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GC-TOF-MS based metabolomics and ICP-MS based metallomics of cucumber (Cucumis sativus) fruits reveal alteration of metabolites profile and biological pathway disruption induced by nano copper†

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Copper-based nanoparticles have wide application in agriculture as fungicides and bactericides. Due to the rapid increase in their use, it is imperative to understand their environmental impact, especially on agricultural crops. In this study, inductively coupled plasma mass spectrometry (ICP-MS) based metallomics and gas chromatography-time of flight mass spectrometry (GC-TOF-MS) based metabolomics were applied to detect metal and molecular changes in cucumber plants exposed to various environmentally-relevant levels of nano copper (nCu at 0, 200, 400, and 800 mg kg⁻¹) until full maturity. Metallomics studies showed nCu caused the perturbation of Fe uptake in leaves. In fruit, Ca, K, S, P, Zn and Mg increased after exposure to 400 and 800 mg kg⁻¹ nCu. Metabolomics and partial least-squares discriminant analysis (PLS-DA) revealed that the metabolite profile in cucumber fruits was distinctively altered due to exposure to nCu. A number of metabolites were either up-regulated (proline, glycine, valine, pelargonic acid, arachidic acid, xylose, benzoic acid) or down-regulated (citric acid, myo-inositol, ornithine, 1-kestose) in response to nCu exposure. Biological pathway analysis showed a number of C and N related pathways were changed, especially galactose metabolism and the tricarboxylic acid cycle, indicating C and N metabolism was perturbed by nCu. This study showed GC-TOF-MS based metabolomics combined with ICP-MS based metallomics provide the necessary preliminary data to conduct further mechanistic investigations.

Nano impact

With the increasing use of copper-containing nano-pesticides in agriculture, a comprehensive understanding of their implications for terrestrial plants, especially edible crops, is needed. GC-TOF-MS based metabolomics and ICP-MS based metallomics were applied to investigate the response at the molecular level of cucumber plants exposed to nano-Cu. Fruit metabolomics revealed that the metabolite profiles were significantly changed due to exposure to nano-Cu. Several metabolites, including 1-kestose, xylose, fructose, ornithine, citrulline, glycine, proline, oxoproline, methionine, aspartic acid, citric acid, glutaric acid, shikimic acid, benzoic acid, palgencic acid, arachidic acid, and caprylic acid were either down- or up-regulated. Nano-Cu also significantly changed the concentration of Ca, K, S, P, Zn, and Mg. The biological pathway of galactose metabolism and the tricarboxylic acid cycle were significantly disrupted.

† Electronic supplementary information (ESI) available: Soil properties data are shown in Table S1. A summary of analysis of variance for effect of nCu on the biomass, Cu and Fe content are presented in Table S2. The concentration of metabolites in cucumber fruit analyzed by GC-TOF-MS are shown in Table S3. Effect of nCu on biomass of root, stem, leaf and fruit are shown in Fig. S1. Water-extractable Cu in soil solutions are shown in Fig. S2. Score plot and loading plot of mineral nutrient analyzing by PLS-DA are shown in Fig. S3 and S4; VIP scores and pathway impact are shown in Fig. S5. See DOI: 10.1039/c6en00093b
Introduction

The use of copper-based nanoparticles (NPs) in agriculture as fungicides and bactericides is increasing rapidly due to their relatively low toxicity and higher efficiency in delivering the active component (Cu\(^{2+}\)). There are numerous copper-containing pesticides on the market, e.g., copper sulfate (Bordeaux mixture), cuprous oxide (e.g., Nordox), copper hydroxide (e.g., Kocide, Champ), and nano copper (nCu). The U.S. Department of Agriculture (USDA) maintains an official list of synthetic substances that can be used for organic farming. According to this list, copper-based materials are allowed for use in organic crop production.\(^1\) However, more and more evidence indicates that copper-based nanoparticles (5–20 mg L\(^{-1}\)) induce phytotoxicity in various plants, such as bean,\(^2\) lettuce,\(^4\) alfalfa,\(^4\) cilantro,\(^5\) and cucumber.\(^6\) The adverse impact includes decreased root and shoot elongation, disturbed mineral nutrients homeostasis, decreased photosynthesis rate, inhibited antioxidant enzyme activities.\(^3,4,7,8\) So far the molecular mechanism underlying those physiological changes is not well understood.

In recent years, "omics" have become a promising methodology for studying plant responses to abiotic and biotic stress.\(^9\) Transcriptomics-based gene expression and proteomics-based protein production have been applied to evaluate the changes of plants to external stressors at the molecular level.\(^10–15\) Unlike transcriptomics and proteomics, which reveal what might be happening in plant tissues, metabolomics profiling can tell what already happened. Metabolites are the end product of gene expression,\(^16\) and the changes of metabolites are regarded as ultimate responses of plant to stress.\(^17\) Thus, environmental metabolomics is becoming a powerful tool to investigate the response of plants to various stressors, e.g., water, light, temperature, and high levels of metals.\(^18\) Recently, Pidatala et al.\(^1\) employed LC-MS/MS based metabolomics to elucidate the stress response mechanism of lettuce to lead. They observed several key metabolic pathways, including sugar and amino acid metabolism, that were disturbed by lead.\(^19\) Our recent study,\(^20\) applying GC-TOF-MS based metabolomics and PLS-DA multivariate analysis, also revealed the profile of metabolites in root exudates was significantly altered by nCu. A number of amino acids were up-regulated to defend against an excess of copper. More recently, we determined that nCu altered the nutritional supply of cucumber fruit,\(^21\) using \(^1\)H NMR and GC-MS based metabolomics. Those studies demonstrate that metabolomics is a powerful tool to investigate the stress response of plant to contaminants. Therefore, the present study was designed to generate a mechanistic understanding of the effects of nCu on cucumber plants by applying untargeted GC-TOF-MS based metabolomics. Fruit metabolic profiling provides information on low molecular weight metabolites, which not only directly reflects fruit nutrient levels, but also generates a more comprehensive understanding of the metabolic network and the biological pathways impacted by NPs.\(^22\) ICP-MS based metalomics were also performed to supply the elemental changes under nCu stress. All those techniques provide a comprehensive insight into the overall stress response mechanism of cucumber plants to nCu, which helps understand their long-term impact in terrestrial environments.

Materials and methods

Characteristics of nCu

Detailed characterization of nCu (U.S. Research Nanomaterials) employed here was presented in a previous study.\(^23\) Briefly, the primary particle size is 40 nm and the hydrodynamic diameter is 2590 ± 1138 nm in deionized (DI, Barnstead nanopure) water at pH 7 (0.5 mM phosphate buffer), at similar concentrations as in this study. The surface charge, expressed as zeta potential (in 0.5 mM buffer solutions), is +10.9 ± 4.0, −29.4 ± 0.8 and −40.8 ± 1.7, at pH 4, 7 and 11, respectively.

Plant exposure and greenhouse conditions

Cucumber (Cucumis sativus) seeds were purchased from Seed Savers Exchange (Iowa, USA). The soil was collected from the Natural Reserve System of UC Santa Barbara (Sedgwick) and was selected for the current experiment. The soil composition is shown in Table S1.\(^*\) NCu was suspended in nanopure water and sonicated for 30 minutes before being applied to the soil. The final concentration of nCu in soil was 0 (control), 200 (low), 400 (medium) and 800 mg kg\(^{-1}\) (high). These (total Cu) concentrations are within the range of those predicted for biosolids applied to soils\(^24\) or due to the application of copper-based nanopesticides.\(^25\) Each treatment had four replicates. In each replicate, pairs of cucumber seedlings were grown in 3.0 L Poly-Tainer containers. The cucumber plants were grown from April 2, 2015 until harvest on June 28, 2015. The temperature in the greenhouse was controlled to be 25.5–30.0 °C during the day and 17.7–18.9 °C at night. At harvest, the fresh weight of root, stem, leaf and fruit from each treatment was recorded. In addition, the weight of each mature fruit from each treatment was recorded. Only the mature fruits (with weight above 100 g) were selected for metabolomics and metabolomics analysis. The number of matured cucumber fruits from each treatment were 6 (control), 5 (low), 3 (medium) and 6 (high); fruits from each treatment were split in two parts, to use for metabolomics and for metabolomics analyses.

Gas exchange measurement

30 days after sowing, the tenth leaf (from the base of the plant) produced by each sampled plant was analyzed for leaf photosynthetic rate, stomatal conductance, transpiration, and instantaneous water use efficiency (WUEi) by using a portable IR gas exchange analyzer (IRGA, LiCor 6400; Licor, Lincoln, Nebraska, USA) with a LiCor 6400–40 fluorometer light source. The measurements were recorded on sunny days between 10:00 and 12:00 h. The following LI-COR settings were used: light source: 6400–40 fluorometer; stability:
measurements of CO₂ and H₂O were stable for at least 15 s with a change in slope of <1 prior to recording the gas exchange rates; stomatal ratio: 0.5; flow rate: 500 μmol s⁻¹; PAR (PAR in the IRGA) = 1500.

ICP-MS analysis

At harvest, the cucumber tissues (root, stem, leaf and fruit) were oven-dried for 7 days at 60 °C. The oven-dried tissues were ground to powder and digested with HNO₃ and H₂O₂ (1:4) using a microwave oven system (Multiwave Eco, Anton Parr). The digestion method was based on EPA 3051. Standard reference materials, NIST 1547 (peach leaves) and 1570a (spinach leaves), were also digested and analyzed as samples. The recoveries for all elements were between 90% and 99%.

The mineral nutrient elements were analyzed using inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7900, Agilent Technologies, Santa Clara, CA).

Gas chromatography time-of-flight mass spectrometer-mass spectrometry (GC-TOF-MS) analysis of cucumber fruit

At harvest, the fresh cucumber fruits were immediately placed in liquid N₂, and lyophilized (Labconco, MO, USA). The freeze-dried fruit samples were delivered to the Genome Center Core Services at University of California Davis to analyze metabolites via gas chromatography time-of-flight mass spectrometer-mass spectrometry (GC-TOF-MS). A description of the analytical method has been reported previously. Partial least-squares discriminant analysis (PLS-DA) was run based on GC-TOF-MS data using online resources (http://www.metaboanalyst.ca/). PLS-DA is a supervised clustering method, which uses a multiple linear regression technique to maximize the separation between groups and help to understand which variables carry the class separating information. More details regarding data acquisition, data processing and data reporting are provided in the ESI.

Biological pathway analysis

Metabolic pathway analysis was performed on all identified metabolites using MetaboAnalyst 2.0 (ref. 30) to identify disturbed biological pathways. The impact value threshold calculated for pathway identification was set at 0.1.

Statistical analysis

Biomass, and mineral nutrients data were analyzed by one-way ANOVA, and group means were compared by conducting Tukey’s Honestly Significant Difference test using IBM SPSS Statistics 22. A probability of p ≤ 0.05 was considered to be significant. Photosynthesis rate, transpiration rate, stomatal conductance, and water use efficiency were analyzed with a
Results and discussion
Photosynthesis and biomass accumulation
Photosynthesis is one of the physiological processes most sensitive to environmental stresses. In order to monitor the physiological changes during development without damaging the plant tissue, photosynthetic rate (Photo), transpiration rate (Trans), stomatal conductance (Cond) and instantaneous water use efficiency (WUEi, Photo/Trans) of cucumber leaves were measured 30 days after sowing. Fig. 1 shows that photosynthetic rate decreased in all nCu treatments compared to the control, but only the low and medium group were statistically significant (Table S2†). Previous studies showed that nano copper depleted PSII action centers, leading to photosynthesis inhibition and disruption of the repair cycle. In addition, stomatal conductance and transpiration rates tended to increase in nCu treatments compared to the control. The actual mechanism for Cu to increase stomatal conductance and transpiration rate is still unknown. One possible explanation is that Cu can trigger the formation of reactive oxygen species (ROS) through redox process cycling between Cu²⁺ and Cu³⁺. It is not known if nCu by itself can trigger ROS. Previous work has shown that ROS open the stomata, increasing stomatal conductance and therefore the transpiration rate. WUEi is defined as the ratio of the rate of carbon assimilation to the rate of transpiration. The decline in carbon assimilation rate and the increase in transpiration rates in response to exposure to nCu resulted in a statistically significant decline in WUEi (p < 0.05) (Fig. 1, Table S2†).

Statistical analysis showed the biomasses of root, stem, leaf and fruit were not statistically impacted by nCu, even though the trend shows obvious decrease in all tissues (Fig. S1 and Table S2†).

Copper bioaccumulation in different tissues
Previous studies demonstrated that nCu has comparatively higher solubility than its oxidized forms, and is sensitive to aqueous matrix pH. Therefore, we hypothesized that Cu would bioaccumulate in cucumber tissues, including fruits. As shown in Fig. 2 and Table S2†, Cu concentrations in all tissues in nCu treatment groups were significantly higher than that in the control (p < 0.05), which indicates Cu is taken up by the roots and translocated even to the fruits. In both control and nCu treated plants, Cu was most abundant in the roots, although there was significantly more bioaccumulation in the nCu exposed plants. This is consistent with previous reports that copper is sequestered primarily in the root compartment.

It is noteworthy that the translocation factors (Cu in shoots/Cu in roots) of nCu/Cu ions in control plants is 0.33, but it decreased to 0.21, 0.22 and 0.23 in the plants exposed to 200, 400 and 800 mg kg⁻¹ nCu, respectively. In the control treatments, plants uptake ionized Cu from the soil. One would expect a similar translocation factor in the nCu treated group if ionized Cu was translocated in a similar manner. The reduced translocation rate suggests that cucumber plants grown in nCu treated soils not only took up Cu ions, but also nCu. But we believe that most of the nCu just adsorbed on the root surface. Confocal microscopy and μ-XRF evidenced that ZnO and CeO₂ NPs are mainly distributed on the outer layer of epidermis cells or trapped in epidermis cell walls; only a small portion of NPs could penetrate the epidermis cell wall and translocate to upper plant tissues via the transpiration stream. In addition, high ionic strength in the soil aqueous matrix probably accelerated the aggregation of nCu. Moreover, nCu or released Cu ions may complex with soil clay minerals or organic matter. All these factors reduced the possibility of small sized and free nCu existing in soil.

Thus, Cu in the stems, leaves and fruits is likely from solubilized Cu leached from nCu. In order to evaluate the ability of nCu for releasing Cu ions into soil, additional experiments were conducted (detailed information is shown in ESI†): soil was spiked with 200 mg kg⁻¹ of CuCl₂·2H₂O and nCu separately. Cucumber seedlings were allowed to grow in these spiked soil samples for 10 days, and water soluble Cu (which is regarded as the most bioavailable form of Cu) was determined via ICP-MS after removing the plants on day 10. After 10 days, the water-extractable Cu in the control (non-spiked media), 200 mg kg⁻¹ CuCl₂ and 200 mg kg⁻¹ nCu treatment was 1.8, 43 and 24 mg kg⁻¹, respectively (Fig. S2†). Thus, within 10 days nCu released a considerable amount of ionized Cu into the soil for plant uptake. These significant differences of bioavailable Cu (ionic or nano) between exposure conditions are likely because of the nCu’s high stability and slow release rate.
to copper salts or nCu highlight the challenge in conducting comparisons. Simply exposing plants to “equivalent” amounts of copper may lead to very different results.

**Mineral nutrient homeostasis**

Root, stem, and leaves supply and transport mineral nutrients. Results showed that nCu did not disturb any mineral nutrient homeostasis in these tissues except Fe. The Fe concentration in root and leaves were significantly decreased ($p \leq 0.05$) by nCu at all concentrations (Fig. 3 and Table S2†). In roots, Fe decreased by 67%, 54%, and 66% compared to the control, when exposed to 200, 400 and 800 mg kg$^{-1}$ nCu, respectively. In leaves, Fe decreased by 56%, 43% and 54% compared to the control. In addition, Fe concentrations in stems also tended to decrease when exposed to nCu, but this was not statistically significant. Previous studies demonstrated a competitive relationship between Cu and Fe uptake; it was observed that in *Arabidopsis thaliana* and *Cucumis sativus*, a low Fe supply led to increased Cu concentration in leaves, while high Cu supply lowered leaf Fe concentration.43 The authors found that the presence of Cu affected the activity of ferric reductase. At high Cu supply, ferric reductase (an indicator of Fe demand) activity was inhibited. This led to decreased demand for Fe by the plant and subsequently less Fe accumulation.43

Another possible explanation for low Fe is that increased Cu results in more efficient or rapid synthesis of Cu proteins that replace Fe proteins, thus reducing Fe demand and generating a feedback inhibition of ferric-chelate reductase activity or a decreased shoot-to-root demand signal. For example, in *Arabidopsis*, Fe-SOD (superoxide dismutase) expression decreased under high Cu supply due to the replacement of FeSOD proteins with CuSOD proteins.43,44 Iron is an essential cofactor for enzymes involved in numerous cellular processes. The nCu induced Fe deficiency may make plants more susceptible to Cu toxicity.

**Fruit metal profiling analysis**

To better understand the mineral nutrient content in cucumber fruit produced by plants grown at different concentrations of nCu, a PLS-DA analysis was performed on the mineral nutrient data. The score plot (Fig. S3†) showed a clear discrimination among the four groups (0, low, medium and high). The control group was separated from the medium and high groups along PC2, which contributed 21.5% of the total variability. The loading plot (Fig. S4†) indicates that the variables responsible for the separation are Ca, K, S, P, Zn, and Mg concentrations. A one-way ANOVA analysis also revealed that the concentration of these elements in plants exposed to nCu was significantly different from the control ($p \leq 0.05$). Except K, all the nutrients were higher in the medium and high groups compared to the control. This indicates that nCu at some level increases mineral nutrient accumulation in fruit, although the mechanism is currently unknown.
Even though Fe uptake was inhibited by nCu in root, stem and leaf, surprisingly, Fe concentration in cucumber fruits was not affected by nCu. It is possible that Fe was translocated through the phloem sap to the reproductive organs to maintain and ensure fruit development. Notably, Ca concentration increased in cucumber fruits in a concentration-dependent manner with increasing nCu levels. One possible reason is that Ca$^{2+}$ accumulation in fruits is determined by Ca$^{2+}$ content in the xylem sap, and Ca flux to the xylem through the apoplastic pathway is influenced markedly by transpiration.\textsuperscript{45,46} The increased transpiration rate due to exposure to nCu delivers more Ca to the leaves; it is then transported to the fruits through phloem loading.\textsuperscript{47} In contrast, Ca in the roots tended to decrease with increasing nCu exposure. One explanation is that higher transpiration rates deplete Ca from the roots. Another explanation is that different stresses cause signal-specific changes in cellular Ca$^{2+}$ levels, which functions as a messenger in modulating diverse physiological processes that are important for stress adaptation.\textsuperscript{48}

Metabolites profile of cucumber fruits

GC-TOF-MS detected 239 metabolites in cucumber fruits; 107 metabolites were identified. Table S3\textsuperscript{†} presents the relative abundance of the identified compounds. In order to visualize the differences between control and nCu treated plants, the 107 identified compounds were analyzed by PLS-DA using online resources (http://www.metaboanalyst.ca).\textsuperscript{49,50} The score plot (Fig. 4) shows that cucumber fruits exposed to different concentrations of nCu were clearly separated along the first principal axis (PC1), which explained 19.8% of the total variability. This suggests nCu considerably altered cucumber fruit metabolic profiles.

In order to further identify the metabolites responsible for this separation, the parameters of variable importance in projection (VIP) were determined. VIP is the weighted sum of the squares of the PLS-DA analysis, which indicates the importance of a variable to the entire model.\textsuperscript{31} A variable with a VIP above 1 is regarded as important.\textsuperscript{31} Forty compounds were identified as discriminating metabolites (VIP > 1) between treatments of different concentrations of nCu (Fig. S5\textsuperscript{†}). Those clearly altered metabolites include carbohydrates (1-kestose, xylose, and fructose), amino acids and their derivatives (ornithine, citrulline, glycine, proline, oxoproline, methionine, and aspartic acids), carboxylic acids (citric, glutaric, shikimic, benzoic, and pelargonic acids) and fatty acids (arachidic, linolenic, and caprylic acids). Univariate statistical analysis (T-test) indicates that nine metabolites (proline, glycine, ornithine, arachidic acid, caprylic acid, xylose, 1-kestose, myo-inositol, 2,5-digydroxypyrazine) were significantly different from the control ($p < 0.05$) (Table S4\textsuperscript{†}). All of them were overlapped with metabolites with high VIP score.

Several of the identified metabolites have been reported to relate to biotic or abiotic stress in previous studies, e.g. proline, glycine, ornithine and myo-inositol.\textsuperscript{18,21,52–54} They may also play an important role in increasing the tolerance of the cucumber plant to excess Cu. It is noteworthy that a few amino acids (e.g., proline, glycine, asparagine) were up-regulated in response to exposure to nCu. They possibly act as chelators of Cu ions to decrease the free Cu in tissues.

Both univariate and multivariate analysis showed glycine was significantly up-regulated in response to nCu. The relative abundance of glycine in cucumber fruit exposed to nCu increased 35–76% compared to the control. In our previous study,\textsuperscript{20} we also found that glycine in cucumber root exudate was up-regulated in response to nCu. It is possible that glycine directly acts as a Cu chelator in plant tissues. Another possible explanation is that glycine is one of the main precursors of reduced glutathione (GSH), which is an important antioxidant and Cu chelator.\textsuperscript{55,56} The enhanced level of

![Fig. 4](image_url) Partial least square (PLS) analysis of metabolites from cucumber fruits extract as affected by different concentrations of nCu. The data are based on the concentrations of 107 metabolites in cucumber fruits using GC-TOF-MS analysis.
glycine may indicate a detoxification process. Proline, as a multifunctional amino acid, plays diverse roles for plants combating stressors. Proline not only can act as a metal chelator, but also participates in antioxidative defense and as an osmolyte under water deficit. It has also been reported that proline is an effective scavenger of ROS. Moreover, proline has been shown to contribute to the detoxification of $O_2^•$ radicals by increasing activity of SOD in Solanum nigrum under Cd stress. Benzoic acid is also involved in the plant’s antioxidative defense system. It is reported that benzoic acid is capable of scavenging $H_2O_2$ and neutralizing free radicals. The up-regulation of benzoic acid may indicate the defense system is activated.

It is interesting to note that some of the up-regulated metabolites (valine, proline, glycine, pelargonic acid and benzoic acid) were also up-regulated in cucumber root exudate in response to nCu in a hydroponic system. Citric acid was also down-regulated in root exudate. Further work is needed to determine whether other plants respond in similar manner.

Biological pathways impacted by nCu

The alteration of metabolite concentrations reflects the inhibition or activation of a specific metabolic pathway. Metabolite pathway analysis can provide a more complete view of the stress response of plants to a contaminant. Therefore, metabolic pathway perturbation was analyzed using MetaboAnalyst 2.0 considering all the identified metabolites. nCu perturbed 15 metabolic pathways (Fig. 5). Five of these pathways (galactose metabolism, inositol phosphate metabolism, tricarboxylic acid (TCA) cycle, glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism) are related to carbohydrate metabolism. Six pathways (arginine and proline metabolism; lysine biosynthesis; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; tyrosine metabolism; glycine, serine and threonine metabolism) are related to amino acid synthesis and metabolism. In addition, alpha-linolenic acid metabolism, isoquinoline alkaloid biosynthesis, and methane metabolism were also disturbed; these pathways are related to lipid metabolism, biosynthesis of other secondary metabolites, and energy metabolism, respectively. These results indicate that accumulated nCu may have a significant impact on carbohydrate and amino acid metabolism for cucumber plants.

Carbohydrate metabolism. Galactose metabolism is the most significantly disrupted pathway by nCu, with 5 metabolites affected in this pathway including galactitol, glycerol, myo-inositol, galactose-6-phosphate, and fructose. All of these metabolites were down-regulated except fructose; this indicates galactose metabolism was inhibited by nCu.

The TCA cycle is the second most significantly disrupted pathway (Fig. 6). Succinic acid, fumaric acid, citric acid and pyruvic acids, which are TCA cycle upstream intermediates, were significantly changed (e.g. succinic, fumaric, and pyruvic acids increased; citric acid decreased) by exposure to nCu at all levels. Maleic acid, a trans-isomer of fumaric acid, was also increased. In the TCA cycle, acetyl-coenzyme A (acetyl-CoA) is a key to the conversion of oxaloacetate to citric acid in the mitochondria. The down-regulated citric acid is likely evidence that nCu influences the availability of acetyl-CoA.

Interestingly, most of the metabolites related to carbohydrate metabolism are reported to be a response to iron deficiency in the leaves and xylem sap of several plant species, including peach, tomato, and sugar beet. It has been demonstrated that the response of leaves to iron deficiency is up-regulation of carbohydrates and TCA cycle metabolites and N-related metabolites. Combining the present results (iron deficiency and changes in metabolites) and a previous study, this may indicate that iron deficiency induced by nCu contributes in part to changes in cucumber fruit metabolite profiles.

Amino acid metabolism. Arginine and proline metabolism is apparently the most disrupted pathway (Fig. 6). There are eight metabolites implicated in this pathway and three of them are significantly changed, including proline, ornithine, and citrulline. Ornithine and citrulline are also involved in the urea cycle, which suggests a perturbed urea cycle. The second disrupted pathway is glycine, serine, and threonine metabolism, with five metabolites affected in this pathway: homoserine, threonine, serine, glycine and pyruvic acid. Among them, glycine and pyruvic acid are significantly
increased due to plant exposure to nCu at all levels. This may indicate this pathway was activated.

Isoleucine, valine, and leucine, which are branched-chain amino acids (BCAA), were up-regulated due to nCu exposure (Fig. 6). It has been reported that these amino acids share several common enzymes.65 The up-regulation may indicate that the related enzymes have been activated. Pyruvate, a precursor of valine and leucine, was also up-regulated. It is reported that BCAA may serve as an oxidative phosphorylation energy source during plant stress.66 Therefore, the up-regulation of BCAA may indicate an adaptation process of cucumber plants to stress induced by nCu.

Food safety and fruit quality

As mentioned before, Cu was present in cucumber fruit at an average concentration of 21.3, 27.4, 28.9, and 31.5 mg kg\(^{-1}\) for control, 200, 400 and 800 mg kg\(^{-1}\) treatments. This indicates that Cu uptake did not increase linearly with exposure. It is likely that the binding of Cu ions to soil clay minerals decreased their bioavailability. In addition, most of the Cu was sequestered in root tissues. Therefore, the likelihood of Cu over-accumulation in fruit is low.

According to US Department of Agriculture, annual per capita consumption of fresh cucumbers in the United States is 3.0 kg in 2013, which means average daily cucumber consumption is \(~8.2\) g per person-day (fresh weight). The average cucumber water content is 95%, so the daily consumption is 0.41 g dry weight per person-day. Thus, daily personal Cu intake from cucumber used in this study would be 10.0, 11.2, 11.8, and 12.9 \(\mu\)g from control, low, medium and high treatments. According to the Food and Nutrition Board at the U.S. Institute of Medicine of the National Academies, the recommended average requirement for Cu is 700 \(\mu\)g per person-day, with a tolerable upper intake level of 10 mg per person-day.67 Therefore, Cu intake from consumption of cucumber exposed to nCu enriched soil would be within the recommended Cu levels, even at the higher application level. Hence, consumption of nCu treated cucumbers, even at the high level, represents no significant added risk to consumers.

Fruit quality is affected by, among others, sugars and fatty, amino, and carboxylic acids. The profile alteration of these nutrients may result in flavor and nutritional supply changes induced by exposure to nCu. Organoleptic analysis was beyond the scope of this work.

Conclusions

ICP-MS based metallomics and GC-TOF-MS based metabolomics demonstrated that exposure of cucumber plants to nCu throughout their entire life cycle can result in significant changes in metal and nutrient bioaccumulation and metabolite profiles. The subsequent metabolic pathway analysis revealed carbon and nitrogen metabolism are significantly disturbed, affecting the levels of amino acids, carbohydrates and other important biomolecules. We demonstrated that metabolite and metallomic profiling provides information that could be used to launch more detailed investigations of specific effects or mechanisms of response from exposure to nanomaterials and other chemicals. The specific contribution of nCu or Cu ions to the observed changes is not known. To elucidate the contribution of ionized Cu from nCu on the observed molecular changes is challenging, an issue we plan to address in future studies. In addition, after having identified...
the metabolite changes induced by nCu, it will be interesting to study the up-stream genes controlling and regulating the identified metabolites, to provide additional insight into the response of plants to NPs.

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