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Comparative Metabolic Response between Cucumber (*Cucumis sativus*) and Corn (*Zea mays*) to a $Cu(OH)_2$ Nanopesticide

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Supporting Information

ABSTRACT: Due to their unique properties, copper-based nanopesticides are emerging in the market. Thus, understanding their effect on crop plants is very important. Metabolomics can capture a snapshot of cellular metabolic responses to a stressor. We selected maize and cucumber as model plants for exposure to different doses of $Cu(OH)_2$ nanopesticide. GC-TOF-MS-based metabolomics was employed to determine the metabolic responses of these two species. Results revealed significant differences in metabolite profile changes between maize and cucumber. Furthermore, the $Cu(OH)_2$ nanopesticide induced metabolic reprogramming in both species, but in different manners. In maize, several intermediate metabolites of the glycolysis pathway and tricarboxylic acid cycle (TCA) were up-regulated, indicating the energy metabolism was activated. In addition, the levels of aromatic compounds (4-hydroxycinnamic acid and 1,2,4-benzenetriol) and their precursors (phenylalanine, tyrosine) were enhanced, indicating the activation of shikimate-phenylpropanoid biosynthesis in maize leaves, which is an antioxidant defense-related pathway. In cucumber, arginine and proline metabolic pathways were the most significantly altered pathway. Both species exhibited altered levels of fatty acids and polysaccharides, suggesting the cell membrane and cell wall composition may change in response to $Cu(OH)_2$ nanopesticide. Thus, metabolomics helps to deeply understand the differential response of these plants to the same nanopesticide stressor.

KEYWORDS: *crop plant, metabolomics, metabolites profile, uptake, stress, defense*

INTRODUCTION

The application of nanomaterials (NM) in agriculture aims to reduce the amount of plant protection products applied to crops by increasing their effectiveness.[1,2] Copper ions (as Cu^{2+} and Cu^{+}) have a long history of use as fungicides by affecting the activity of several enzymes, thereby preventing germination of fungal spores.[2] There is increased interest in copper-based nanopesticides in the market, particularly in organic farming.[3] However, copper is a redox active metal and can generate reactive oxygen species (ROS) through the Fenton reaction, which may damage lipids, proteins, and DNA.[4] Therefore, the potential risk of copper-based nanopesticides on crop plants needs to be thoroughly understood, and determining the metabolic response can help to elucidate the implications of using these novel materials.

Metabolomics is the field of science concerned with the study of low molecular mass metabolites within a cell, tissue, or biofluid.[5] Low molecular mass metabolites are the end products of cellular regulatory processes, and their levels are regarded as the ultimate response of organisms to environmental stressors.[6] Exposure of organisms to environmental stressors will result in changes to gene expression and protein production, which are amplified at the level of the metabolome.[5] Thus, metabolomics is generally a more sensitive indicator than other omics. Application of metabolomics to characterize the interaction of living organisms with their environment is defined as environmental metabolomics.[7] In the past decade, emerging evidence shows that environmental metabolomics is a powerful and promising approach to study organism responses to environmental stressors.[5] Using nuclear magnetic resonance (NMR) or mass spectrometry (MS) based metabolomics, the simultaneous identification and quantification of hundreds or thousands of plant metabolites can be approached. Multivariate approaches, such as principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and univariate analysis (*t* test, one-way ANOVA) based on the metabolite data set serve to screen out discriminating and significantly changed metabolites. Quantitative information on metabolite levels enables a comprehensive assessment of plant response and adaptation to specific stressors.[8] Recently, environmental metabolomics has proven to be a promising tool to study the metabolic responses of various organisms (snail,[9] seagrass,[10] mussel,[11] fish[12]) after exposure to different environmental stressors. However, there are currently few studies[13,14] using metabolomics to study crop plant exposure to nanomaterials, particularly nanopesticides.

Maize and cucumber are not only important crop plants but also model organisms for basic and applied research in plant biology.[15] Moreover, cucumber and maize are typical C3 and C4 plants, respectively. C4 plants are more efficient in photosynthesis than C3 plants. Differences in photosynthetic efficiency

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may lead to different metabolic responses to the same stressor. In this study, these two species were exposed to environmentally relevant concentrations of a commercial $Cu(OH)_2$ nanopesticide via foliar application, as recommended by the manufacturer. A gas chromatography−time-of-flight-mass spectrometry (GC-TOF-MS)-based nontargeted metabolomics approach was used to determine the levels of low molecular weight metabolites. Multivariate (PLS-DA) and univariate (*t* test) analyses were used to identify the metabolites contributing to the differences between the control group and exposed groups and potential biomarkers. The metabolite information was then used to elucidate the underlying signals, defense and damage responses. Combining the results of metabolomics and phenotypes, biochemical networks were created to provide a mechanistic insight into the metabolic pathways of the response of these two species to $Cu(OH)_2$ nanopesticide. This study focused on the potential effect on metabolic profiles of these two crop plants; additional studies are needed to estimate the potential risks to humans or ecological receptors from exposure during application of the nanopesticide, which for humans can be mitigated using proper personal protective equipment, or via ingestion of foods with nanopesticide residues.

MATERIALS AND METHODS

$Cu(OH)_2$ Nanopesticide. The $Cu(OH)_2$ nanopesticide used in this study was in the form of a commercial biocide (Kocide 3000, DuPont). The primary particle size is from ∼50 to >1000 nm.[16,17] The hydrodynamic diameter of Kocide 3000 in NanoPure water (pH 7) is 1532 ± 580 nm, and the zeta potential is −47.6 ± 43 mV, measured via dynamic light scattering (Malvern Zetasizer Nano ZS-90). The micronized particles in Kocide 3000 are made up of $Cu(OH)_2$ nanosheets, bound together by an organic composite that dissociates in water.[16] Copper content in Kocide 3000 is 26.5 ± 0.9%; other elements detected by SEM-EDS are C, O, Na, Al, Si, S, and Zn.[16] Inductively coupled plasma mass spectrometry (ICP-MS) detected additional elements present in Kocide 3000, such as Ce, Ca, Fe, Mg, K, and P (Table S1).

Plant Growth and Experimental Design. Cucumber (*Cucumis sativus*, A & C Picking OG) and corn (*Zea mays*, OG Stowell's Evergreen) seeds were purchased from Seed Savers Exchange (Decorah, IA, USA). The soil was composed of sand (Quikrete Washed Plaster Sand), Sunshine Advanced Growing Mix#4 (SunGro Horticulture), vermiculite (Therm-O-Rock), coco coir (Canna), and perlite (Therm-O-Rock), at a ratio of 1:3:1:2:2 by volume. In addition, 4-4-4 fertilizer was added at 0.4%. Eighteen pots of cucumber seedlings and 18 pots of corn seedlings were grown in a greenhouse for 3 weeks before foliar application of the $Cu(OH)_2$ nanopesticide suspension. The temperature in the greenhouse was maintained at 28 °C by day and at 20 °C by night. On day 22, the $Cu(OH)_2$ nanopesticide suspensions were applied to corn or cucumber leaves three times per day for 7 days using a portable manual sprayer. The $Cu(OH)_2$ nanopesticide concentrations in the applied suspensions were 0, 100, and 1000 mg/L, with six replicates per exposure level. $Cu(OH)_2$ nanopesticide suspensions (100 and 1000 mg/L) were prepared in NanoPure water and sonicated (Branson 8800, Danbury, CT, USA) in a temperature-controlled bath (20 °C) for 30 min prior to application. The total doses of $Cu(OH)_2$ nanopesticide applied, via foliar spray, were 0, 2.5, and 25 mg per cucumber plant and 0, 10, and 100 mg per corn plant. Dose levels generally followed manufacturer recommendations, but a higher dose was applied to maize and a lower dose to cucumber, taking into account that maize leaves are hydrophobic (and some of the applied drops could runoff) and cucumber leaves are hydrophilic (retaining more of the applied dose). More details regarding the foliar spray procedure are provided in the Supporting Information.

Gas Chromatography−Time of Flight-Mass Spectrometry Analysis of Metabolites in Leaves. Freeze-dried tissue samples were analyzed via GC-TOF-MS at the Genome Center Core Services, University of California—Davis, to identify the metabolites present. Sample pretreatment, analytical method, and instrument have been described by Fiehn et al.[18,19] Briefly, an Agilent 6890 gas chromatograph (Santa Clara, CA, USA) containing an Rtx-5Sil MS column (30 cm length × 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl/5% diphenylpolysiloxane) with an additional 10 mm integrated guard column was used to run the samples, controlled using Leco ChromaTOF software version 2.32 (http://www.leco.com). Quantification was determined from peak height using the unique ion as default. Metabolites were unambiguously assigned by the BinBase identifier numbers using retention index and mass spectrum as the two most important identification criteria. More details regarding data acquisition, data processing, and data reporting are provided in the Supporting Information.

Multivariate Analysis and Biological Pathway Analysis. PLS-DA is a supervised clustering method, which uses a multiple linear regression technique to maximize the separation between groups and helps to understand which variables carry the class-separating information.[20] PLS-DA was run on the GC-TOF-MS data using online resources (http://www.metaboanalyst.ca/).[21] Variable importance in projection (VIP) is the weighted sum of squares of the PLS-DA analysis, which indicates the importance of a variable to the entire model.[20] A variable with a VIP >1 is regarded as significant.[22] In addition, univariate statistical analyses (*t* test) were also conducted using online resources (http://www.metaboanalyst.ca/) taking 0.05 as a threshold value. Biological pathway analysis was performed on the basis of all identified metabolites data using MetaboAnalyst 2.0.[10] The impact value threshold calculated for pathway identification was set at 0.1.[22]

RESULTS AND DISCUSSION

Metabolite Profiles in Cucumber and Corn Leaves. The metabolite profiles of unexposed maize and cucumber leaves were obtained via GC-TOF-MS-based metabolomics. A total of 543 metabolites were detected, and on the basis of retention index and mass spectrum, 178 metabolites were identified by the BinBase identifier. To compare the metabolite profiles of the two species, an unsupervised multivariate method using principal component analysis (PCA) was performed on the data sets of maize and cucumber leaves. The model was not provided with any prior information concerning the identity of the samples.[23] The grouping of the samples in a PCA scores plot is based on the similarities between their metabolic profiles.

The PCA scores plot (Figure S1) shows that the metabolite profiles of unexposed maize and cucumber were clearly separated along the first principal component (PC1), which explained 66.8% of the total variance. This indicates that the metabolite patterns are quite different for these two species. We then ran PLS-DA, a supervised multivariate analysis, based on the unexposed (control) maize and cucumber data sets to determine the discriminating compounds (Figure S2). The discriminating compounds (Figure S3) include a number of primary metabolites, such as soluble sugars (maltose, fructose, glucose, and sucrose), organic acids (quinic, pipecolinic, aconitic, shikimic, mucic, linolenic, citric, succinic, and fumaric acids), amino acids (isoleucine, valine, leucine, phenylalanine, tryptophan, threonine, proline, and asparagine), polyamines (spermidine), and other metabolites (urea and phosphate). Notably, all of the soluble sugars (sucrose, fructose, and glucose) are more abundant in maize leaves relative to cucumber leaves. Sucrose is the primary product of photosynthesis and the initial form of transported sugar in higher plants.[24] Glucose and fructose are two important reducing monosaccharides and are the degradation products of sucrose.[25] The higher levels of soluble sugars in maize leaves indicates that maize has a higher carbon fixation capacity compared to cucumber. The reason may be the higher

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photosynthesis efficiency of C4 plants (corn) relative to C3 plants (cucumber).[26] Interestingly, the intermediates of the tricarboxylic acid (TCA) cycle (e.g., citric, succinic, and fumaric acids) were found to be more abundant in cucumber leaves compared to maize leaves. The TCA cycle provides precursors of certain amino acids as well as the reducing agent nicotinamide adenine dinucleotide (NADH), which is used in numerous other biochemical reactions. Cucumber leaves had higher levels of several amino acids (isoleucine, valine, leucine, threonine, proline, and asparagine), which are synthesized on the basis of the TCA cycle intermediates as precursors, compared to corn leaves. The higher levels of TCA cycle intermediates and TCA cycle derived amino acids in cucumber suggest that cucumber leaves favor more N metabolism relative to C metabolism than corn. In addition, two cyclohexanecarboxylic acids (shikimic and quinic acids) were found more abundant in maize leaves relative to cucumber leaves. Shikimic and quinic acids are precursors of shikimate-phenylpropanoid biosynthesis, which generates a number of aromatic compounds such as phenols with antioxidant ability. It is possible that the maize shikimate-phenylpropanoid pathway is more active to be able to generate more defense-related metabolites, which would serve to prepare against oxidative stress under adverse conditions.

Alteration of Metabolite Profile of Maize Leaves Exposed to $Cu(OH)_2$ Nanopesticide. To elucidate the impact of $Cu(OH)_2$ nanopesticide on the metabolite profile of maize leaves, PLS-DA was performed on the maize data set. The score plot (Figure 1) indicates that the 10 and 100 mg $Cu(OH)_2$ nanopesticide groups are clearly separated from the control group, which indicates that the metabolite profiles were markedly altered after exposure to the $Cu(OH)_2$ nanopesticide, especially at a 100 mg dose. In response to foliar exposure to $Cu(OH)_2$ pesticide, many metabolites were significantly (VIP $\geq$ 1) up-regulated (Figure S4), such as amino acids (valine, lysine, leucine, methionine, proline, alanine, and serine), organic acids (4-hydroxycinnamic, saccharic, isocitric, mannonic, isohexonic, maleic, aconitic, galactonic, ferulic, and glycolic acids), phosphate-related compounds (pyrophosphate, glycerol-α-phosphate, and fructose-6-phosphate), and other metabolites (1-monostearn, adenosine, and 3-phosphoglycerate). Only three metabolites (arachidic acid, phytol, and methionine) were down-regulated in response to $Cu(OH)_2$ nanopesticide. Univariate statistical analyses were also conducted in a complementary manner. Results showed that 10 metabolites had the most significant changes ($p < 0.05$) (Figure 2), including 1-monostearin, pyrophosphate, *myo*-inositol, saccharic acid, phytol, β-gentiobiose, mannonic acid NIST, glycerol-α-phosphate, fructose-6-phosphate, and galactonic acid. These significantly up- or down-regulated metabolites may be byproducts of stress metabolism or signal transduction molecules or play other roles to enhance defense or tolerance. Exploring the changes in the levels of these metabolites helps to understand the underlying mechanism of the acclimation process or the defense response of maize plants in response to $Cu(OH)_2$ nanopesticide.

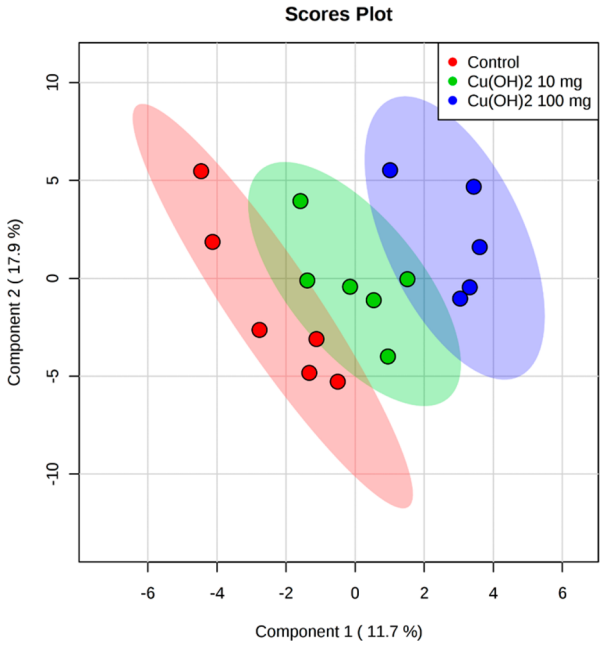

Figure 1. Partial least-squares discriminate analysis (PLS-DA) score plots of metabolic profiles in maize leaves foliar sprayed with different doses of $Cu(OH)_2$ nanopesticide (0, 10, and 100 mg).

As a simple polyol, *myo*-inositol has been reported to play an important role in signal transduction.[27] For physiological purposes, *myo*-inositol acts as a compatible solute (osmolyte) facilitating the retention of water in the cytoplasm. In addition, *myo*-inositol acts as a scavenger of active oxygen to protect the cellular structures.[28] Obata et al.[29] reported that the importance of *myo*-inositol in plant stress tolerance is related to its function as a precursor of many metabolites involved in abiotic stress tolerance. The reason for the up-regulation of *myo*-inositol in this study is unclear, but either role will assist adaptation of maize leaves to stress induced by the $Cu(OH)_2$ nanopesticide. Phytol is a chlorophyll degradation product that accumulates during plant senescence.[30] Phytol levels were unchanged in the 10 mg treatment; however, the levels decreased significantly at a dose of 100 mg of $Cu(OH)_2$. The reduced level of phytol may be an indicator of decreased chlorophyll degradation. The up-regulation of pyrophosphate, glycerol-α-phosphate, and fructose-6-phosphate indicates that the glycolysis pathway was activated. This pathway provides energy for plant growth and supports the antioxidative system such as catalase (CAT), superoxide dismutase (SOD), and other antioxidant enzymes.[31,32] The up-regulation of these glycolysis pathway intermediates may indicate the energy demand increased to support antioxidant defenses.

Global Metabolic Responses to $Cu(OH)_2$ Nanopesticide in Maize Leaves. Changes in metabolite abundance in a tissue reflect an inhibition or activation of specific metabolic pathways, which also represent metabolic rearrangements.[31] Biological pathway analysis via MetaboAnalyst[22] revealed that the most relevant pathway influenced by foliar exposure of maize leaves to $Cu(OH)_2$ nanopesticide was inositol phosphate metabolism (Figure S7). This is reflected in the 1.5-fold ($p < 0.01$) increase in *myo*-inositol levels when corn leaves were exposed to 100 mg of $Cu(OH)_2$ nanopesticide.

Although metabolites in other pathways were significantly altered (up or down), MetaboAnalyst indicated that the impact to other pathways was not statistically significant. However, it is useful to consider a more global view of the impacts to the interconnected network of C and N metabolism pathways (Figure 3). Most of the discriminating metabolites were involved in this network, which helps to provide a comprehensive vision of the impacts.

Effects on C Metabolism. (a) Glycolysis Pathway and TCA Cycle. As shown in Figure 3, several glycolysis pathway

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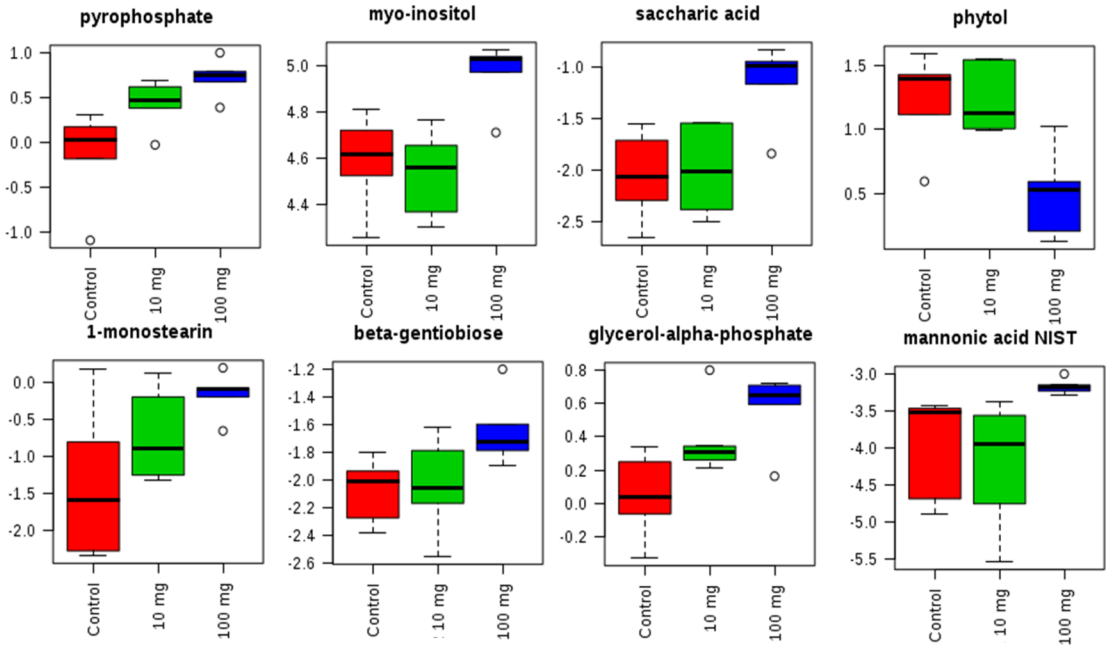

Figure 2. Relative abundance of significantly changed metabolites in 4-week-old corn plants exposed to different doses of $Cu(OH)_2$ nanopesticide (0, 10, and 100 mg) (n = 6). The Y-axis indicates absolute signal from GC-TOF-MS.

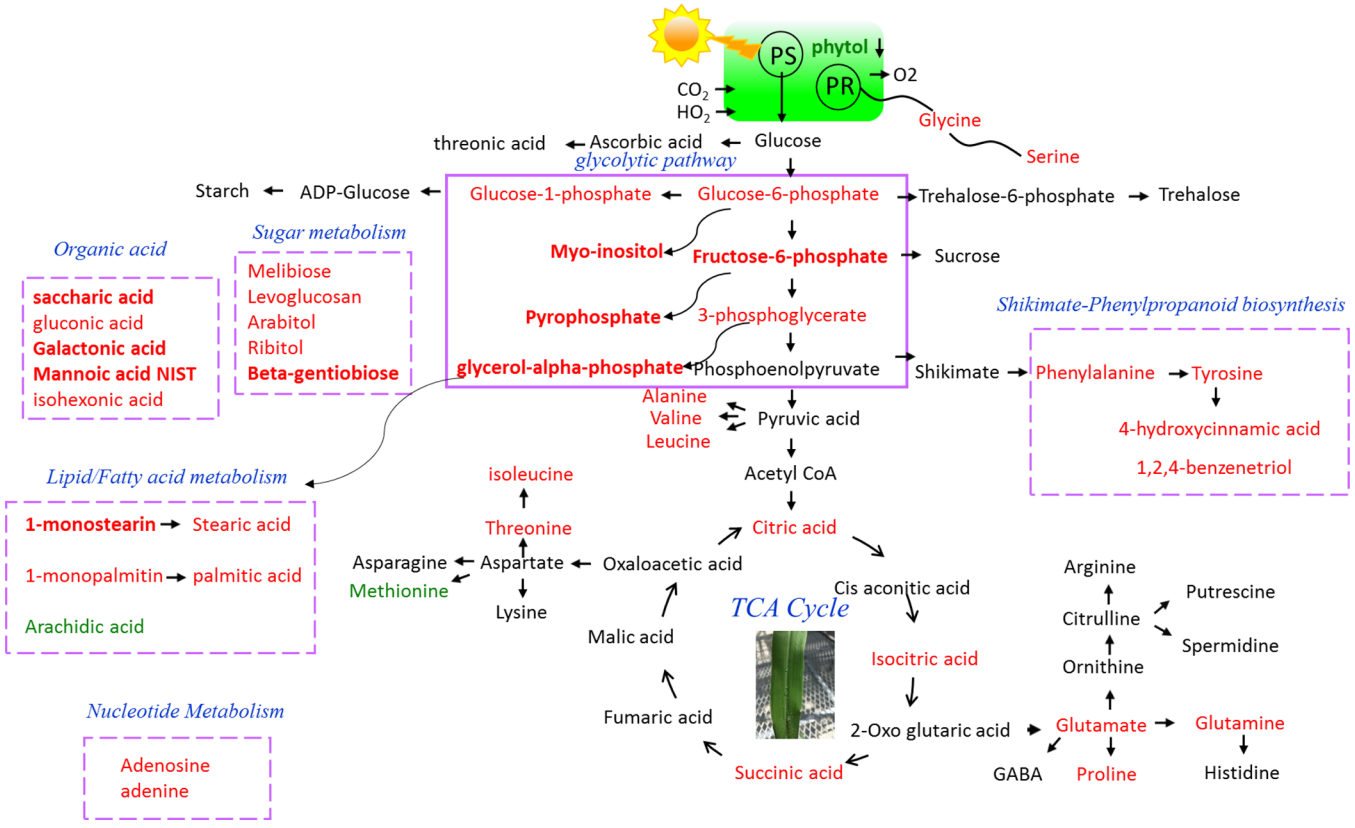

Figure 3. Schematic diagram of proposed metabolic pathways of maize. Central metabolic pathways (glycolytic pathway, TCA cycle, and shikimate-phenylpropanoid biosynthesis) and other metabolite biosynthetic pathways (sugar, amino acid, lipids, and fatty acid) are shown. Red and green circles indicate that the metabolites increased or decreased in response to $Cu(OH)_2$ nanopesticide.

intermediates (glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, glycerol-α-phosphate, *myo*-inositol, and pyrophosphate) were up-regulated in response to $Cu(OH)_2$ nanopesticide. Almost all of these glycolysis pathway intermediates were up-regulated in exposed leaves in a dose-dependent manner, except *myo*-inositol. The glycolysis pathway is responsible for the production of accessible energy.[25] In addition, some of the TCA cycle intermediates (citric, isocitric, and succinic acids) were up-regulated in maize leaves in response to $Cu(OH)_2$ nanopesticide exposure, which suggest the TCA

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cycle was up-regulated. The TCA cycle is also an energy-related pathway. The up-regulation of intermediates of energy metabolism (inositol phosphate, glycolysis, and TCA cycle) indicates that maize attempted to accumulate energy, which might be used to maintain normal physiological processes or increase defense-related activities. An alternative explanation for the up-regulation of citric acid is that the plants tried to chelate excess copper using citrate.

(b) Shikimate-Phenylpropanoid Pathway. The shikimate-phenylpropanoid pathway generates numerous antioxidants (e.g., flavonoids, phenols, and lignins) and their precursors (e.g., aromatic amino acids and shikimic acid) to scavenge or inhibit the synthesis of ROS in plant cells under biotic or abiotic stress, thereby protecting cell proteins, membrane lipids, DNA, and other cellular components from serious injury.[33] In exposed maize leaves, 1,2,4-benzenetriol and 4-hydroxycinnamic acid were up-regulated (Figure S4). The level of 1,2,4-benzenetriol enhanced 1.5-fold compared to the control after treatment with 10 and 100 mg of $Cu(OH)_2$ nanopesticide. The abundance of 4-hydroxycinnamic acid in corn leaves exposed to 10 and 100 mg $Cu(OH)_2$ increased by >2-fold. These two compounds are able to quench ROS. The up-regulation of antioxidants may be a response to overproduction of ROS due to foliar exposure to the $Cu(OH)_2$ nanopesticide. As mentioned before, copper can trigger the overproduction of ROS through the Fenton reaction.[4] In addition, increased levels of aromatic amino acids such as phenylalanine and tyrosine, which are precursors of 1,2,4-benzenetriol and 4-hydroxycinnamic acid and other secondary metabolites, indicate that the shikimate-phenylpropanoid pathway was up-regulated. The increased content of defense-related metabolites (e.g., 4-hydroxycinnamic acid, β-sitosterol, 1,2,4-benzenetriol, and dehydroascorbic acid) and their precursors (e.g., phenylalanine and tyrosine) from the shikimate-phenylpropanoid pathway indicates that the plant defense system was activated. These antioxidant compounds are up-regulated to scavenge excessive ROS and protect cells from damage. A similar up-regulation of low molecular weight antioxidants (3,4-dihydroxycinnamic acid, dehydroascorbic acid, *cis*-caffeic acid, and chlorogenic acid) was observed in lettuce (*Lactuca sativa*) after foliar exposure to $Cu(OH)_2$ nanopesticide.[14]

(c) Fatty Acid Metabolism. Fatty acids are critical components of cellular membranes. The alteration of fatty acids may affect cell survival under stress conditions through the remodeling of membrane fluidity.[34] It is known that 1-monostearin and 1-monopalmitin (monoacylglycerols, lipid component) are potential precursors for membrane lipids.[19] We found that 1-monostearin significantly increased 2.3-fold at the 100 mg dose (Figure 2). In addition, 1-monopalmitin also increased at this higher dose. Interestingly, two saturated fatty acids (stearic acid and palmitic acid), which are the downstream products of 1-monostearin and 1-monopalmitin, were also up-regulated in response to 100 mg of $Cu(OH)_2$ nanopesticide. This suggests that a dose of 100 mg of $Cu(OH)_2$ nanopesticide induced the alteration of fatty acid composition: the saturated fatty acid increased. Lipids play a crucial role in the regulation of membrane fluidity and signal transduction.[35] Thus, this metabolic shift might be a membrane protection mechanism.

Effects on N Metabolism. Exposure of maize leaves to 100 mg of $Cu(OH)_2$ nanopesticide induced the increase of alanine, valine, and leucine approximately 2-fold compared to the control. These branched-chain amino acids (BCAA) are derived from pyruvate. It is reported that BCAA may serve as an oxidative phosphorylation energy source during plant stress.[36] Therefore, the up-regulation of BCAA may indicate an adaptation process of maize plants to stress induced by the $Cu(OH)_2$ nanopesticide. In addition, some amino acids (isoleucine, threonine, proline, glutamate, and glutamine) derived from TCA cycle intermediates also increased in response to $Cu(OH)_2$ nanopesticide in a dose-dependent way. Among them, proline and glutamic acid at 100 mg of $Cu(OH)_2$ nanopesticide increased 2.5- and 1.6-fold, respectively, compared to the control. Proline accumulated in many plant species in response to environmental stress.[37] The function of proline could be either as a compatible solute or as a signaling molecule.[37] The up-regulation of proline may have an osmoprotective function to guard maize from damage by $Cu(OH)_2$ nanopesticide and its transformation products.

Glutamine and glutamate are involved in nitrogen metabolism that regulates ammonium assimilation in plants.[38] The increase in glutamine and glutamate might imply a higher capacity of nitrogen assimilation through the glutamine synthetase/glutamate synthase pathway.[38] Glutamate is also a precursor for chlorophyll synthesis in developing leaves.[39] The up-regulation of glutamic acid might be a mechanism to improve the chlorophyll content. The increase in amino acids may also contribute to increased protein synthesis.[40]

The increased levels in glycine (Gly) and serine (Ser), two essential amino acids formed during photorespiration,[41] may reflect an up-regulation of this process. Gluconic acid and saccharic acid are products of glucose oxidation. Their up-regulation indicates a plant shift to respiration. Because cell C metabolism is dependent on the balance between photosynthesis and respiration, a shift to respiration means net C loss.

Metabolite Profile Changes in Cucumber Leaves Exposed to $Cu(OH)_2$ Nanopesticide. Similar to maize, the PLS-DA scores plot (Figure 4) revealed clear differences in the metabolite profiles of cucumbers exposed by foliar application to 2.5 and 10 mg of $Cu(OH)_2$ nanopesticide relative to the control. This indicates the metabolite profile in cucumber leaves was altered due to this exposure. Metabolites with significantly

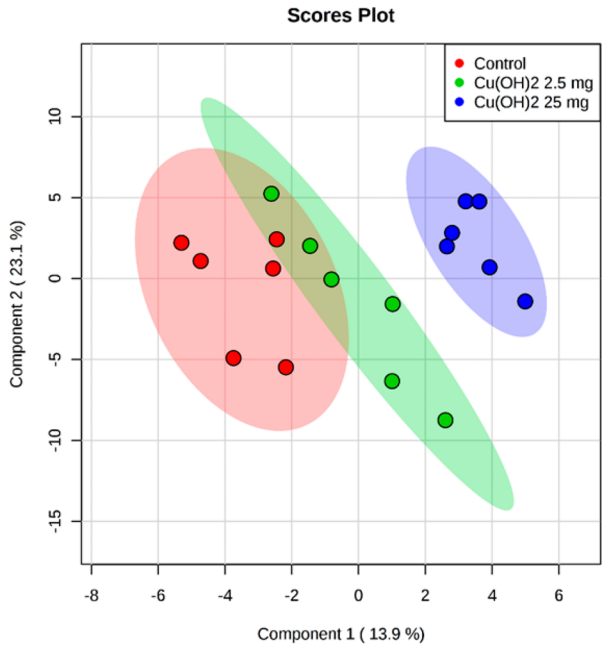

Figure 4. PLS-DA scores plots of metabolic profiles in cucumber leaves foliar sprayed with different doses of $Cu(OH)_2$ nanopesticide (0, 10, and 100 mg).

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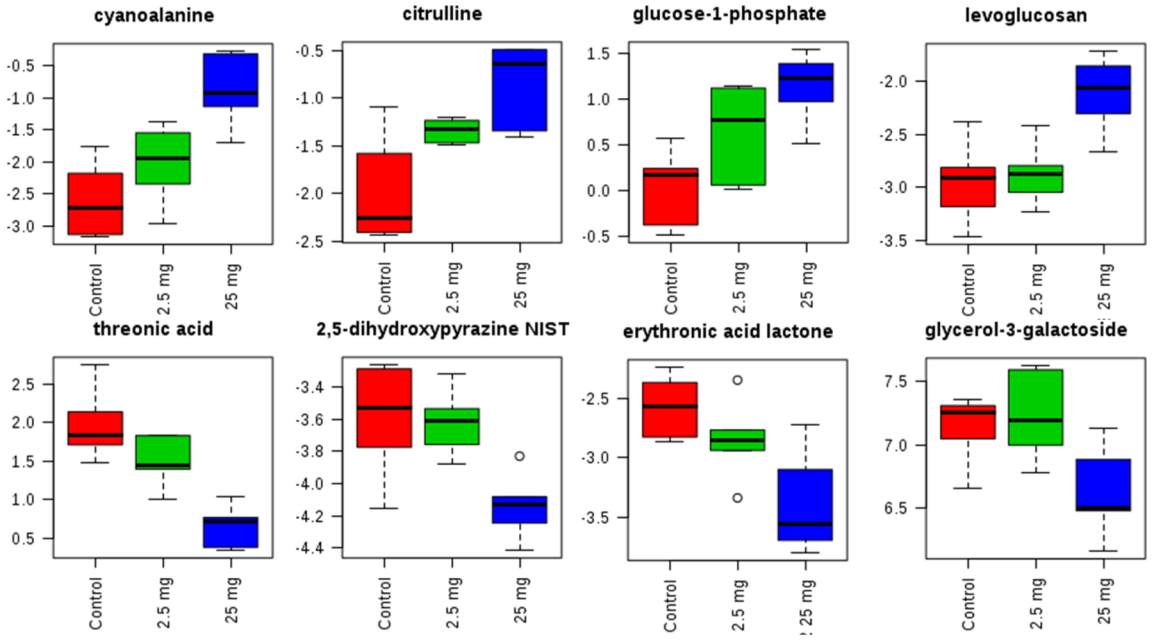

Figure 5. Relative abundance of significantly changed metabolites in 4-week-old cucumber plants exposed to different doses of $Cu(OH)_2$ nanopesticide (0, 2.5, and 25 mg) (n = 6). The Y-axis indicates absolute signal from GC-TOF-MS.

altered levels (based on VIP) are shown in Figure S7. Among them, 11 metabolites were down-regulated, including ribitol, threitol, raffinose, xylose, fumaric acid, threonic acid, 2-ketoglucose dimethylacetal, erythronic acid lactone, *N*-acetylmannosamine, and 2,5-dihydroxypyrazine NIST. A total of 29 metabolites were up-regulated, including sugars and sugar alcohols (sucrose, glucose, levoglucosan, isomaltose, sorbitol, xylitol, and arabitol), amino acids (*N*-acetylaspartic acid, cyanoalanine, asparagine, oxoproline, and glutamine), organic acids (oxalic acid, lauric acid, and lactic acid), phosphate compounds (glucose-1-phosphate, glucose-6-phosphate, and β-glycerolphosphate), phenols (quinic acid and shikimic acid), amines (putrescine and citrulline), and phenolics (4-hydroxycinnamic acid and 1,2,4-benzenet). A univariate one-way ANOVA showed that exposure to the $Cu(OH)_2$ nanopesticide significantly altered the levels of 20 metabolites ($p < 0.05$) (Figure S8). Even though the dose levels were lower for cucumber plants, the number of metabolites up- or down-regulated in cucumber leaves was greater than in maize leaves, in which 11 metabolites changed significantly. When the threshold p value is reduced to 0.01, eight metabolites were still significantly altered, with four down-regulated (threonic acid, 2,5-dihydroxypyrazine NIST, erythronic acid lactone, and glycerol-3-galactoside) and four up-regulated (cyanoalanine, citrulline, glucose-1-phosphate, and levoglucosan) (Figure 5). These significant alterations of the levels of these metabolites indicate they can be potential biomarkers of $Cu(OH)_2$ nanopesticide stress and may play an important role in increasing the tolerance of cucumber plant to similar stressors.

Cyanoalanine levels increased in a dose-dependent way with $Cu(OH)_2$ nanopesticide exposure. In leaves exposed to 25 mg of $Cu(OH)_2$, cyanoalanine levels were almost 3-fold greater than the control ($p < 0.01$). Previous studies have shown that abiotic stresses increase production of ethylene, a stress-related phytohormone,[42] and raise "stress cyanide" production.[43] β-Cyanoalanine is a byproduct of ethylene biosynthesis, and its role is to remove cyanide resulting from ethylene synthesis in plants because cyanide is a toxic chemical that readily binds to metalloenzymes, inhibiting primary metabolic processes. The up-regulation of cyanoalanine may be an indicator of activation of ethylene biosynthesis. Thus, this metabolite might be related to signal transduction.

$Cu(OH)_2$ nanopesticide at doses of 2.5 and 25 mg significantly increased the levels of citrulline (p = 0.045 and 0.028, respectively) in cucumber leaves (Figure 2). Citrulline is a precursor of polyamine biosynthesis. We also observed a significant increase in the levels of putrescine, an important polyamine, after exposure to 2.5 and 25 mg of $Cu(OH)_2$ nanopesticide (p = 0.088 and 0.004, respectively). It is reported that putrescine plays an important role in plant tolerance under stress conditions such as cold or drought.[44] Putrescine was shown to alleviate plant stress by reducing H_2O_2 and MDA levels, by increasing peroxidase and catalase enzyme activity and proline levels[45,46] Tun et al.[47] reported that putrescine levels were positively correlated with reduced levels of hydrogen peroxide and lipid peroxidation. Thus, the up-regulation of citrulline and putrescine may be a plant strategy to defend against oxidative stress and improve plant tolerance to this exposure.

It is noteworthy that oxalic acid started to respond even at a 2.5 mg of $Cu(OH)_2$ dose. The levels of oxalic acid in cucumber leaves treated with 2.5 and 25 mg of $Cu(OH)_2$ were 2-fold greater than the control. Oxalic acid is a strong copper chelator[48,49] It is possible that cucumber leaves up-regulate oxalic acid levels to chelate excess copper ions to reduce damage.

Down-regulation of threonic acid was also dose-dependent (Figure 5). The levels of threonic acid in cucumber leaves exposed to 25 mg of $Cu(OH)_2$ nanopesticide decreased significantly ($p < 0.01$) by 64% compared to the control. Threonic acid is an oxidized form of an important antioxidant, namely, ascorbic acid. The lower levels of threonic acid may indicate ascorbic acid has been decreased in tissues to defend against overproduction of ROS generated by $Cu(OH)_2$ and its transformation byproducts. An alternative explanation is that under stress conditions, threonic acid is oxidized to threarate,[50] a biochemically compatible compound[51] that increases in concentration to increase cellular osmolality.[52] The reduced

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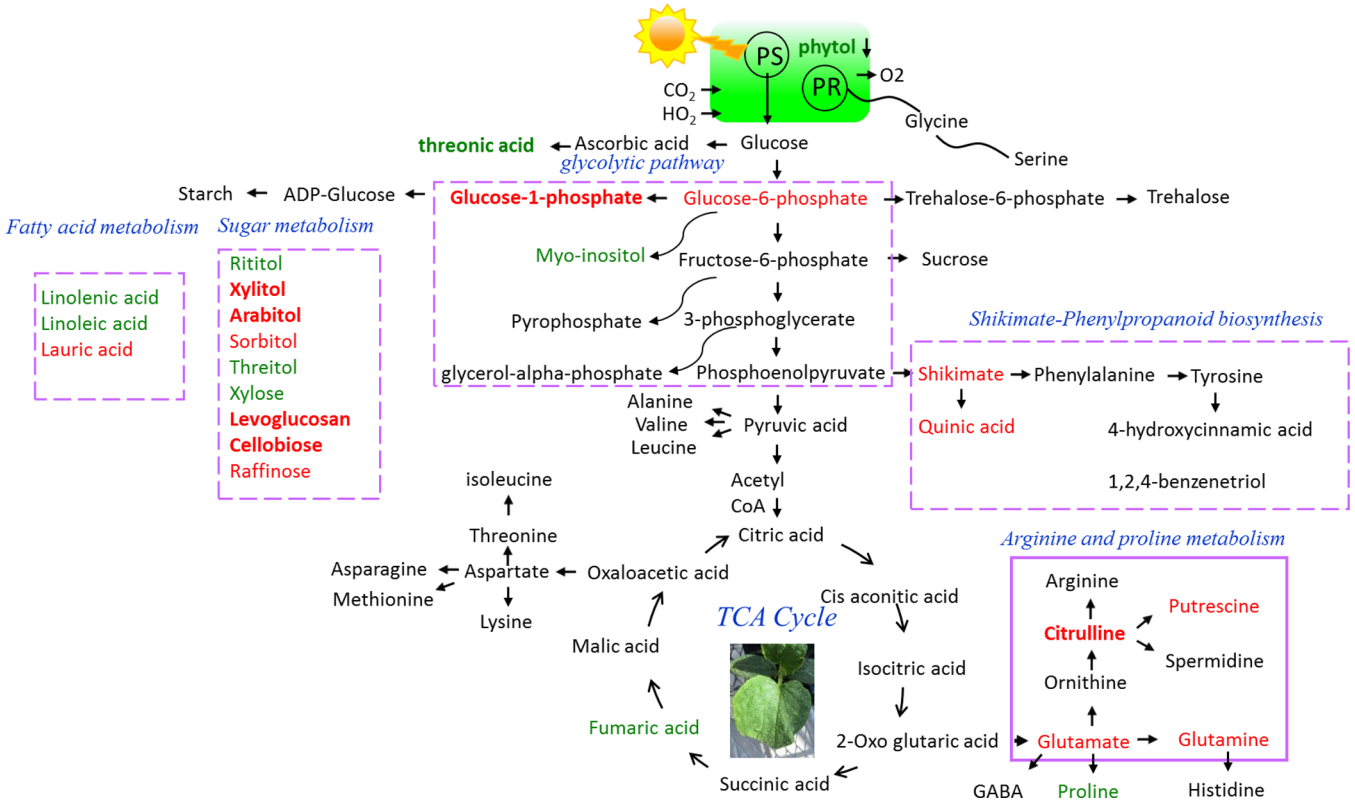

Figure 6. Schematic diagram of proposed metabolic pathways of cucumber. Central metabolic pathways (glycolytic pathway, TCA cycle, and shikimate-phenylpropanoid biosynthesis) and other metabolite biosynthetic pathways (sugar, amino acid, lipids, and fatty acid) are shown. Red and green circles indicate that the metabolites increased or decreased in response to $Cu(OH)_2$ nanopesticide.

level of threonic acid in cucumber leaves may reflect a protective response to $Cu(OH)_2$-induced ROS stress.

Pathway Mapping in Cucumber Leaves Exposed to $Cu(OH)_2$ Nanopesticide. MetaboAnalyst was used to determine the most statistically significant pathways. In contrast to the response of maize leaves, the arginine and proline metabolic pathway was the most significantly perturbed ($p < 0.05$) in cucumber leaves exposed to $Cu(OH)_2$ nanopesticide (Figure S9). Six metabolites in this pathway were up- or down-regulated including glutamate, proline, glutamine, aspartic acid, citrulline, putrescine, and fumaric acid. The up-regulation of putrescine may result in the observed down-regulation of proline because they share a common precursor (glutamate).

Global Metabolic Response of Cucumber Plant to $Cu(OH)_2$. In Figure 6, we present a more complete vision of the metabolite network changes for the central metabolism biosynthetic pathway, including the glycolysis and TCA cycle, shikimate-phenylpropanoid biosynthesis, and amino acid, fatty acid, and sugar metabolism. The intermediates of the TCA cycle were unchanged except for fumaric acid, which decreased in a dose-dependent way with exposure to $Cu(OH)_2$. Two glycolysis pathway intermediates (i.e., glucose-1-phosphate and glucose-6-phosphate) were up-regulated. Energy metabolism, in particular, glycolysis and the TCA cycle, generates significant amounts of energy transfer compounds (e.g., ATP and NADH) and precursors for various plant physiological processes through the oxidative decomposition of carbohydrates using a series of enzymes in the plant cell.[53] Because the TCA cycle was barely perturbed, this suggests that the incremental energy consumed by the defense system may come from the glycolysis pathway.

Sugar Metabolism. We found that the levels of rititol, xylitol, arabitol, sorbitol, and threitol were altered at the 25 mg dose level (Figure S7). These metabolites are sugar polyols from reduced forms of aldose and ketose sugars. Sugar polyols have been reported to act as osmoprotectants. The accumulation of sugar polyols may help to maintain cell hydration levels and cellular functions. Because cellobiose, xylose, and raffinose are polysaccharides located in the cell wall, the elevated levels of cellobiose and raffinose suggest that plant cell walls were decomposed to produce small sugar molecules such as osmolytes, to protect the cell membrane and plant proteins.[25] Xylose production was down-regulated after exposure to the $Cu(OH)_2$ nanopesticide. Therefore, the changes of compositions of sugar and sugar polyols maybe a protective mechanism for cucumber plants in response to $Cu(OH)_2$ nanopesticide.

Shikimate-Phenylpropanoid Biosynthesis. Aromatic amino acids (phenylalanine and tyrosine), serving as precursors for a wide range of secondary metabolites, were unchanged at both doses of $Cu(OH)_2$ nanopesticide. Interestingly, their precursor, shikimate, markedly increased 2-fold at 2.5 mg of Cu, but decreased when dosing increased to 25 mg. In addition, quinate, a metabolite synthesized in a lateral branch of the shikimate biosynthesis pathway, had a tendency very similar to that of shikimate. This indicates that low doses of $Cu(OH)_2$ may accelerate the synthesis of aromatic amino acids, but higher doses may inhibit the shikimate-phenylpropanoid pathway.

Fatty Acid Metabolism. In cucumber, exposure to 25 mg of $Cu(OH)_2$ nanopesticide resulted in the down-regulation of two unsaturated fatty acids (linolenic and linoleic acids) ($p < 0.05$). Lauric acid, a saturated fatty acid, was up-regulated ($p = 0.051$) at 25 mg of $Cu(OH)_2$ nanopesticide. Membrane fluidity is

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positively associated with the level of unsaturated fatty acids and is important for proper cellular metabolism and function.[54] Reduced levels of unsaturated fatty acids and increased saturated fatty acid levels may indicate an alteration of cell membrane structure in cucumber leaves.

Conclusions. Metabolomics demonstrated that both species undergo some metabolic reprograming in response to the $Cu(OH)_2$ nanopesticide, but the responses were largely distinct. In general, maize up-regulated some energy-related pathways (glycolytic pathway and TCA cycle) and the antioxidant defense-related shikimate-phenylpropanoid biosynthesis to cope with oxidative stress induced by $Cu(OH)_2$ nanopesticide. In contrast, cucumber plants up-regulated N metabolism (arginine and proline metabolism). Exposure to the nanopesticide resulted in fatty acid composition changes in both species, up-regulating saturated fatty acids and down-regulating unsaturated fatty acids. In addition, low molecular weight antioxidants appear to play an important role in an early defense system of maize in response to this stressor. In general, the metabolites that exhibited altered levels appear to play an important role in antioxidant defense and enhancing tolerance of these two crops to the $Cu(OH)_2$ nanopesticide. The more global view of the metabolic pathway network suggests that there are several pathways that are activated to minimize the impact but that there are significant differences between these two phenotypes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01306.

PCA and PLS-DA score plots of metabolic profiles in maize and cucumber leaves without exposure to $Cu(OH)_2$ nanopesticide (Figured S1 and S2); VIP scores from PLS-DA of control cucumber and maize leaves (Figure S3); VIP scores from PLS-DA of maize leaves metabolites (Figure S4); *t* test significantly changed metabolites in maize leaves in response to $Cu(OH)_2$ nanopesticide (Figure S5); perturbed biological pathway in maize leaves (Figure S6); VIP scores from PLS-DA of cucumber leaf metabolites (Figure S7); *t* test significantly changed metabolites in cucumber leaves in response to $Cu(OH)_2$ nanopesticide (Figure S8); perturbed biological pathway in cucumber leaves (Figure S9) PDF)

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Notes

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