Molecular Response of Crop Plants to Engineered Nanomaterials

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Molecular Response of Crop Plants to Engineered Nanomaterials

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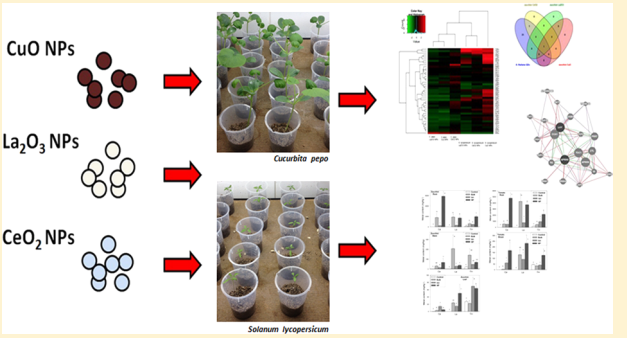
Supporting Information

ABSTRACT: Functional toxicology has enabled the identification of genes involved in conferring tolerance and sensitivity to engineered nanomaterial (ENM) exposure in the model plant *Arabidopsis thaliana* (L.) Heynh. Several genes were found to be involved in metabolic functions, stress response, transport, protein synthesis, and DNA repair. Consequently, analysis of physiological parameters, metal content (through ICP-MS quantification), and gene expression (by RT-qPCR) of *A. thaliana* orthologue genes were performed across different plant species of agronomic interest to highlight putative biomarkers of exposure and effect related to ENMs. This approach led to the identification of molecular markers in *Solanum lycopersicum* L. and *Cucurbita pepo* L. (tomato and zucchini) that might not only indicate exposure to ENMs (CuO, CeO_2, and La_2O_3) but also provide mechanistic insight into response to these materials. Through Gene Ontology (GO) analysis, the target genes were mapped in complex interatomic networks representing molecular pathways, cellular components, and biological processes involved in ENM response. The transcriptional response of 38 (out of 204) candidate genes studied varied according to particle type, size, and plant species. Importantly, some of the genes studied showed potential as biomarkers of ENM exposure and effect and may be useful for risk assessment in foods and in the environment.



INTRODUCTION

The application of nanotechnology has occurred across many sectors: health and medicine, communications and electronics, energy production, water treatment, and food production and agriculture. The increases have been exponential in the last 10 years, with even greater use predicted in the future.[1] There is a general consensus in the scientific community that our understanding of the fate and effects of these materials in the environment has lagged behind and is not adequate for accurate risk assessment. Important steps forward have been made in the last 3−4 years, especially on the properties that determine the behavior and the distribution of engineered nanomaterials (ENMs) in the environment[2−4] as well as the particle-size-dependent process of bioaccumulation and the trophic transfer within food chains.[5,6] The current state of knowledge regarding plants and ENMs interactions at both the physiological- and molecular-response level, including uptake, toxicity, and cellular compartmentalization, has been reviewed,[7,8] and there is now some understanding of which plant organs, tissues, cells, and organelles are involved in response, as well as of the molecular pathways associated with more general toxicity (e.g., DNA damage, ROS production, protein misfolding, etc.).[9] However, given the wide range of ENMs used (composition, size, shape, coating, etc.) and of their effects, it remains difficult to highlight consistent end points commonly shared in response to different classes of these materials. Unlike the situation for humans,[10] biomarkers for exposure, effects, and ENM susceptibility are unknown in plants. Typical biomarkers of exposure often involve the measurement of metabolites or other physiological parameters that reflect the biological dose and effect, showing directly or indirectly the physiological implications of exposure. Biomarkers of effects show changes at the cellular and molecular levels and reflect the expression of genes or the abundance of proteins under experimentally controlled conditions. Considering this potential, biomarkers of effects can be usefully applied as a tool for the assessment of toxicity.[11] Biomarkers of susceptibility indicate the constitutive responsiveness to contaminant exposure, such as through tolerance and resistance pathways as described for nonsensitive and

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hypersensitive phenotypes.[12] Some or all of these categories of biomarkers comprehensively reflect the "whole" organism response to ENM exposure and provide valuable knowledge for the determination of actual risk.[13,14]

Whole-genome studies performed in *Arabidopsis thaliana* (L.) Heyhn[15−17] revealed some of the main biological processes involved in the response to different ENMs. Marmiroli et al.[18] screened *A. thaliana* mutants for tolerance to cadmium sulfide quantum dots (CdS QDs) and subsequently combined physiological and genetic characterization of the phenotypes with a genome-wide transcriptomic analysis. A systems biology approach led to identification in the wild-type line (accession Ler-0) of approximately 200 impacted genes, including those involved in metabolic functions, detoxification and stress response, transport, protein synthesis, and DNA repair. This approach also enabled a determination of the mechanistic basis of CdS QDs tolerance and the key genes involved in the plant's response to exposure. Lastly, a comparison showed that the response to cadmium ions and to CdS QDs were clearly different at both the molecular and the physiological level.

A total of three different types of metal oxide nanoparticles (NPs) were used in the current study: copper oxide (CuO NPs), cerium oxide (CeO_2 NPs), and lanthanum oxide (La_2O_3 NPs). In addition, corresponding bulk and ion controls were included. These particles were chosen as model analytes given their properties and potential for wide-scale usage. CuO NPs are used in catalysis, superconducting materials, thermoelectric and sensing materials, propellant, glass, and ceramics.[19] In addition, CuO NP interactions with plant species of agronomic interest have been initiated.[20,21] CeO_2 NPs are utilized in catalysis, electrolyte and electrode materials, UV and infrared absorbents, and oxidation- and heat-resistant coatings.[22] Studies performed with CeO_2 NPs on *A. thaliana* and *Phaseolus vulgaris* L. showed variable effects on growth, physiological response, and nutritional quality.[23,24] La_2O_3 NPs are an emerging material used as a magnetic nanoparticle for electronic devices, in laser crystals and optics, and for catalysis, propellants, and biosensors. Recent studies have also focused on the toxicity and trophic transfer of La_2O_3 NPs.[25,26]

Data obtained from *A. thaliana* guided our comparative analysis in other plants, which can then provide important information required for assessing the environmental and public health risks related to ENM exposure. The primary aim of this work was the identification of biomarkers for exposure and effects in plants exposed to several ENMs. Previously identified candidate genes were tested in two species of agricultural interest, tomato and zucchini, whose genomes have been characterized.[27,28] This approach enabled us to use a diverse set of ENMs and plants, the intent being to identify genes consistently modulated regardless of particle type and species. Following ortholog identification, a transcriptional approach was applied to validate the "plant-specific" targets found in *A. thaliana* and to find genes commonly involved in response to ENMs; these genomic analyses were coupled with elemental and physiological analyses.

EXPERIMENTAL SECTION

Plants and NP Treatments. Copper oxide (CuO) nanopowder (99% purity, 40 nm particle size) and lanthanum oxide (La_2O_3) nanopowder (99.99% purity; 10−100 nm particle size range) were purchased from U.S. Research Nanomaterials, Inc. (Houston, TX). Cerium oxide (CeO_2) nanopowder (<25 nm particle size, BET) was purchased from Sigma-Aldrich (St. Louis, MO). NPs in deionized water were characterized for average particle size and ζ potential as well as by scanning and transmission electron microscopy (SEM−TEM) (Figure S1−S3). Equivalent bulk materials and metal salts (copper sulfate, cerium chloride, and lanthanum chloride) were purchased from Sigma-Aldrich.

Zucchini (*Cucurbita pepo* L., cv Costata Romanesco) seeds were purchased from Johnny's Selected Seeds (Albion, ME); tomato (*Solanum lycopersicum* L., cv Isis Candy Cherry) seeds were purchased from Seed Saver Exchange (Decorah, IA). Seeds were germinated in vermiculite for 7 d and were then transferred into 30 g of vermiculite containing a solution of 0 mg L^{-1} (untreated control) or 500 mg L^{-1} of bulk or NP CuO, La_2O_3, or CeO_2. In accordance with previous studies from the literature that highlight concerns over the greater availability and toxicity of select elements in the pure ionic form,[29] the concentration used for the metal salts was equal to the 10% of the metal content in NP and in bulk-material treatments: 71 mg L^{-1} of $CeCl_3$, 75 mg L^{-1} of $LaCl_3$, or 158 mg L^{-1} of $CuSO_4 \cdot 5H_2O$ was used. To minimize particle aggregation, we sonicated bulk and NP solutions by Fisher Scientific Model 505 Sonic Dismembrator (Fisher Scientific, Waltham, MA) at 40% amplitude for 60−120 s. For all experiments, seedlings were exposed for 21 d at 24 °C, a relative humidity of 30%, and under a 16 h photoperiod (light intensity 120 μM m^{-2} s^{-1} photosynthetic photon flux). Samples containing 10% Hoagland's solution (Phytotechnology Laboratories, Shawnee Mission, KS) in tap water were used to water the seedlings only after transplantation. A total of 10 replicates for each treatment were included for each species.

Physiological Analysis and Metal Content. Plants after 21 d of treatment with 0−500 mg L^{-1} NPs, bulk materials, or metal salts were harvested and thoroughly washed with deionized water to remove any vermiculite or residual particles. All plants were still undergoing vegetative growth at harvest. Primary root and shoot length and fresh mass were measured. Because of abundant biomass, zucchini shoots were divided into stems and leaves; for tomato, more limited biomass resulted in the analysis of whole shoot tissues. After being dried at 60 °C for 48 h, the tissues' dry mass was determined in five independent replicates. Digestion of 0.1 g of samples occurred in two steps: the first step involved 2.5 mL of 65% HNO_3 for 45 min at 115 °C, and then 1 mL of 30% H_2O_2 was added for 20 min at 115 °C. The digestion protocol was performed using a SCP Science DigiPREP MS digestor (SCP SCIENCE, Baie D'Urfé, Quebec, CND). The resulting solution was diluted to a final volume of 50 mL prior to analysis on an Agilent ICP-MS CE 7500 (Agilent Technologies, Santa Clara, CA). Ce (140 amu), La (139 amu), and Cu (63 amu) contents were quantified through a four-point calibration curve based on reference material standards (SPEX CertiPrep, Metuchen, NJ).

Gene-Expression Analysis. To compare the results obtained with that of *A. thaliana* under CdS QDs treatment,[18] we extracted total RNA from 0.1 g of fresh plant material (derived from the whole plant) using a Sigma-Aldrich Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO); a total of three independent biological replicates per treatment were used. Total RNA sample quality and quantity was assessed by a Thermo Scientific Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and gel electrophoresis. The process of two-step reverse transcription was performed on 1 μg of the total RNA extracted using the Qiagen QuantiTect Reverse Transcription kit (Qiagen, Velno, The Netherlands).

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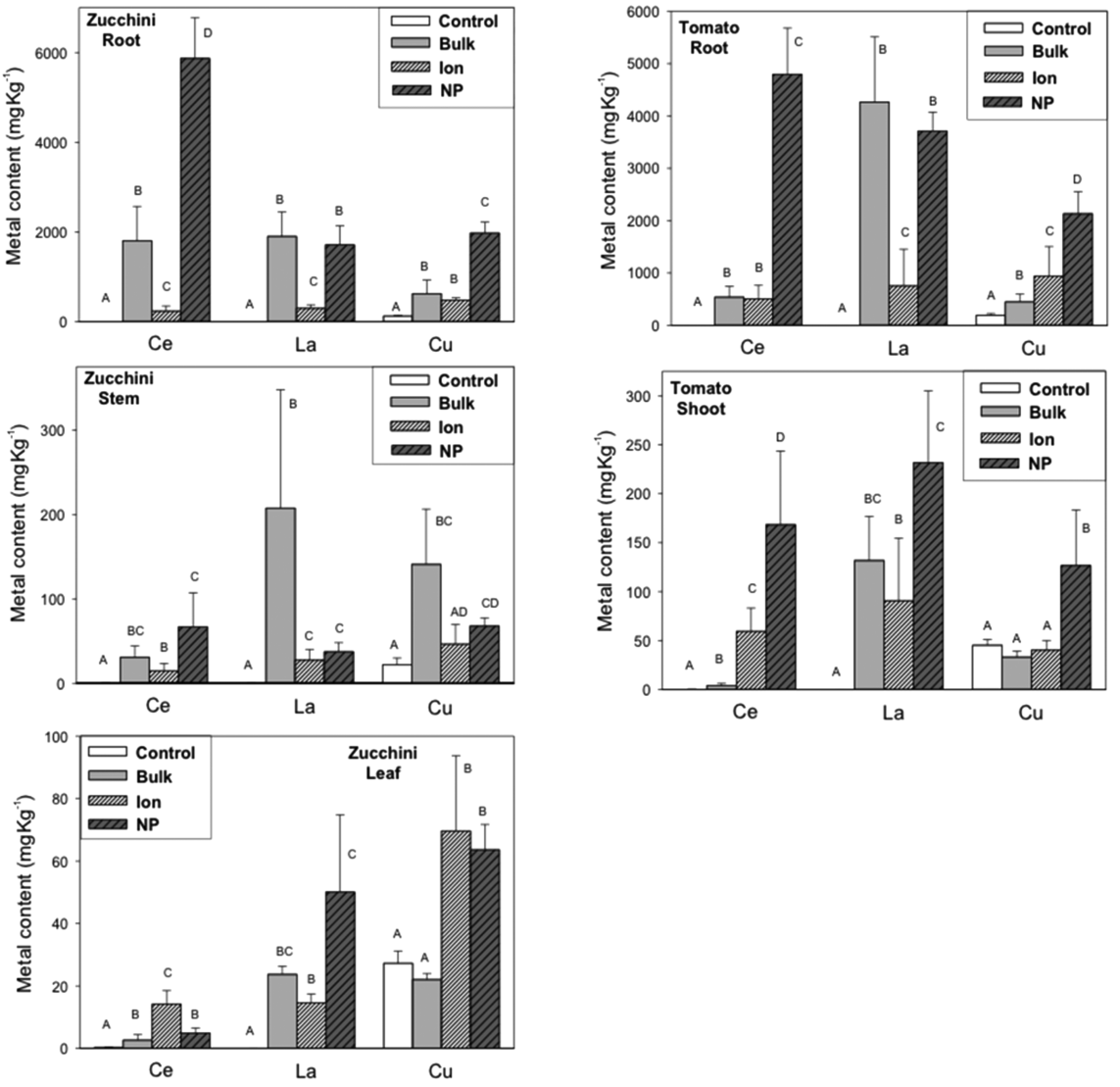

Figure 1. ICP-MS data related to Ce, La, and Cu content (mg kg^{-1}) from treatment with 0 or 500 mg L^{-1} bulk or NP CuO, La_2O_3, or CeO_2 in zucchini root, stem, and leaf tissues (left) and tomato roots and shoots (right). Metal salts at 10% of the metal content in NP and bulk-material treatments were used as ions: 71 mg L^{-1} of $CeCl_3$, 75 mg L^{-1} of $LaCl_3$, or 158 mg L^{-1} of $CuSO_4{\cdot}5H_2O$, respectively. Within a tissue and element, bars with different letters are significantly different (one-way ANOVA followed by a Tukey multiple comparison test on log-transformed data).

Reverse-transcription real-time PCR (RT-qPCR) was carried out using the Bio-Rad SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) in an optical 96 well plate with the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). On the basis of previous work,[18] *A. thaliana* ortholog gene coding sequences (CDS) were obtained through the BLAST tool of Cucurbigene database resource (http://cucurbigene.net/) and Sol Genomics Network database resource (http://solgenomics.net/) for *C. pepo* and *S. lycopersicum*, respectively. A $1{\cdot}e^{-20}$ (E-value) threshold with the query sequence (of *A. thaliana*) was used to identify the orthologous coding sequences in zucchini and tomato: a total of 46 orthologs were identified in both of the species (Table S1a). Specific primers for each selected gene transcript were designed (Table S1b) using the Primer3 software (primer3.-ut.ee); the thermal profile for RT-qPCR amplifications was: 95 °C for 10′, 95 °C for 15″, and 60 °C for 60″ for 40 cycles. Confirmation of the single amplicon in each reaction was performed by a dissociation-curve step. Relative expression was estimated through ΔΔCt method using *β*-actin of *C. pepo* and *S. lycopersicum* as the housekeeping gene.

Statistical and Network Analysis. For tissue-element content and biomass and root-length values, a two-tail Student *t* test was used in paired comparisons, and for grouped comparisons, data were log-transformed to achieve normality and were evaluated by a one-way ANOVA with all pairwise Tukey's multiple comparison test (Systat SigmaPlot 13.0 software). A two-tail Student *t* test was also applied to RT-qPCR results. The R software (https://www.r-project.org/) was used for the gene clustering and principal component analysis (PCA) of different treatments. Venn diagrams were generated by the Venny bioinformatics tool (http://bioinfogp.cnb.csic.es/tools/venny/). Network analysis, based on Gene Ontology (GO) classes, was performed using the GeneMANIA data service (http://www.genemania.org/) to highlight colocalization, coexpression, and physical or genetic interaction data regarding the genes studied.

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RESULTS AND DISCUSSION

Physiological Analyses. The physiological response of zucchini and tomato to NP exposure (500 mg L^{-1} of CuO, La_2O_3, or CeO_2 NPs) is shown in Tables S2−S4 and clearly varied by species. CuO, La_2O_3, and CeO_2 NPs had no effect on zucchini total biomass (fresh weight) as compared to untreated controls, whereas in tomatoes, plant biomass significantly decreased (59−78%) upon exposure to both CuO and La_2O_3 NPs (Table S2). A differential response dependent on plant type was also evident when measuring plant moisture content and root and shoot length. In tomatoes, root and shoot water content was unaffected by NP exposure, but the moisture content of zucchini leaves decreased significantly (20−22%) with CuO and La_2O_3 NP treatments (Table S3). Zucchini stems were increased (15%) by CeO_2 NPs; CuO NPs significantly increased (44%) root length. Conversely, in tomatoes, both CuO NPs and La_2O_3 NPs treatment significantly reduced root (68−75%) and shoot (42−47%) length as compared to untreated controls (Table S4). The different physiological response in the two horticultural species to ENMs was noteworthy, particularly with La_2O_3 and CuO NPs treatment. Zucchini seems to be largely unaffected by the treatments, whereas tomato was more sensitive to ENMs. These findings agree somewhat with previous work, in which several reports show that exposure to CeO_2 NPs did not impact physiological parameters in agricultural crops.[9] However, treatment with La_2O_3 or CuO NPs has been shown to inhibit root and shoot elongation and biomass as well as induce reactive-oxygen-species (ROS) production and programmed cell death.[21,30]

In zucchini, treatment with CuO, La_2O_3, CeO_2 (bulk materials) and $CeCl_3$, $LaCl_3$, and $CuSO_4$ had little effect on total biomass, with the only statistically significant decrease occurring under $CuSO_4$ treatment (38%) (Table S5). A significant decrease in moisture content was shown in stems of zucchini when treated with $CuSO_4$ and CuO (−21 and −24%), and CuO treatment led to a 33% increase in leaf moisture. Root length was also affected (−20 and −45%) by $LaCl_3$ and $CuSO_4$ (Tables S6a and S7a). Similar to what was observed for NPs, zucchini are largely unaffected by the bulk materials and metal salts, with the exception of $CuSO_4$. Conversely, in tomatoes, all metal salts ($CeCl_3$, $LaCl_3$, and $CuSO_4$) caused a significant decrease in biomass: −68% for $CeCl_3$, −56% for $LaCl_3$, and −64% for $CuSO_4$. Bulk La_2O_3 also reduced tomato biomass (−76%) (Table S5). Significant differences were not shown for tomato moisture content, but a significant decrease of tomato shoot length was evident after treatment with $CeCl_3$ (−35%), La_2O_3 (−36%), and CuO (−34%) as well as for roots (−48%) treated with CuO (Tables S6b and S7b). Thus, tomatoes were more sensitive to treatments with metal salts and with bulk La_2O_3, which agrees with the results from the La_2O_3 NP and CuO NP treatment; exposure to bulk or NP CeO_2 was generally less toxic.

Metal-Content Data. The Ce, La, and Cu content in zucchini roots and shoots is shown in Figure 1. Root content of all the three elements in both horticultural species was significantly increased by bulk, ion, and NP treatment. For the NPs, the Ce and Cu content did not vary significantly across species; zucchini and tomato root Ce content was 5870 and 4790 mg kg^{-1}, and root Cu content was 1970 and 2130 mg kg^{-1}, respectively. However, root La content under NP exposure differs significantly across the two plant species. Zucchini and tomato-root La levels were 1700 and 3710 mg kg^{-1}, respectively. Zucchini and tomato shoot Ce, La, and Cu content increased after NPs treatment, showing that the translocation of all three elements after NP exposure was quite high: in tomato shoots, the Ce, La, and Cu concentrations were 169, 232, and 127 mg kg^{-1}, respectively. For zucchini, stem Ce, La, and Cu concentrations under NP exposure were 66.8, 37.5, and 67.8 mg kg^{-1}, respectively; the leaf values were 4.92, 50.1, and 63.6 mg kg^{-1}, respectively. In the cells of zucchini leaves from CuO NPs exposed plants, SEM−TEM-EDS analysis showed the presence of Cu aggregates, including those in the nanometer size range (Figure S4; EDS data in Figure S5). Zucchini seems to effectively translocate La from roots to both stems and leaves, but for Ce, another element in the lanthanide series, the relative movement from the stems to the leaves was minimal. These Ce data disagree with previous findings[5] that showed that zucchini-leaf Ce content is greater than in stems. However, a comparison between the two studies is confounded by the different exposure conditions, including media (soil versus vermiculite), concentration, and duration. It is important to note that Ce and La accumulation in tomato shoots was 2.5-fold greater than that in zucchini; in the case of La, this correlated with the greater phytotoxicity observed in tomatoes upon exposure to La_2O_3 NPs.

ICP-MS analyses of bulk material and metals salts showed that particle size and element form significantly impacted content (Figure 1 and Tables S8−S9). For zucchini roots, Ce was present at 234 and 1805 mg kg^{-1} for $CeCl_3$ and bulk CeO_2, respectively; values of Ce are significantly lower than after NP exposure. In zucchini stems, the concentration of Ce was 14.8 and 30.6 mg kg^{-1} for ion and bulk treatment, respectively, whereas leaf content was 14.1 and 2.8 mg kg^{-1}. The ionic Ce was translocated equally in stem and leaves, as compared to the bulk and NP CeO_2 treatments, whereas leaf Ce content was lower than in stems. These results correlate with the decreased biomass observed in the Ce salt treatment. Analogous results are reported for La, with concentrations in zucchini roots after treatment with ion and bulk of 296 and 1805 mg kg^{-1}, respectively. Although the ion concentration was significantly lower than the NPs level, the bulk and NPs values are statistically equivalent. The stem content was 27.3 and 207 mg kg^{-1}, respectively, and the leaf content was 14.6 and 23.6 mg kg^{-1}, respectively. Similar to the La_2O_3 NP treatment, La translocation to the shoots varied little across all treatments, in agreement with the results from the physiological analyses, and further demonstrates the different in planta behavior of La and Ce.[30] Treatment with $CuSO_4$ and CuO (bulk) in zucchini produced a similar effect to the NP exposure, with Cu content several-fold lower than that of Ce. The root Cu content was 480 and 622 mg kg^{-1} for $CuSO_4$ and CuO (bulk) treatments, respectively; the Cu content in the NPs exposure was significantly greater than with bulk and ion. The concentrations reported in stems were 46.4 and 140 mg kg^{-1} and 69.6 and 22 mg kg^{-1} in the leaves. The evidence of an increased Cu leaf content for the ion treatment may partially explain a decrease in biomass after $CuSO_4$ exposure, although similar levels were detected after NP treatment, and no biomass effects were noted. Results in tomatoes for bulk and ion exposure are shown in Figure 1 and Table S9. Root Ce content was 503 and 508 mg kg^{-1} for $CeCl_3$ and CeO_2 (bulk) treatments; both levels were significantly lower than that of NP exposure. Roots La levels were 755 and 4268 mg kg^{-1} in plants treated with $LaCl_3$ and La_2O_3 (bulk), respectively; similarly to what was observed for

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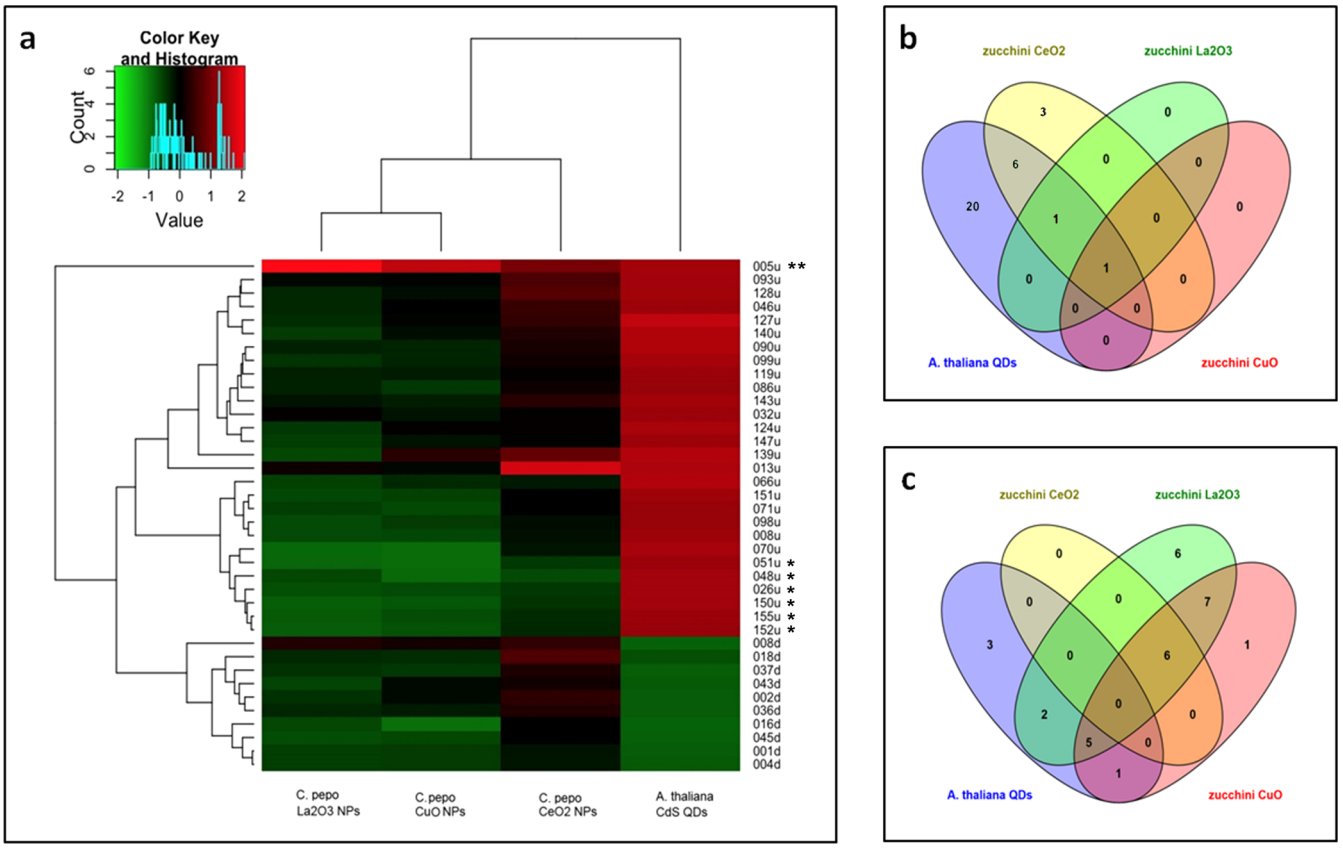

Figure 2. Comparison between *A. thaliana* and *C. pepo*. Heatmap (a) and Venn diagrams of the genes up-regulated (b) and down-regulated (c) of *A. thaliana* (Marmiroli et al., 2014) compared with *C. pepo* treated with 500 mg L^{-1} of CuO NPs, 500 mg L^{-1} La_2O_3 NPs, or 500 mg L^{-1} CeO_2 NPs. Signals were normalized on the untreated control (data not shown). In the heatmap, down-regulated genes are reported in green, whereas up-regulated genes are shown in red. Genes not significantly different from the expression levels of the untreated control are reported in black; (*), genes commonly regulated in all the treatments; (**), genes commonly regulated also with *A. thaliana*.

zucchini, La content in bulk and NPs treatments were statistically equivalent, but ion treatment resulted in lower La content. The Cu root content was 941 and 447 mg kg^{-1} for $CuSO_4$ and CuO (bulk) treatment, respectively. Levels of La after NP exposure were significantly higher. It was noted that La levels in tomato shoots exceeded those in zucchini, and this correlates with the observed physiological effects. With Ce in ionic and NPs form, this resulted in a greater translocation to the shoots than with bulk CeO_2 and correlated with observed toxicity. The literature supports these findings, where the phytotoxicity of La_2O_3 NPs is greater than CeO_2 NPs, likely caused by the higher level of dissolution and La^{3+} release that occurred in the plant (cucumber).[30,31] The lower dissolution rate of CeO_2 NPs correlates with the decreased level of translocation to the leaves and with the lower impact on plant growth and ROS generation.[31] Translocation of Cu after NP exposure was 3-fold higher than in bulk and ion treatments, in partial agreement with reports for alfalfa roots and shoots[21] and for maize.[32]

Gene-Expression Analysis of the ENM Treatments. Gene-expression analysis was conducted to identify up- and down-regulated genes for the two plant species treated with the three metals. Table S10 shows the genes of interest and the relevant literature for reference. From the 204 *A. thaliana* genes reported in Marmiroli et al. (2014),[18] 71 were isolated as zucchini orthologs, but only 46 were found both in zucchini and in tomatoes. Of those, a total of 38 orthologue genes were used for the study.

Expression analyses performed with RT-qPCR for treated and untreated zucchini are shown in Figure 2, along with results from Marmiroli et al. (2014)[18] for *A. thaliana* exposed to CdS QDs (Figure 2). Heatmaps (Figure 2a) and Venn diagrams (Figure 2b,c) showed a trend of general down-regulation upon exposure to La_2O_3 NPs or CuO NPs (500 mg L^{-1}) relative to untreated controls. Treatment with CeO_2 NPs produced few changes in the level of expression of most of the analyzed genes. It is also interesting that the expression profile for the treatments with the two lanthanides, La_2O_3 and CeO_2, were quite different. In fact, the response to La_2O_3 and CuO NP treatments were actually quite similar (Figure 2a), confirming results obtained in the physiological analyses (Figure 1 and Tables S2−S4). Of the 38 genes analyzed, 7 were simultaneously expressed (either up- or down-regulated) in zucchini across the CuO, La_2O_3, and CeO_2 NPs treatments (Figure 2). When we take into consideration the results from the *A. thaliana* treatments from Marmiroli et al. (2014),[18] only one gene was simultaneously expressed and modulated (up-regulated in this case) across all treatments (Figure 2b). Of the seven genes in common, one, 026u (*GTP2*), was down-regulated and encodes for a glucose 6-phosphate transporter 2 located in the chloroplast and in the vacuolar membrane. Another common down-regulated gene was 048u (*SNRK2-9*), which encodes a member of SNF1-related serine and threonine protein kinases that are calcium- and calmodulin-dependent and is involved in osmotic stress response. A third down-regulated gene was 051u (*PLP2*), which encodes for a lipid acyl

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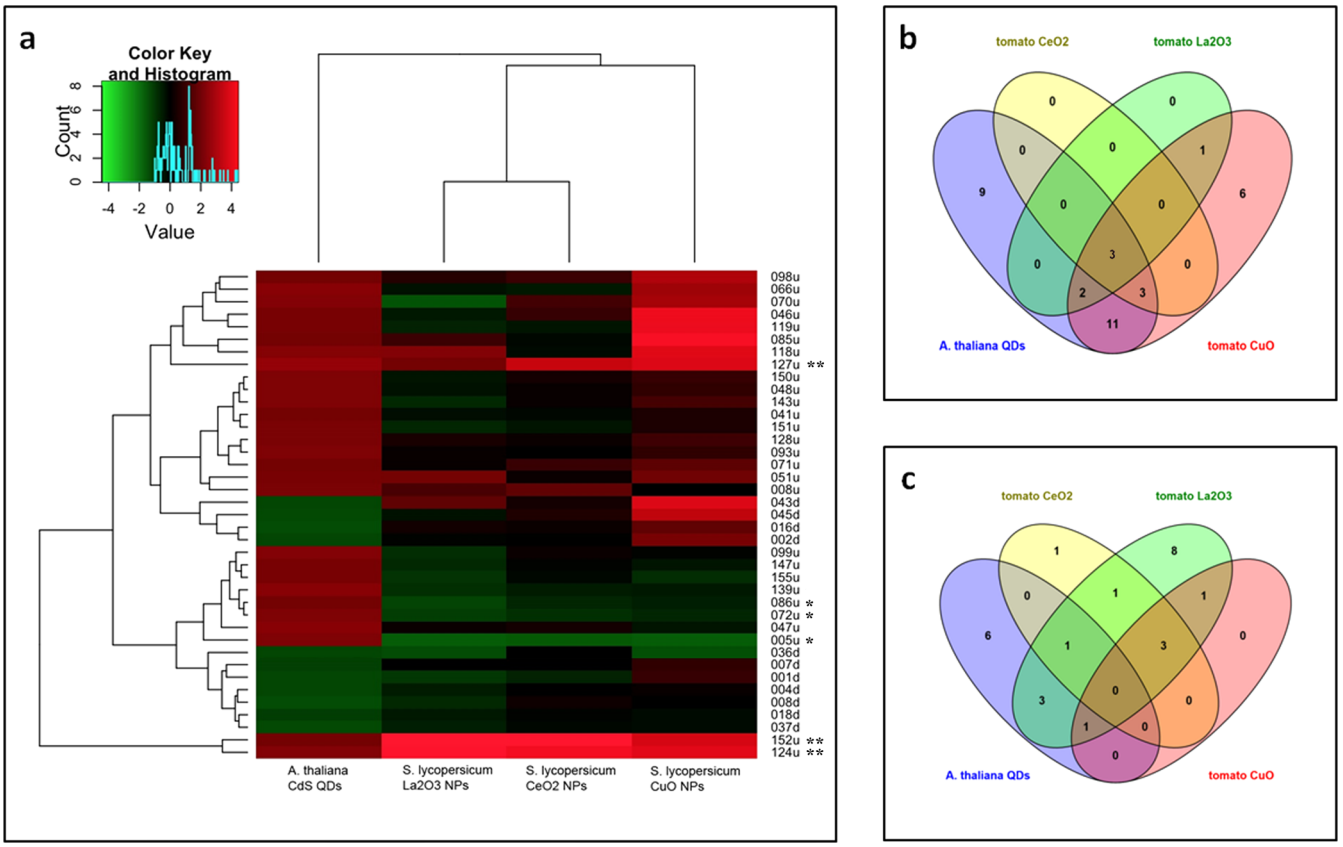

Figure 3. Comparison between *A. thaliana* and *S. lycopersicum*. Heatmap (a) and Venn diagrams of the genes up-regulated (b) and down-regulated (c) of *A. thaliana* (Marmiroli et al., 2014) compared with *S. lycopersicum* treated with 500 mg L^{-1} of CuO NPs, 500 mg L^{-1} La_2O_3 NPs, or 500 mg L^{-1} CeO_2 NPs. Signals were normalized on the untreated control (data not shown). In the heatmap, down-regulated genes are reported in green, whereas up-regulated genes are shown in red. Genes not significantly different from the expression levels of the untreated control are reported in black; (*), genes commonly regulated in all the treatments; (**), genes commonly regulated also with *A. thaliana*.

hydrolase with wide substrate specificity. This gene plays a role in cell death and contributes to resistance against viral infection and cadmium toxicity. The gene 150u (*RPS12A*) is a chloroplast gene that encodes a ribosomal protein S12. Gene 152u (*ORF31*) encodes for an electron carrier located in chloroplast endomembrane system and in the cytochrome b6f complex. In literature, *ORF31* was reported as highly responsive under high-level H_2O_2 conditions. The gene was coexpressed with *ZAT12*, a zinc-finger protein involved in abiotic stress response, such as heat and oxidative stress. These findings not only underlie the central role of the chloroplast in cellular response to ROS and abiotic stress (Figure S6) but also the putative role of *ORF31* as a biomarker of abiotic stress response, such as during ENM treatment. Gene 155u (*PSBN*) encodes a photosystem II (PSII) low-molecular-weight protein that is located on thylakoid membrane. Several chloroplast genes are among those commonly down-regulated by NP exposure. The only gene up-regulated in all four the treatments (including *A. thaliana*) was 005u (*BIP3*), which encodes for Heat Shock Protein 70 (HSP70) with ATP-binding function. This gene is involved in protein folding, as well as response to heat stress and pollen-tube growth, and is located in the lumen of the endoplasmic reticulum. In *A. thaliana*, *BIP3* is also involved in transcriptional regulation as a part of the mediator complex. As shown in the gene network reported in Figure S7, this gene also physically interacts with BZIP28, a putative membrane-tethered transcriptional factor that is up-regulated in response to heat. BIP3 gene product also physically interacts with ERDJ3A and ERDJ3B, two DNAJ domain proteins strictly related to the modulation of heat stress response and pollen germination. *BIP3* is also coexpressed in *A. thaliana* with *PNP-A* (Plant Natriuretic Peptide A), which was reported in Marmiroli et al. (2014)[18] as one of the more differentially expressed genes in *A. thaliana* CdS QDs resistant mutant *atnp01* (Figure S7). PNPs are a class of systemically mobile molecules that may function as a component of plant defense response and systemic acquired resistance (SAR).

Results from the RT-qPCR analysis of treated and untreated tomato are shown in Figure 3, along with results from Marmiroli et al. (2014)[18] for *A. thaliana* exposed to CdS QDs. Different from the response observed in *C. pepo*, treatments with the lanthanide CeO_2 and La_2O_3 NPs gave quite similar results in terms of differential gene expression. Tomato response to CuO NPs produced a more consistent pattern of up-regulation. For tomatoes, a total of six genes were up- or down-regulated with the CeO_2, La_2O_3, and CuO NPs treatments; three of these genes (Figure 2) were also up-regulated in *A. thaliana* from Marmiroli et al. (2014).[18] The first down-regulated gene was 072u (*SKS13*), which encodes for an endomembrane system protein involved in oxidoreductase activity and copper-ion binding; *SKS13* was down-regulated in all Cu treatments (NPs, bulk, and ion), suggesting that it may not be specifically involved in response to ENM exposure. A second down-regulated gene was 086u (*At3g59845*), which encodes a cytosolic Zn^{2+} ion binding dehydrogenase family protein known to be involved in the

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response to oxidative stress. Similar to zucchini, 005u (*BIP3*) was commonly down-regulated in tomatoes across the three treatments, suggesting a possible central role in response to EMN exposure. Regarding genes commonly overexpressed in tomatoes and *A. thaliana*, the first one is 152u (*ORF31*), which was also up-regulated in zucchini exposures. A second gene commonly up-regulated in tomatoes and *A. thaliana* was 124u (*PRR5*), which codes for a pseudoresponse regulator whose mutation affects circadian-associated biological events, acting as a transcriptional repressor of *CCA1* and *LHY* (001d). As observed in *A. thaliana* (Figure S8), 001d was down-regulated in tomatoes upon exposure to CeO_2 and La_2O_3 NPs (Figure 3a). *LHY* (Late Elongated Hypocotyl) and *CCA1* (Circadian Clock Associated1) belong to the MYB-transcription-factor family and are involved in the regulation of circadian rhythm and oxidative stress response in plants. The importance of this finding is reinforced by the activity of *At1g13880* (*ELM2*), a gene found to be a key element in the tolerance and resistance of the CdS QDs of *A. thaliana* mutant *atnp01*; this gene is also a putative MYB-transcription factor, highlighting the central role of this transcriptional-regulator family in response to ENMs. The gene 127u (*GGCT2;1*) was up-regulated in tomatoes across all exposures (as well as in *A. thaliana*); this gene encodes a γ-glutamyl cyclotransferase 2;1 (GGCT2;1) that belongs to the ChaC-like family protein and is involved in Cd^{2+} and Pb^{2+} response. It is interesting to observe that some of the genes differentially modulated in the two species (*GTP2*, *PLP2*, and *BIP3* and *SNRK2-9*, *LHY*, and *GGCT2;1*, respectively) were reported in literature as coexpressed in *A. thaliana* under hypoxia and bacterial infection conditions (Table S10).

Comparison of the results for tomato and zucchini samples exposed to CeO_2, La_2O_3, and CuO NPs treatments with that of *A. thaliana* exposed to CdS quantum dots shows that in spite of a large phylogenetic distance, the general response of tomatoes is more similar to *A. thaliana* than it is with zucchini plants (Figure S9). In both zucchini and tomatoes, treatment with CeO_2 NPs caused only small changes in gene expression, and this agrees with the physiological responses, which showed that CeO_2 NPs were significantly less toxic than La_2O_3 and CuO NPs. These findings agree with soil-based studies showing greater La_2O_3 phytotoxicity and La accumulation in exposed plants.[26] Interestingly, La_2O_3 had different effects on the two plant species at both the physiological and the genetic level. These findings correlate with published data on response to ENM exposure, both with regard to species-specific response (one material, many species) and with different nanomaterials on a single species.[9,15,33] With regard to CuO NPs, the different effects observed in tomato and zucchini plants indicate two different response pathways to the same exposure: tomatoes respond with a general up-regulation of genes mainly involved in copper and cadmium response (similar to what was seen in *A. thaliana* upon treatment with CdS QDs), whereas the zucchini response showed a general down-regulation of nearly all genes studied.

Considering the three plant species (zucchini, tomato, *A. thaliana*) and different ENM exposures, only two genes were consistently modulated, although in opposite ways (according to *A. thaliana* results): *BIP3* (005u) and *ORF31* (152u) (Figure S9SI). Their biological interactions, as described in SI Figure S6−S7, point out how the two genes are involved in abiotic stress response (not only to heat and oxidative stress but potentially also to ENMs exposure; Table S10) and chloroplast functionality and appear to play a central role in ENMs toxicity and response;[34,35] these genes can be considered potential biomarkers for ENM exposure. Principal component analysis (PCA) performed on zucchini and tomato in different treatments (Figure S10) showed the major components of variability to be related to a "plant-specific" response (response of zucchini and tomato) and to a "nanospecific" response (gene-expression level related to the treatment); these represent 80.8% of the total variance observed. Again, among the genes analyzed, 005u (*BIP3*), 152u (*ORF31*), and 124u (*PPR5*) were the most responsive out of the 38 genes analyzed in both species, representing the major determiners of the variance observed.

Comparison with Bulk and Ion. RT-qPCR on the putative biomarkers isolated from the NP exposure (Figures 2 and 3) was tested on the two species upon exposure to bulk CuO, La_2O_3, and CeO_2, as well as to the corresponding metal salts. Of the seven simultaneously expressed genes in zucchini (Figure 2), the three NP treatments all group separately from the corresponding bulk and ionic exposures. This ENMs difference is clearly greatest for NP CuO, followed by La_2O_3, suggesting that at least for these seven genes, much of the effects observed with NP exposure are indeed "nanospecific" (Figure S11). For CeO_2, the bulk and ion transcriptomic response groups fairly closely to NP, indicating that for these selected genes, much of the differential response shown in Figure 2 is traceable to Ce exposure in general and therefore not size-specific. In addition, *BIP3* (005u) and *ORF31* (152u) were the only two genes in which the expression level was significantly different across all genes for the NP exposure (Figure S11). There is a potential for 005u and 152u to serves as candidate biomarker genes for NP exposure in zucchini and possibly other related plants.

The scenario was different when we considered the transcriptomic response of tomato in the presence of NP, bulk, and ion treatments (Figure S12). As with zucchini, CuO NPs caused a response separate from the two other forms of Cu, suggesting that much of the change (at least in these seven genes) with the exposure to ENMs is size-specific and likely mechanistically distinct from the Cu ion. Unlike zucchini, the two lanthanides behave differently, with bulk and NP La_2O_3 grouping more closely together and CeO_2 having a more significant "nanospecific" effect on transcription. Specifically, *BIP3* (005u), *SKS13* (072u), and 086u (Zn^{2+}-ion-binding dehydrogenase) were consistently down-regulated across all the treatments. 152u (*ORF31*) was up-regulated in tomatoes upon exposure to NPs CeO_2, La_2O_3, and CuO but also in the presence of bulk CuO, $CuSO_4$, and $LaCl_3$. The overall picture observed in tomatoes seems to be more complex than in zucchini and will require further analyses and subsequent additional study to be fully understood, including exposures at lower NPs concentrations and in soil-based systems over longer periods of time.

"Nanospecific" Response. This study elucidates the involvement of some of the molecular mechanisms of response triggered in plants upon ENM exposure (response to abiotic stress), also highlighting the primary role of the chloroplast. The results showed different responses between zucchini and tomatoes upon exposure to NP CeO_2, La_2O_3, and CuO, both at the physiological and molecular level. In addition, the toxicity of CeO_2 NPs was lower (Figure 1), partially because of low translocation to the leaf. Conversely, La_2O_3 NPs produced greater toxicity, especially in tomatoes, and this correlates with

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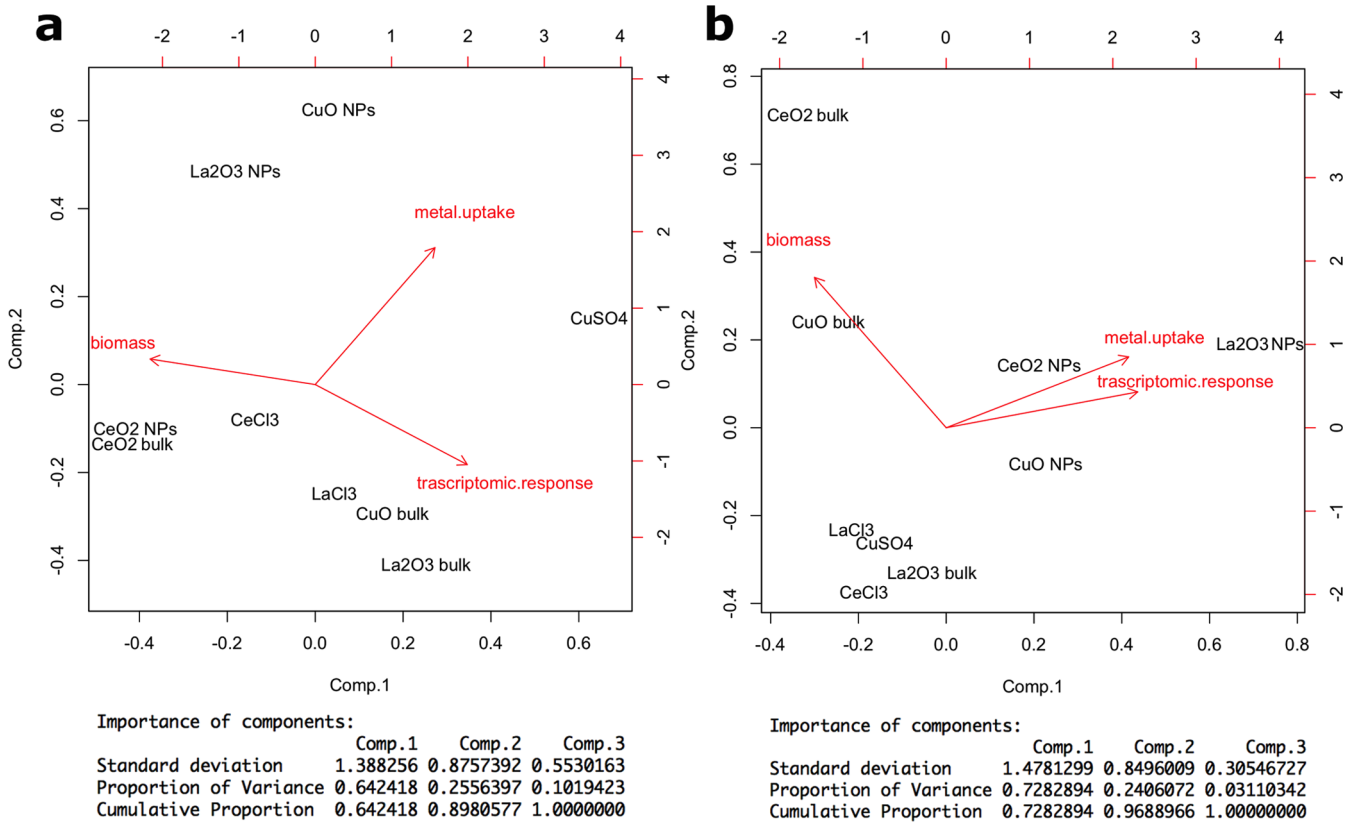

Importance of components:

	Comp.1	Comp.2	Comp.3
Standard deviation	1.388256	0.8757392	0.5530163
Proportion of Variance	0.642418	0.2556397	0.1019423
Cumulative Proportion	0.642418	0.8980577	1.0000000

Importance of components:

	Comp.1	Comp.2	Comp.3
Standard deviation	1.4781299	0.8496009	0.30546727
Proportion of Variance	0.7282894	0.2406072	0.03110342
Cumulative Proportion	0.7282894	0.9688966	1.00000000

Figure 4. Principal component analysis regarding uptake, physiological response (biomass), and transcriptomic response in zucchini (a) and tomato (b). The first two components (response based on particle type and element) represents the 64.2% and 25.5% of the total variance observed in zucchini and the 72.8% and 24.0% in tomatoes, respectively. The overall nanoparticles response, which was unique in most of the cases, underlying the "nanospecificity" of the phenomenon observed.

the high uptake and translocation to the shoots (Figure 1, Table S1). CuO NPs response at physiological level was similar to that of La_2O_3 NPs, whereas the transcriptomic response differed on the basis of species. Exposure to equivalent bulk and ionic materials confirmed the general fact that NPs are accumulated to a greater extent but that equivalent metal ions (salts) often exert the greatest toxicity. It is important to point out that in the targeted transcriptional analysis, the effect from NP exposure differs from that of the equivalent bulk and ion materials. After analyzing the overall effects observed in the experiments performed (biomass, metal uptake, and gene expression), we determined that the degree of the "nanobased" specificity was represented as 89% and 96% of the total variance observed in zucchini and tomatoes, respectively (Figure 4). This nanoresponse does depend on the specific particle type and element (nano, bulk, or ion form) but may indicate a unique mechanism of toxicity for the ENMs. The high variability in response based both on particle type and plant species (Figures 4 and S10) supports the contention of Aslani et al. (2014),[33] who recommends assessing NP exposure, effects, and risk on a case-by-case basis. The current work shows some consistent molecular responses related to oxidative stress and points out the potential role of the chloroplast in the ENMs reponse[34,35] (Figures S7, S11, and S12) in both zucchini and tomatoes (partially). Further investigations are underway to verify if 005u and 152u are true molecular biomarkers of exposure and effects in a wide range of plant species, with the objective of developing new and effective strategies to assess the risk of ENM in the environment and the food supply.

The integration of "-omics" data sets and a systems biology approach can facilitate a multitargeted method, allowing the identification of key regulatory "hubs" in complex gene networks.[36] The application of these biomarkers opens new perspectives for ENM screening and monitoring in one or more plant species, may facilitate the determination of dose−response relationships, and may help to complete risk assessment efforts in species of agricultural interest[37] and their environmental implications.[38] The biomarkers approach can reflect the genetic linkage to ENMs response expressed phenotypically as acquired tolerance and resistance or susceptibility of plants to specific substances. Biomarkers can also be a tool for selection and improvement of plant varieties as well as for other efforts such as biofortification[39] or for plants to facilitate heavy metal phytoremediation.[40] Investigation of other species of agronomic interest, phylogenetically close and distant from the species studied (e.g., lettuce, rice, maize, and wheat), is currently underway. In addition, the role of the rhizosphere in the ENM management or the impact of co-contamination (ENM and otherwise) remains largely unknown.[41,42] Moreover, this work demonstrates that the application of high-throughput methodologies such as transcriptomics, but also (potentially) proteomics[43] and metabolomics,[44] coupled with analytical and physiological data can bridge the gap between genotype and phenotype regarding plant response to ENM exposure.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01816.

Additional details on NP characterization and SEM-EDS and TEM-EDS sample preparation. Tables showing 46 *A. thaliana* orthologs and primers used for RT-qPCR experiments; biomass, water content, and length measurements and metal content of tomato and zucchini upon NPs and bulk and ion treatments; and a list of supplementary references. Figures showing SEM and TEM images of NPs and zucchini leaf; a network analysis of the 0005u, 152u, and 124u genes; a heatmap of the genes analyzed in tomatoes and zucchini upon NP treatment; principal component analysis of the transcriptional responses of zucchini and tomato; and heatmaps of the genes analyzed in tomatoes and zucchini upon bulk and ion treatments and their comparison with NP treatment. (PDF)

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Notes

The authors declare no competing financial interest.

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