

Alleviation of Copper Toxicity in *Arabidopsis thaliana* by Silicon Addition to Hydroponic Solutions

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ABSTRACT. Copper (Cu) is an essential micronutrient for plants and is the a.i. in pesticides for some pathogens and algae. Elevated doses of Cu can cause toxicity in plants. While silicon (Si) is reported to alleviate the toxicity of some heavy metals, its role in reducing the symptoms induced by excess Cu is unclear. Therefore, the role of Si in plant response to Cu stress was investigated in *Arabidopsis thaliana* (L.) Heyn.]. Based on plant symptoms (a reduction of leaf chlorosis as well as increased shoot and root biomass) and a reduction of phenylalanine ammonia lyase [PAL (EC 4.3.1.5), a stress-induced enzyme] activity in the shoot, Si was found to alleviate copper stress. Real-time reverse transcriptase-polymerase chain reaction analyses indicated that the RNA levels of two *Arabidopsis* copper transporter genes, *copper transporter 1 (COPT1)* and *heavy metal ATPase subunit 5 (HMA5)* were induced by high levels of Cu, but were significantly decreased when Si levels were also elevated. Taken together, our findings indicate that Si addition can improve the resistance of *Arabidopsis* to Cu stress, and this improvement operates on multiple levels, ranging from physiological changes to alterations of gene expression.

The transition metal copper (Cu) is an essential element for all organisms. Copper functions as a cofactor within plant cells in a variety of physiological processes, including electron transfer in photosynthesis, mitochondrial respiration, superoxide scavenging, lignification of cell walls, and ethylene sensing (Epstein and Bloom, 2005; Fox and Guerinet, 1998; Marschner, 1995). In addition to its role as an essential element, Cu is a potentially toxic agent at supraoptimal levels due to its ability to generate harmful reactive oxygen species (Sancenón et al., 2004).

Electrolytically generated Cu, cupric sulfate, and copper hydroxide, as the active component of pesticides, bactericides, and fungicides, has been used in agriculture to control diseases and algae (Kaplan, 1999; Scheck and Pscheidt, 1998). For example, over 1.5 million kilograms of copper hydroxide was used on over 50 crops in California in 2005 (Kegley et al., 2007). Recent survey data indicated that the use of electrolytically generated Cu to control diseases and algae was increasing in the greenhouse industry (Zheng et al., 2004). Proper calibration of Cu generators and following labeling guidelines should reduce the risk of plants being exposed to excess Cu. However, the increased use of Cu-containing products carries an inherent risk of causing Cu toxicity.

To overcome Cu toxicity, plants possess a sophisticated homeostatic network controlling Cu uptake, utilization, and detoxification (for reviews, see Harrison et al., 2000; Puig and Thiele, 2002; Rees and Thiele, 2004). Regulation of uptake

through the plasma membrane is a key step controlling plant Cu homeostasis. This function is accomplished by a family of cell-surface, high-affinity Cu transporters, denoted copper transporter (COPT) (Sancenón et al., 2003). Subsequent intracellular distribution of Cu is mediated by Cu chaperones, which are soluble Cu-binding proteins that direct the metal to its final destination. A plant ATX1-like protein participates in the delivery of Cu to a Cu-transporting P-type ATPase located at the secretory pathway membranes, which is responsible for proper acquisition of Cu by membrane- and Cu-containing proteins (Lin and Culotta, 1995; Lin et al., 1997). Among heavy metal-transporting P-type ATPases (HMAs), the protein encoded by the *HMA5* gene is mainly expressed in pericycle cells (Birbaum et al., 2003), and it is speculated to play a role in Cu transport from roots to shoots (Andrés-Colás et al., 2006). It is likely that other factors also play a role in Cu homeostasis.

The roles and requirements for silicon (Si) in plant biology have been debated for more than 150 years despite numerous reports describing its beneficial properties (Epstein, 1994, 1999; Fauteux et al., 2006; Williams and Vlamis, 1957). The most positive and consistent effects of Si are the alleviation of abiotic and biotic stress (Datnoff et al., 2007; Williams and Vlamis, 1957). For example, Si application can balance nutrient levels, especially heavy metals, in plant tissues. This is accomplished through the suppression of uptake of some nutrients, such as Mn (Horst and Marscher, 1978; Iwasaki et al., 2002a, 2002b, 2002c; Rogalla and Römheld, 2002a, 2002b), or by facilitating the uptake of others, such as Zn (Epstein and Bloom, 2005; Marschner, 1995). Compared with studies examining the effect of Si on Mn and Zn, the effect of Si on Cu toxicity has not been studied widely. Therefore, the objective of this study was to examine the effects of Si on Cu detoxification with the model plant, *Arabidopsis*. Cu toxicity was examined by a variety of parameters ranging from morphological responses to changes in gene expression.

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Materials and Methods

PLANT GROWTH CONDITIONS AND TREATMENTS. Seed holders were prepared by cutting off the conical end of 0.5-mL microcentrifuge tubes (PCR Reaction Cap Strips; Fisher Scientific, Hanover Park, IL). Pieces of dry packing foam previously cleaned with hydrochloric acid and rinsed with distilled water were inserted into the tubes. The foam-filled tubes were saturated with nutrient solution under vacuum and placed into holes in small micropipette tip racks. Concentrations of elements and their primary sources in the nutrient solution were as follows: macronutrients (mM) 1.25 K (KNO₃), 2.45 N [KNO₃, Ca(NO₃)₂, and NH₄NO₃], 0.5 Mg (MgSO₄), 0.5 Ca [Ca(NO₃)₂], 0.5 P (KH₂PO₄), and 0.1 Si (K₂SiO₃); micronutrients (μM) 5 Mn (MnSO₄), 0.08 Mo [(NH₄)₆Mo₇O₂₄], 0.5 Zn (ZnSO₄), 30 B (H₃BO₃), 0.12 Cu (CuSO₄), and 50 Fe (Fe-EDTA). The pH was adjusted and maintained at a value of 5.6. Before sowing, the racks with tubes were floated in a tray of nutrient solution. Arabidopsis ecotype Sf-1 seeds were suspended in the same nutrient solution described above and were loaded individually with a micropipette onto the top of the foam-filled seed holders. Sf-1 is an ecotype of Arabidopsis that is originally from San Feliu, Spain. The seeds were obtained from the Ohio State Arabidopsis Biological Resource Center. Sf-1 is very similar to the standard ecotype, Col-0, in its physiology and morphology.

After sowing, the tray with seed holders was covered with aluminum foil and was incubated at 4 °C for 2 d to synchronize germination. Afterward, the tray was moved to a growth chamber maintained at 20 °C and a relative humidity of 70% under a 16-h photoperiod. Photosynthetic photon flux was set at 100 μmol·m⁻²·s⁻¹. Nineteen days after sowing, plants were transferred into 4.5-L opaque circular (20 cm i.d. × 16.5 cm in height) plastic containers (12 plants per container) and were grown with continued aeration in a growth chamber. Nutrient solutions were replaced weekly. Thirty-five days after sowing (16 d after transfer), plants were treated with additional Cu and Si. Four treatment regimes were examined: control [CK (0.12 μM Cu and 0.10 mM Si)], elevated Si (0.12 μM Cu and 1.5 mM Si), elevated Cu (30 μM Cu and 0.10 mM Si), and elevated Cu and Si [Cu + Si (30 μM Cu and 1.5 mM Si)] with four replications (one replication consists of one hydroponic container). Arabidopsis growth experiments were repeated five times. The planting dates for these experiments varied from 15 Nov. 2006 to 25 June 2007, and the harvesting dates varied from 28 Dec. 2006 to 6 Aug. 2007.

After 3 d of treatment, shoots and roots were harvested, rinsed with distilled water, blotted dry, and fresh weight was determined. In the initial Arabidopsis growth experiments, tissue was dried in a forced air oven at 55 °C for 3 d and was used for tissue analysis. In the remainder of the Arabidopsis growth experiments, the fresh tissue was immediately frozen in liquid nitrogen and stored at -80 °C for subsequent use. Average and standard deviations for fresh weight and for all other data reported below were determined using Excel (Office 2003; Microsoft, Redmond, WA), which calculates these values as described in Neter et al. (1996).

ELEMENTAL ANALYSIS. Dried tissue was ground separately in a stainless steel mill to pass through a 1-mm (20-mesh) screen, and 0.15 g was digested in a microwave digester (MARS Express II; CEM Corp., Matthews, NC) using a modified EPA method [EPA method 3051 (Nelson, 1988): HNO₃ digestion at

200 °C with an additional peroxide digestion step]. Total nutrient concentrations for P, K, Ca, S, Mg, B, Cu, Fe, Mn, and Zn were determined by inductively coupled plasma optical emission spectroscopy (model IRIS Intrepid II; Thermo Fisher Scientific, Waltham, MA). A quality control was run every 10 samples and if any element was determined to be more than 10% higher or lower than the standard value, the instrument was recalibrated. Tomato (*Solanum lycopersicum* L.) standards [National Institute of Standards and Technology (NIST, Gaithersburg, MD) reference material 1573 (Sharpless and Gill, 2000)] were compared every 20 samples and tomato and spinach (*Spinacia oleracea* L.) standards [NIST reference material 1570a (Sharpless and Gill, 2000)] were compared every 40 samples. Total N was analyzed with a total carbon-hydrogen-nitrogen analyzer (model 2400; Perkin-Elmer, Waltham, MA).

Total Si concentration was quantified according to Frantz et al. (2008). Briefly, 0.15 g of dried, ground sample was mixed with 3.0 mL of 7.5 M KOH in a 55-mL Teflon vessel. The solution was heated in a microwave by increasing the temperature to 200 °C over a period of 15 min, and the solution was then held at that temperature for an additional 15 min. After the digested material cooled to room temperature, 2 mL of 30% H₂O₂ was added. The temperature of the samples was increased to 200 °C over 15 min and was held at 200 °C for an additional 5 min. After cooling, the solution was filtered (Whatman No. 2), and 1 mL of the filtrate was diluted with 9 mL of deionized water (18 megaohm purity) and was analyzed by inductively coupled plasma optical emission spectroscopy. A quality control was run every 10 samples, and if Si was determined to be more than 10% higher or lower than the standard value, the instrument was recalibrated with a blank and a high-standard solution of 9.985 mg·L⁻¹ with a background matrix of 0.75 M KOH. Every 20 samples, a laboratory-grown standard of rice (*Oryza sativa* L.) straw containing 9.6 g·kg⁻¹ (dry weight) was analyzed as a reference (L. Datnoff, personal communication).

PHENYLALANINE AMMONIA LYASE [PAL (EC 4.3.1.5)] ENZYME ASSAYS. PAL activity was measured by the method described in Liang et al. (2005) with some modifications. Briefly, 1.0 g of fresh leaf segments were homogenized in liquid nitrogen in 5 mL of 50 mM borate buffer (pH 8.8) containing 5.0 mM thioalcohol and 1 mM EDTA. The homogenate was thawed and centrifuged at 9000 g_n for 25 min at 4 °C. A reaction mixture containing 3.8 mL of 50 mM borate buffer, pH 8.8, and 1.0 mL of 20 mM phenylalanine was added to 0.2 mL of the crude enzyme extract. After incubating at 60 °C for 60 min, the reaction was ended by adding 1 mL of 600 mM HCl. Absorbance of the colored trans-cinnamate was recorded at 290 nm. One unit of enzyme activity was defined as the absorbance increase by 0.01 A₂₉₀ units per hour. Enzyme activity was expressed as units per milligram of protein. The soluble protein concentrations of the extracts were measured by the method of Bradford (1976) using bovine serum albumin as a standard.

GENE EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR). Total RNA was isolated from Arabidopsis tissues with the PureLink™ Plant RNA Purification Reagent (Invitrogen™, Carlsbad, CA) according to the manufacturer's specifications. The samples were digested with TURBO DNase (TURBO DNA-free™ kit; Ambion, Austin, TX), according to the

manufacturer's specifications, to remove contaminating DNA, and the RNA was quantified by ultraviolet spectrophotometry. The integrity of RNA was visually assessed by formaldehyde-agarose gel (1%) electrophoresis.

Two micrograms of total RNA, isolated from arabidopsis roots and shoots, was first converted to cDNA by RT (Invitrogen) using random hexamer primers for arabidopsis genes: *multi-copper oxidase type I family protein (MCO)* and *esterase lipase thioesterase family protein (ELT)*. For the arabidopsis *COPT1* and *HMA5* genes, total root RNA was converted to cDNA by SuperScriptII reverse transcriptase (Invitrogen) using anchored oligo(dT)₂₀ (Roche Applied Science, Indianapolis) primers according to the manufacturer's specifications.

Gene-specific primers (Table 1) were designed based on arabidopsis cDNA sequences obtained from TAIR (Garcia-Hernandez et al., 2002), which were imported into PrimerSelect software (DNASTAR, Madison, WI). At least one primer of each pair was designed to span an exon-exon junction. An exon-exon junction is the site where two exons are joined in the mature RNA but which are separated by an intron in the genomic DNA. Hence, the PCR products obtained in this analysis are only from an mRNA template and not from DNA because the primer binding site is interrupted by an intron in the genome. Actin was used as an internal control.

PCR were performed in 96-well plates (TempPlate, Scientific, Ocala, FL) using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) to monitor dsDNA synthesis. Reactions contained 12.5 µL of 2× SYBR Green Supermix, 2 µL of cDNA, and 400 nM each of gene-specific primer in a final volume of 25 µL. A master mix, excluding cDNA, was prepared before dispensing the reaction mixture into individual wells of 96-well PCR plates to reduce pipetting errors. All RT-PCR experiments were performed multiple times. The cDNA products generated from the *ELT* and *MCO* genes were from two biological replications done in triplicate, while those amplified from the *COPT1* and *HMA5* genes were from three biological replicates performed in duplicate. Reactions were performed using the iCycler Real-time PCR Detection System (Bio-Rad) with the two-step amplification plus melting curve protocol. The reaction conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of: 94 °C for 30 s (denaturation), 57 °C (*ELT*, *COPT1*, and *HMA5*) or 58 °C (*MCO*) for 30 s (annealing) and 72 °C for 30 s (elongation). Actin primers were used in the same 96-well plates and were simultaneously amplified along with the other genes by the same program, to serve as an internal control. The values for

threshold cycle (C_t) determination were generated automatically by iCycler software. The parameter C_t (threshold cycle) is defined as the amplification cycle number at which the fluorescence, due to the formation of a PCR product, passes a fixed threshold. *ELT*, *MCO*, *COPT1*, and *HMA5* expression levels were reported as fold change ($2^{-\Delta C_t}$ (treated-calibrator)) in which treated C_t values were normalized with the corresponding C_t values of actin; the calibrator C_t values were the normalized control C_t values. Calculations were performed as described by Dorak (2006). The fold change and the standard deviation were calculated with Excel.

Results and Discussion

SOLUBLE Si AND FOLIAR AND ROOT DAMAGE INDUCED BY EXCESS Cu. Arabidopsis appeared healthy and showed no obvious stress symptoms when grown under control levels of Cu (0.12 µM) and Si (0.10 mM) (Fig. 1A). Leaf tissue nutrient concentrations indicated no deficiencies or toxicities based on comparisons of recommendations for related species [*Brassica juncea* (L.) Czern., *Brassica oleracea* L., *Brassica perviridis* L.H. Bailey, and *Brassica rapa* L.] (Table 2; Mills and Jones, 1996) with the possible exception of N and Mn, which were slightly above the highest reported value for N and Mn in these species. Plants also grew well under elevated (1.5 mM) Si conditions, and tissue nutrient concentrations for everything except Si were not different from the controls.

In contrast, arabidopsis plants grown under elevated Cu conditions (30 µM) showed reduced growth and chlorosis starting at the tips of older leaves (Fig. 1, A and B). Occasionally, the older leaves became completely chlorotic. The plant chloroplast is particularly sensitive to Cu toxicity (Abdel-Ghany et al., 2005; Shikanai et al., 2003). Moreover, Cu is a cofactor in the ethylene-signaling pathway (Hirayama et al., 1999). Excess Cu supply could intensify ethylene responses and hasten aging, which would explain leaf chlorosis as well as the reduction of shoot fresh weight (Fig. 1B) (see below).

The tissue from plants grown under elevated Cu showed significant differences in concentrations for many nutrients (Table 2): the macronutrient (N, P, K, Ca, S, and Mg) concentrations were significantly different from the control and the elevated Si treatment concentrations; the micronutrients B, Cu, Mn, and Zn were also significantly different from the control and the elevated Si treatments, with Cu concentrations being significantly elevated above those treatments. These changes in tissue nutrient concentrations are indicative of

Table 1. Primers used in these experiments to examine the gene expression of *multi-copper oxidase type I family protein (MCO)*, *esterase lipase thioesterase family protein (ELT)*, *copper transporter 1 (COPT1)*, *heavy metal ATPase 5 (HMA5)*, and *actin*.

Primer name	Sequence (5'-3'); (forward, upper; reverse, lower)	Product size (bp)	Locus no. (Garcia-Hernandez, et al., 2002)
<i>MCO</i>	GCTCGAGCTATCAGGACCAACCTT CAAGTCTGATCGTTCGGGTGATGT	103	At1g21860
<i>ELT</i>	CCCCACAGACTAGCTGGAGCAGT CCACGGAACATAATGAGCCACTTT	147	At5g22460
<i>COPT1</i>	CGAAGGAGGAGGACATCACCACAT GACGGCGAGAAAGAAGACAAAGAT	148	At5g59030
<i>HMA5</i>	CGCTGGATCTGTTGAAAAGGCTATCA TCTCCACATCGACAGAGTTAGGGTA	116	At1g63440
<i>Actin</i>	CAACCGTATTGTGCTGGATTCTGG GCAAGGTCAAGACGGAGGATGG	99	At3g18780

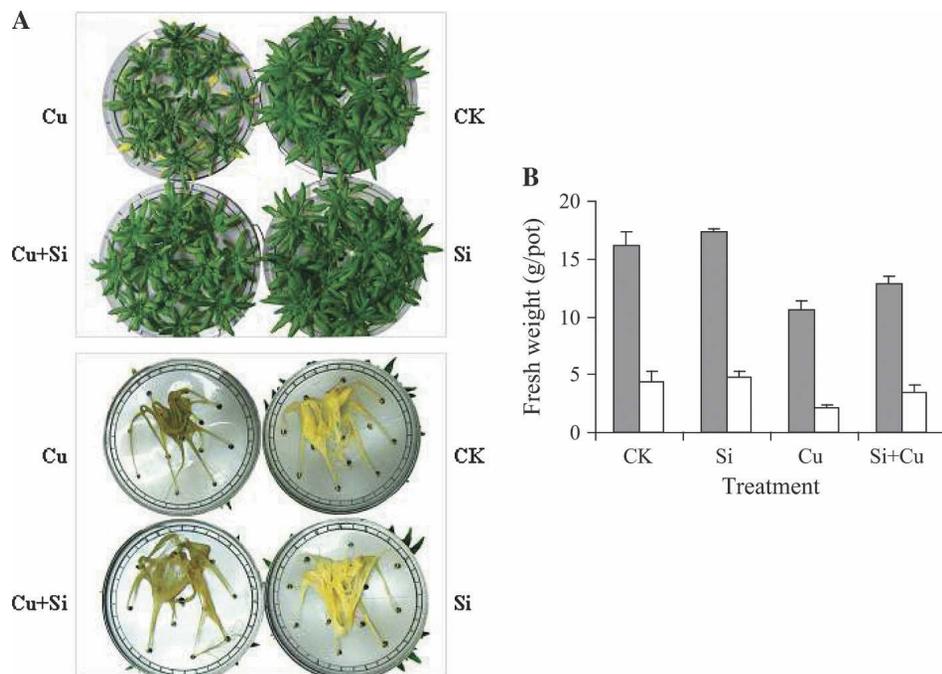


Fig. 1. Responses of Arabidopsis shoots and roots to elevated Si and Cu. Arabidopsis plants were treated with Si and Cu and analyzed after 3 d. The treatments were: control [CK (0.12 μ M Cu + 0.10 mM Si)], elevated Si [Si (0.12 μ M Cu + 1.5 mM Si)], elevated Cu [Cu (30 μ M Cu + 0.10 mM Si)], and elevated Cu and Si [Cu + Si (30 μ M Cu + 1.5 mM Si)]. (A) Arabidopsis shoot (upper) and root (lower) growth under different conditions; (B) changes in shoot (shaded) and root (white) fresh weight under different conditions. Error bars indicate SD (n = 4).

general stress but do not fall outside the “normal” ranges for the recommendations for *Brassica* species (Mills and Jones, 1996).

Elevated Si treatment alleviated the reduction of shoot and root fresh weight caused by elevated Cu alone (Fig. 1B) and reduced leaf chlorosis caused by Cu toxicity. Tissue nutrient concentrations recovered nearly to control levels for all nutrients, with the exception of Cu concentrations; supplemental Si did not change total Cu concentrations in the leaf tissue.

Arabidopsis grown under control hydroponic and elevated Si conditions showed robust root growth and roots appeared

whitish-yellow (Fig. 1A). In contrast, the root weight of plants grown under elevated Cu was less and the roots were dark brown, indicating the presence of phenolics or deposition of metal oxides on the root surface. Roots grown under elevated Cu and supplemented with Si appeared light brown, but were more vigorous than plants grown under elevated Cu without supplemental Si. These data were also reflected in the fresh weight measurements (Fig. 1B). Hence, Si partially alleviated Cu stress in roots, permitting better growth.

When grown in elevated Cu, Arabidopsis shoots had elevated total Cu levels whether Si concentration was elevated or not (Table 2). The improved growth and decrease in visual stress symptoms when additional Si is present suggest that under elevated Cu conditions, Si is influencing the distribution or bioavailability of Cu within leaves. This is analogous to the reported effects of elevated Si on the distribution of Mn; soluble Si reduced Mn toxicity in several plant

species (Horst and Marscher, 1978; Iwasaki et al., 2002a, 2002b, 2002c; Rogalla and Römheld, 2002a, 2002b) not because of a reduction in Mn uptake or translocation (Horst and Marschner, 1978), but because the distribution of Mn throughout the leaves was more diffuse. As a result, toxic levels of Mn did not accumulate in particular groups of cells. More recent studies attributed these protective effects to the deposition of Si in plant cell walls, which provided additional binding sites for metals and thus reduced their apoplastic bypass flow (Ma and Yamaji, 2006). Thus, in our experiments, the concentration of free Cu could be suppressed as a consequence of the

Table 2. Arabidopsis leaf tissue nutrient concentrations. Arabidopsis plants were treated with Si and Cu and harvested after 3 d. The treatments were: control [CK (0.12 μ M Cu + 0.10 mM Si)], elevated Si [Si (0.12 μ M Cu + 1.5 mM Si)], elevated Cu [Cu (30 μ M Cu + 0.10 mM Si)], elevated Cu and Si [Cu + Si (30 μ M Cu + 1.5 mM Si)]. Values are averages of four replicates with each replicate consisting of 12 plants. Data were analyzed using a two-way analysis of variance.

Treatment	N	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	Si
	Leaf tissue concentration (g·kg ⁻¹)						Leaf tissue concentration (mg·kg ⁻¹)					
CK	62.3 a	10.3 a ^z	44.7 a	31.1 a	9.7 a	11.0 a	59.9 a	9.1 b	196.3 a	260.8 a	72.4 a	84.2 b
Si	62.7 a	9.9 a	45.8 a	28.7 ab	9.1 ab	10.5 ab	56.2 a	6.9 b	75.3 a	233.3 ab	63.1 ab	452.2 a
Cu	47.5 b	7.1 c	28.3 b	21.1 c	6.9 c	7.9 c	46.0 b	14.7 a	80.2 a	164.4 c	52.3 b	102.4 b
Cu + Si	52.9 b	8.6 b	41.4 a	24.6 bc	8.0 bc	9.3 bc	59.8 a	16.5 a	53.1 a	188.3 bc	50.6 b	565.9 a
<i>Brassica</i> spp. ^y	20–55	2.5–10	20–90	13–55	1.7–20	2.8–13	12–100	2–30	31–563	25–250	15–250	Not reported
Significance												
Si	NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	****
Cu	****	****	***	****	****	***	NS	****	NS	***	**	NS
Si × Cu	NS	**	**	*	*	*	**	NS	NS	NS	NS	NS

^yRange of values taken from Mills and Jones (1996) for the highest and lowest surveyed values from *B. juncea*, *B. oleracea*, *B. perviridis*, and *B. rapa*.

^zMeans within columns followed by the same letter are not significantly different ($\alpha = 0.05$) based on Tukey’s pairwise comparison.

NS, *, **, ****Nonsignificant or significant at $P \leq 0.05$, 0.01, 0.001, or 0.0001, respectively.

formation of Si cross-linked binding pools for Cu in the cell walls, analogous to the studies on Mn (Iwasaki et al., 2002b; Rogalla and Römheld, 2002b). However, not all cases of Si alleviation of Mn toxicity are due to sequestration of Mn within the cell wall. For example, Iwasaki et al. (2002c) reported that in cowpea [*Vigna unguiculata* (L.) Walp.], the sensitivity of Mn-toxicity symptoms was not correlated with the Mn concentration within the apoplastic solution, but was highly correlated with the water-soluble Si in the apoplast.

SI AND PAL ACTIVITY. An almost ubiquitous feature of plant response to incompatible environmental conditions is the activation of phenylpropanoid biosynthetic pathway in which PAL catalyzes the first committed step (Schuster and Rétey, 1995). The downstream products of PAL activity in plants have a beneficial effect on host responses to pathogen invasion, wounding reactions, chilling susceptibility, and adverse environmental stress. In arabidopsis, PAL activity was induced by excess Cu in roots and shoots when compared with CK- and Si-treated plants (Fig. 2). Even though PAL activity was elevated in the shoots of arabidopsis grown in excess Cu, it was reduced to near-control levels in arabidopsis grown in excess Cu + Si (Fig. 2).

The decrease in PAL activity in the shoots of Si-supplemented plants grown in excess Cu suggests that these plants are not experiencing the same level of stress as plants grown in elevated Cu alone. The mechanism for this reduced stress may be from the formation of Si-phenol complexes (Rogalla and Römheld, 2002c) and their subsequent cross linkage with wall polymers. Si-mediated extension of wall-binding sites for Cu could buffer the free apoplastic Cu, analogous to the findings of Maksimovic et al. (2007). Si addition has been reported to decrease the concentration of plant-borne substrates of anti-oxidative enzymes, such as ferulic acid and coniferyl alcohol under 100 μM Mn (Maksimovic et al., 2007). Thus, the decreased levels of these phenolic compounds may down-regulate PAL activity, as a feedback adjustment. This would result in fewer substrates for plant oxidative enzymes and a reduction of oxidative stress.

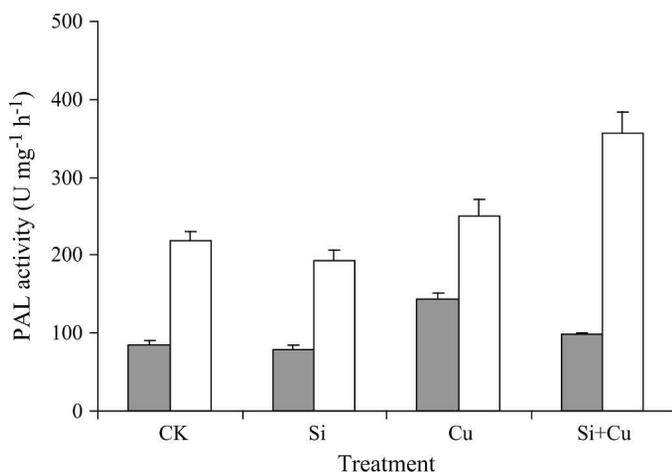


Fig. 2. Effects of Si and Cu treatments on arabidopsis phenylalanine ammonia-lyase (PAL) activity in shoots (shaded) and roots (white). Plants were treated for 3 d under hydroponic conditions. The treatments were: control [CK (0.12 μM Cu + 0.10 mM Si)], elevated Si [Si (0.12 μM Cu + 1.5 mM Si)], elevated Cu [Cu (30 μM Cu + 0.10 mM Si)], and elevated Si and Cu [Si + Cu (1.5 mM Si + 30 μM Cu)]. PAL activity is expressed as units per milligram of total protein per hour. Error bars indicate SD (n = 12).

The situation was different in roots. PAL activity in roots was higher under elevated Cu conditions than in control or elevated Si treatments, but the PAL activity of arabidopsis grown under elevated Si and Cu was greater than with either nutrient alone (Fig. 2). One possible explanation for this is that Si helped stimulate accumulation of polymerized phenolics in roots by stimulating PAL activity (Cherif et al., 1992) to resist toxicity by direct exposure to Cu. As mentioned above for shoots, Si addition to roots could result in the formation of Si-phenolic complexes, thereby reducing the concentration of free phenols (Rogalla and Römheld, 2002c). The sequestration of phenolic compounds by Si would reduce the available substrates for oxidative browning enzymes involved in the expression of Cu-toxicity symptoms. Therefore, although PAL activity was elevated by Si supplementation at high Cu concentration, the roots were not as brown as with elevated Cu alone (Fig. 1A). However, Si is mainly deposited in shoots (Yoshida, 1965). Hence, the role of Si in roots might be to stimulate plant defenses to protect the roots against Cu toxicity, but it may not function in the same way as in shoots. Roots possess an endodermis, which regulates nutrient uptake into the vascular system, while most shoots do not. Therefore, the deposition of Si in the endodermal layer could act as a natural barrier to shoot Cu uptake, as proposed for Cd (Kirkham, 2006). If Cu uptake into the vascular system is blocked, this would result in accumulation of Cu in epidermal and cortical cells, inducing stress responses such as increased PAL activity.

SI AND THE EXPRESSION OF MCO AND ELT. To investigate the beneficial effects of Si on Cu toxicity at the molecular level, we examined the expression of arabidopsis genes influenced by Si. Si treatment alone is reported to affect the expression of two arabidopsis genes: multicopper oxidase (*MCO*, At1g21860.1) and a particular esterase called *ELT* (At5g22460.1) (Fauteux et al., 2006). The *Escherichia coli* Escherich *MCO* gene product safeguards the periplasm and plasma membrane from Cu-induced damage (Rensing and Grass, 2003). Perhaps *MCO* plays a similar plasma membrane-stabilizing role in plants. Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of carboxyl ester bonds.

The pattern of expression in shoots and roots differed for these genes (Fig. 3). For example, Si caused a slight elevation of *MCO* expression in shoots and had no effect in roots (Fig. 3, A and B). In contrast, *ELT* expression was more variable in Si-treated shoots and was not different from the controls (Fig. 3C). However, the expression of *ELT* in Si-treated roots was elevated above that of the controls (Fig. 3D). Treatment with elevated Cu or elevated Cu along with elevated Si caused a decrease in *MCO* expression in shoots when compared with controls (Fig. 3A). Interestingly, increased Cu caused an increase in *MCO* expression in roots above that of the controls, although treatment with Si did not alter the level of expression (Fig. 3B). Thus, toxicity caused by excess Cu induced *MCO* expression in roots, possibly to protect the plasma membranes of root cells regardless of whether Si was present or not. Compared with roots (that are directly exposed to excess Cu), Cu stress in shoots was obviously much less (based on visual observation and PAL activities), and as a result, the expression pattern detected in shoots was not the same as in roots (Fig. 3A). Hence, elevated Cu appears to reduce *MCO* expression in shoots, while elevating it in roots.

Contrary to *MCO*, *ELT* expression in shoots and roots was increased by 30 μM Cu alone or in combination with elevated

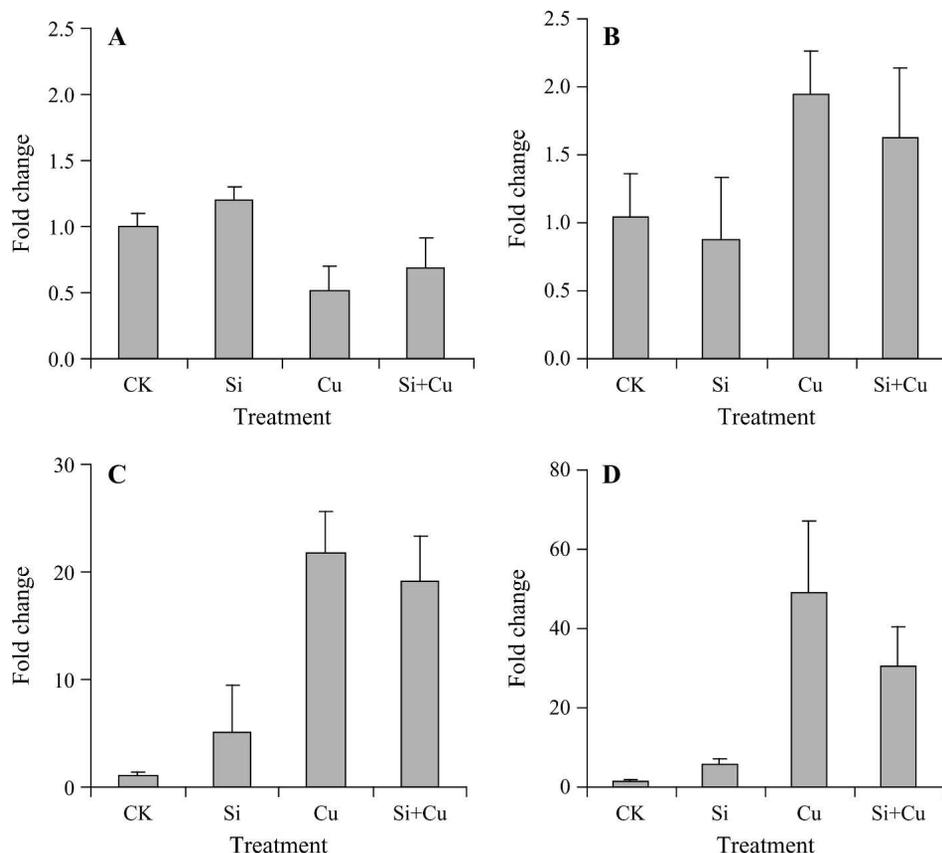


Fig. 3. Real-time RT-PCR analysis of *multi-copper oxidase type I family protein (MCO)* and *esterase lipase thioesterase family protein (ELT)* gene expression in Arabidopsis shoots and roots after 3 d of Cu and Si treatment. The treatments were: control [CK (0.12 μ M Cu + 0.10 mM Si)], elevated Si [Si (0.12 μ M Cu + 1.5 mM Si)], elevated Cu [Cu (30 μ M Cu + 0.10 mM Si)], and elevated Si and Cu [Si + Cu (1.5 mM Si + 30 μ M Cu)]. (A) *MCO* expression in shoots; (B) *MCO* expression in roots; (C) *ELT* expression in shoots; (D) *ELT* expression in roots. Actin was used to correct for variations in RNA levels. Error bars indicate SD (n = 12).

Si. Thus, *ELT* expression was more strongly induced by Cu than by Si in roots and shoots. In contrast, elevated Cu reduced *MCO* expression in shoots, while elevating it in roots.

SI AND THE EXPRESSION OF GENES INVOLVED IN CU TRANSPORT. Arabidopsis leaf Cu levels increased as Cu concentrations were elevated in the hydroponic solution. Cu uptake and transport relies on specific proteins. Therefore, we examined the expression of two genes involved in Cu transport: *COPT1* and *HMA5* (Birbaum et al., 2003; Sancenón et al., 2003). Because both genes encode transporters that are located in root cells, expression of these genes was only examined in roots.

COPT1 encodes an Arabidopsis plasma membrane transporter playing a key role in the uptake of Cu from the surrounding growth medium into roots (Kampfenkel et al., 1995). Plants exposed to elevated Si showed an increase in *COPT1* expression when compared with controls (Fig. 4A). Arabidopsis exposed to elevated Cu levels showed an even greater increase in *COPT1* expression, which has been reported previously (Sancenón et al., 2004). In contrast, when Si as well as Cu levels were elevated in the hydroponic solution, a reduction of *COPT1* expression compared with elevated Cu alone was observed.

The Arabidopsis *HMA5* gene encodes a heavy metal transport ATPase subunit (Andrés-Colás et al., 2006). *HMA5* is primarily expressed in root pericycle cells and is thought to play

a role in the export of Cu from roots to shoots. *HMA5* is induced by Cu (Andrés-Colás et al., 2006), and we observed this as well (Fig. 4B). Si has a suppressive effect on the expression of *HMA5* in the presence of elevated and control levels of Cu. In fact, treatment of Arabidopsis with elevated Si and Cu decreased *HMA5* expression to control levels in roots. Hence, Si reduced the expression of two Cu transporter genes in roots.

Taking these data together, we propose a potential model for Si alleviation of Cu toxicity in Arabidopsis. The levels of Cu entering the shoots grown under elevated Cu conditions cause leaf tissue to experience stress, resulting in increased PAL activity. The increase in PAL activity leads to elevated production of phenolic compounds that then bind to Cu and reduce its bioavailability. When Si is also elevated, we speculate that a portion of the Si taken up by the shoots is incorporated into the cell wall and provides additional Cu-binding sites. In this way, the Cu bioavailability is decreased, reducing stress in shoots, with a subsequent decline in PAL activity. This would also explain why shoots show reduced stress, but still contain large amounts of total Cu.

Under elevated Cu conditions, the stress in roots causes an increase in PAL activity to produce secondary metabolites that bind to Cu and reduce its bioavailability. Our data show that PAL activity of roots is higher than in shoots, probably because roots are more directly exposed to higher levels of Cu than shoots. However, Si and Cu can bind to phenolic compounds and may compete for binding sites. Hence, more phenolic compounds would be needed and PAL activity would rise even higher in the plants treated with elevated Si and Cu than with elevated Cu alone. The increased phenolics in roots would give rise to the brown appearance in plants treated with elevated Cu or both elevated Cu and Si. Alternatively, the color may be due to the deposition of a metal oxide on the root surface. Because the root cells may have multiple binding sites for Cu by Si and phenolic compounds, the amount of free Cu accessible to the apoplast would decrease. Because the *COPT1* and *HMA5* transporter genes are induced by Cu, the reduction in bioavailable Cu to the roots due to its sequestration in the apoplast by Si and phenolic compounds or the deposition of a metal oxide would cause the expression of these genes to decline. The reduction in expression induced by Si does not lead to lower leaf Cu levels. This may be because the reduction in *COPT1* and *HMA5* expression may occur late in the response to high Cu. This would lead to further protection of roots against Cu, while the Cu already present within the shoot would be retained.

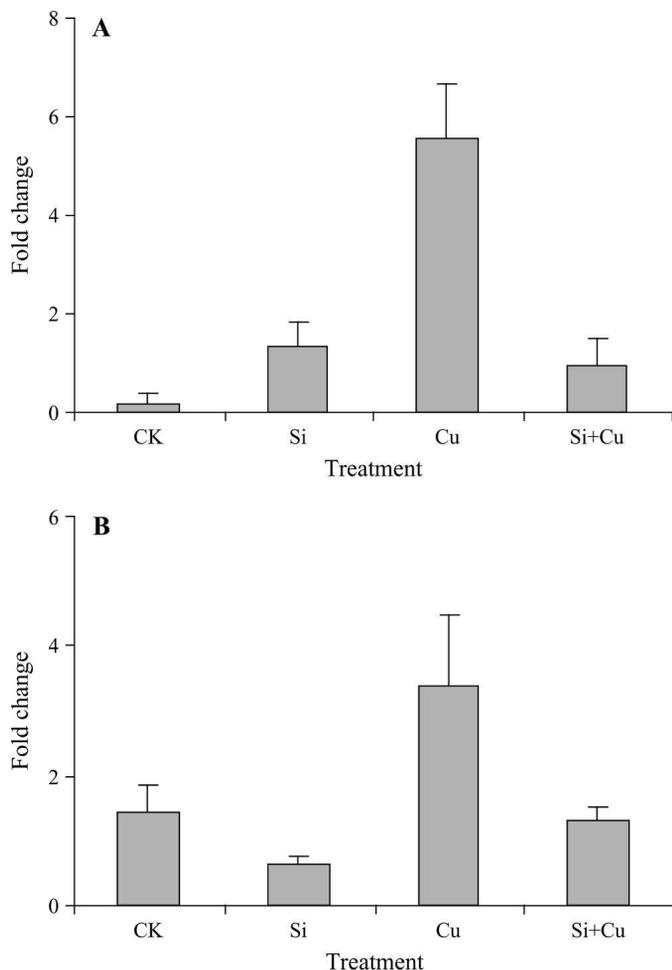


Fig. 4. Expression of *copper transporter 1 (COPT1; A)* and *heavy metal ATPase 5 (HMA5; B)* genes in *Arabidopsis* roots after treatment for 3 d with various combinations of Si and Cu under hydroponic conditions. The treatments were: control [CK (0.12 μ M Cu + 0.10 mM Si)], elevated Si [Si (0.12 μ M Cu + 1.5 mM Si)], elevated Cu [Cu (30 μ M Cu + 0.10 mM Si)], and elevated Si and Cu [Si + Cu (1.5 mM Si + 30 μ M Cu)]. Actin was used to correct for variations in RNA levels. Averages are shown with error bars indicating sd (n = 12).

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