Journal of Plant Nutrition

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lpla20

Silicon modulates expression of pathogen defense-related genes during alleviation of copper toxicity in *Nicotiana tabacum*

Christopher Flora , Sushant Khandekar , Jennifer Boldt & Scott Leisner

To cite this article: Christopher Flora , Sushant Khandekar , Jennifer Boldt & Scott Leisner (2021) Silicon modulates expression of pathogen defense-related genes during alleviation of copper toxicity in *Nicotiana tabacum* , Journal of Plant Nutrition, 44:5, 723-733, DOI: 10.1080/01904167.2020.1849296

To link to this article: https://doi.org/10.1080/01904167.2020.1849296

JOURNAL OF PLANT NUTRITION
2021, VOL. 44, NO. 5, 723–733
https://doi.org/10.1080/01904167.2020.1849296

Taylor & Francis
Taylor & Francis Group

Silicon modulates expression of pathogen defense-related genes during alleviation of copper toxicity in *Nicotiana tabacum*

Christopher Flora[a], Sushant Khandekar[a], Jennifer Boldt[b], and Scott Leisner[a]

[a]Department of Biological Sciences, University of Toledo, Toledo, OH, USA; [b]USDA-Agricultural Research Service, Application Technology Research Unit, Toledo, OH, USA

ABSTRACT

Plants treated with metals better fend off infection by pathogens. This activity is mediated, at least in part, by the activation of the salicylic acid (SA) signaling pathway. Previous work in our laboratory demonstrated that silicon (Si) could alleviate copper (Cu) toxicity stress in *Nicotiana tabacum*. However, how Si affected expression of genes involved in SA biosynthesis and signaling in *N. tabacum* under Cu stress was unclear. Therefore, we investigated this at two time points. Roots of plants treated with Cu toxicity for one week exhibited increased transcript levels for *Isochorismate Synthase1*, a gene encoding a key enzyme involved in SA biosynthesis and the addition of Si further enhanced expression. Microarray analysis identified a number of disease defense-associated genes up-regulated in *N. tabacum* roots after one week of Cu toxicity treatment and further increased when plants were also supplemented with Si. However, the effect of Si on expression of these genes was lost after three weeks of treatment, indicating these effects are dynamic.

Abbreviations: BLAST: basic local alignment search tool; Chi: chitinase; HRP: horseradish peroxidase; HSD: honest significant difference; ICS1: isochorismate synthase 1; MHS: modified Hoagland's solution; NCBI: national center for biological information; PVDF: polyvinylidene fluoride; PR protein: pathogenesis-related protein; RO: reverse osmosis; ROS: reactive oxygen species; RT-qPCR: reverse transcriptase-quantitative polymerase chain reaction; SA: salicylic acid; SAR: systemic acquired resistance; SEM: standard error of the mean; UBC: ubiquitin conjugating enzyme

ARTICLE HISTORY
Received 23 May 2020
Accepted 17 August 2020

KEYWORDS
Cu; metal toxicity; PR-protein; salicylic acid; Si; tobacco

Introduction

Certain metals such as copper (Cu) are required for normal plant growth (Printz et al. 2016). In plants, Cu mainly functions in electron transport within mitochondria and chloroplasts, controlling the cellular redox state, and remodeling of the cell wall. However, if intracellular Cu concentrations become too high, the metal can cause a variety of detrimental effects including stunting, leaf chlorosis correlated with decreased leaf chlorophyll content, and enhanced oxidative stress (Ducic and Polle 2005; Pätsikkä et al. 2002). Many of these effects are likely due to free Cu forming reactive oxygen species (ROS) through Fenton and Haber Weiss reactions (Hasan et al. 2017).

CONTACT Scott Leisner Scott.Leisner@utoledo.edu Department of Biological Sciences, University of Toledo, 2801 West Bancroft Street, Mail Stop 601, Toledo, OH 43606, USA.

Supplemental data for this article is available online at https://doi.org/10.1080/01904167.2020.1849296

Metal-induced ROS affect plant morphology (growth), physiology (photosynthesis and metabolism), and biochemistry (membrane leakage) (Noctor, Reichheld, and Foyer 2018).

One unexpected plant response to metals, described in the literature, is protection against biotic stress (Horváth, Szalai, and Janda 2007; Poschenrieder, Tolrà, and Barceló 2006). Plants exposed to elevated levels of metals, including Cu, are better able to reduce stress from biotic sources. Some plants that accumulate metals also build up high levels of the plant signaling molecule salicylic acid (SA). Thus, responses of plants to metal toxicity and pathogen defense appear to be interconnected. Interestingly, plants exposed to SA resist metal toxicity better than controls. SA is a phenolic compound that regulates a variety of physiological processes (Janda and Ruelland 2015). One of the more widely-studied responses regulated by SA is plant defense; plants produce SA as a local and systemic signal to defend against pathogens. SA is a major signal in the systemic acquired resistance (SAR) pathway (Métraux et al. 1990). Plant SAR is somewhat akin to immunity in animals: initial exposure of a plant to a pathogen results in broad spectrum resistance at sites remote from the first introduction of the etiologic agent.

A set of genes, characteristic of plant pathogen defense pathways activated by SA, are those that encode the pathogenesis-related (PR) proteins (Janda and Ruelland 2015). The PR proteins are typically classified into families by homology and their proposed functions. However, the function of the first PR protein, PR-1, is not fully understood (Fang et al. 2019). PR-1 is encoded by three different genes: *PR-1a, PR-1b,* and *PR-1c*. Many of the other PR genes encode chitinases thought to provide protection against both insects and fungi. The insect exoskeleton contains high levels of chitin, as does the fungal cell wall, so attack on these structures by chitinases would have a detrimental effect on both types of plant pests. Chitinases are typically grouped into acidic, neutral and basic enzymes based on their physical properties.

In addition to metals, the element silicon (Si) can also help plants tolerate stress (Coskun et al. 2019; Debona, Rodrigues, and Datnoff 2017). Silicon is the second most abundant element in soils but concentrations vary depending upon soil type and properties (Epstein 1994). Soil weathering releases Si into the soil solution in the form of monosilicic acid (H_4SiO_4)—a monomeric uncharged molecule that exists below pH 9 (Debona, Rodrigues, and Datnoff 2017). Plants absorb Si from the soil solution as H_4SiO_4 and ferry it from roots to shoots using specific transporter proteins (Ma et al. 2006, 2007). The path of Si translocation within the root, from epidermal cells to the xylem, as well as translocation of Si from roots to shoots, can vary with the plant species (Ma and Yamaji 2015a, 2015b). Furthermore, the levels of Si accumulated by the plant can vary with species (Coskun et al. 2019). In general, plants are divided into three different categories based on foliar Si: high (>10% dry weight (dw)), medium (0.1%–10% dw), and low (<0.1% dw), respectively (Hodson et al. 2005; Jones and Handreck 1967). Upon translocation from the xylem to its final endpoint, silicic acid can be deposited in the plant cell wall or in specialized cells as solid, amorphous, hydrated silica (Ma and Yamaji 2015a, 2015b).

Silicon supplementation provides plants with many beneficial effects including: increased photosynthetic activity, crop yield, along with resistance to stressors such as insects, disease, drought, cold, and mineral toxicity (Debona, Rodrigues, and Datnoff 2017). While these beneficial effects are thought to be confined to mainly high Si accumulator plants (Coskun et al. 2019), low accumulating plants can also benefit by Si supplementation (Zellner, Frantz, and Leisner 2011). Silicon mediates disease resistance via a variety of mechanisms (Coskun et al. 2019; Vivancos et al. 2015). It can also alleviate toxicity to metals and this may be mediated through several proposed mechanisms (Adrees et al. 2015; Debona, Rodrigues, and Datnoff 2017; Liang et al. 2007).

Taken together, it appears that both metals and Si can help plants deal with biotic stress (Debona, Rodrigues, and Datnoff 2017; Liang et al. 2007). Furthermore, Si can help plants to better withstand metal toxicity. How Si and metals together modulate expression of plant biotic defense genes is currently poorly understood. To investigate this, we examined the responses of

Nicotiana tabacum (tobacco), a low Si accumulator, to Si and Cu toxicity at two time points to follow up their dynamics at the RNA level.

Materials and methods

Plant propagation

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38, from the Plant Biology Section, Cornell College of Agriculture and Life Sciences, Ithaca, NY) seeds were individually sown on pieces of foam (derived from Black Open Cell Polyurethane Foam Strips, 1/8", 2", 72", Grainger, Akron, OH) inserted individually into the pipette tip holes of 1000 µL pipette tip boxes (ThermoFisher Scientific, Hanover Park, IL). The foam was then suspended over reverse osmosis (RO; 18 MΩ) water in the pipette tip box. The box was placed in a Conviron E7/2 growth chamber (set to 20 °C and 45% humidity) under 16 h fluorescent light (70 µmol m^{-2} s^{-1} photosynthetically active radiation; PAR) and 8 h dark (Flora et al. 2019; Li, Leisner, and Frantz 2008). Upon germination, plants were supplemented with modified Hoagland's solution (MHS) at a pH of 5.7 until the two-leaf stage. Seedlings were then transferred into 4.5 L opaque plastic buckets (5 quart silver promotional pail with lid, purchased from Encore Plastics Corporation, Cambridge, OH; one plant per bucket) filled with 4 L of continuously aerated MHS and placed in a Conviron E7/2 growth chamber (20 °C and 45% humidity) under 16 h light (100 µmol m^{-2} s^{-1} PAR) and 8 h dark. Modified Hoagland's solution was replaced every seven days. Upon development of 4–6 true leaves, plants were subjected to MHS with the four treatments listed below (four plants per treatment) in a completely randomized design for 7 or 21 d and then harvested. Treatments were as follows: control (MHS), Si (MHS with elevated Si as 1.0 mM K_2SiO_3), Cu (MHS with elevated Cu as 35 μM $CuSO_4$) and Cu + Si (MHS with elevated Cu and elevated Si). Hydroponic solution pH was monitored and remained at 5.7 throughout the course of the experiments. Plants were harvested and analyzed independently per treatment after 7 or 21 d, respectively. Upon harvest, leaves were removed, weighed, and flash frozen in liquid nitrogen. Roots were cut at the root–shoot interface, rinsed three times with 0.1 N HCl, then once with water to remove excess Si, and blotted dry. Root fresh weight was recorded and tissue was flash frozen in liquid nitrogen. Total leaf and root tissue from each sample was independently homogenized by grinding in a mortar and pestle in liquid nitrogen. All tissue collected was stored at −80 °C until further analysis.

RNA isolation and analysis

Total RNA was isolated from frozen ground root tissue (100 mg) from each individual plant using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with on-column DNase digestion, according to the manufacturer's specifications. Total RNA was eluted in 50 µL RNase-free H_2O and purified RNA concentrations were measured using a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Total RNA from the roots of three plants per treatment was pooled and sent for microarray analysis (MOgene, St. Louis, MO, USA). Purified total RNA was used to produce Cy3 labeled cDNA. Labeled cDNA was hybridized to a 4 × 44 K tobacco microarray chip (Agilent, Santa Clara, CA, USA) at 65 °C overnight using the Gene Expression Hybridization kit (Agilent) according to manufacturer's specifications. The microarray chip was washed after hybridization using the Gene Expression Wash Pack (Agilent), and 0.005% Triton X-102 was added according to manufacturer's specifications. The microarray chip was treated with Stabilization and Drying solutions (Agilent), and scanned using the Agilent Technologies Scanner. Data were analyzed using Agilent Feature Extraction Software (version 12.1).

Gene specific primers (Supplemental Table 1) were designed for quantitative real-time reverse-transcriptase-polymerase chain reaction (qRT-PCR) analyses based on the *N. tabacum* TN90 transcriptional assembly (Philip Morris International R&D). For each gene, the mRNA sequence was input into the National Center for Biological Information (NCBI) Primer-Basic Local Alignment Search Tool (BLAST) program with an optimal annealing temperature of 60 °C and the best predicted primer pairs were chosen for further analysis. Once designed, the primer sequences were used to interrogate the *N. tabacum* Refseq mRNA database (taxid: 4097) to identify other potential off-target binding sites. The predicted primer pairs that did not show off-target potential but would amplify the appropriate gene were synthesized (Integrated DNA Technologies, Coralville, IA, USA). The specificity of the primers was then confirmed by PCR of *N. tabacum* cDNA produced as described previously, and PCR product size was confirmed by agarose gel electrophoresis.

One step RT-qPCR was performed using the iTaq Universal One-Step RT-qPCR Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's specifications. Each reaction was performed in triplicate per biological sample (three biological samples were analyzed per treatment) and data analysis was performed using Bio-Rad CFX Manager software (Bio-Rad). 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).

Protein isolation and gel blot analysis

Proteins were extracted from 500 mg of frozen root tissue by grinding in 3 mL of protein extraction buffer (100 mM Tris–HCl pH 8.8, 150 mM NaCl, 1 mM EDTA, 10% w/v glycerol, 1:1000 protease inhibitor cocktail) and samples were sonicated on ice (20 s on/off three times at 10 W). Protein samples were filtered through a 0.22 µm polyvinylidene fluoride (PVDF) syringe-driven filter into a sterile conical tube on ice. Protein lysate concentrations were determined using Bradford Protein Quantification Reagent (Biorad) according to the manufacturer's specifications. Protein lysate concentrations were equilibrated using cold protein extraction buffer. Equal amounts of protein lysate were loaded onto a 12.5% polyacrylamide gel and separated by gel electrophoresis using a BioRad mini-protean gel electrophoresis unit, according to the manufacturer's specifications. Proteins were electrophoretically transferred to a PVDF membrane using a BioRad Criterion Blotter according to the manufacturer's specifications. Protein gel blot analysis was performed using PR-1 primary antibody (Agrisera, Vannas, Sweden; 1:1000 dilution in 5% milk blocking buffer) overnight at 4 °C. Goat anti-rabbit secondary antibody (ThermoFisher, Waltham, MA, USA) conjugated to Horseradish Peroxidase (HRP; 1:10,000 dilution) was used to detect the primary antibody using HyGlo Chemiluminescent HRP antibody detection reagent (Denville Scientific, Holliston, MA, USA) according to the manufacturer's specifications. Membranes were exposed to autoradiography film (Denville Scientific) for 10 s and the film was developed. Protein bands were quantified using ImageJ (NIH, Bethesda, MD, USA).

Results

One week of exposure to Cu toxicity inhibits plant growth and cannot be reversed by Si

N. tabacum treated with Si for one week showed growth similar to controls as reflected by both leaf and root fresh weight (Figure 1). In contrast, plants treated with either Cu or Cu + Si were stunted to a similar degree and smaller than controls. Leaf and root fresh weight were both reduced in plants treated with Cu or Cu + Si.

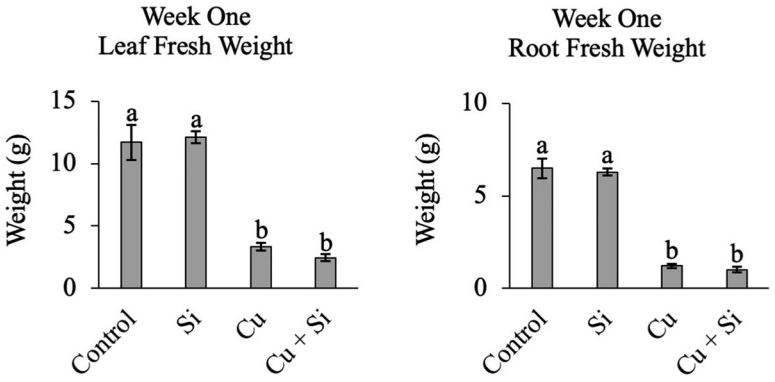

Figure 1. Physiological parameters of *Nicotiana tabacum* after one week exposure to the four treatments. Leaf (left) and root (right) fresh weight of *N. tabacum* treated with control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for 7 d then harvested. Values are mean ± SE ($n = 8$). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $p < 0.05$.

Exposure of N. tabacum to Cu toxicity causes changes in SA biosynthetic gene expression that are influenced by Si

After one week of Si treatment, *Isochorismate Synthase 1 (ICS1)* transcript levels in *N. tabacum* roots analyzed by RT-qPCR were the same as controls (Figure 2). *ICS1* mRNA levels were increased five-fold above control levels in the roots of Cu-treated plants and increased even more (fifteen-fold) in plants treated with Cu + Si. To determine if this effect persisted over time, expression of *ICS1* was also measured after three weeks of treatment. After three weeks, root *ICS1* transcripts remained at control levels for Si treated plants. Roots of plants exposed to Cu treatment still showed elevated (three-fold above control) *ICS1* transcript levels, but those of Cu + Si treated plants were not significantly different from controls.

The *Pathogenesis-Related protein-1* (*PR-1*) genes are induced by the SA response and encode the polypeptide, PR-1 (Fang et al. 2019). PR-1 is synthesized from three genes: *PR-1a, PR-1b* and *PR-1c*. Transcripts for all three *PR-1* genes (isoforms) were identified in our microarray analysis (Supplemental Table 2). To confirm the microarray data, total root RNA was isolated from plants grown under the four treatments and analyzed by RT-qPCR. All three *PR-1* isoforms showed similar patterns of mRNA expression (Table 1), and the expression of *PR-1a* is shown as a representative example in Figure 2. At one week of treatment, all three *PR-1* isoforms showed a reduction in mRNA levels in roots of plants treated with Si (Figure 2, Table 1). In contrast root mRNA levels for all three isoforms increased above control levels under Cu treatment and increased even more in plants exposed to Cu + Si. At three weeks of treatment, *PR-1* isoform mRNA levels still showed a reduction in roots from Si-treated plants, compared to controls and an increase caused by Cu (Table 1; Figure 2). In contrast, with the one week data, roots from plants treated with Cu + Si for three weeks showed reductions of *PR-1* isoform transcripts to either control or near-control levels. PR-1 protein levels examined by Western Blot analysis accurately reflected the transcript levels (Figure 2).

Exposure of N. tabacum to Cu toxicity causes changes in pathogen defense-related gene expression that are influenced by Si

We performed microarray analyses on mRNA isolated from *N. tabacum* root tissue after one week of treatment to identify candidate genes in potential signaling pathways that may be influenced by Si in plants under Cu toxicity. Using the Agilent 4 × 44 K tobacco microarray, many genes showing differential expression were identified. Only genes varying by ten-fold or more in

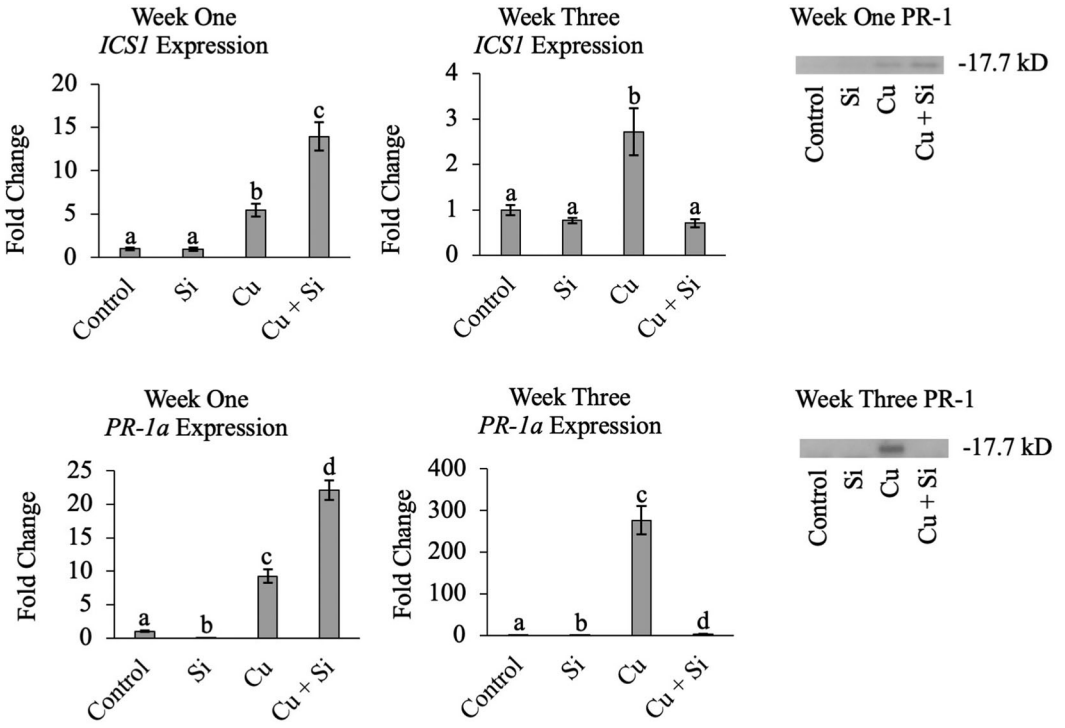

Figure 2. Salicylic acid biosynthetic gene *Isochorismate Synthase (ICS1)* and *Pathogenesis Related protein-1* (*PR-1*) expression in *Nicotiana tabacum* roots under copper toxicity and supplemented with silicon. *N. tabacum* were treated with control, Si (1 mM Si), Cu (35 μM $CuSO_4$), or Cu + Si (35 μM $CuSO_4$ + 1 mM Si) for one and three weeks. Left panels, *ICS1* and *PR-1a* transcript levels in roots as determined by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Target gene expression was normalized to *Ubiquitin Conjugating Enzyme 2* (*Ubc2*). Values are mean ± SE ($n = 3$). Data were analyzed by ANOVA and different letters represent statistically significant differences with Tukey's HSD at $p < 0.05$. Right panels, total protein was isolated from roots under the four treatments, after one and three weeks, protein levels were determined and equal amounts of total proteins were loaded onto a gel, separated by gel electrophoresis and examined by protein gel blot analysis.

one of the treatments compared to controls, were subjected to further study and totaled 86 (Supplemental Table 2). The largest group of up-regulated genes (Figure 3) were those increased by Cu + Si treatment (69 genes), followed by Cu alone (56 genes), with Si alone only showing increased expression of five genes. The largest group of down-regulated genes were those reduced by Si treatment alone (49 genes), followed by Cu + Si (16 genes) and Cu alone (15 genes). The 86 genes identified by this analysis were roughly classified, based on their functions, into 15 categories, although some genes could be classified in more than one category (Figure 3; Supplemental Table 2). Around 2% of the regulated genes play a role in either protein import or proteolysis. Nearly 5% encode oxygenase genes, while over 6% represent transcription factors. Some genes appear to be involved in cell wall function (6%), whereas others likely play a role in membrane processes (8%). Almost 11% encode proteins involved in some form of electron transport. Over 14% of the genes encode enzymes involved in either phenolic compound (7%) or terpene/alkaloid (7%) metabolism. By far, the largest class of genes identified by the microarray, over 25%, appear to play a role in plant disease-associated resistance responses. To investigate further, expression patterns of several of these disease defense-associated genes were assessed by RT-qPCR after one and three weeks of treatment to determine if mRNA levels varied through time.

The expression pattern of *Acidic chitinase III* (*AChiIII*) mimicked that of *PR-1* (Table 1). After one week of treatment, roots of plants treated with Si alone showed a reduction in *ChiIII*

Table 1. Reverse transcriptase-quantitative polymerase chain reaction data for plant defense-related genes in *Nicotiana tabacum* in response to silicon and copper treatment.

Week one			
Gene	Si	Cu	Cu + Si
AChIII	0.13 ± 0.01^{b}	4.4 ± 0.5^{c}	13.1 ± 1.8^{d}
ICS1	0.94 ± 0.18^{a}	5.4 ± 0.7^{b}	13.9 ± 1.6^{c}
PR-1a	0.02 ± 0.001^{b}	9.3 ± 1.0^{c}	22.1 ± 1.5^{d}
PR-1b	0.05 ± 0.001^{b}	13.4 ± 1.5^{c}	29.8 ± 3.1^{d}
PR-1c	0.03 ± 0.003^{b}	12.5 ± 1.7^{c}	42.7 ± 4.8^{d}
PR-3	0.16 ± 0.01^{b}	4.2 ± 0.6^{c}	9.5 ± 1.2^{d}
PR-4a	0.03 ± 0.003^{b}	17.3 ± 1.0^{c}	47.5 ± 6.6^{d}
PR-4b	0.04 ± 0.004^{b}	52.3 ± 1.9^{c}	91.2 ± 4.3^{d}
PR-P	0.04 ± 0.002^{b}	12.2 ± 1.7^{c}	25.6 ± 4.4^{d}
PR-Q	0.18 ± 0.02^{b}	57.1 ± 8.2^{c}	87.3 ± 11.7^{d}
PR-R	0.11 ± 0.01^{b}	20.1 ± 2.5^{c}	51.2 ± 7.1^{d}
Week three			
Gene	Si	Cu	Cu + Si
AChIII	1.72 ± 0.19^{a}	20.7 ± 3.0^{b}	2.9 ± 0.22^{c}
ICS1	0.77 ± 0.06^{a}	2.7 ± 0.5^{b}	0.7 ± 0.10^{a}
PR-1a	0.51 ± 0.08^{b}	276.0 ± 34.0^{c}	3.3 ± 0.27^{d}
PR-1b	0.41 ± 0.11^{b}	353.0 ± 20.1^{c}	5.2 ± 0.61^{d}
PR-1c	0.13 ± 0.02^{b}	151.0 ± 21.6^{c}	1.5 ± 0.57^{a}
PR-3	2.17 ± 0.17^{b}	1.9 ± 0.1^{b}	14.6 ± 1.79^{c}
PR-4a	3.37 ± 0.52^{b}	65.7 ± 7.8^{c}	0.9 ± 0.09^{a}
PR-4b	7.57 ± 0.004^{b}	31.1 ± 4.7^{c}	2.2 ± 0.19^{d}
PR-P	13.40 ± 1.53^{b}	18.0 ± 2.2^{c}	1.8 ± 0.06^{d}
PR-Q	0.43 ± 0.05^{b}	31.9 ± 3.9^{c}	2.8 ± 0.13^{d}
PR-R	0.42 ± 0.05^{b}	113.0 ± 15.2^{c}	1.2 ± 0.04^{a}

Nicotiana tabacum were propagated under four treatments (control, Si, Cu, and Cu + Si), root tissues was harvested at two time points and transcript levels were measured for plant pathogen defense-related genes by reverse-transcriptase-quantitative polymerase chain reaction. Transcript levels (± standard deviation) are reported relative to controls, which were set to 1.0. *ICS1* and *PR-1a*, reported in Figure 2, are provided as references. Different letters represent significant differences ($p < 0.01$), same letters are not significantly different, values with letter a are not significantly different from controls.

transcript levels relative to controls, while Cu treated plants showed an elevation in mRNA levels that increased further in plants grown under Cu toxicity and supplemented with Si. At three weeks of treatment, roots of plants treated with Si alone showed control levels of *AChiIII* mRNA. While transcript levels were still elevated under Cu toxicity, they returned to near-control levels in plants grown under Cu toxic conditions when supplemented with Si.

The expression pattern of *PR-3* was nearly identical to that of *PR-1* at one week of treatment (Table 1). However, at three weeks of treatment the pattern was different. The mRNA levels of *PR-3* in roots of plants treated with either Si or Cu were slightly elevated compared to controls. The *PR-3* transcript levels remained highly elevated in plants exposed to Cu + Si.

PR-4a and *PR-4b* showed nearly identical patterns of expression to *PR-1* in roots of plants at one week of treatment (Table 1). After three weeks of treatment, both *PR-4a* and *PR-4b* showed higher transcript levels in roots of plants provided with Si compared to controls and both transcripts were further elevated in roots of Cu-treated plants. In Cu + Si-treated plants, mRNA levels for both transcripts dropped to control (*PR-4a*), or were slightly elevated above control (*PR-4b*) levels.

PR-P, *PR-Q*, and *PR-R* encode basic endo-chitinases under SA pathway regulation (Rawat et al. 2017). All three genes were identified by our microarray. After one week of treatment, root transcript levels for all three genes were similar to *PR-1*: a slight reduction in expression by Si, compared to control, an increase in expression in Cu-treated plants, and further increases in mRNA levels in roots of plants treated with Cu + Si (Table 1). After three weeks of treatment, the patterns of expression for two of the three genes (*PR-Q* and *PR-R*) were similar to *PR-1*. However, in the roots of plants treated with Cu + Si, transcript levels of *PR-R* returned to control

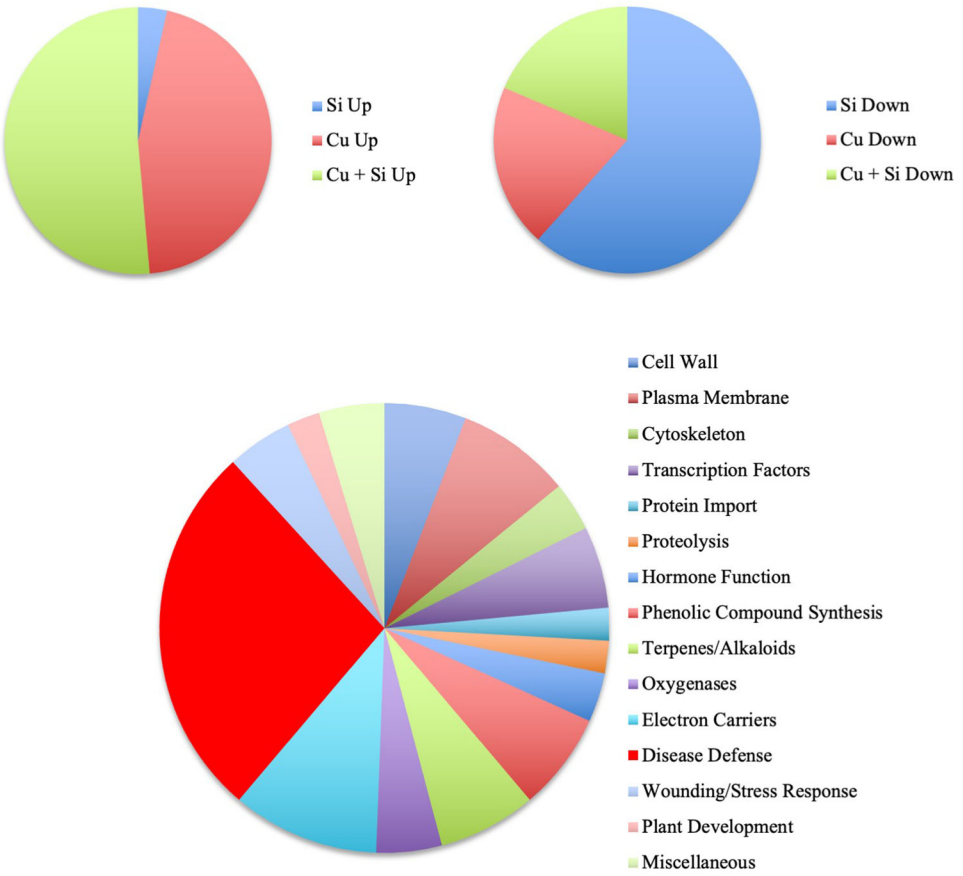

Figure 3. Microarray results from roots of plants treated with Si, Cu or Cu + Si for one week. Upper left, up-regulated genes compared to control; upper right, down-regulated genes more compared to control under three different conditions: Si alone, blue; Cu, red; Cu + Si, green. Bottom panel, predicted functions of genes varying by 10-fold or more relative to control in microarray after 7 d treatment; key to gene functions given to right of pie chart.

levels, while *PR-Q* transcript levels remained slightly elevated above controls, although not to the levels of Cu-treated plants alone. At three weeks of treatment, expression of *PR-P* showed a somewhat different pattern. *PR-P* transcript levels were still elevated in roots of plants treated with Cu and declined to levels slightly above controls in plants treated with Cu + Si. However, plants treated with Si alone showed increased *PR-P* transcript levels.

Discussion

Silicon (Si) is a beneficial element (Epstein 1994) for plants, although the mechanisms behind these benefits are unclear. Previously, Si was proposed to confer benefits primarily to plants that accumulated large amounts of the element (Coskun et al. 2019). Plants that accumulated small amounts of Si were not expected to accrue benefits from supplementation. Yet, the literature indicates that even plants that accumulate small amounts of Si can derive benefits from it (Debona, Rodrigues, and Datnoff 2017). Indeed, we previously showed that Si supplementation alleviated the effects of Cu toxicity on *N. tabacum*, a low Si accumulator (Flora et al. 2019). However, that study was conducted over a period of three weeks and whether the beneficial effects of Si on *N. tabacum* occurred earlier than three weeks was not investigated. Often, plant responses are temporally regulated (Kim et al. 2014; Vivancos et al. 2015). Therefore, in the current study, we

investigated the responses occurring in *N. tabacum* after one week of treatment compared to data obtained after three weeks of treatment (Flora et al. 2019). Treating hydroponically-grown *N. tabacum* with high Cu for one week showed reductions in both foliar and root fresh mass that were not alleviated by Si. Hence, it appears to take longer than one week of treatment with Si to alleviate Cu toxicity. Interestingly, even though Si did not alleviate the Cu toxicity growth effects at one week of treatment, it still influenced *N. tabacum* physiological responses.

Previous studies have shown that treatment of plants with metals can provide beneficial effects by inhibiting biotic stress (Poschenrieder, Tolrà, and Barceló 2006). Although most of these studies were conducted with metal hyperaccumulators, one study showed that treatment of *N. tabacum* with cadmium could inhibit *Turnip vein clearing virus* infections. Models have been proposed for how metals can protect plants against biotic stress involving the production of and signaling mediated by SA. However, how Si modulates these responses was unclear. Therefore, we examined the SA biosynthetic pathway. SA is synthesized by two distinct routes, one via the phenylpropanoid pathway and the other via the shikimate pathway (Janda and Ruelland 2015). In the latter pathway, ICS is the key enzyme. *Arabidopsis thaliana* harbors two *ICS* genes: *ICS1* which is the major player in anti-bacterial SA-mediated responses and *ICS2* which is a minor contributor.

In the roots of *N. tabacum* treated for one week with Cu, *ICS1* expression was strongly induced above control levels, which was consistent with other reports (Horváth, Szalai, and Janda 2007; Poschenrieder, Tolrà, and Barceló 2006). Si + Cu appeared to further enhance *ICS1* expression. However, by three weeks of treatment, while the roots of plants treated with Cu still showed an increase in *ICS1* transcript levels, the addition of Si to Cu-treated plants reduced expression back to control levels. Hence, Cu toxicity consistently increased *ICS1* transcript levels, but the addition of Si to Cu-treated plants caused either a further increase or a decrease to control levels depending on the length of exposure to the treatments. The data imply that Si responses can be quite dynamic and may help explain some of the contradictory observations in the literature (Coskun et al. 2019).

Increases in *ICS1* transcript levels likely correlate with elevated levels of SA (Janda and Ruelland 2015). Therefore, one might expect Cu-treated plants to show elevated SA levels in *N. tabacum* roots that are either hyper-elevated or reduced by Si, depending on the time of analysis. In *Oryza sativa* (rice; a high Si accumulator), SA levels were elevated at one, five, and ten days after Cu treatment (Kim et al. 2014). However, Si caused a reduction in SA levels in plants under Cu treatment at all three time-points. Our data predict a similar scenario in *N. tabacum* (a low Si accumulator) at three weeks of treatment. Thus, these data suggest that Si-mediated reductions of SA levels require a longer time frame in *N. tabacum* than in rice, which may reflect the plants' differing Si accumulation status.

To obtain a more global picture of *N. tabacum* responses to these chemical elements, microarray analysis of roots was performed after one week of treatment. Of the 86 genes we identified that responded strongly to at least one of the treatments, one-quarter were involved in pathogen defense. Many of these genes encoded members of the PR-proteins. We validated expression levels of several of these genes by RT-qPCR. The expression patterns of all three *PR-1* genes were similar, showing increased transcript levels in roots of plants exposed to Cu treatment and even further enhancement of mRNA levels in plants treated with Cu + Si. At three weeks of treatment, Cu still led to increased *PR-1* expression, while roots of plants treated with Cu + Si showed a reduction in *PR-1* transcript levels either slightly above or at control levels. The changes in expression were also reflected by *PR-1* protein levels. The same was mostly true of the other PR proteins identified by our microarray: *PR-4a, PR-4b, PR-P, PR-Q,* and *PR-R*. As might be expected, the levels of *PR* transcripts generally matched those of *ICS1* at both time points. This suggests that the amounts of *ICS1* transcripts are most likely reflecting SA levels and the "classic" indicators of SA response (Fang et al. 2019; Horváth, Szalai, and Janda 2007; Rawat et al. 2017)

supports this hypothesis. Thus, these data suggest that Cu treatment increases SA-based responses in *N. tabacum*, which matches other systems. Silicon further regulates the SA-response pathway in a time-dependent manner.

It is possible that Si modulation of the SA pathway could contribute to the changes observed in growth of *N. tabacum* under Cu stress. Most *A. thaliana* mutants that constitutively over-accumulate SA show a dwarf phenotype (Janda and Ruelland 2015). This is presumably because limited metabolites are being directed toward plant defense rather than growth. In our studies, at one week of treatment, the SA pathway was induced by Cu toxicity and further enhanced by Si, while at three weeks, the SA pathway was down-regulated by Si compared to Cu toxicity alone. Hence the recovery mediated by Si in plants under Cu toxicity treated for three weeks could be contributed to, in part, by a reduction of the SA pathway. Others have suggested that SA does not play a role in Si-mediated responses (Vivancos et al. 2015), whereas our data suggest that it may. Our data and that of Vivancos et al. (2015) may indicate that different species use Si to deploy signaling molecules in different ways to help protect the plant against stress.

In summary, one week of Si treatment was not sufficient to alleviate Cu toxicity in *N. tabacum*. Cu toxicity activated the SA signaling pathway in *N. tabacum* roots. Finally, Si appeared to modulate Cu-induced activation differentially in a time-dependent manner.

Acknowledgments

The authors thank MOgene, St. Louis, MO, for performing the microarray analysis. The authors also thank Wendy Zellner (Department of Biological Sciences, University of Toledo) for her assistance with the manuscript and the University of Toledo Plant Science Research Center. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the University of Toledo or the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Conflict of interest

No conflict of interest was reported by the authors.

Funding

This work was supported in part by US Department of Agriculture-Agricultural Research Service Specific Cooperative Agreement (grant number: 58-5082-6-012).

References

Adrees, M., S. Ali, M. Rizwan, M. Zia-Ur-Rehman, M. Ibrahim, F. Abbas, M. Farid, M. F. Qayyum, and M. K. Irshad. 2015. Mechanisms of silicon-mediated alleviation of heavy metal toxicity in plants: A review. *Ecotoxicology and Environmental Safety* 119:186–197.

Coskun, D., R. Deshmukh, H. Sonah, J. G. Menzies, O. Reynolds, J. F. Ma, H. J. Kronzucker, and R. R. Bélanger. 2019. The controversies of silicon's role in plant biology. *The New Phytologist* 221 (1):67–85. doi: 10.1111/nph.15343.

Debona, D., F. A. Rodrigues, and L. E. Datnoff. 2017. Silicon's role in abiotic and biotic plant stresses. *Annual Review of Phytopathology* 55:85–107. doi: 10.1146/annurev-phyto-080516-035312.

Ducic, T., and A. Polle. 2005. Transport and detoxification of manganese and copper in plants. *Brazilian Journal of Plant Physiology* 17 (1):103–112.

Epstein, E. 1994. The anomaly of silicon in plant biology. *Proceedings of the National Academy of Sciences of the United States of America* 91 (1):11–17.

Fang, L.-J., R.-L. Qin, Z. Liu, C.-R. Liu, Y.-P. Gai, and X.-L. Ji. 2019. Expression and functional analysis of a PR-1 gene, MuPR1, involved in disease resistance response in mulberry (*Morus multicaulis*). *Journal of Plant Interactions* 14 (1):376–385.

Flora, C., S. Khandekar, J. Boldt, and S. Leisner. 2019. Silicon alleviates long-term copper toxicity and influences gene expression in *Nicotiana tabacum*. *Journal of Plant Nutrition* 42 (8):864–878.
Hasan, M. K., Y. Cheng, M. K. Kanwar, X.-Y. Chu, G. J. Ahammed, and Z.-Y. Qi. 2017. Responses of plant proteins to heavy metal stress—A review. *Frontiers in Plant Science* 8:1492. doi: 10.3389/fpls.2017.01492.
Hodson, M. J., P. J. White, A. Mead, and M. R. Broadley. 2005. Phylogenetic variation in the silicon composition of plants. *Annals of Botany* 96 (6):1027–1046.
Horváth, E., G. Szalai, and T. Janda. 2007. Induction of abiotic stress tolerance by salicylic acid signaling. *Journal of Plant Growth Regulation* 26 (3):290–300.
Janda, M., and E. Ruelland. 2015. Magical mystery tour: salicylic acid signalling. *Environmental and Experimental Botany Complete* 114 (114):117–128.
Jones, L. H. P., and K. A. Handreck. 1967. Silica in soils, plants, and animals. In *Advances in agronomy*, ed. A. G. Norman, vol. 19, 107–149. New York: Academic Press.
Kim, Y.-H., A. Latif Khan, D.-H. Kim, S.-Y. Lee, K.-M. Kim, M. Waqas, H.-Y. Jung, J.-H. Shin, J.-G. Kim, and I.-J. Lee. 2014. Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, *Oryza sativa* low silicon genes, and endogenous phytohormones. *BMC Plant Biology* 14:13.
Li, J., S. M. Leisner, and J. Frantz. 2008. Alleviation of copper toxicity in *Arabidopsis thaliana* by silicon addition to hydroponic solutions. *Journal of the American Society for Horticultural Science* 133 (5):670–677.
Liang, Y., W. Sun, Y.-G. Zhu, and P. Christie. 2007. Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: A review. *Environmental Pollution* 147 (2):422–428. doi: 10.1016/j.envpol.2006.06.008.
Ma, J. F., K. Tamai, N. Yamaji, N. Mitani, S. Konishi, M. Katsuhara, M. Ishiguro, Y. Murata, and M. Yano. 2006. A silicon transporter in rice. *Nature* 440 (7084):688–691.
Ma, J. F., and N. Yamaji. 2015a. Silicon uptake and accumulation in higher plants. *Trends in Plant Science* 20 (7): 435–497. doi: 10.1016/j.tplants.2015.04.007.
Ma, J. F., and N. Yamaji. 2015b. A cooperative system of silicon transport in plants. *Trends in Plant Science* 20 (7): 435–442.
Ma, J. F., N. Yamaji, N. Mitani, K. Tamai, S. Konishi, T. Fujiwara, M. Katsuhara, and M. Yano. 2007. An efflux transporter of silicon in rice. *Nature* 448 (7150):209–212. doi: 10.1038/nature05964.
Métraux, J. P., H. Signer, J. Ryals, E. Ward, M. Wyss-Benz, J. Gaudin, K. Raschdorf, E. Schmid, W. Blum, and B. Inverardi. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science (New York, N.Y.)* 250 (4983):1004–1006. doi: 10.1126/science.250.4983.1004.
Noctor, G., J.-P. Reichheld, and C. H. Foyer. 2018. ROS-related redox regulation and signaling in plants. *Seminars in Cell & Developmental Biology* 80:3–12. doi: 10.1016/j.semcdb.2017.07.013.
Pätsikkä, E., M. Kairavuo, F. Sersen, E.-M. Aro, and E. Tyystjärvi. 2002. Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. *Plant Physiology* 129 (3):1359–1367. doi: 10.1104/pp.004788.
Poschenrieder, C., R. Tolrà, and J. Barceló. 2006. Can metals defend plants against biotic stress? *Trends in Plant Science* 11 (6):288–295. doi: 10.1016/j.tplants.2006.04.007.
Printz, B., S. Lutts, J.-F. Hausman, and K. Sergeant. 2016. Copper trafficking in plants and its implication on cell wall dynamics. *Frontiers in Plant Science* 7:601. doi: 10.3389/fpls.2016.00601.
Rawat, S., S. Ali, B. Mittra, and A. Grover. 2017. Expression analysis of chitinase upon challenge inoculation to alternaria wounding and defense inducers in *Brassica juncea*. *Biotechnology Reports (Amsterdam, Netherlands)* 13:72–79.
Schmidt, G. W., and S. K. Delaney. 2010. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. *Molecular Genetics and Genomics* 283: 233–241.
Vivancos, J., C. Labbé, J. G. Menzies, and R. R. Bélanger. 2015. Silicon-mediated resistance of arabidopsis against powdery mildew involves mechanisms other than the salicylic acid (SA)-dependent defence pathway. *Molecular Plant Pathology* 16 (6):572–582. doi: 10.1111/mpp.12213.
Zellner, W., J. Frantz, and S. Leisner. 2011. Silicon delays tobacco ringspot virus systemic symptoms in *Nicotiana tabacum*. *Journal of Plant Physiology* 168 (15):1866–1869. doi: 10.1016/j.jplph.2011.04.002.