It suggests that a possible SIZ1-dependent controlling mechanism cannot only regulate Cu partition but also influences the uptake and translocation of Zn and Mn. Interestingly, a reduction in K accumulation was also found in the Cu hyperaccumulator *Erica andevalensis* (Oliva et al., 2010). Excess Cu can also induce K efflux from roots in many plant species such as *Agrostis capillaries*, *Silene vulgaris*, *Mimulus guttatus*, and wheat (*Triticum aestivum*; De Vos et al., 1991; Strange and Macnair, 1991; Quartacci et al., 2001). The reduced accumulation of K could be due to the impairment of Cu distribution in the *siz1* mutant.

In general, Cu accumulation levels are correlated with Cu hypersensitivity. Overexpression of Cu uptake transporters, *COPT1* and *COPT3*, caused a hypersensitive phenotype to Cu (Andrés-Colás et al.,

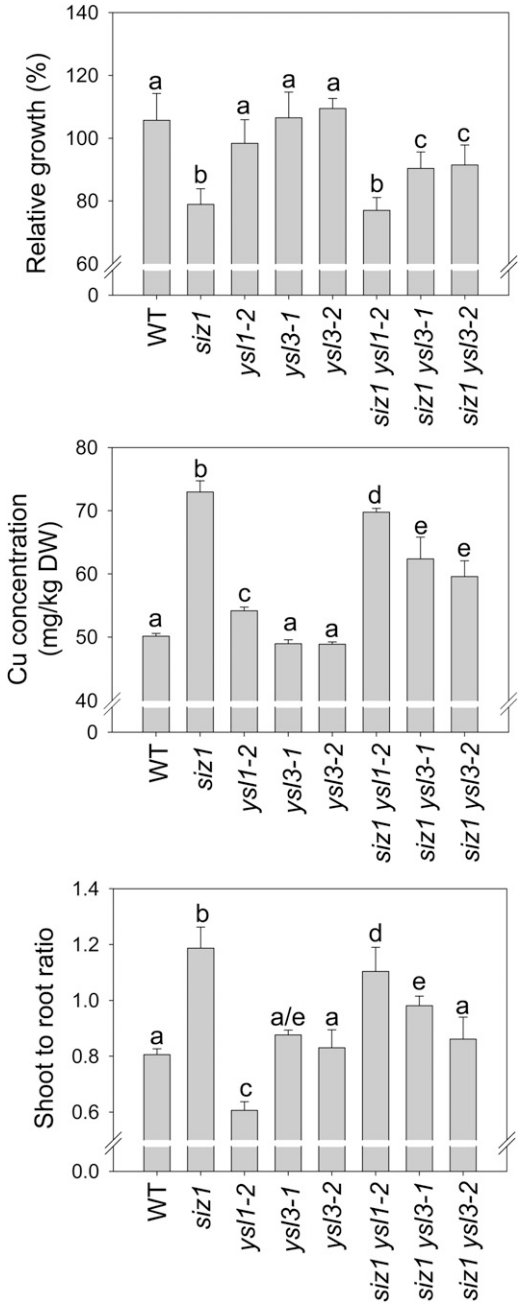

Figure 7. Phenotypes of *siz1*, *ysl1*, and *ysl3* single mutants as well as *siz1ysl1*and *siz1ysl3* double mutants under excess Cu. One-week-old seedlings (wild type [WT], *siz1-2* [*siz1*], *ysl1-2*, *ysl3-1*, *ysl3-2*, *siz1ysl1-2*, *siz1ysl3-1*, and *siz1ysl3-2*) were transferred and grown vertically for 5 d on half-strength MS, treated with 35 μM $CuSO_4$ for 5 d, and dried before analysis of Cu in shoot and root tissues by ICP-OES. A, Relative growth of shoots normalized to their growth on half-strength MS medium. Data represent means plus SD from six repeats with 20 plants. B, Shoot Cu contents. C, Shoot-to-root ratio of Cu concentration. Means and error bars were calculated from three biological repeats. Letters a through e indicate independent groups of statistical significance. DW, Dry weight.

2010). The *hma5* mutant accumulates high Cu levels in the root and shows Cu hypersensitivity in the root (Andrés-Colás et al., 2006). A common feature of Cu toxicity in most plants is inhibition of primary root growth and a reduction in biomass (Lequeux et al., 2010). We observed that the reduction in primary root growth was lower in the *siz1* mutant than in the wild type under conditions of excess Cu. On the other hand, the shoot of *siz1* is more sensitive to excess Cu (Fig. 1; Supplemental Fig. S1). These phenotypes can be rationalized by the Cu accumulation levels.

We also observed that the Cu distribution was anomalous in the *siz1* mutant. The abnormal distribution could be due to differences in either Cu uploading in the root or Cu unloading in the shoot. Hence, we examined the expression patterns of Cu-related transporters (Colangelo and Guerinot, 2006; Puig et al., 2007; Yruela, 2009; del Pozo et al., 2010). Among them, we found that the expression of *YSL1* and *YSL3* in shoot differed largely between *siz1* and the wild type (Figs. 5 and 6). Previous studies suggested that YSL1 and YSL3 function in the remobilization of Cu and Zn from senescing leaves and are required for the formation of pollen and Fe, Zn, and Cu loading in seed development (Himelblau and Amasino, 2001; Curie et al., 2009; Chu et al., 2010). The roles of *YSL1* and *YSL3* are redundant in Fe transport and transfer of metal micronutrients to or from vascular tissues (Le Jean et al., 2005; Waters et al., 2006; Chu et al., 2010). By creating *siz1ysl1* and *siz1ysl3* double mutants, we were able to demonstrate that these two genes that are highly expressed in the *siz1* mutant play a role downstream of the action of *SIZ1*, causing the irregular Cu distribution (Fig. 7). Our results further support a functional role of YSL1 and YSL3 in Cu transport.

In yeast experiments, nicotianamine (NA)-metal complexes were demonstrated to constitute substrates of YSL2 transporters (DiDonato et al., 2004). NA possesses affinities to various metal ions, including Cu, Fe, nickel, Mn, cobalt, and Zn. NA-Cu could be the major NA-metal complex in the xylem due to the low pH value in this transport tissue (Rellán-Alvarez et al., 2008; Curie et al., 2009). Indeed, the Cu-NA complex has been found in xylem saps and is likely an intermediate for the translocation of Cu from roots to shoots (Pich et al., 1994; Rellán-Alvarez et al., 2008; Curie et al., 2009). Although there is no evidence that YSL1 and YSL3 possess a direct function on NA-Cu or Cu transport so far, transgenic plants overexpressing *YSL3* accumulated more Cu in leaves (Chu et al., 2010). In our study, we found that the Cu concentration in the shoot and the shoot-to-root ratio of Cu concentration were reduced in the *siz1ysl3* and *siz1ysl1* double mutants as compared with *siz1* (Fig. 7). In addition, the *siz1ysl3* double mutant is more efficient than *siz1ysl1*, as *siz1ysl3* contains a lower Cu concentration in shoot and has a lower shoot-to-root ratio of Cu concentration, suggesting that *YSL3* plays a major role in Cu accumulation in the shoot (Fig. 7). This result supports the conclusion that *YSL3* is important for Cu distribu-

tion controlled by SIZ1 under excess Cu. Together, the high expression of *YSL3* and *YSL1* in the *siz1* mutant could be responsible for the high accumulation of Cu in shoots.

In conclusion, we have found that sumoylation is involved in excess Cu tolerance and distribution. In *siz1*, Cu overly translocates to the shoot, and this translocation is accompanied by an anomalously high expression of *YSL1* and *YSL3*. The sumoylation induced by excess Cu is specifically mediated by SIZ1. Therefore, we suggest that the SUMO E3 ligase *SIZ1* is required for excess Cu tolerance and distribution. Our data support the notion that a regulatory pathway exists that prevents excessive translocation of Cu from root to shoot under Cu stress by down-regulation of *YSL1* and *YSL3* that is mediated by SIZ1-dependent sumoylation. Although sumoylation of multiple substrates was observed, similar to sumoylation induced by other environmental stresses, we hypothesize that the control of at least one component involved in Cu distribution requires the regulation of SIZ1-mediated sumoylation; this component is possibly an upstream component in the regulatory mechanism of *YSL1* and *YSL3* expression.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) and *siz1* mutant plants were used. Seeds of the T-DNA insertion lines *siz1-2* (SALK_065397), *siz1-3* (SALK_034008), *ysl1-2* (SALK_034534), *ysl3-1* (SALK_064683C), and *ysl3-2* (SALK_045218) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. Homozygote T-DNA insertion mutants were confirmed using specific primers. To create *siz1-2ysl1-2*, *siz1-2ysl3-1*, and *siz1-2ysl3-2* double mutants, we crossed *ysl1-2*, *ysl3-1*, and *ysl3-2* with *siz1-2*, respectively. The T-DNA insertion and gene expression were confirmed by PCR and RT-PCR, respectively. Primers are listed in Supplemental Tables S2 and S3. Seeds were surface sterilized with 70% ethanol for 5 min and then treated with 1.2% bleach containing 0.02% SDS for 15 min, rinsed five times with sterilized water, and kept in darkness at 4°C for 4 d to break dormancy. Treated seeds were plated on half-strength Murashige and Skoog (MS) medium (half-strength MS salt, pH 5.7, 1% Suc, and 0.35% phytagel) before the excess Cu treatments indicated in the figure legends. Soil-grown plants were obtained by sowing seeds in pots containing a mixture of organic substrate, vermiculite, and mica sheet (9:1:1, v/v/v). In all cases, plants were subjected to a 16-h-light (70 μmol m^{-2} s^{-1})/8-h-dark cycle at 22°C.

Plasmid Construction, and Expression and Purification of Recombinant SUMO1

The full-length Arabidopsis *SUMO1* (At4g26840) cDNA was generated by RT-PCR from total RNA isolated from Arabidopsis ecotype Columbia as the template. The forward primer 5′-GGTGGTCATATGTCTGCAAACCAGGAG-3′ and reverse primer 5′-GCCACCAGTCTGATGGAGCATCG-3′ were designed to introduce *Nde*I and *Sma*I restriction sites at the predicted start codon and the C-terminal diglycine motif, respectively. The RT-PCR product was then cloned into the vector pTYB2 with an in-frame fusion to chitin-binding protein (CBD) at the C terminus. Expression of the CBD-SUMO-1 fusion protein was induced in *Escherichia coli* BL21 (DE3) culture by the addition of isopropylthio-β-galactoside (0.3 mM). Cells were grown at 28°C for an additional 4 h after induction and lysed using a cell disruptor (Constant Systems). Total soluble protein was applied to a chitin column according to the manufacturer's recommendations (New England Biolabs). The bound protein was treated with 30 mM dithioerythritol for 48 h at 25°C. Then, the resulting intein cleavage product was eluted with the elution buffer.

Production and Purification of Anti-SUMO1 Antibody

The SUMO1-containing elution was boiled in 2× SDS sample buffer for 10 min and analyzed on a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen). After Commassie Brilliant Blue staining, the SUMO1-containing gel band was excised and used as the antigen by injecting it directly into a rabbit. The antibody raised against full-length recombinant Arabidopsis SUMO1 was further purified using the PVDF method. The recombinant SUMO1 protein was run on a SDS-PAGE gel and then transferred to a PVDF membrane. The membrane was stained with Ponceau S and rinsed with distilled, deionized water to destain. The band corresponding to the region of the correct molecular mass was excised and collected. The membrane strip was blocked with 5% dry milk in 1× phosphate-buffered saline (PBS; pH 7.4) buffer for 1 h at room temperature and then washed three times for 5 min in 1× PBST (PBS with 0.1% Tween 20). The membrane strips containing recombinant SUMO1 were then incubated with 1 mL of antiserum in 5 mL of 1× PBS buffer for 1 h at room temperature. After incubation, the depleted antiserum was removed and the membrane was washed three times for 15 min in 1× PBS buffer. To strip the antibody off the membrane, the membrane was incubated with 1.5 mL of ImmunoPure IgG elution buffer, pH 2.8 (Pierce), for 5 min at room temperature. The eluted antibody was transferred to a new microcentrifuge tube containing 100 μL of 1 M Tris (pH 9.5). After neutralization, the antibody was dialyzed against 1× PBS buffer with a centrifuged filtrate tube (Vivaspin 500, VS0111; Sartorius Stedium Biotech). The purified antibody is able to recognize subnanogram amounts of recombinant SUMO1 (Supplemental Fig. S2).

Plant Protein Extraction and Immunoblot Analysis of SUMO1 Conjugates

Samples were extracted with extraction buffer (2× SDS sample buffer containing 20 mM *N*-ethylmaleimide, 100 mM $Na_2S_2O_5$, and one tablet of protease inhibitor cocktail [Roche Applied Science] per 50 mL). Samples were centrifuged at 12,000*g* for 10 min, and the protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific). Total proteins (200 μg) were separated on a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen) and transferred to a PVDF membrane (Immobilon-P; Millipore). For immunodetection, the membrane was blotted with 5% fat-free milk and PBST for 1 h, incubated with 1:10,000-diluted purified anti-SUMO1 antibody, washed with PBST, and incubated for 1 h with 1:10,000-diluted secondary antibody (peroxidase-conjugated goat anti-rabbit IgG; Millipore). The membrane was washed five times for 10 min each with PBST solution before development. Specific protein bands were visualized using the Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore).

Elemental Analysis

Elemental analysis was conducted according to the procedure described previously (Lin et al., 2009). Harvested plant samples were washed with $CaCl_2$ and water and dried for 3 d before digestion. Microwave-digested samples were analyzed by ICP-OES (OPTIMA 5300; Perkin-Elmer).

RNA Isolation and qPCR

Frozen shoot and root tissues (approximately 100 mg) were ground in liquid nitrogen using a tissue homogenizer (SH-48, J&H Technology) to which 1 mL of TRIzol reagent was immediately added. Samples were mixed briefly and incubated for 30 min at room temperature. Subsequently, chloroform (200 μL) was added to the sample and the mixture was vigorously shaken for 30 s. The samples were centrifuged at 15,000*g* at 4°C for 15 min, and the upper aqueous phase was carefully transferred to a new tube. RNA was precipitated by the addition of 0.5 mL of isopropanol and incubation at −80°C for 30 min. Following centrifugation at 15,000*g* at 4°C for 15 min, the resulting pellet was washed twice with 75% ethanol. The residual ethanol was evaporated in a chemical fume hood for 10 min. RNA was redissolved in 30 μL of diethyl pyrocarbonate-treated water. The concentration of the RNA was determined at 260 nm on a NanoDrop ND-1000 Spectrophotometer (Isogen Life Science).

Subsequently, 2 μg of RNA was treated with RQ1 RNase-Free DNase (Promega), and the reaction buffer was replaced with 5× First-Strand RT buffer (Invitrogen). The cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). qPCR analyses were conducted with SYBR Green I dye (ABI). The expression of *ACTIN2* (*ACT2*) was used as the internal control for all tested genes. The sequences of primers used for qPCR are listed in Supplemental Table S1. The efficiency of primers was tested based on the manufacturer's instructions. Relative expression was calculated using a previously described method (Livak and Schmittgen, 2001).

Statistical Analysis

Assessments of statistical difference between controls and treatments were made based on two-sample *t* tests and methods of multiple comparisons (Montgomery, 2009).

To control overall levels of confidence in multiple comparisons, Fisher's protected LSD method was used throughout (Fig. 7), as it is one of the most commonly used multiple testing procedures. All possible pairwise comparisons were performed once the *F* test rejected the hypothesis that all group means are equal in the one-way ANOVA. In this study, the outcome of Fisher's LSD coincides with that of Duncan's multiple range tests. As a result, statistical significance in the pairwise differences between samples was used to determine the group membership of an individual sample.

Supplemental Data

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

Supplemental Figure S1. Phenotypes of two *siz1* mutants grown on excess Cu.

Supplemental Figure S2. Antibody production.

Supplemental Figure S3. Sumoylation of shoot and root proteins under Cu stress.

Supplemental Figure S4. Expression of Cu-related transporter genes in the wild type and *siz1* under excess Cu conditions.

Supplemental Table S1. List of Cu-related transporter families and primers used for real-time PCR in Arabidopsis.

Supplemental Table S2. Primers used to confirm T-DNA insertion lines.

Supplemental Table S3. Primers used in RT-PCR.

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