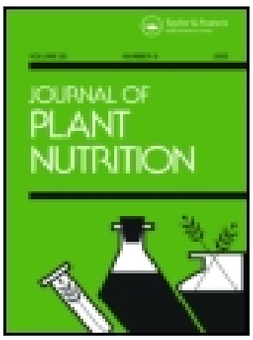

Journal of Plant Nutrition

ISSN: 0190-4167 (Print) 1532-4087 (Online) Journal homepage: https://www.tandfonline.com/loi/lpla20

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To cite this article: Christopher Flora, Sushant Khandekar, Jennifer Boldt & Scott Leisner (2019): Silicon alleviates long-term copper toxicity and influences gene expression in *Nicotiana tabacum*, Journal of Plant Nutrition, DOI: 10.1080/01904167.2019.1589508

To link to this article: https://doi.org/10.1080/01904167.2019.1589508

Published online: 27 Mar 2019.

JOURNAL OF PLANT NUTRITION
https://doi.org/10.1080/01904167.2019.1589508

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Silicon alleviates long-term copper toxicity and influences gene expression in *Nicotiana tabacum*

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ABSTRACT

Silicon (Si) is beneficial for plant growth and aids in stress tolerance. In this study, the effects of Si on long-term copper (Cu) toxicity in the low Si accumulator *Nicotiana tabacum* were evaluated. Silicon supplementation alleviated growth inhibition in roots and shoots of *N. tabacum* exposed to Cu toxicity. Alleviation of Cu toxicity correlated with increased Si accumulation in roots and leaves, suggesting *N. tabacum* contains a stress-regulated mechanism for Si transport. Root Cu concentration decreased in Si-supplemented plants exposed to Cu toxicity. Interestingly, *Copper Transporter 1* (*COPT1*) expression decreased in roots of Si-supplemented plants exposed to Cu toxicity, which may contribute to Cu uptake reduction. Decreases in ethylene (ET) biosynthetic gene expression were previously implicated in Si-mediated stress alleviation. In the present study, Si-mediated alleviation of Cu toxicity corresponded with increased ET biosynthetic gene expression.

Abbreviations: ΦPSII: effective quantum efficiency; ACC: 1-aminocyclopropane-1-carboxylic acid; ACO: 1-aminocyclopropane-1-carboxylic acid oxidase; ACS: 1-aminocyclopropane-1-carboxylic acid synthase; ADC: arginine decarboxylase; ANOVA: analysis of variance; AP2: apetala 2; COPT: copper transporter; CuAO: copper diamine oxidase; DHCA: dicyclohexylammonium sulfate; EREBP: ethylene responsive element binding protein; ERF: ethylene responsive factor; ET: ethylene; GSH: glutathione; HMA: heavy metal ATPase; HSD: honest significant difference; ICP-OES: inductively coupled plasma optical emission spectroscopy; LCF: leaf chamber fluorometer; MT: metallothionein; ODC: ornithine decarboxylase; PA: polyamine; PAO: polyamine oxidase; PAR: photosynthetically active radiation; PC: phytochelatin; PCS: phytochelation synthase; RT-qPCR: reverse transcriptase-quantitative polymerase chain reaction; SAM: s-adenosylmethionine; SAMDC: s-adenosylmethionine decarboxylase; SAMS: S-adenosylmethionine synthetase; SEM: standard error of the mean; SPDS: spermidine synthase; SPMS: spermine synthase; TRSV: Tobacco ringspot virus; UBC: ubiquitin conjugating enzyme

ARTICLE HISTORY
Received 26 June 2018
Accepted 10 July 2018

KEYWORDS
copper; ethylene; metal toxicity; polyamine; silicon; tobacco

Introduction

Copper (Cu), an essential micronutrient in plants, functions as a co-factor in several physiological processes including cellular respiration, photosynthesis, lignification, and ethylene (ET) perception (Printz et al. 2016). However, Cu is toxic at high concentrations due to generation of reactive

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oxygen species via the Haber-Weiss reaction (Sancenon et al. 2003). Plants possess homeostatic networks which control Cu transport and facilitate detoxification to prevent toxicity (Printz et al. 2016). Copper transport across the plasma membrane and into the root system is accomplished by cell surface, high-affinity Cu transporters (COPTs) such as COPT1 (Sancenon et al. 2003). Once Cu enters root cells, it binds with chaperones that direct it to heavy metal P-Type ATPases (HMAs) for distribution throughout the plant (Andres-Colas et al. 2006). For example, HMA5 transports Cu into the xylem. To maintain low free cytosolic concentrations of Cu, plants employ chelators such as metallothioneins (MTs) and phytochelatins (PCs) (Ducic and Polle 2005). MTs are small metal-binding proteins, while PCs are small organic molecules that bind metals and are synthesized by PC synthase (PCS). *Metallothionein* and *PC Synthase* (*PCS*) expression is induced by Cu in *Arabidopsis thaliana* (arabidopsis). However, other factors can influence expression of chelator genes. For example, *MT2* expression increased further when Cu-treated plants were supplemented with Si (Khandekar and Leisner 2011). Furthermore, Si supplementation has alleviated Cu toxicity in arabidopsis (Li, Frantz, and Leisner 2008), and has been reported to alleviate heavy metal toxicity in a variety of plant species (Adrees et al. 2015).

Silicon alleviates heavy metal toxicity through several proposed mechanisms (Adrees et al. 2015; Debona, Rodrigues, and Datnoff 2017). The formation of silicate complexes in the soil can increase pH and change the chemical form of metals, reducing bioavailability. Silicon can also alleviate heavy metal toxicity by affecting metal compartmentalization within plants, increasing antioxidant activity, and altering expression of Cu homeostasis genes. For example, Cu-treated arabidopsis supplemented with Si exhibited expression changes for *superoxide dismutase*, *COPT1*, and *HMA5* compared to Cu-treated plants (Khandekar and Leisner 2011; Li, Frantz, and Leisner 2008). Silicon-mediated changes in gene expression likely employ signaling molecules. Ethylene and polyamines (PAs) were recently implicated in Si-mediated alleviation of salt stress in *Sorghum bicolor* (*S. bicolor*), a high Si-accumulator (Yin et al. 2016).

A diverse range of plant developmental and physiological processes involve ET, and many stress conditions result in higher ET production (Keunen et al. 2016). Synthesis of ET occurs through the consecutive action of three enzymes: S-adenosylmethionine (SAM) synthetase (SAMS) converts methionine to SAM, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) produces ACC from SAM, and ACC oxidase (ACO) converts ACC to ET. Once produced, ET diffuses throughout the plant and binds to receptors localized in the membrane of the endoplasmic reticulum. Ethylene signaling results in the expression and activation of ET response factors (ERFs), transcription factors that modulate expression of genes involved in hormonal and redox pathways (Dietz, Vogel, and Viehhauser 2010; Wang, Li, and Ecker 2002).

Polyamines are small aliphatic polycations ubiquitously distributed in living organisms (Hussain et al. 2011). Three main PAs exist in plants: putrescine (Put), spermidine (Spd), and spermine (Spm). Accumulation of PAs occurs in response to abiotic stress, largely due to increased de novo synthesis, and expression of PA biosynthetic genes are representative of PA content within plants (Liu et al. 2015). Synthesis of plant PAs begins with the activity of either arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), since both enzymes can catalyze Put production. Decarboxylation of SAM occurs via SAM decarboxylase (SAMDC). Decarboxylated SAM (dSAM) then serves as an aminopropyl donor in the conversion of Put to Spd or Spd to Spm, through Spd synthase (SPDS) or Spm synthase (SPMS) activity, respectively (Alcazar et al. 2010). Whether SAM is converted to ACC for ET biosynthesis or dSAM for PA biosynthesis determines the fate of these two biosynthetic pathways (Pandey et al. 2000). Therefore, SAM is a common precursor in competitive demand. Furthermore, the ratio of PA biosynthesis to catabolism is considered an important factor in the induction of abiotic stress tolerance (Moschou, Paschalidis, and Roubelakis-Angelakis 2008). Spermidine and Spm are catabolized to Put by polyamine oxidases (PAOs), and Put is broken down by Cu diamine oxidase (CuAO) (Liu et al. 2015).

As mentioned above, ET and PAs have been implicated in Si-mediated alleviation of salt stress in *S. bicolor* (Yin et al. 2016), which accumulates high amounts of Si (>1% dry weight) (Hodson et al. 2005). Specifically, Yin et al. (2016) observed reduced production of ACC and decreased *ACS* expression. However, PAs accumulated in NaCl + Si-treated plants and *ADC* expression increased. These data suggest a shift towards PA synthesis rather than ET synthesis. Furthermore, inhibition of Spm synthesis abolished Si-mediated salt tolerance suggesting PAs play a direct role in Si-mediated alleviation of stress. However, it is unknown if Si alleviates Cu toxicity in low Si accumulators such as *N. tabacum*, and if the mechanism of Si-mediated stress alleviation is similar to *S. bicolor*. Therefore, the present study investigates whether Si alleviates Cu toxicity in *N. tabacum*, examines expression of genes involved in Cu homeostasis, as well as genes involved in biosynthesis of ET and PAs.

Materials and methods

Plant material and growth conditions

Tobacco (*N. tabacum* L. cv. *Wisconsin 38*) seeds were sown onto cotton-filled pipette tip trays (1000 µL pipette tips) over water in a pipette tip box and placed in a Conviron CMP5090 (Winnipeg, Manitoba, CA) growth chamber (20 °C and 45% humidity) under 16 h light (70 µmol m^{-2} s^{-1} photosynthetically active radiation; PAR) and 8 h dark. Upon germination, plants were supplemented with nutrient solution containing 1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.1 mM NH_4NO_3, 0.5 mM $MgSO_4$, 0.5 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 5 µM $MnSO_4$, 0.08 µM $(NH_4)_6Mo_7O_{24}$, 0.5 µM $ZnSO_4$, 30 µM H_3BO_3, 0.12 µM $CuSO_4$, and 50 µM Fe-EDTA; pH 5.7 (Li, Frantz, and Leisner 2008) until the two-leaf stage. Seedlings were then transferred into 4.5 L plastic buckets (1 plant per bucket, Encore Plastics Corp., Cambridge, OH, USA) with 4 L of continuously aerated nutrient solution and placed in a growth chamber (20 °C and 45% humidity) under 16 h light (100 µmol m^{-2} s^{-1} PAR) and 8 h dark. Nutrient solution was replaced every 7 d. Upon development of 4–6 true leaves, nutrient solution treatments commenced, and plants were grown for 21 d and then harvested. Treatments were as follows: Control (no Si), Si (elevated Si as 1.0 mM K_2SiO_3), Cu (elevated Cu as 35 µM $CuSO_4$), and Cu + Si (elevated Cu as 35 µM $CuSO_4$, and elevated Si as 1.0 mM K_2SiO_3). Hydroponic solution pH was monitored and remained at 5.7 throughout the course of the experiments. Eight plants per treatment were examined as individuals, and experiments were repeated at least three times.

Plant harvest and morphological measurements

Eight plants per treatment were harvested and analyzed independently. Upon harvest, leaves were removed, weighed, and flash frozen in liquid nitrogen. Stem length was measured from apical meristem to root-shoot interface. A piece of string was placed along stems to follow their contours, cut to match the stem length, and measured with a ruler. Roots were cut at the root-shoot interface, rinsed three times with 0.1 N HCl to remove excess Si, and blotted dry (Frantz et al. 2008). Root length was measured from root-shoot interface to root apical meristem, root fresh weight was recorded, and roots were flash frozen in liquid nitrogen. Total leaf and root tissue from each sample was independently homogenized by grinding in liquid nitrogen. All tissue collected was stored at −80 °C until further analysis. Data presented are representative of one experiment and were subjected to one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD). Each experiment was repeated three times with similar results, and $P < 0.05$ was considered statistically significant.

Measurement of photosynthetic parameters

Chlorophyll fluorescence measurements were recorded with the LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) fitted with a leaf chamber

fluorometer (LCF) for three randomly selected plants per treatment on day 21 prior to harvest. For dark-adapted chlorophyll fluorescence measurements, the first fully expanded leaf below the apical meristem was fitted with a dark-adapting clip for 30 min. The shutters were opened as the LCF was clamped to the leaf. Minimum (F_o) and maximum (F_m) fluorescence were recorded and the ratio of variable (F_v; F_m-F_o) to maximum fluorescence (F_v/F_m) was calculated.

Effective quantum efficiency (Φ_{PSII}) was determined after dark-adapted leaves were exposed to light for a minimum of 30 min. Light intensity in the LCF was set to 300 µmol m^{-2} s^{-1} PAR (90:10 red:blue from light-emitting diodes). Light-adapted maximum (F_m') and steady-state (F_s) fluorescence was recorded, and Φ_{PSII} was calculated [(F_m'-F_s)/F_m']. Data presented are representative of one experiment and were subjected to one-way ANOVA with Tukey's HSD. Each experiment was repeated three times, and $P < 0.05$ was considered statistically significant.

Elemental analysis

Homogenized leaf and root tissue from four randomly selected plants per treatment was dried at 60 °C for 7 d. For total elemental analysis, 0.15 g of dried tissue per sample was digested using EPA method 3051 (Nelson, 1988). Total concentration of Cu was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; iCAP 6300 Duo, Thermo Electron Corp., Waltham, MA, USA). Total Si concentration was determined via KOH digestion and ICP-OES analysis (Frantz et al. 2008). Data presented are representative of one experiment and were subjected to one-way ANOVA with Tukey's HSD. Each experiment was repeated three times, and $P < 0.05$ was considered statistically significant.

RNA extraction and RT-qPCR

Total RNA was isolated from 100 mg frozen root tissue from three randomly selected individual plants per treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with on column DNase digestion according to the manufacturer's specifications. Purified RNA concentrations were measured using a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and RNA integrity was assessed via formaldehyde-agarose gel electrophoresis. Gene specific primers (Table S1) were designed using the *N. tabacum* TN90 transcriptional assembly (Philip Morris International R&D), synthesized (Integrated DNA Technologies, Coralville, IA, USA), and specificity was confirmed via agarose gel electrophoresis. One step RT-qPCR was performed using the iTaq Universal One-Step RT-qPCR Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's specifications with 100 ng purified RNA per reaction. Each reaction was performed in triplicate and data analysis was performed using Bio-Rad CFX Manager software. Expression of target genes was normalized to *Ubiquitin Conjugating Enzyme 2*, which remains stable in *N. tabacum* under abiotic stress conditions (Schmidt and Delaney 2010). Data presented are representative of one experiment and were subjected to one-way ANOVA with Tukey's HSD. Each experiment was repeated three times with similar results, and $P < 0.05$ was considered statistically significant.

Results

Silicon alleviates Cu-induced growth inhibition in N. tabacum

To determine if Si alleviated prolonged Cu toxicity in *N. tabacum*, growth (Figure 1) was monitored for plants under extended treatment (21 d) with Si (1 mM Si), Cu (35 µM $CuSO_4$), or Cu + Si (35 µM $CuSO_4$ + 1 mM Si). Control and Si-treated plants were approximately the same size, while Cu-treated plants displayed significant stunting. However, Cu + Si-treated plants

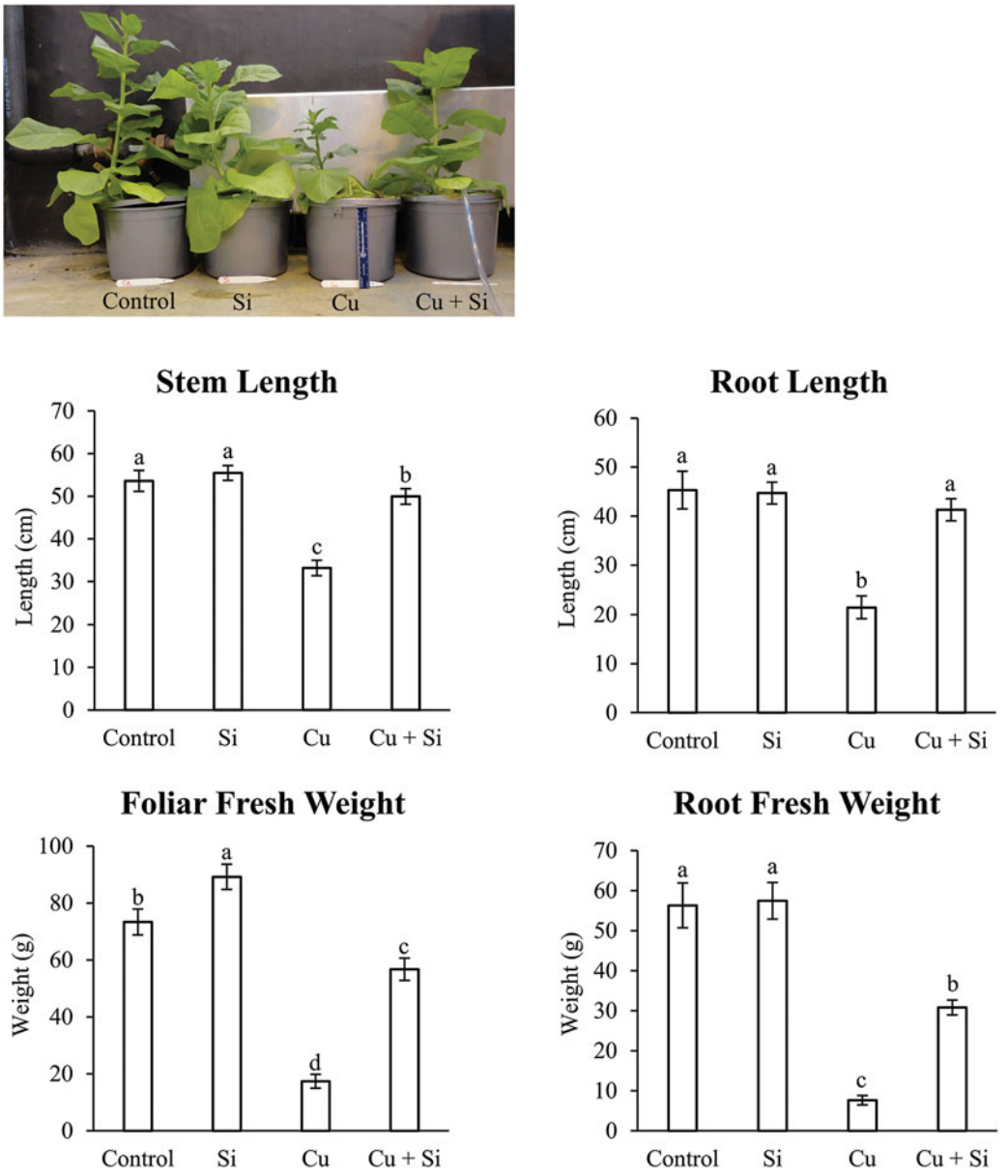

Figure 1. Silicon (Si) alleviates copper (Cu) toxicity in *Nicotiana tabacum*. Morphological parameters of *Nicotiana tabacum* treated with Control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 21 d then harvested. Values are mean ± SEM (n = 8). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

Table 1. Maximum quantum yield (F_v/F_m) and effective quantum yield (Φ_{PSII}) values of leaves from *Nicotiana tabacum* treated with Control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 21 d.

Treatment	F_v/F_m	Φ_{PSII}
Control	0.760 ± 0.009^{a}	0.492 ± 0.026^{a}
Si	0.720 ± 0.019^{ab}	0.416 ± 0.037^{a}
Cu	0.711 ± 0.006^{b}	0.354 ± 0.065^{a}
Cu + Si	0.740 ± 0.005^{a}	0.432 ± 0.027^{a}

Values are mean ± SEM (n = 3). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

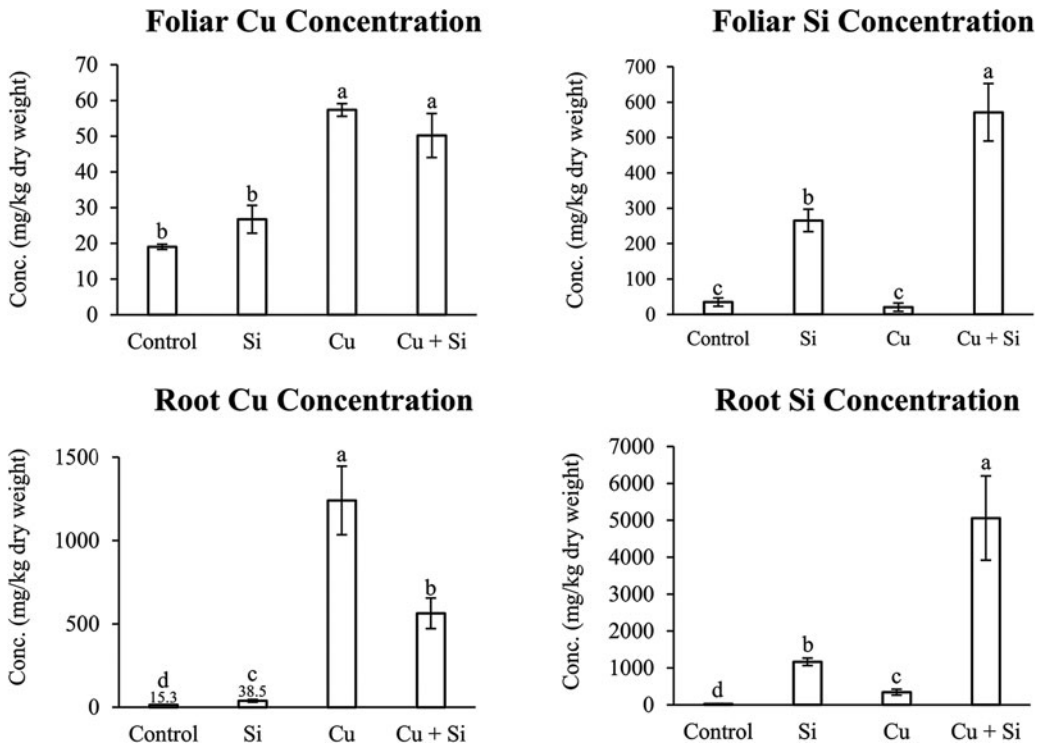

Figure 2. Copper (Cu) and silicon (Si) concentration in *Nicotiana tabacum*. Concentrations of Cu and Si in *Nicotiana tabacum* leaves treated with Control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 21 d then harvested. Concentrations were determined by ICP-OES. Values are mean ± SEM (n = 4). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

exhibited growth recovery compared to Cu-treated plants. Compared to control, stem and root length of Si-treated plants was similar, but both were significantly reduced for Cu-treated plants. The stem and root length of Cu + Si-treated plants partially recovered (Figure 1). The foliar fresh weight of Si-treated plants was significantly higher than the control, while the foliar fresh weight of Cu-treated plants was significantly reduced. Foliar fresh weight of Cu + Si treated plants significantly recovered but did not reach control levels. Root fresh weight of Si-treated plants was similar to control, while root fresh weight was significantly reduced in Cu-treated plants. The root fresh weight of Cu + Si-treated plants was greater compared to Cu-treated plants, but did not reach control levels. Overall, growth parameters measured were reduced in Cu-treated plants compared to controls, and Si helped plants recover from Cu toxicity.

Silicon increases chlorophyll fluorescence in N. tabacum under Cu toxicity

To examine the effect of prolonged Cu toxicity on photosystem II efficiency, chlorophyll fluorescence was recorded in both dark- and light-adapted plants (Table 1). Dark-adapted Si-treated plants showed similar F_v/F_m values compared to control, while dark-adapted Cu-treated plants exhibited lower F_v/F_m relative to control, but not compared to Si-treated plants. Dark-adapted Cu + Si-treated plant F_v/F_m was also similar to control levels. In light-adapted plants, Φ_{PSII} was similar across all treatments. Hence, Si supplementation did aid photosynthetic efficiency in plants exposed to Cu toxicity.

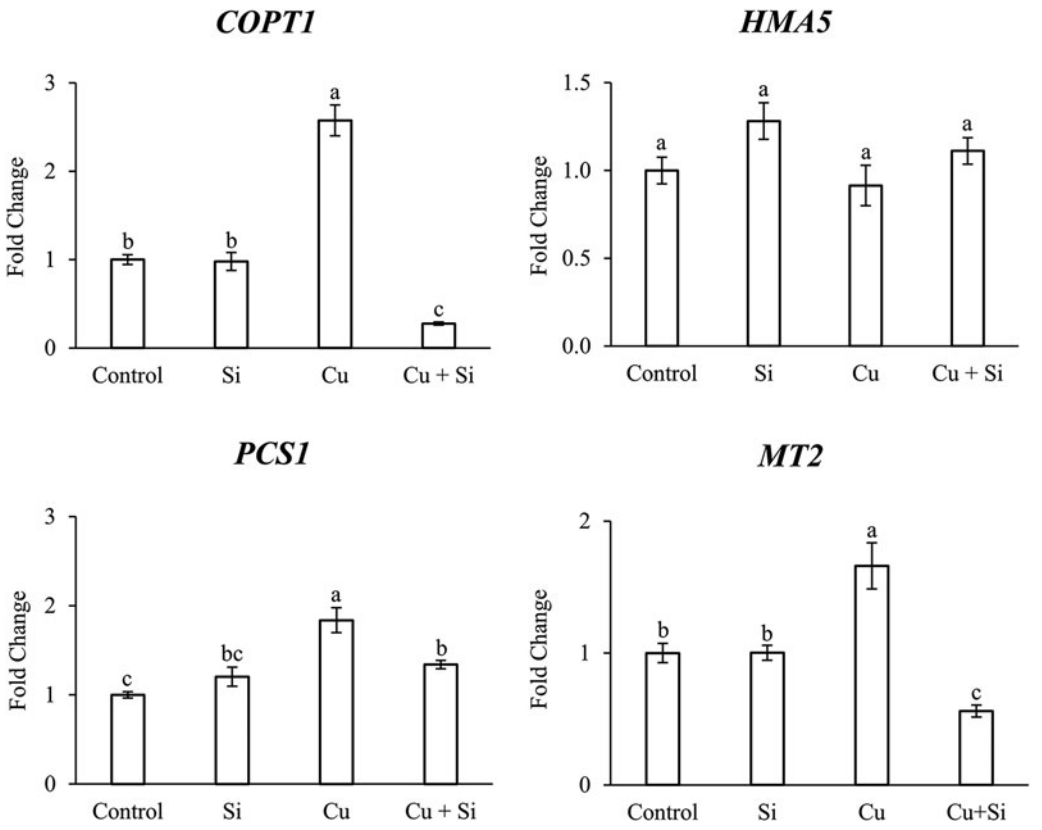

Figure 3. Expression of copper (Cu) transporter and detoxification genes. *Nicotiana tabacum* treated with Control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 21 d then harvested. *Copper Transporter 1* (*COPT1*), *Heavy Metal ATPase 5* (*HMA5*), *Phytochelatin Synthase 1* (*PCS1*), and *Metallothionein 2* (*MT2*) expression in roots was determined by RT-qPCR. All target gene expression was normalized to *Ubiquitin Conjugating Enzyme 2* (*Ubc2*). Values are mean ± SEM (n = 3). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

Silicon and Cu concentrations change in Cu + Si-treated plants

To investigate Si-mediated alleviation of Cu toxicity, Cu and Si concentrations within foliar and roots tissue were determined (Figure 2). The foliar Cu concentration of Si-treated plants was similar to control, but that of Cu-treated plants significantly increased. The foliar Cu concentration of Cu + Si-treated plants was similar to Cu-treated plants indicating Si supplementation did not prevent foliar Cu accumulation under Cu toxicity.

In roots of Si-treated plants (Si-treated roots), the Cu concentration was slightly elevated compared to control, while that in the roots of Cu-treated plants (Cu-treated roots) was even greater. Unlike foliar tissue, the Cu concentration of roots from Cu + Si treated plants (Cu + Si-treated roots) was significantly lower than in Cu-treated plants. Thus, Si supplementation appeared to cause a reduction in root Cu uptake. However, the Cu concentration within the Cu + Si roots was much higher than in controls, indicating that high levels of the element were still being acquired by roots.

In Si-treated plants, foliar Si concentration significantly increased compared to control, while that of Cu-treated plants did not. The foliar Si concentration of Cu + Si-treated plants was significantly higher compared to all other treatments. Therefore, Cu toxicity appeared to cause an increase in foliar Si accumulation when supplemental Si was also provided. A similar observation was made in root tissue. The Si concentration in Si-treated roots increased compared to control, and the Si concentration in Cu-treated roots also increased, but not to the level of Si-treated

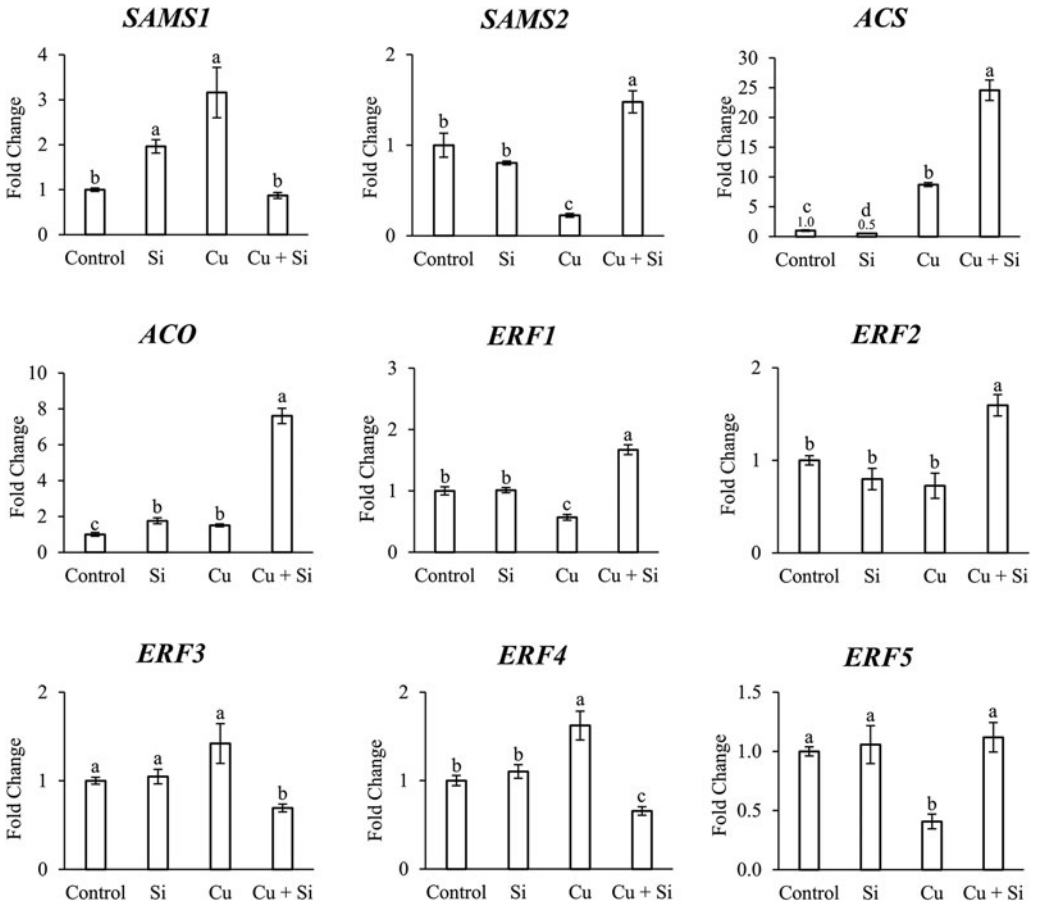

Figure 4. Expression of ethylene biosynthetic and transcription factor genes. *Nicotiana tabacum* treated with Control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 21 d then harvested. *S-adenosylmethionine* (SAM) *Synthetase 1* (*SAMS1*), *SAMS2*, *1-aminocyclopropane-1-carboxylic acid* (ACC) *Synthase* (*ACS*), *ACC Oxidase* (*ACO*), *Ethylene Responsive Factor 1* (*ERF1*), *ERF2*, *ERF3*, *ERF4*, and *ERF5* expression in roots was determined by RT-qPCR. All target gene expression was normalized to *Ubiquitin Conjugating Enzyme 2* (*Ubc2*). Values are mean ± SEM (n = 3). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

plants. In Cu + Si-treated roots the Si concentration was significantly higher than all other treatments. Hence, as in foliar tissue, Cu toxicity appeared to cause an increase in root Si levels.

Cu and Si influence COPT1 and MT2 gene expression

To understand how Si affects Cu homeostasis, Cu transporter (*COPT1* and *HMA5*) and chelator (*MT2* and *PCS1*) gene expression was examined in *N. tabacum* roots (Figure 3). In Si-treated roots, *COPT1* expression was similar to control, while *COPT1* expression increased 2.5-fold in Cu-treated roots. However, *COPT1* expression decreased 4-fold in Cu + Si treated roots compared to control. Expression of *HMA5* did not vary from control levels across all treatments.

Compared to the control, *PCS1* expression in Si-treated roots was similar, but increased 1.5-fold in Cu-treated roots. However, Cu + Si-treated roots exhibited increased *PCS1* expression compared to control, but not to the level of Cu-treated roots. Expression of *MT2* was similar to control in Si-treated roots but increased approximately 2-fold in Cu-treated roots. In Cu + Si-treated roots, *MT2* expression decreased 2-fold compared to control. Taken together, Si

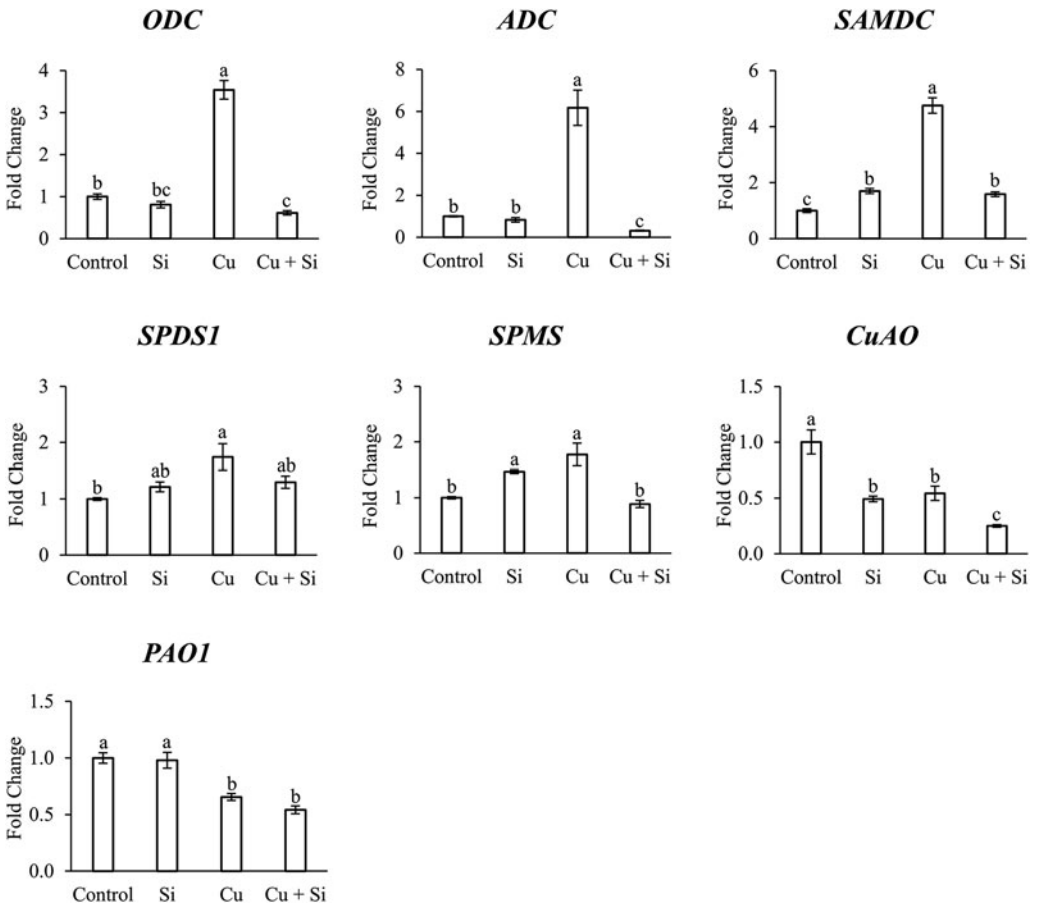

Figure 5. Expression of polyamine biosynthetic and catabolic genes. *Nicotiana tabacum* treated with Control, Si (1 mM Si), Cu (35 µM $CuSO_4$), or Cu + Si (35 µM $CuSO_4$ + 1 mM Si) for 21 d then harvested. *Ornithine Decarboxylase* (*ODC*), *Arginine Decarboxylase* (*ADC*), *S-adenosylmethioneine* (SAM) *Decarboxylase* (*SAMDC*), *Spermidine Synthase 1* (*SPDS1*), *Spermine Synthase* (*SPMS*), *Copper Diamine Oxidase* (*CuAO*), and *Polyamine Oxidase 1* (*PAO1*) expression in roots was determined by RT-qPCR. All target gene expression was normalized to *Ubiquitin Conjugating Enzyme 2* (*Ubc2*). Values are mean ± SEM (n = 3). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $P < 0.05$.

supplementation appears to decrease expression for *COPT1* and the chelator genes (*PCS1* and *MT2*) in plants exposed to Cu toxicity.

Copper and Si influence expression of genes involved in ET biosynthesis and signaling

Ethylene acts as a plant stress hormone (Keunen et al. 2016). Since Si alleviated stress in our system, perhaps this element affects ET synthesis. In Si-treated roots, *SAMS1* and *ACO* expression increased 2-fold relative to control, while *ACS* expression decreased 2-fold compared to control (Figure 4). Expression of *SAMS2* remained constant. Expression of *SAMS1*, *ACS*, and *ACO* increased 3-, 10-, and 2-fold in Cu-treated roots, respectively, compared to control. However, *SAMS2* expression decreased 4-fold in Cu-treated roots relative to control. In Cu + Si-treated roots, *SAMS2*, *ACS*, and *ACO* expression increased 1.5-, 25-, and 8-fold compared to control, respectively. Hence, Cu + Si-treated roots showed increased ET biosynthetic gene expression (except for *SAMS1*) compared to Cu-treated plants.

ERFs are transcription factors that modulate ET responses (Dietz, Vogel, and Viehhauser 2010; Fujimoto et al. 2000). Therefore, expression for several *ERFs* was monitored to understand how

Si may affect ET signaling under Cu toxicity (Figure 4). In Si-treated roots, expression for all *ERFs* (*ERF1-5*) examined was similar to control. Expression of *ERF1* and *ERF5* decreased 2-fold in Cu-treated roots compared to control, while *ERF2* and *ERF3* expression remained similar to control. However, *ERF4* expression increased 1.5-fold in Cu-treated roots relative to control. In Cu + Si-treated roots, *ERF1* and *ERF2* expression increased 1.5-fold compared to control, while *ERF5* expression remained constant. However, *ERF3* and *ERF4* expression decreased 2-fold in Cu + Si-treated roots relative to control. In addition, *ERF1*, *ERF2*, and *ERF5* expression increased, while *ERF3* and *ERF4* gene expression decreased in Cu + Si-treated roots compared to Cu-treated roots.

Copper and Si influence expression of genes involved in PA biosynthesis and catabolism

Polyamine biosynthesis and accumulation is associated with abiotic stress alleviation and has been implicated in Si-mediated stress alleviation responses (Yin et al. 2016). Therefore, expression of PA biosynthetic genes was examined in *N. tabacum* roots under extended Cu toxicity (Figure 5). In Si-treated roots, *SAMDC* and *SPMS* expression increased approximately 2-fold, while *ODC*, *ADC*, and *SPDS1* expression was similar to control. However, expression of all PA biosynthetic genes increased in Cu-treated roots compared to control: *ODC* (4-fold), *ADC* (6-fold), *SAMDC* (5-fold), *SPDS1* (2-fold), and *SPMS* (2-fold). Expression of *ADC* and *ODC* decreased 4- and 2- fold in Cu + Si-treated roots respectively, relative to control, while *SAMDC* gene expression increased 2-fold. In addition, *SPDS1* and *SPMS* expression in Cu + Si-treated roots was similar to control. Overall, expression of PA biosynthetic genes increased in Cu-treated roots, but decreased in Cu + Si-treated roots.

Polyamine abundance in plants is also modulated by catabolism (Moschou, Paschalidis, and Roubelakis-Angelakis 2008). Expression of *CuAO* decreased 2-fold in Si-treated roots relative to control, while *PAO1* gene expression remained constant (Figure 5). Both *CuAO* and *PAO*1 expression decreased 2-fold in Cu-treated roots compared to control. In addition, *PAO1* and *CuAO* expression decreased 2- and 4-fold in Cu + Si-treated roots relative to control, respectively. Thus, PA catabolic gene expression either was reduced (*CuAO*) or remained the same (*PAO1*) in Si supplemented roots exposed to Cu toxicity compared to Cu toxicity alone.

Discussion

Numerous studies have demonstrated Si to enhance heavy metal stress tolerance in plants, especially in Si accumulators (Debona, Rodrigues, and Datnoff 2017). However, if and how Si mediates alleviation of Cu toxicity in low Si accumulators, such as *N. tabacum*, is unclear. In the present study, we show Si does indeed alleviate Cu toxicity.

Silicon may alleviate Cu toxicity and promote growth by positively affecting photosynthesis. The Cu + Si-treated plants showed a recovery of growth and chlorophyll fluorescence compared to Cu-treated plants. Surprisingly, Φ_{PSII} values did not significantly change across all treatments, although the Φ_{PSII} value trend corresponded to F_v/F_m. Insignificant differences in Φ_{PSII} values may be accounted for by increased variability in light-adapted chlorophyll fluorescence due to xanthophyll cycle activation (Vaz and Sharma 2011). Similarly, Cu-treated bean plants exhibited greatly impacted growth but only a small decrease in photosynthetic efficiency (Cook et al. 1998).

Interestingly, foliar Si concentrations increased in Cu + Si-treated plants compared to Si-treated plants. Previously, we showed *Tobacco ringspot virus* (*TRSV*) infection induced Si accumulation in *N. tabacum* leaves, which corresponded with decreased *TRSV* symptomology (Zellner, Frantz, and Leisner 2011). Taken together, these data suggest *N. tabacum* possesses a stress-regulated mechanism for modulating Si transport; however, Si transporters have yet to be discovered in *N. tabacum*. The increased foliar Si accumulation may be part of a general response

and aid in stress alleviation. Silicon may aid plant heavy metal stress tolerance by affecting metal compartmentalization throughout foliar and/or root tissues. In *Cucumis sativas*, for example, Si alleviated Mn toxicity by sequestering Mn in foliar cell walls (Rogalla and Romheld 2002). In our study, foliar Cu concentrations did not vary between Cu− and Cu + Si-treated plants. We did not quantify where Cu localized in cells; therefore, it is possible Si binds Cu and sequesters it in foliar cell walls to alleviate Cu toxicity in *N. tabacum*.

We observed higher Si concentrations in Cu + Si-treated roots compared to Si-treated roots, which suggests *N. tabacum* roots also accumulate Si in response to Cu stress. Furthermore, Si-mediated alleviation of Cu toxicity in roots corresponded with a 2-fold decrease in Cu concentration relative to Cu-treated plants. This reduction in root Cu concentration by Si could be due to at least two mechanisms. The first mechanism involves Cu-silicate complex formation in the hydroponic solution, reducing metal bioavailability. We believe this is unlikely since $CuSO_4$ does not precipitate with Si below pH 6.0 (Leggett 1978), and our nutrient solution pH was maintained at 5.7 throughout our experiments. A second mechanism entails Si reducing Cu uptake by affecting Cu transporters. In a previous study, *COPT1* and *HMA5* expression in arabidopsis roots decreased in Cu + Si-treated plants compared to Cu-treated plants (Li, Frantz, and Leisner 2008). Therefore, we hypothesized Si may similarly influence Cu transport in *N. tabacum* roots by affecting the expression of Cu transporters *COPT1* and *HMA5*. Indeed, *COPT1* expression significantly decreased in Cu + Si-treated roots compared to Cu-treated roots although *HMA5* expression remained constant. Taken together, our data suggest Si may affect Cu transport into *N. tabacum* roots, but not shoots under Cu toxicity.

Plants produce small proteins (MTs) and organic molecules (PCs) to sequester metals, including Cu, thereby reducing their free concentration and protecting cells from toxicity (Hasan et al. 2017). Phytochelatins are produced from glutathione (GSH) by phytochelatin synthase (PCS), while MTs are produced by MT genes. Previously, we demonstrated increased *MT2* expression in leaves of Cu + Si-treated arabidopsis compared to Cu-treated plants (Khandekar and Leisner 2011). However, in the present study, Cu + Si-treated roots showed a reduction in both *PCS1* and *MT2* expression relative to Cu-treated roots. Differences in *MT2* expression across studies may be attributed to species, the analysis of different tissues, plant age, or durations of exposure. Surprisingly, overexpression of *PCS1* in leaves and roots of transgenic arabidopsis resulted in hypersensitivity to cadmium (Cd) and zinc (Zn) even though PC production increased 2-fold compared to wild type plants (Lee et al. 2003). Therefore, plants could possess other protective mechanisms against heavy metal toxicity which are affected by Si. One possible mechanism could be pumping Cu out of the roots and into the nutrient solution through H+/Cu(II) antiporters (Parrotta et al. 2015). Perhaps Si reduces *MT2* and *PCS1* expression in *N. tabacum* roots to increase free Cu and efflux capability, which could contribute to the reduced Cu levels in Cu + Si-treated roots. Another possible mechanism could be that if a certain concentration of Cu is required to induce the expression of *MT2* and *PCS1*, the reduction of root Cu levels by Si may not allow those genes to become induced.

Many studies have established Si to regulate gene expression in response to heavy metal toxicity (Adrees et al. 2015) and biosynthesis of ET and PAs was recently implicated in Si-mediated tolerance to salt stress in *S. bicolor*, a high Si accumulator (Yin et al. 2016). The role of ET and PAs in Si-mediated alleviation of Cu toxicity in *N. tabacum* was currently unknown. Therefore, we examined expression of ET and PA biosynthetic genes in tobacco roots under Cu toxicity with and without Si.

Ethylene is a gaseous plant hormone synthesized by the concerted action of three enzymes: SAMS, ACS, and ACO (Keunen et al. 2016). The *N. tabacum* genome encodes two SAMS genes: *SAMS1* and *SAMS2*. Our data show expression of *SAMS1* and *SAMS2* are oppositely regulated, and may enable redundant SAM production in roots under different conditions. Expression of *ACS* and *ACO* increased in Cu + Si-treated roots relative to Cu-treated roots, which suggests the

potential for increased ET production in Cu + Si-treated roots relative to Cu-treated roots. Perhaps Si somehow stimulates ET production to alleviate long-term Cu toxicity. Various reports have discussed potential ET involvement in plant tolerance to toxic metals, and plant genotypes which emit more ET were suggested to be more resistant to metal stress (Lu and Kirkham 1991). However, *ACS* expression decreased in NaCl + Si-treated *S. bicolor* (Yin et al. 2016), suggesting changes in ET production in response to stress/Si is species dependent. Interestingly, a recent report in *Solanum lycopersicum* demonstrated *ACO* expression increased in response to Si supplementation (Ghareeb et al. 2011). We also observed amplified *ACO* expression in Si-treated *N. tabacum* roots. Taken together, these data further suggest Si can influence expression of ET biosynthetic genes.

Once produced, ET binds to endoplasmic reticulum receptors and initiates a signaling cascade which results in the activation/repression of many genes through the activity of ERFs (Keunen et al. 2016). ERFs belong to the APETALA2/ET response element binding protein (AP2/EREBP) transcription factor family, which mediate hormonal and redox signaling during abiotic stress (Dietz, Vogel, and Viehhauser 2010). These transcription factors typically bind to a GCC box element present within the promoters of ET-regulated genes (Fujimoto et al. 2000). Various ERFs exhibit different effects on transcription. Arabidopsis *ERF1*, *ERF2*, and *ERF5* transcriptionally activated GCC box containing promoters in vitro, while *ERF3* and *ERF4* were repressive (Fujimoto et al. 2000). Interestingly, expression of putative transcriptionally-activating ERFs is increased in response to heavy metals. Arabidopsis exposed to Cd for 2 h showed increased *ERF1*, *ERF2* and *ERF5* expression, which may be indicative of higher ET production as a short-term stress response (Weber, Trampczynska, and Clemens 2006). Transgenic studies over-expressing ERFs in arabidopsis (ERF1) in roots and leaves mimic the effects of ethylene over-production (Berrocal-Lobo, Molina, and Solano 2002). In *N. tabacum*, overexpression of an ERF from *Lycium chinense* results in plants that displayed greater tolerance to Cd stress than non-transformed controls (Guan et al. 2015). Hence, ERFs affect stress tolerance. In our studies with *N. tabacum*, expression of the putative transcription activators, *ERF1*, *ERF2*, and *ERF5* increased in Cu + Si-treated roots relative to Cu-treated roots, while expression of the putative repressors *ERF3* and *ERF4* decreased. Taken together, these data suggest that an increase in ET production and concomitant signaling in *N. tabacum* roots is affected by Si and may be involved in Si-mediated alleviation of Cu-toxicity.

Polyamines are synthesized by pathways in competition with ET production (as both use a common metabolic intermediate, SAM) and often accumulate in response to stress (Liu et al. 2015). Polyamine accumulation is dependent on the ratio of synthesis to degradation, and PA synthesis corresponds with expression of biosynthetic genes. In our study, expression of all examined PA biosynthetic genes in Cu + Si-treated tobacco roots, decreased relative to Cu-treated roots. These data suggest that Cu + Si-treated roots may contain decreased PA levels relative to Cu-treated roots and thus, these signaling molecules may not play a major role in Si alleviation responses.

If PAs play a minor role, then it is likely that ET is the major player in Si alleviation of Cu stress responses in *N. tabacum*. These data are in contrast with the studies in *S. bicolor*, in which NaCl + Si-treated seedlings showed increased expression of PA biosynthetic genes relative to NaCl-treated seedlings and a reduction in ET biosynthetic gene expression (Yin et al. 2016). Differences in Si-mediated stress response signaling could be explained in several ways. First, *S. bicolor* is a high Si accumulator, while *N. tabacum* is a low Si accumulator plants with different Si accumulation status may utilize different mechanisms of Si-mediated stress tolerance. Second, Si-mediated salt resistance was studied in *S. bicolor* seedlings, whereas the present study was conducted with vegetative *N. tabacum* under Cu toxicity. Plants at different developmental stages and/or under different stress conditions may use different signaling pathways in Si-mediated stress alleviation. Finally, *S. bicolor* gene expression was measured after 1 d, while tobacco expression was measured after 21 d. It is possible plants favor one pathway early in Si-mediated stress alleviation, and a different one long term.

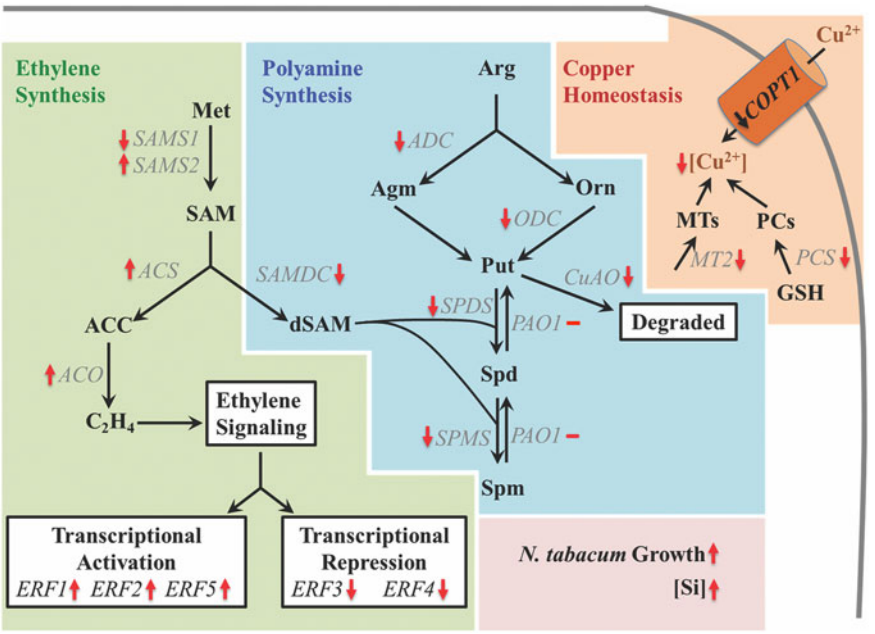

Figure 6. Silicon (Si)-mediated alleviation of copper (Cu) toxicity. Silicon-supplemented *Nicotiana tabacum* exposed to extended Cu toxicity exhibited increased expression of ethylene biosynthesis genes (green shading) and decreased expression of polyamine biosynthesis genes (blue shading). Expression of genes involved in Cu transport and sequestration (orange shading) are reduced in Cu + Si conditions relative to Cu alone. Silicon-supplemented plants show lower Cu concentration in roots, but not in leaves when exposed to Cu toxicity. Silicon concentration increases in both root and leaf tissue, which correlates with an increase in plant growth (pink shading). Black arrows indicate catalytic steps, black words indicate metabolites, gray words indicate genes encoding catalytic enzymes, and vertical arrows (red) indicate if a quantity is increased (upward arrow) or decreased (downward arrow) in Cu + Si treated plants relative to Cu alone. The horizontal line next to *PAO1* indicates no change in expression under Cu and Cu + Si conditions.

Conclusion

Silicon supplementation alleviated the detrimental effects of extended Cu toxicity in the low Si accumulator *N. tabacum*. Our data suggest Si-mediated alleviation of Cu toxicity may be, in part, due to a reduction in root uptake of Cu involving decreased *COPT1* expression. Both foliar and root Si levels increased under Cu toxicity, suggesting *N. tabacum* contains a stress-regulated mechanism for Si transport and improved Si accumulation may be part of *N. tabacum*'s general response to stress. In addition, Si-mediated alleviation of Cu toxicity in *N. tabacum* roots was associated with elevated expression of ET biosynthetic genes and a reduction in expression of PA biosynthetic genes (Figure 6), which indicates the ET pathway may play a role in Si-mediated stress alleviation responses. These data are in contrast with data from *S. bicolor* in which Si alleviation of salt stress was correlated with a decrease in ET biosynthetic gene expression and an increase in expression of PA biosynthetic genes. Our findings serve as a foundation for extensive genomic, proteomic, and metabolomic investigation of Si involvement in these pathways.

Acknowledgments

The authors thank Lirim Shemshedini and Wendy Zellner, both in the Department of Biological Sciences at the University of Toledo, for critical comments on the manuscript. The authors also thank Douglas Sturtz of the USDA-ARS for his assistance with ICP-OES, and the University of Toledo Plant Science Research Center.

Funding

This work was supported by USDA-ARS Specific Cooperative Agreement: 58-5082-6-012.

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