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Authors

Zhang, Huiling
Du, Wenchao
Peralta-Videa, Jose R
et al.

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Metabolomics Reveals How Cucumber (*Cucumis sativus*) Reprograms Metabolites To Cope with Silver Ions and Silver Nanoparticle-Induced Oxidative Stress

Huiling Zhang,[†] Wenchao Du,[†] Jose R. Peralta-Videa,[‡] Jorge L. Gardea-Torresdey,[‡] Jason C. White,[§] Arturo Keller,[∥] Hongyan Guo,[†] Rong Ji,*[,†] and Lijuan Zhao*[,†]

[†]State Key Laboratory of Pollution Control and Resource Reuse, School of Environment, Nanjing University, Nanjing 210023, China

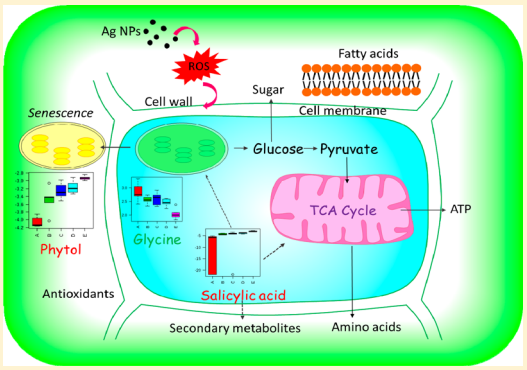
[‡]Chemistry Department, The University of Texas at El Paso, 500 West University Avenue, El Paso, Texas 79968, United States

[§]Department of Analytical Chemistry, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504, United States

[∥]Bren School of Environmental Science & Management, University of California, Santa Barbara, California 93106-5131, United States

Supporting Information

ABSTRACT: Due to their well-known antifungal activity, the intentional use of silver nanoparticles (AgNPs) as sustainable nanofungicides is expected to increase in agriculture. However, the impacts of AgNPs on plants must be critically evaluated to guarantee their safe use in food production. In this study, 4-week-old cucumber (*Cucumis sativus*) plants received a foliar application of AgNPs (4 or 40 mg/plant) or Ag^+ (0.04 or 0.4 mg/plant) for 7 days. Gas chromatography−mass spectrometry (GC-MS)=based nontarget metabolomics enabled the identification and quantification of 268 metabolites in cucumber leaves. Multivariate analysis revealed that all the treatments significantly altered the metabolite profile. Exposure to AgNPs resulted in metabolic reprogramming, including activation of antioxidant defense systems (upregulation of phenolic compounds) and downregulation of photosynthesis (upregulation of phytol). Additionally, AgNPs enhanced respiration (upregulation of tricarboxylic acid cycle intermediates), inhibited photorespiration (downregulation of glycine/serine ratio), altered membrane properties (upregulation of pentadecanoic and arachidonic acids, downregulation of linoleic and linolenic acids), and reduced inorganic nitrogen fixation (downregulation of glutamine and asparagine). Although Ag ions induced some of the same metabolic changes, alterations in the levels of carbazole, lactulose, raffinose, citraconic acid, lactamide, acetanilide, and *p*-benzoquinone were AgNP-specific. The results of this study offer new insight into the molecular mechanisms by which cucumber responds to AgNP exposure and provide important information to support the sustainable use of AgNPs in agriculture.



INTRODUCTION

Land degradation, declining soil organic matter, low nutrient use efficiency, and climate change are all serious challenges to modern agriculture, compromising our ability to produce sufficient food to support the world's ever-increasing population.[1] Nanotechnology is emerging as a promising strategy to sustainably increase food production[2] and has been used to develop new agrochemical products aimed at promoting plant growth and productivity.[3] Nanoenabled products can also help to reduce the amount of applied plant protection products (PPP) and fertilizers, thereby reducing environmental impacts while saving valuable water and energy inputs.

Given the resistance that a number of pests have exhibited toward traditional pesticides, silver nanoparticles (AgNPs) has begun received significant attention as a novel nanopesticide. AgNPs, with a broad spectrum of antimicrobial activity, have been found to effectively limit a number of plant diseases. Jo et al.[4] reported that Ag ions and AgNPs had a significant negative impact on the colony formation of two plant pathogenic fungi (*Bipolaris sorokiniana* and *Magnaporthe grisea*). Although the U.S. Environmental Protection Agency (EPA) granted a

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conditional registration for the first nanosilver pesticide, it should be noted that this original application was for textile use and not as a plant protection product.[5] Importantly, significant uncertainty remains concerning the application of AgNPs in agriculture. Therefore, research on the toxicity of AgNPs to nontarget species such as terrestrial plants is needed to ensure sustainable use in food production efforts.

The phytotoxicity of AgNPs to various species, including *Crambe abyssinica*,[6] *Arabidopsis*,[7] *Lycopersicum esculentum* and *Zea mays*,[8] *Cucurbita pepo*,[9] and *Phaseolus radiatus* and *Sorghum bicolor*,[10] has been reported in a number of studies in recent years. Most of this work has focused on the impact of exposure on physiological end points such as root elongation, transpiration rate, and photosynthesis. A smaller number of studies have sought to characterize the underlying toxicity and detoxification mechanisms of plants exposed to AgNPs. Using a microarray approach, Kaveh et al.[11] detected gene expression changes in *Arabidopsis thaliana* exposed to 5 mg/L AgNPs and observed that the thalianol biosynthetic pathway was involved in defense against stress. In addition, a number of reports have demonstrated that AgNPs trigger the overproduction of reactive oxygen species (ROS), causing oxidative stress in human hepatoma cells,[12] rat alveolar macrophages,[13] single-cell green algae,[14] and plants.[15] Others have addressed how plants alleviate AgNP-induced oxidative stress. Ma et al.[6] found that glutathione (GSH) and related peptides protect plants from Ag-induced nanotoxicity. Uncovering the mechanistic strategies that plants employ to defend against AgNP toxicity will be highly informative in efforts to sustainably use these materials as plant protection products.

Low molecular weight metabolites are the final product of gene expression; therefore, changes in a cell's metabolite profile may be a powerful strategy for assessing biological activities.[16] Metabolomics, defined as "the technology geared towards providing an essentially unbiased, comprehensive qualitative and quantitative overview of the metabolites present in an organism,"[17] has been shown to be a powerful tool to facilitate an understanding about how plants respond and alleviate various stressors at the molecular level.[18−20]

In this study, 4-week-old cucumber plants were foliar-exposed to AgNPs for 1 week. A control of $AgNO_3$ was included for an ion comparison. The measured physiological parameters included biomass, chlorophyll content, and lipid peroxidation. In addition, gas chromatography−mass spectrometry (GC-MS)-based metabolomics was used to provide new insight into the metabolic response of the exposed plants. A mechanistic assessment of phytotoxicity and detoxification mechanisms will not only advance understanding of environmental implications of these materials but also provide important baseline knowledge for the sustainable use of AgNPs in agriculture.

EXPERIMENTAL SECTION

Nanoparticles and Plant Growth. Ag nanopowder was procured from Pantian Nano Material Co., Ltd. (Shanghai, China). The original size was 20 nm. The hydrodynamic diameter of AgNPs in ultrapure water was 199.83 ± 10.15 nm at 10 mg/L and 174.67 ± 4.51 nm at 100 mg/L. The ζ potential was 6.23 ± 1.45 mV, measured via dynamic light scattering (Zetasizer Nano ZS, Malvern). The pH was 7.05 ± 0.03 for ultrapure water, 5.26 ± 0.05 for 10 mg/L AgNP solution, and 5.67 ± 0.04 for 100 mg/L AgNP solution. Equivalent silver salt ($AgNO_3$) was purchased from Sigma−Aldrich.

Cucumber (*Cucumis sativus*) seeds (Zhongnong 28 F1) were purchased from Hezhiyuan Seed Corp. (Shandong, China). Potting soil (Miracle-Gro, Beijing) with a nutrient composition of 0.68% N, 0.27% P_2O_5, and 0.36% K_2O was used in this study. The seeds were sown at a depth of 1 cm in plastic planters (14 cm × 14 cm × 13 cm). Plants were maintained in a greenhouse at a 28/20 °C day/night cycle. The relative humidity and illumination in the greenhouse were 60% and 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively.

Exposure Assay. Foliar exposure was initiated when the cucumber seedlings were 4 weeks old. Five treatments were established, including control (no Ag ions or AgNPs), 0.04 and 0.4 mg of Ag ion/plant, and 4 and 40 mg of AgNPs/plant. Previous study[7] and our preliminary experiments showed that approximately 1% Ag ions release from AgNPs over 7 days; therefore, 0.04 and 0.4 mg of Ag ion/plant was set up parallel to the 4 and 40 mg of AgNPs/plant treatments. Five replicate plants (one plant/pot) were grown for each treatment. The stock solution of 10 and 100 mg of AgNPs/L and 0.1 and 1 mg of $AgNO_3$/L were prepared in nanopure water. Before application, the AgNP suspension was sonicated (KH-100DB, Hechuang Utrasonic, Jiangsu, China) at 45 kHz for 30 min in cool water to approach a well-dispersed solution. The foliar application was made 3 times/day for a 7-day exposure period by use of a hand-held spray bottle, with total volume of 400 mL/plant during 7 days applied, yielding approximate total delivered masses of 0.04 and 0.4 mg of Ag ion/plant and 4 and 40 mg of AgNPs/plant.

Biomass and Silver Content Analysis. At harvest, plants were thoroughly washed with deionized water to remove any residual particles. Plants were separated into leaves, stems, and roots. It has to be pointed out that the petioles were not separated with blades and were counted as leaf biomass in this study. Tissues for metal content analysis were dried at 70 °C for 72 h. A sample of approximately 0.02 g of dried tissue was microwave-digested (Milestone Ethos Up) in a mixture of 8 mL of H_2O_2 and 2 mL of HNO_3 (4/1 v/v) at 160 °C for 40 min. The resulting digest was diluted to a final volume of 50 mL prior to analysis. Ag content was quantified by inductively coupled plasma mass spectrometry (ICP-MS) (NexION-300, PerkinElmer).

Lipid Peroxidation. Lipid peroxidation in leaves was measured by the thiobarbituric acid reactive substances (TBARS) assay.[21] Malondialdehyde (MSA), which is the final product of fatty acid degradation, is indicative of lipid peroxidation. Briefly, 0.2 g of fresh cucumber leaves was mixed with 4 mL of 0.1% trichloroacetic acid; the mixture was then centrifuged at 10 000 rpm for 15 min. A 1-mL aliquot of the supernatant was mixed with 2 mL of 20% trichloroacetic acid and 2 mL of 0.5% thiobarbituric acid (TBA), and then the mixture was heated in water bath at 95 °C for 30 min. After cooling, the UV absorbance was measured at 532 and 600 nm (UV-1800, Shimadzu Corp., Kyoto Japan). Lipid peroxidation was expressed as nicromoles of MDA equivalent per gram of fresh weight.

Metabolite Analysis in Cucumber Leaf Extracts. Leaf metabolites were analyzed by gas chromatography−mass spectrometry (GC-MS). Details on sample preparation, GC-MS analysis, and multivariate analysis are shown below.

Sample Preparation. At harvest, cucumber leaves were thoroughly rinsed with tap water and nanopure water to

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remove the residual soil or particles from the surface. Leaves were blotted dry with Kimwipes. Fresh leaves were ground into power in liquid nitrogen, and 60 mg of tissue was transferred to a 1.5 mL Eppendorf tube containing two small steel balls. Then 360 μL of cold methanol and 40 μL of 2-chloro-L-phenylalanine (0.3 mg/mL), dissolved in methanol as internal standard, were added to each sample. The samples were placed at −80 °C for 2 min and sonicated at 60 Hz for 2 min. An aliquot of 200 μL of chloroform was added to the samples, which were then vortexed and amended with 400 μL of water. The samples were vortexed again and ultrasonicated at ambient temperature for 30 min. The samples were centrifuged at 13900*g* for 10 min at 4 °C. A quality control (QC) sample was prepared by mixing aliquots of all samples (a pooled sample). A 300 μL aliquot of supernatant was transferred to a glass sampling vial for vacuum-drying at room temperature; 80 μL of 15 mg/mL methoxylamine hydrochloride in pyridine was then added. The resultant mixture was vortexed vigorously for 2 min and incubated at 37 °C for 90 min. Eighty microliters of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (with 1% trimethylchlorosilane) and 20 μL of *n*-hexane were then added, and the sample was vortexed vigorously for 2 min prior to derivatization at 70 °C for 60 min. The samples were placed at ambient temperature for 30 min before GC-MS analysis.

Gas Chromatographic–Mass Spectrometric Analysis. The derivatized samples were analyzed by using an Agilent 7890B gas chromatography system coupled to an Agilent 5977A mass-selective detector (Agilent Technologies Inc., Santa Clara, CA). The column employed was a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm; Agilent J&W Scientific, Folsom, CA, and Agilent Technologies, Santa Clara, CA). Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min through the column. The initial oven temperature was 60 °C, ramped to 125 °C at a rate of 8 °C/min, to 210 °C at a rate of 4 °C/min, to 270 °C at a rate of 5 °C/min, to 305 °C at a rate of 10 °C/min, and finally held at 305 °C for 3 min. The injection volume was 1 μL with the injector temperature 260 °C in splitless mode. The temperatures of MS quadrupole and ion source (electron impact) were set to 150 and 230 °C, respectively. The collision energy was 70 eV. Mass data were acquired in a full-scan mode (m/z 50−500), and the solvent delay time was set to 5 min. The QC samples were injected at regular intervals (every 10 samples) throughout the analytical run.

Multivariate Statistical Analysis. Unsupervised principal component analyses (PCA) and a supervised partial least-squares discriminant analysis (PLS-DA) clustering method were run on GC-MS data via online resources (http://www.metaboanalyst.ca/).[22] Before PCA and PLS-DA analysis, data normalization (normalization by sum) has been done for general-purpose adjustment for difference among samples, and data transformation (log transformation) was conducted to make individual features more comparable. PLS-DA uses a multiple linear regression technique to maximize the separation between groups; this helps to understand which variables carry the class-separating information.[23] 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.[23] A variable with a VIP greater than 1 is regarded as responsible for separation, defined as a discriminating metabolite in this study.[24] Biological pathway analysis was performed on GC-MS data by use of MetaboAnalyst 2.0.[25] The impact value threshold calculated for pathway identification was set at 0.1.[24]

RESULTS AND DISCUSSION

Accumulation of Silver Nanoparticles and Silver Ions and Effect on Physiological End Points. Exposure to AgNPs and Ag ions resulted in significant accumulation of Ag in cucumber tissues (Figure S1). It must be noted that the Ag detected in leaves is likely from the direct foliar application and includes Ag both attached to the leaf surface and in the tissues. However, incidental contamination of the soil during the foliar application process may have occurred, and as such, some fraction of Ag in the stem and root tissues could be the result of soil to plant transfer. However, such determination is out of the scope of this study.

Visible Symptoms. The 0.04 mg Ag^+ treatment did not induce overt toxicity in the leaves (Figure S2). However, the 0.4 mg Ag/plant treatment induced leaf yellowing by day 4, which is indicative of leaf chlorotic damage and senescence. AgNPs at both concentrations (4 and 40 mg/L) caused similar leaf damage, with the higher dose also causing dehydration (Figure S2).

Lipid Peroxidation. Malondialdehyde (MDA) content in cucumber leaves exposed to 4 and 40 mg of AgNPs significantly increased (28.6% and 44.93%, respectively; $p \leq 0.05$) as compared to the control (Figure 1). MDA is an end

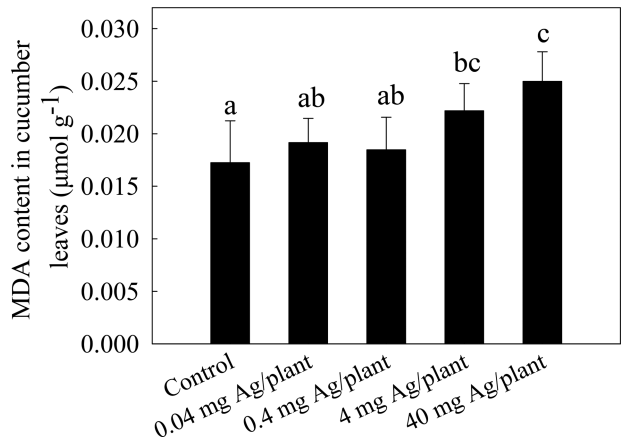

Figure 1. Biochemical detection of lipid peroxidation in cucumber leaves exposed to different doses of AgNPs and Ag^+ for 7 days. All data show the means of five replicates. Error bars represent standard deviation. Means with same letters are not significantly different [Tukey's honest significant difference (HSD) multiple comparison at $p < 0.05$].

product of polyunsaturated fatty acid oxidation, which directly reflects the extent of lipid damage induced by oxidative stress. Higher MDA levels are indicative of an increase in lipid peroxidation, especially in leaves that have high levels of polyunsaturated fatty acids.[26] Here, the MDA increase indicates potentially significant membrane damage as a function of AgNP exposure.

Importantly, the Ag ion treatment at both doses (0.04 and 4 mg/L) did not induce lipid peroxidation relative to the controls. Navabpour et al.[26] reported that silver nitrate resulted in increased lipid peroxidation and cellular damage in *A. thaliana*. Although this differs from our results, it should be noted that their dosing was 1 mM $AgNO_3$ (169 mg/L), which was 16.9 times higher than the 10 mg/L concentration used in

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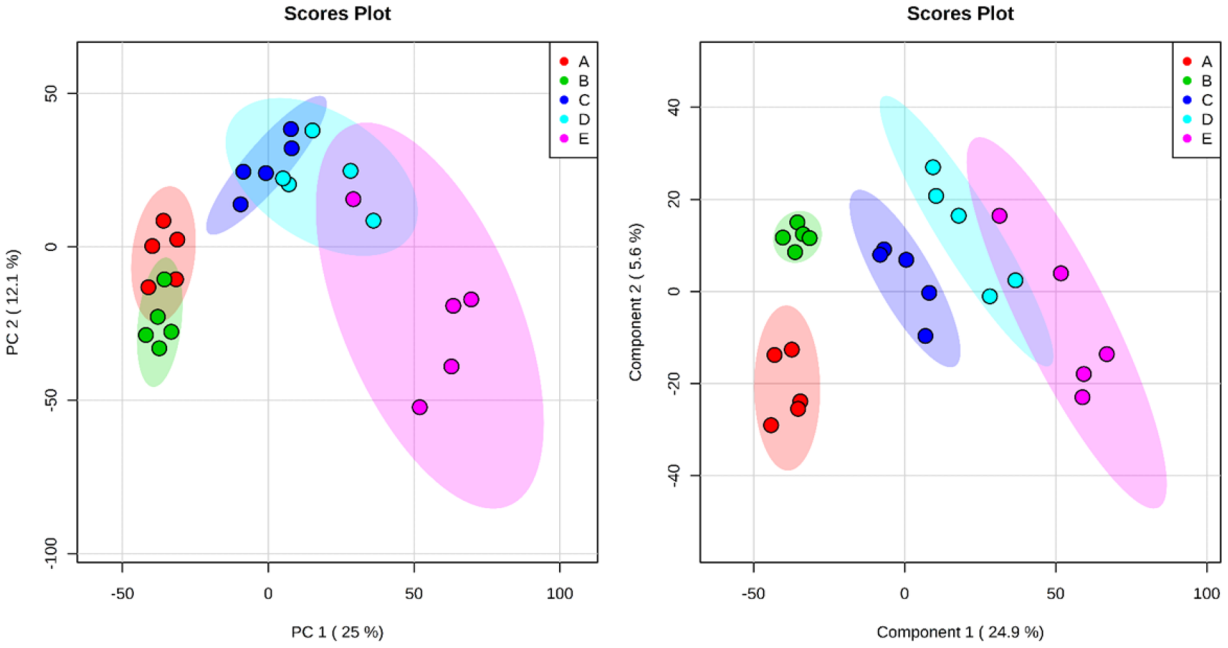

Figure 2. (Left) Principal component analysis (PCA) and (right) partial least-squares discriminate analysis (PLS-DA) score plots of metabolic profiles in cucumber leaves treated with different doses of AgNPs (4 and 40 mg/plant) and Ag^+ (0.04 and 0.4 mg/plant). A–E represent control, 0.04 mg and 0.4 mg of Ag ions, and 4 and 40 mg of AgNPs, respectively.

the current study. However, this does suggest that the damage noted in the AgNP treatment may be a result of ion release and may not be the result of nanoparticle exposure.

Biomass. Figure S3 presents the average fresh biomass of root, stem, and leaf and the total biomass of cucumber plants exposed to AgNPs or Ag^+ for 7 days. Although lipid peroxidation and visible toxicity symptoms were observed with the 4 and 40 mg AgNPs treatments, there were generally no significant changes in biomass of root, stem, and leaf compared with the controls and Ag^+/AgNP-treated plants, except that 100 mg/L AgNPs significantly ($p < 0.05$) decreased root biomass compared to other treatments (Figure S3, top). In addition, there were no difference between control and Ag groups in total biomass (Figure S3, bottom). This lack of correlation may highlight the more sensitive nature of end points that focus on molecular changes within the plant tissues as compared to more gross measures such as biomass.

Metabolic Response of Cucumber to Silver Nanoparticles and Silver Ions. The physiological alterations noted above are clearly the result of intracellular metabolic changes. A total of 268 metabolites were identified and semiquantified on the basis of their mass spectral fingerprints and retention-index matches. First, an unsupervised clustering method, principal component analysis (PCA), was performed on the GC-MS data by use of MetaboAnalyst, which provides a general overview of the clustering information between groups. This model was used without any sample designation.[27] The grouping of the samples in a PCA scores plot is based on the similarities between sample or treatment metabolic profiles.[28] The PCA scores plot (Figure 2, left) shows that there was no noticeable separation between the 0.04 mg of Ag ion group (group B) and the control (group A); however, the other treatments (0.4 mg of Ag^+, 4 and 40 mg of AgNPs) were clearly separated from the control group along the first principal component (PC1), which explained 25% of the total variance. Importantly, no clear separation was observed between 0.4 mg Ag^+ (group C) and 4 mg AgNPs (group D) in the PCA model. PLS-DA, a supervised clustering method, generally provides greater discrimination power compared to PCA.[29] To maximize the separation between groups, PLS-DA multivariate analysis based on the GC-MS data set was performed. The score plot (Figure 2, right) shows that all AgNPs and Ag^+ groups were clearly separate from the control and that this separation along PC1 was generally dose-dependent. These results indicate that both AgNPs and Ag^+ altered the metabolic profile of cucumber leaves. The responsive metabolites were subsequently isolated on the basis of VIP score >1. The 40 metabolites that lead to separation of the control and all Ag-treated groups are shown in Figure S4. In order to further discriminate Ag ion and NPs response, a metabolic data set of Ag^+ groups versus control and AgNPs versus control PLS-DA analyses were conducted separately. In addition, Ag ion-specific and AgNP-specific metabolites were screened out separately (Figures S5 and S6). Univariate statistical analysis (analysis of variance, ANOVA) was performed to detect metabolites significantly changed by AgNPs and Ag^+, which may be omitted by a multivariate analysis. The combined multivariate and univariate results for the differentially regulated metabolites as a function of Ag ions or AgNPs are listed in a visualized square (Figure S7). A significant overlap of differentially impacted metabolites was observed in response to AgNPs and Ag^+ (76 significant metabolite changes: 21 downregulated and 55 upregulated), suggesting that a significant fraction of AgNP-induced stress may originate predominantly from toxicity of the released ion. That being said, there were some metabolite changes that were NPs specific, which does indicates a nanoscale particle effect. These findings are consistent with Kavel et al.,[11] who note that both Ag ion and the NP contribute to the observed toxicity of AgNPs to *A. thaliana*. The metabolites that were significantly changed according to the PLS-DA and one way ANOVA (Figure S7) were then classified into different groups on the

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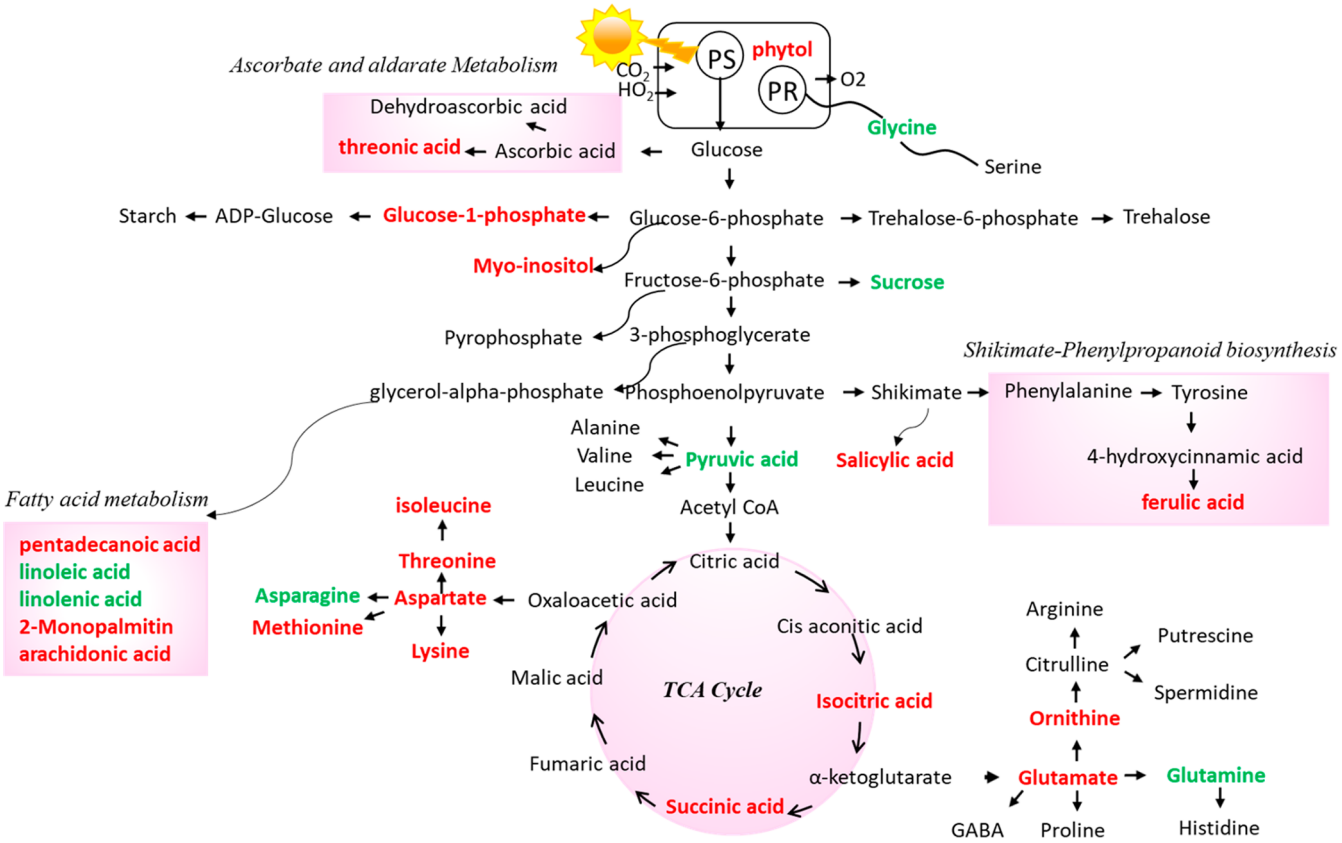

Figure 3. Schematic diagram of proposed metabolic pathways in cucumber leaves exposed to AgNPs. Red and green represent up- and downregulated metabolites, respectively.

basis of their metabolic functions and pathways; a discussion of these data follows.

Metabolites Changed by both Silver Ions and Silver Nanoparticles. Among the 93 significantly changed metabolites, 76 (nearly 82%) were due to both Ag^+ and AgNP exposure, highlighting the importance of ion release to plant response.

Phytol. Phytol, a degradation product of chlorophyll, was significantly increased (1.5−2.2-fold) by exposure to both Ag^+ and AgNPs in a dose-dependent manner (Figure 3). The increase in phytol accumulation is indicative of chlorophyll degradation. As noted, the increased MDA (Figure 1) suggests oxidative stress and lipid peroxidation. Plants have evolved convergent mechanisms to cope with stress that are, in general, energetically demanding.[30] Such energetic demands may require the disassembly of organelles or organelle components to overcome stress-induced damage and to adequately distribute cellular resources. Degradation of chloroplasts and recycling of their nutrients is known to be important under stress conditions and during leaf senescence.[30] It has been reported[28] that phytol from chlorophyll degradation is used in the biosynthesis of tocopherol, which is an important lipid-soluble antioxidant to protect lipids from oxidative damage. The degradation of chlorophyll to phytol could be an active protective mechanism for cucumber to combat oxidative stress. Phytol response to Cu-induced stress has been previously reported.[20] In addition, phytol has antimicrobial properties and is also induced by water stress,[31] which makes it a general biomarker of plant defense against stress.

Antioxidants: β-Glucosides and Phenolic Acids. Notably, arbutin and salicin are the metabolites with the highest VIP scores (Figure S4), indicating that both compounds contribute significantly to the separation between control and treated groups. Arbutin and salicin were not detected in unexposed control and 0.04 mg Ag^+ treatment. However, 0.4 mg of Ag ions and both AgNP treatments triggered significant ($p < 0.01$) upregulation in the production of these two compounds (Figure 4). Salicin and arbutin are alcoholic and phenolic glycosides, respectively, and they may participate in the defense of cucumber plant against Ag-induced stress, either as free radical scavengers or as signaling molecules.

In addition to glucosides, the presence of a number of aromatic compounds was increased by exposure to AgNPs and Ag ions (Figure 4). The aromatic compounds 4-hydroxyquinazoline (4-HQ), 3-hydroxybenzoic acid (phenolic acid), 1,2,4-benzenetriol (BT), and pyrogallol were significantly ($p < 0.05$) increased by all Ag/AgNP treatments, indicating that they are very sensitive to the presence of this metal. 4-HQ has been found to limit the increase of ROS produced by plasma membrane NADH oxidase.[32] 1,2,4-Benzenetriol (BT) is a polyphenolic metabolite of benzene and has been shown to significantly reduce the generation of ROS in H_2O_2-induced BV-2 cells.[33] Pyrogallol has also been reported to function as an antioxidant.[34] Taguri et al.[35] found that polyphenols having pyrogallol groups also have strong antibacterial activity. Thus, significant increases in the accumulation of these compounds are likely related to the quenching of oxidative stress and to general defense. Somewhat differently, *cis*-2-hydroxycinnamic acid, ferulic acid, and ethyl cinnamate (a modified ester of coumaric acid) were unchanged by 0.04 mg of Ag^+ but were significantly increased by the other Ag treatments (Figure 4). The upregulation of these aromatic compounds may be

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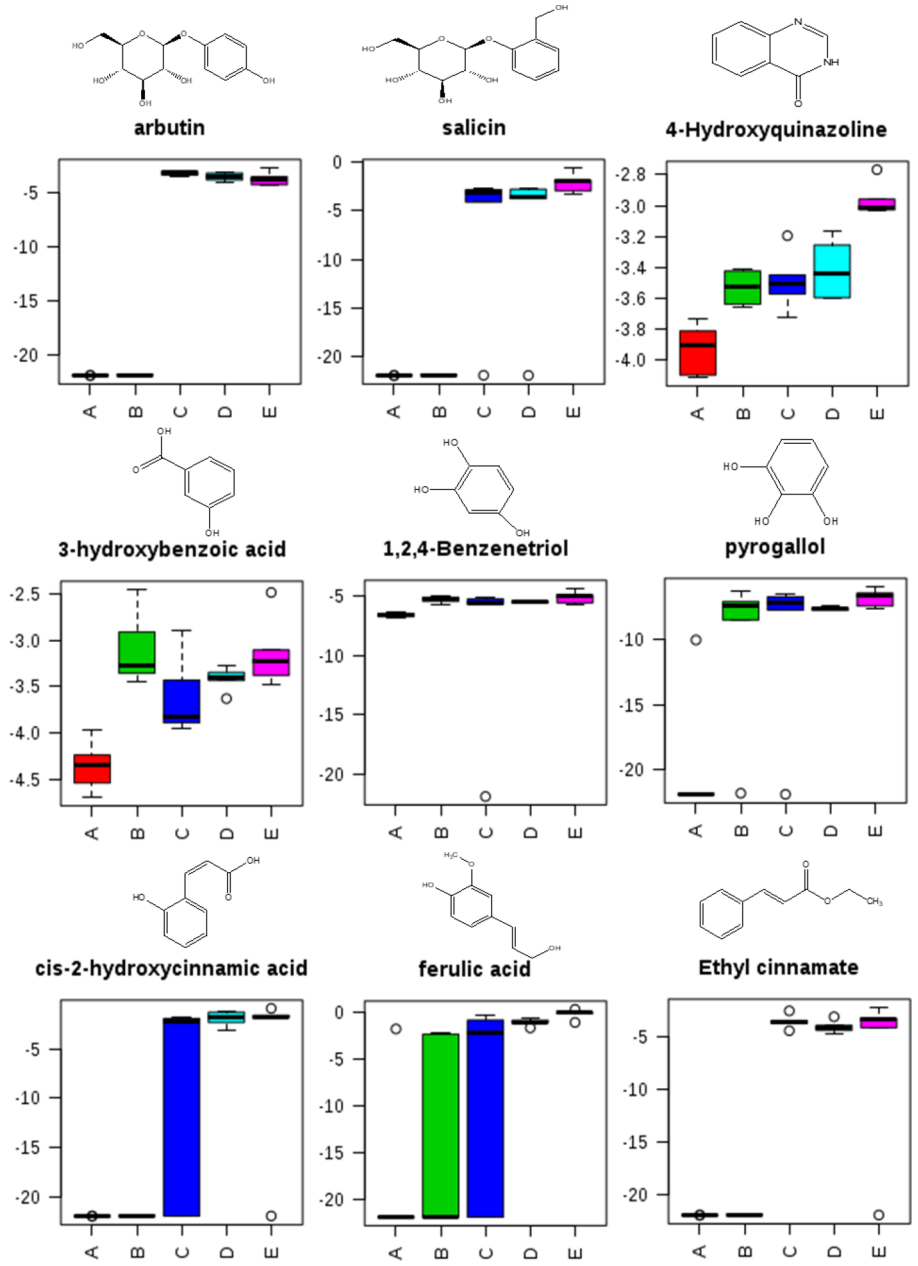

Figure 4. Box plots of relative abundance of antioxidant metabolites in 4-week-old cucumber leaves exposed to different doses of Ag^+ (0.04 and 0.4 mg/plant) and AgNPs (4 and 40 mg/plant) (n = 5). A−E represent control, 0.04 mg and 0.4 mg of Ag ions, and 4 and 40 mg of AgNPs, respectively.

another active protective mechanism employed by cucumber upon Ag stress.

The biosynthesis of antioxidant compounds is believed to result in the scavenging of excess ROS. Biological pathway analysis also reveals that phenylalanine metabolism, which is a stress response-related biological pathway, was disturbed at the dose of 40 mg of AgNPs, (Figure S8). The results clearly indicate that the antioxidant defense system was activated by AgNPs; this probably leads to a switching of cellular energy metabolism from growth to defense, thereby resulting in the accumulation of defense-related metabolites.[36]

Tricarboxylic Acid Cycle. Succinic acid, an intermediate in the tricarboxylic acid (TCA) cycle, was significantly increased in a dose-dependent manner with Ag exposure (Figure 5). With the exception of 0.04 mg of Ag^+, all other treatments significantly ($p < 0.05$) increased succinic levels (1.6−1.7-fold) in cucumber leaves. Isocitric acid, another TCA cycle intermediate, increased 2-fold upon exposure to 40 mg of AgNPs. The upregulation of TCA cycle intermediates may indicate activation of the TCA pathway; biological pathway analysis confirms that the TCA cycle was altered by the 40 mg of AgNPs treatment (Figure S8). The TCA cycle is the core of the cell's respiratory machinery; it is likely that plants upregulate respiration to produce energy for the manufacture of defense compounds needed to address oxidative stress.

Sugars and Sugar Alcohols. Sugars are important signaling molecules in the regulation of plant metabolism, and they are known to accumulate during stress, as well as in senescing leaves.[37] A significant increase ($p \leq 0.05$) in the accumulation of lyxose, levoglucosan, and gentiobiose was identified upon

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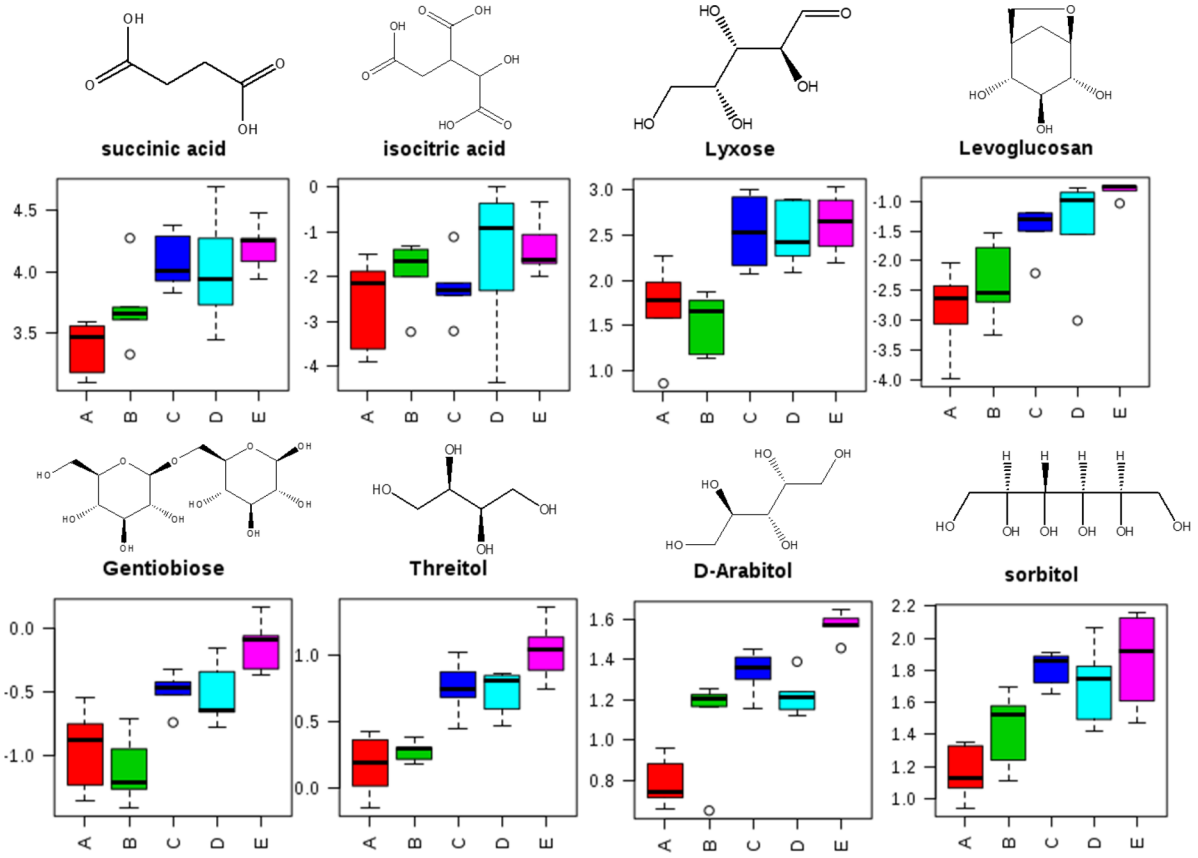

Figure 5. Box plots of relative abundance of carbohydrates in 4-week-old cucumber leaves exposed to different doses of Ag^+ (0.04 and 0.4 mg/plant) and AgNPs (4 and 40 mg/plant) (n = 5). A–E represent control, 0.04 mg and 0.4 mg of Ag ions, and 4 and 40 mg of AgNPs, respectively.

exposure to 0.4 mg of Ag^+, as well as 4 and 40 mg of AgNPs (Figure 5). In addition, several sugar alcohols, including threitol, D-arabitol, and sorbitol, were unaffected at 0.04 mg of Ag^+ but were significantly ($p \leq 0.05$) increased in a dose-dependent fashion with the other Ag treatments (Figure 5). The accumulation of sugar polyols is known to help maintain cellular hydration and functions during leaf senescence;[38] increased polyol content could possibly function in the stress tolerance of plants.

Nitrogen Metabolism: Amino Acids. Amino acids are essential components of plant primary metabolism and play an important role in plant physiological process: acting as osmolytes, modulating stomatal opening, and serving as precursors for the synthesis of defense-related metabolites and signaling metabolites.[39] Results showed that a number of amino acids, namely, ornithine, cycloleucine, norleucine, isoleucine, threonine, methionine, lysine, and β-alanine, were dramatically increased after Ag exposure (Figure 6). For example, cycloleucine was not detected in the control leaves, but levels were significantly increased in a dose-dependent fashion with Ag exposure. The increased accumulation of several amino acids suggests that nitrogen metabolism was disturbed by AgNPs or that significant protein degradation has occurred. One exception is *N*-methyl-DL-alanine, which was unchanged by exposure to 0.04 mg of Ag ions but then decreased in a dose-dependent manner with Ag ions and AgNPs. The reason for these observed changes is unknown.

Glutamine (Gln) and asparagine (Asn) are two important amides and were found unchanged by treatment with 0.04 mg of Ag ions; however, their content decreased in a dose-dependent manner with further treatment. Specifically, exposure to 40 mg of AgNPs resulted in the significant ($p \leq 0.05$) 4- and 15-fold decrease of Gln and Asn, respectively, compared to control. Gln and Asn are the main N-rich amino acids in leaves and are involved in fixing inorganic nitrogen;[40] the reduction in these two amino acids suggests that this important process may be disturbed.

Glycine content was decreased with treatment but serine levels were increased in a dose-dependent manner in Ag-exposed plants (Figure 3). Glycine (Gly) and serine (Ser) are two essential amino acids formed during photorespiration, and the Gly/Ser ratio is commonly used as an indicator of photorespiratory activity.[40] It is interesting to note that the Gly/Ser ratio decreased 3-fold upon exposure to 40 mg of AgNPs, suggesting that photorespiration was inhibited under this condition. Wingler et al.[37] suggested that the photorespiration pathway may provide additional protection against oxidative damage under high light-induced stress by supplying glycine, which can be used for synthesis of the broad defense molecule glutathione. Earlier reports also demonstrated that the Gly/Ser ratio is a sensitive biomarker of leaf senescence, changing significantly even prior to senescence symptoms.[40] In summary, AgNPs and Ag ions induced significant changes in the amino acid composition of cucumber leaves (Figure 3).

Fatty Acids. Pentadecanoic acid, a saturated phospholipid fatty acid, was significantly ($p \leq 0.05$) increased in a dose-dependent manner with both Ag ions and AgNPs (Figure 7). Pentadecanoic acid is a component of the phospholipid bilayer, and increases in its content may be indicative of membrane remodeling or synthesis to adapt to adverse conditions. Given

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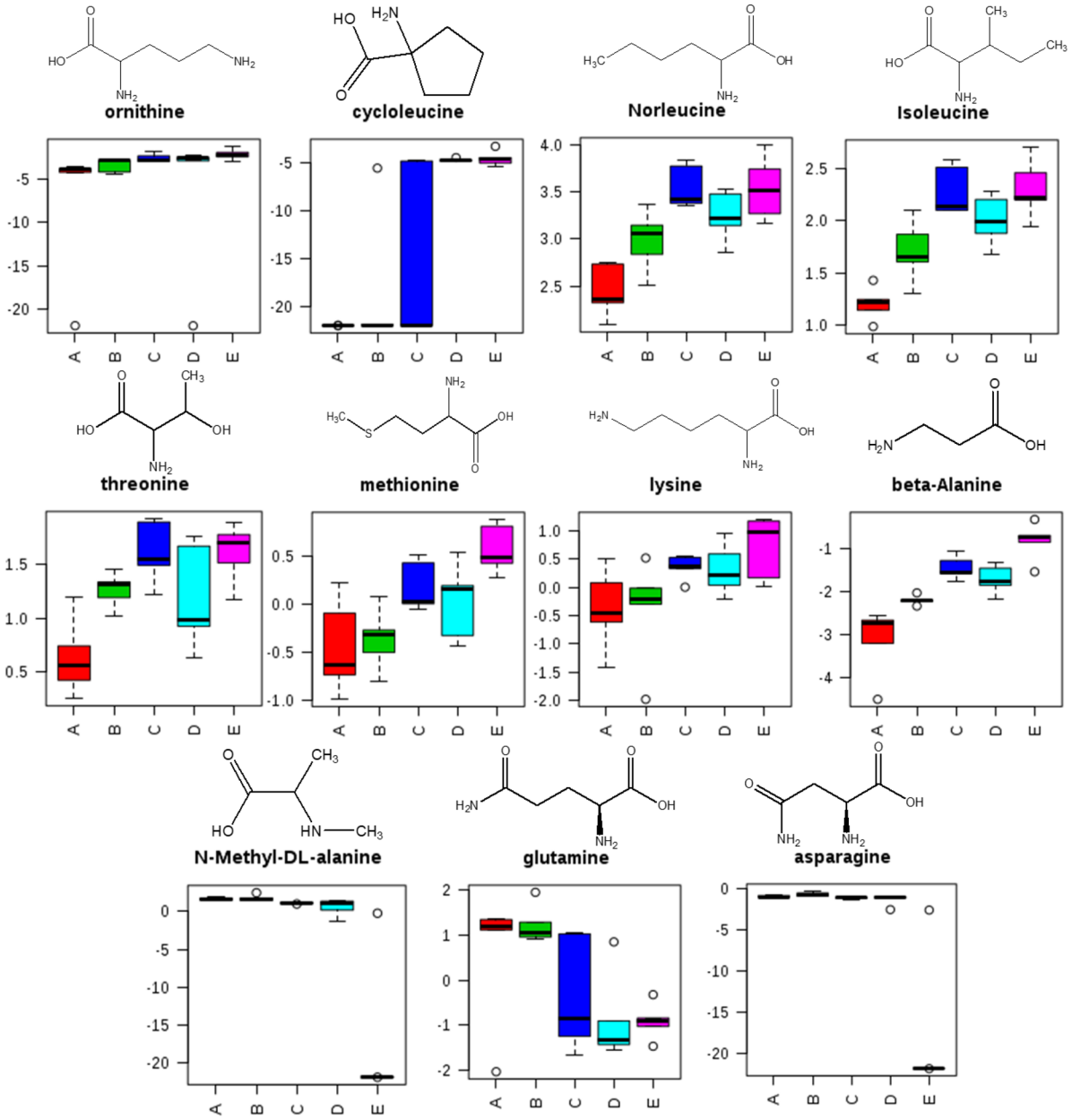

Figure 6. Box plots of relative abundance of amino acids in 4-week-old cucumber leaves exposed to different doses of Ag^+ (0.04 and 0.4 mg/plant) and AgNPs (4 and 40 mg/plant) (n = 5). A−E represent control, 0.04 mg and 0.4 mg of Ag ions, and 4 and 40 mg of AgNPs, respectively.

the increase in MDA content, the upregulation of pentadecanoic acid was possibly for repairing the damaged membrane. The unsaturated fatty acids linoleic and linolenic acids were unchanged by the 0.04 mg of Ag^+ treatment; however, levels were significantly ($p \leq 0.05$) decreased by the other Ag exposures with the 40 mg of AgNPs decrease being approximately 1-fold. It is known that as plants acclimate to biotic and abiotic stress, the response may include a remodeling of membrane fluidity by releasing α-linolenic (18:3) from membrane lipids.[41] This flexible adjustment of membrane fluidity maintains an environment suitable for the function of critical integral proteins during stress. Arachidonic acid, an unsaturated phospholipid fatty acid, was unaffected by 0.04 mg of Ag^+ but was significantly ($p \leq 0.05$) increased by the higher Ag^+ and the AgNP treatments by 1−6-fold. All of these metabolite changes are indicative of Ag-induced disruption of the composition and integrity of lipid membranes. Lipid peroxidation is a chain reaction and is created by free radicals influencing unsaturated fatty acids in cell membranes, leading to their damage. Clearly, one potential reason for the observed up- or downregulation of fatty acid metabolites is the result of lipid peroxidation. Another possibility is that plants adjust the membrane composition to rebuild membrane integrity and to restrict Ag ion permeation into cells. Phospholipids are composed of polar head groups, glycerides, and two fatty acyl chains. We found that diglycerol, the polar headgroup component of phospholipid, was significantly ($p \leq 0.05$) increased 2.3-fold upon exposure to 40 mg of AgNPs (Figure 7). These findings support the hypothesis that cucumber leaves altered intracellular metabolism in order to rebuild membrane integrity.

Salicylic Acid: Signaling Molecule and Plant Hormone. Unlike most metabolites, which were unaffected by 0.04 mg of Ag^+, salicylic acid (SA) was significantly ($p \leq 0.05$) increased

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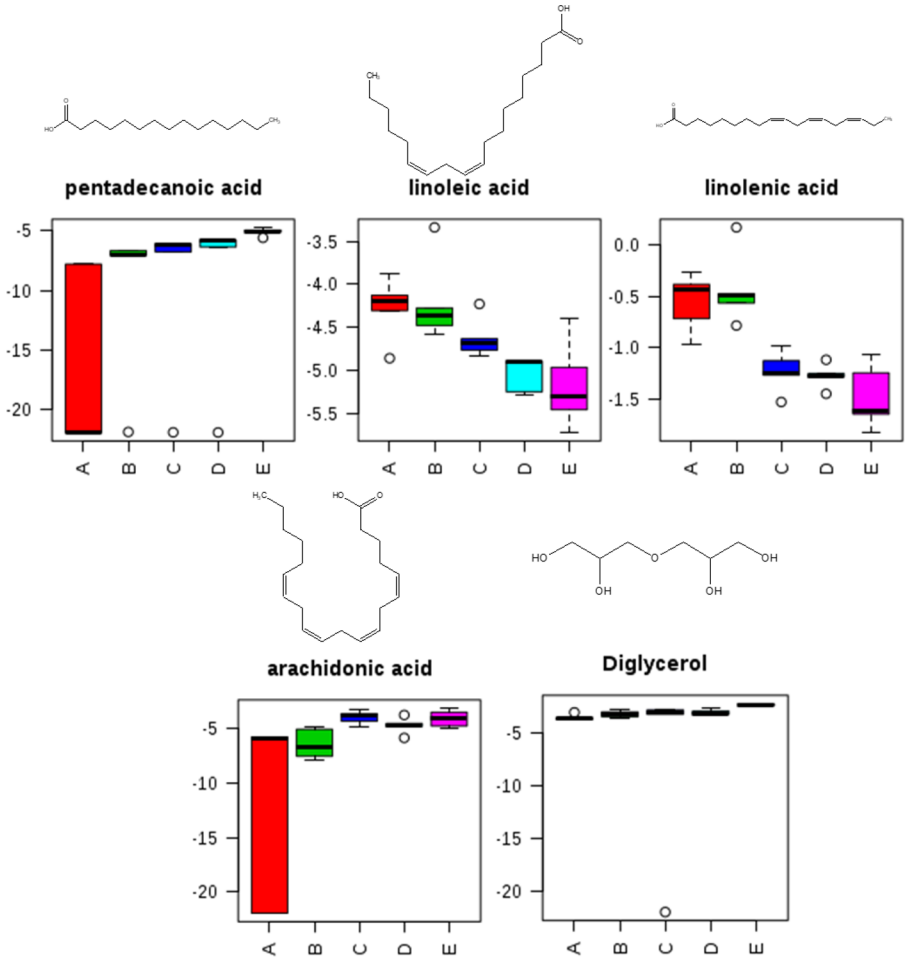

Figure 7. Box plots of relative abundance of fatty acids in 4-week-old cucumber leaves exposed to different doses of Ag^+ (0.04 and 0.4 mg/plant) and AgNPs (4 and 40 mg/plant) (n = 5). A−E represent control, 0.04 mg and 0.4 mg of Ag ions, and 4 and 40 mg of AgNPs, respectively.

at this lower ion dose and was affected in a dose-dependent manner by Ag content in tissues (Figure 3). Notably, a significant ($p \leq 0.01$) 8-fold increase in the accumulation of SA was noted upon exposure to 40 mg of AgNPs. SA is a plant hormone that plays an important role in plant defense. Previous evidence indicates that SA is a signaling molecule for the activation of plant defense response, including systemic acquired resistance (SAR).[42] Similarly, Shulaev et al.[29] demonstrated that SA produced as a result of stress can serve as a signaling molecule, activating systemic defense and acclimation responses. In addition to serving as a signaling molecule, SA has been reported to mediate the phenylpropanoid pathway and to play an important role against pathogens, some insect pests, and other abiotic stresses.[43] It has been reported that SA is synthesized at the site of wounding or pathogen infection in tobacco leaves, possibly serving as an endogenous signal for disease resistance.[44] Taken together, the upregulation of SA is clearly a broad defense-related behavior of cucumber in response to Ag-induced stress. In addition to signaling molecules, AgNPs also induced significant changes in defense-related secondary metabolites; some discussion of this can be found in Supporting Information.

Silver Nanoparticle-Specific Response. Importantly, some metabolites were up- or downregulated in response to AgNPs only, indicating a nanoscale size-specific effect on the plant. The metabolites upregulated specifically by AgNPs include carbazole, raffinose, lactulose, citraconic acid, aspartic acid, dithioerythritol, D-erythronolactone, and *N*-methyl-L-glutamic acid. Among these, carbazole, raffinose, lactulose, and citraconic acid were not detected in control and Ag ion-treated plants but were present at significant levels in AgNP-treated plants (Figure S10). Notably, *N*-methyl-L-glutamic acid increased 116-fold upon exposure to 40 mg of AgNPs as compared to unexposed control. Carbazoles are bioactive compounds with known antioxidative activity.[45] The polysaccharides raffinose and lactulose were not detected in the leaves of unexposed and Ag ion-treated plants; however, their levels were significantly increased at 40 mg of AgNPs. Raffinose is a sugar with no known energetic role; however, it has been reported to accumulate in response to a broad range of abiotic stressors such as drought, extreme temperatures, and salinity.[46] Nishizawa et al.[47] speculated that raffinose is possibly involved in membrane protection and radical scavenging in response to oxidative damage caused by salinity or chilling. Raffinose has been associated with reduction processes in oxidative membrane damage and ROS scavenging,[48] which may explain its increase concurrent with MDA upon exposure to 40 mg of AgNPs.

In addition, a number of metabolites were notably downregulated (8−17-fold change) specifically by AgNPs, including acetanilide, *p*-benzoquinone, 5,6-dihydrouracil, dibenzofuran, oxalic acid, oxamic acid, and lactamide (Figure S11). Remarkably, adrenaline became undetectable in response

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to AgNP exposure. Kaveh et al.[11] also reported that exposure of *A. thaliana* to AgNPs induced some nanoparticle-specific gene responses; the authors reported specific responses to AgNPs, including the induction of genes involved in defense against insects and pathogens (AT1G52040) and wounding (AT2G01520), which are believed to be related to mechanical damage to plant tissues caused by AgNPs. The reason for downregulation of those compounds in our current study is unknown.

Environmental Implications. This work has provided insight into the metabolic response of cucumber to exposure to AgNPs and Ag ions. Our data suggest that AgNPs at 4 and 40 mg/plant induced overt toxicity symptoms and significant lipid peroxidation in cucumber. Additionally, both doses resulted in alteration of the leaf metabolite profiles. A mechanistic understanding of AgNP toxicity is very important for future AgNP applications in agriculture. Metabolomic analysis proved to be a powerful tool and revealed that plants employ multiple strategies for increasing their tolerance of AgNP- or Ag ion-induced oxidative stress, including adjustment of membrane phospholipid composition, increasing the sugar and sugar alcohol levels, and activation of antioxidant defense pathways. These findings provide very valuable information for understanding the molecular mechanisms involved in plant response to AgNP-induced stress and will be useful to efforts at seeking to accurately characterize the risk of these materials in the environment, as well as to the development of sustainable nanoenabled plant protection strategies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b02440.

Additional text discussing other significantly changed secondary metabolites; 11 figures showing Ag concentrations in cucumber root, stem, and leaf, photographs of cucumber leaves with phytotoxicity symptoms, biomass of cucumber, PLS-DA analysis of cucumber leaves, lists and box plots of significantly changed metabolites, and biological pathway analysis (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*(R.J.) Tel: +86 025-8968 0581; fax: +86 025-8968 0581; e-mail: ji@nju.edu.cn.

*(L.Z.) Tel: +86 025-8968 0581; fax: +86 025-8968 0581; e-mail: ljzhao@nju.edu.cn.

ORCID

Jorge L. Gardea-Torresdey: 0000-0002-9467-0536

Jason C. White: 0000-0001-5001-8143

Arturo Keller: 0000-0002-7638-662X

Rong Ji: 0000-0002-1724-5253

Lijuan Zhao: 0000-0002-8481-0435

Notes

The authors declare no competing financial interest.

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