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## 1 Molecular Response of Crop Plants to Engineered Nanomaterials

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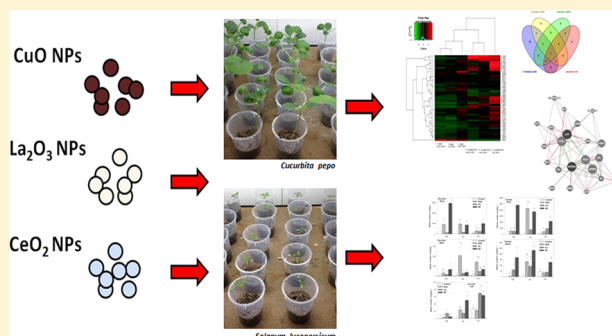
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### 9 **S** Supporting Information

10 **ABSTRACT:** Functional toxicology has enabled the identifica-  
11 tion of genes involved in conferring tolerance and sensitivity to  
12 engineered nanomaterial (ENM) exposure in the model plant  
13 *Arabidopsis thaliana* (L.) Heynh. Several genes were found to be  
14 involved in metabolic functions, stress response, transport,  
15 protein synthesis, and DNA repair. Consequently, analysis of  
16 physiological parameters, metal content (through ICP-MS  
17 quantification), and gene expression (by RT-qPCR) of *A.*  
18 *thaliana* orthologue genes were performed across different plant  
19 species of agronomic interest to highlight putative biomarkers of  
20 exposure and effect related to ENMs. This approach led to the  
21 identification of molecular markers in *Solanum lycopersicum* L. and  
22 *Cucurbita pepo* L. (tomato and zucchini) that might not only indicate exposure to ENMs (CuO, CeO<sub>2</sub>, and La<sub>2</sub>O<sub>3</sub>) but also  
23 provide mechanistic insight into response to these materials. Through Gene Ontology (GO) analysis, the target genes were  
24 mapped in complex interatomic networks representing molecular pathways, cellular components, and biological processes  
25 involved in ENM response. The transcriptional response of 38 (out of 204) candidate genes studied varied according to particle  
26 type, size, and plant species. Importantly, some of the genes studied showed potential as biomarkers of ENM exposure and effect  
27 and may be useful for risk assessment in foods and in the environment.



### 28 ■ INTRODUCTION

29 The application of nanotechnology has occurred across many  
30 sectors: health and medicine, communications and electronics,  
31 energy production, water treatment, and food production and  
32 agriculture. The increases have been exponential in the last 10  
33 years, with even greater use predicted in the future.<sup>1</sup> There is a  
34 general consensus in the scientific community that our  
35 understanding of the fate and effects of these materials in the  
36 environment has lagged behind and is not adequate for accurate  
37 risk assessment. Important steps forward have been made in the  
38 last 3–4 years, especially on the properties that determine the  
39 behavior and the distribution of engineered nanomaterials  
40 (ENMs) in the environment<sup>2–4</sup> as well as the particle-size-  
41 dependent process of bioaccumulation and the trophic transfer  
42 within food chains.<sup>5,6</sup> The current state of knowledge regarding  
43 plants and ENMs interactions at both the physiological- and  
44 molecular-response level, including uptake, toxicity, and cellular  
45 compartmentalization, has been reviewed,<sup>7,8</sup> and there is now  
46 some understanding of which plant organs, tissues, cells, and  
47 organelles are involved in response, as well as of the molecular  
48 pathways associated with more general toxicity (e.g., DNA  
49 damage, ROS production, protein misfolding, etc.).<sup>9</sup> However,

given the wide range of ENMs used (composition, size, shape,  
50 coating, etc.) and of their effects, it remains difficult to highlight  
51 consistent end points commonly shared in response to different  
52 classes of these materials. Unlike the situation for humans,<sup>10</sup>  
53 biomarkers for exposure, effects, and ENM susceptibility are  
54 unknown in plants. Typical biomarkers of exposure often  
55 involve the measurement of metabolites or other physiological  
56 parameters that reflect the biological dose and effect, showing  
57 directly or indirectly the physiological implications of exposure.  
58 Biomarkers of effects show changes at the cellular and  
59 molecular levels and reflect the expression of genes or the  
60 abundance of proteins under experimentally controlled  
61 conditions. Considering this potential, biomarkers of effects  
62 can be usefully applied as a tool for the assessment of toxicity.<sup>11</sup>  
63 Biomarkers of susceptibility indicate the constitutive respon-  
64 siveness to contaminant exposure, such as through tolerance  
65 and resistance pathways as described for nonsensitive and 66

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hypersensitive phenotypes.<sup>12</sup> 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.<sup>13,14</sup>

Whole-genome studies performed in *Arabidopsis thaliana* (L.) Heyhn<sup>15–17</sup> revealed some of the main biological processes involved in the response to different ENMs. Marmioli et al.<sup>18</sup> 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<sub>2</sub> NPs), and lanthanum oxide (La<sub>2</sub>O<sub>3</sub> 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.<sup>19</sup> In addition, CuO NP interactions with plant species of agronomic interest have been initiated.<sup>20,21</sup> CeO<sub>2</sub> NPs are utilized in catalysis, electrolyte and electrode materials, UV and infrared absorbents, and oxidation- and heat-resistant coatings.<sup>22</sup> Studies performed with CeO<sub>2</sub> NPs on *A. thaliana* and *Phaseolus vulgaris* L. showed variable effects on growth, physiological response, and nutritional quality.<sup>23,24</sup> La<sub>2</sub>O<sub>3</sub> 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<sub>2</sub>O<sub>3</sub> NPs.<sup>25,26</sup>

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.<sup>27,28</sup> 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

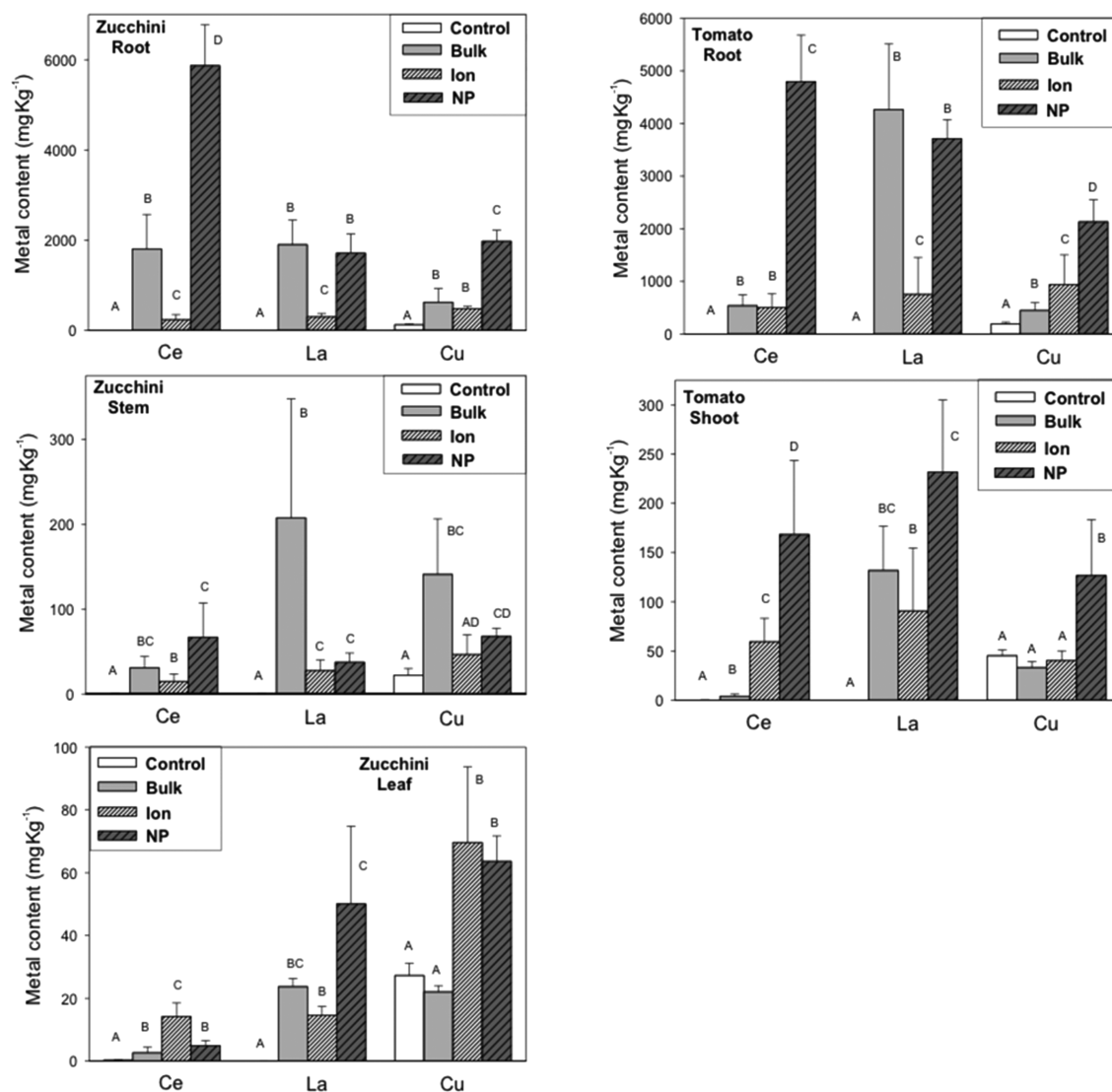
**Plants and NP Treatments.** Copper oxide (CuO) nanopowder (99% purity, 40 nm particle size) and lanthanum oxide (La<sub>2</sub>O<sub>3</sub>) nanopowder (99.99% purity; 10–100 nm particle size range) were purchased from U.S. Research Nanomaterials, Inc. (Houston, TX). Cerium oxide (CeO<sub>2</sub>) 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  $\zeta$  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<sup>-1</sup> (untreated control) or 500 mg L<sup>-1</sup> of bulk or NP CuO, La<sub>2</sub>O<sub>3</sub>, or CeO<sub>2</sub>. 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,<sup>29</sup> 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<sup>-1</sup> of CeCl<sub>3</sub>, 75 mg L<sup>-1</sup> of LaCl<sub>3</sub>, or 158 mg L<sup>-1</sup> of CuSO<sub>4</sub>·5H<sub>2</sub>O 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  $\mu\text{M m}^{-2} \text{s}^{-1}$  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<sup>-1</sup> 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<sub>3</sub> for 45 min at 115 °C, and then 1 mL of 30% H<sub>2</sub>O<sub>2</sub> was added for 20 min at 115 °C. The digestion protocol was performed using a SCP Science DigiPREP MS digester (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,<sup>18</sup> 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  $\mu\text{g}$  of the total RNA extracted using the Qiagen QuantiTect Reverse Transcription kit (Qiagen, Velno, The Netherlands).



**Figure 1.** ICP-MS data related to Ce, La, and Cu content ( $\text{mg kg}^{-1}$ ) from treatment with 0 or 500  $\text{mg L}^{-1}$  bulk or NP CuO,  $\text{La}_2\text{O}_3$ , or  $\text{CeO}_2$  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  $\text{mg L}^{-1}$  of  $\text{CeCl}_3$ , 75  $\text{mg L}^{-1}$  of  $\text{LaCl}_3$ , or 158  $\text{mg L}^{-1}$  of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 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).

192 Reverse-transcription real-time PCR (RT-qPCR) was carried  
 193 out using the Bio-Rad SsoAdvanced Universal SYBR Green  
 194 Supermix (Bio-Rad, Hercules, CA) in an optical 96 well plate  
 195 with the Bio-Rad CFX96 Touch Real-Time PCR Detection  
 196 System (Bio-Rad). On the basis of previous work,<sup>18</sup> *A. thaliana*  
 197 ortholog gene coding sequences (CDS) were obtained through  
 198 the BLAST tool of Cucurbitgene database resource ([http://](http://cucurbitgene.net/)  
 199 [cucurbitgene.net/](http://cucurbitgene.net/)) and Sol Genomics Network database  
 200 resource (<http://solgenomics.net/>) for *C. pepo* and *S.*  
 201 *lycopersicum*, respectively. A  $1 \cdot 10^{-20}$  (E-value) threshold with  
 202 the query sequence (of *A. thaliana*) was used to identify the  
 203 orthologous coding sequences in zucchini and tomato: a total  
 204 of 46 orthologs were identified in both of the species (Table  
 205 S1a). Specific primers for each selected gene transcript were  
 206 designed (Table S1b) using the Primer3 software (primer3-  
 207 ut.ee); the thermal profile for RT-qPCR amplifications was: 95  
 208 °C for 10', 95 °C for 15", and 60 °C for 60" for 40 cycles.  
 209 Confirmation of the single amplicon in each reaction was  
 210 performed by a dissociation-curve step. Relative expression was

211 estimated through  $\Delta\Delta\text{Ct}$  method using  $\beta$ -actin of *C. pepo* and  
 212 *S. lycopersicum* as the housekeeping gene.

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

## 229 ■ RESULTS AND DISCUSSION

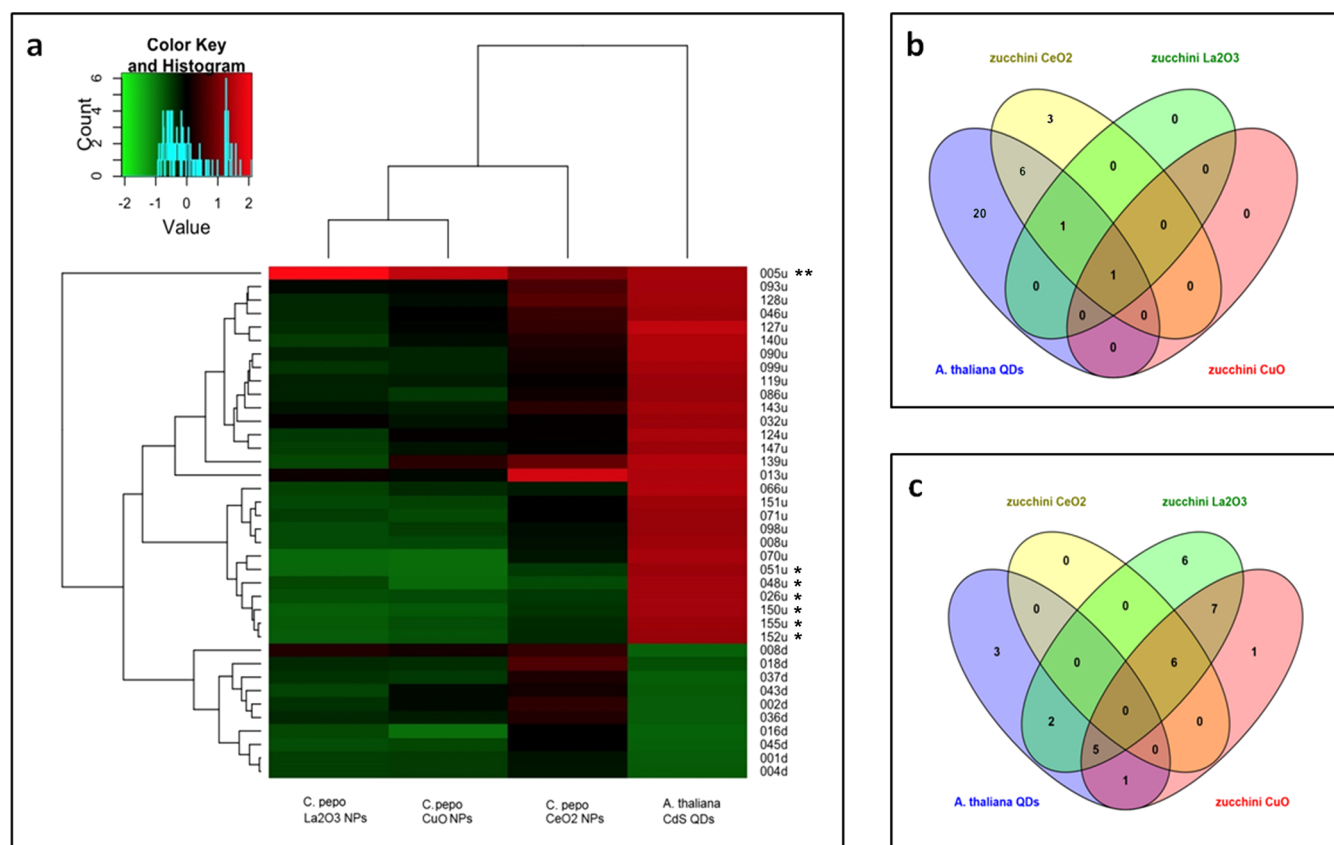
230 **Physiological Analyses.** The physiological response of  
231 zucchini and tomato to NP exposure (500 mg L<sup>-1</sup> of CuO,  
232 La<sub>2</sub>O<sub>3</sub>, or CeO<sub>2</sub> NPs) is shown in Tables S2–S4 and clearly  
233 varied by species. CuO, La<sub>2</sub>O<sub>3</sub>, and CeO<sub>2</sub> NPs had no effect on  
234 zucchini total biomass (fresh weight) as compared to untreated  
235 controls, whereas in tomatoes, plant biomass significantly  
236 decreased (59–78%) upon exposure to both CuO and La<sub>2</sub>O<sub>3</sub>  
237 NPs (Table S2). A differential response dependent on plant  
238 type was also evident when measuring plant moisture content  
239 and root and shoot length. In tomatoes, root and shoot water  
240 content was unaffected by NP exposure, but the moisture  
241 content of zucchini leaves decreased significantly (20–22%)  
242 with CuO and La<sub>2</sub>O<sub>3</sub> NP treatments (Table S3). Zucchini  
243 stems were increased (15%) by CeO<sub>2</sub> NPs; CuO NPs  
244 significantly increased (44%) root length. Conversely, in  
245 tomatoes, both CuO NPs and La<sub>2</sub>O<sub>3</sub> NPs treatment  
246 significantly reduced root (68–75%) and shoot (42–47%)  
247 length as compared to untreated controls (Table S4). The  
248 different physiological response in the two horticultural species  
249 to ENMs was noteworthy, particularly with La<sub>2</sub>O<sub>3</sub> and CuO  
250 NPs treatment. Zucchini seems to be largely unaffected by  
251 treatments, whereas tomato was more sensitive to ENMs.  
252 These findings agree somewhat with previous work, in which  
253 several reports show that exposure to CeO<sub>2</sub> NPs did not impact  
254 physiological parameters in agricultural crops.<sup>9</sup> However,  
255 treatment with La<sub>2</sub>O<sub>3</sub> or CuO NPs has been shown to inhibit  
256 root and shoot elongation and biomass as well as induce  
257 reactive-oxygen-species (ROS) production and programmed  
258 cell death.<sup>21,30</sup>

259 In zucchini, treatment with CuO, La<sub>2</sub>O<sub>3</sub>, CeO<sub>2</sub> (bulk  
260 materials) and CeCl<sub>3</sub>, LaCl<sub>3</sub>, and CuSO<sub>4</sub> had little effect on  
261 total biomass, with the only statistically significant decrease  
262 occurring under CuSO<sub>4</sub> treatment (38%) (Table S5). A  
263 significant decrease in moisture content was shown in stems  
264 of zucchini when treated with CuSO<sub>4</sub> and CuO (–21 and  
265 –24%), and CuO treatment led to a 33% increase in leaf  
266 moisture. Root length was also affected (–20 and –45%) by  
267 LaCl<sub>3</sub> and CuSO<sub>4</sub> (Tables S6a and S7a). Similar to what was  
268 observed for NPs, zucchini are largely unaffected by the bulk  
269 materials and metal salts, with the exception of CuSO<sub>4</sub>.  
270 Conversely, in tomatoes, all metal salts (CeCl<sub>3</sub>, LaCl<sub>3</sub>, and  
271 CuSO<sub>4</sub>) caused a significant decrease in biomass: –68% for  
272 CeCl<sub>3</sub>, –56% for LaCl<sub>3</sub>, and –64% for CuSO<sub>4</sub>. Bulk La<sub>2</sub>O<sub>3</sub> also  
273 reduced tomato biomass (–76%) (Table S5). Significant  
274 differences were not shown for tomato moisture content, but  
275 a significant decrease of tomato shoot length was evident after  
276 treatment with CeCl<sub>3</sub> (–35%), La<sub>2</sub>O<sub>3</sub> (–36%), and CuO  
277 (–34%) as well as for roots (–48%) treated with CuO (Tables  
278 S6b and S7b). Thus, tomatoes were more sensitive to  
279 treatments with metal salts and with bulk La<sub>2</sub>O<sub>3</sub>, which agrees  
280 with the results from the La<sub>2</sub>O<sub>3</sub> NP and CuO NP treatment;  
281 exposure to bulk or NP CeO<sub>2</sub> was generally less toxic.

282 **Metal-Content Data.** The Ce, La, and Cu content in  
283 zucchini roots and shoots is shown in Figure 1. Root content of  
284 all the three elements in both horticultural species was  
285 significantly increased by bulk, ion, and NP treatment. For  
286 the NPs, the Ce and Cu content did not vary significantly  
287 across species; zucchini and tomato root Ce content was 5870  
288 and 4790 mg kg<sup>-1</sup>, and root Cu content was 1970 and 2130 mg  
289 kg<sup>-1</sup>, respectively. However, root La content under NP  
290 exposure differs significantly across the two plant species.

Zucchini and tomato-root La levels were 1700 and 3710 mg  
kg<sup>-1</sup>, 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<sup>-1</sup>, respectively. For zucchini, stem Ce,  
La, and Cu concentrations under NP exposure were 66.8, 37.5,  
and 67.8 mg kg<sup>-1</sup>, respectively; the leaf values were 4.92, 50.1,  
and 63.6 mg kg<sup>-1</sup>, 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<sup>5</sup> 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<sub>2</sub>O<sub>3</sub> 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<sup>-1</sup> for CeCl<sub>3</sub> and bulk CeO<sub>2</sub>,  
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<sup>-1</sup> for ion and bulk treatment, respectively,  
whereas leaf content was 14.1 and 2.8 mg kg<sup>-1</sup>. The ionic Ce  
was translocated equally in stem and leaves, as compared to the  
bulk and NP CeO<sub>2</sub> 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<sup>-1</sup>,  
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<sup>-1</sup>, respectively, and the leaf content was 14.6 and 23.6 mg  
kg<sup>-1</sup>, respectively. Similar to the La<sub>2</sub>O<sub>3</sub> 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.<sup>30</sup> Treatment with CuSO<sub>4</sub> 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<sup>-1</sup> for CuSO<sub>4</sub> 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<sup>-1</sup> and 69.6 and 22  
mg kg<sup>-1</sup> in the leaves. The evidence of an increased Cu leaf  
content for the ion treatment may partially explain a decrease in  
biomass after CuSO<sub>4</sub> 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<sup>-1</sup> for CeCl<sub>3</sub> and CeO<sub>2</sub> (bulk) treatments; both levels were  
significantly lower than that of NP exposure. Roots La levels  
were 755 and 4268 mg kg<sup>-1</sup> in plants treated with LaCl<sub>3</sub> and  
La<sub>2</sub>O<sub>3</sub> (bulk), respectively; similarly to what was observed for 353



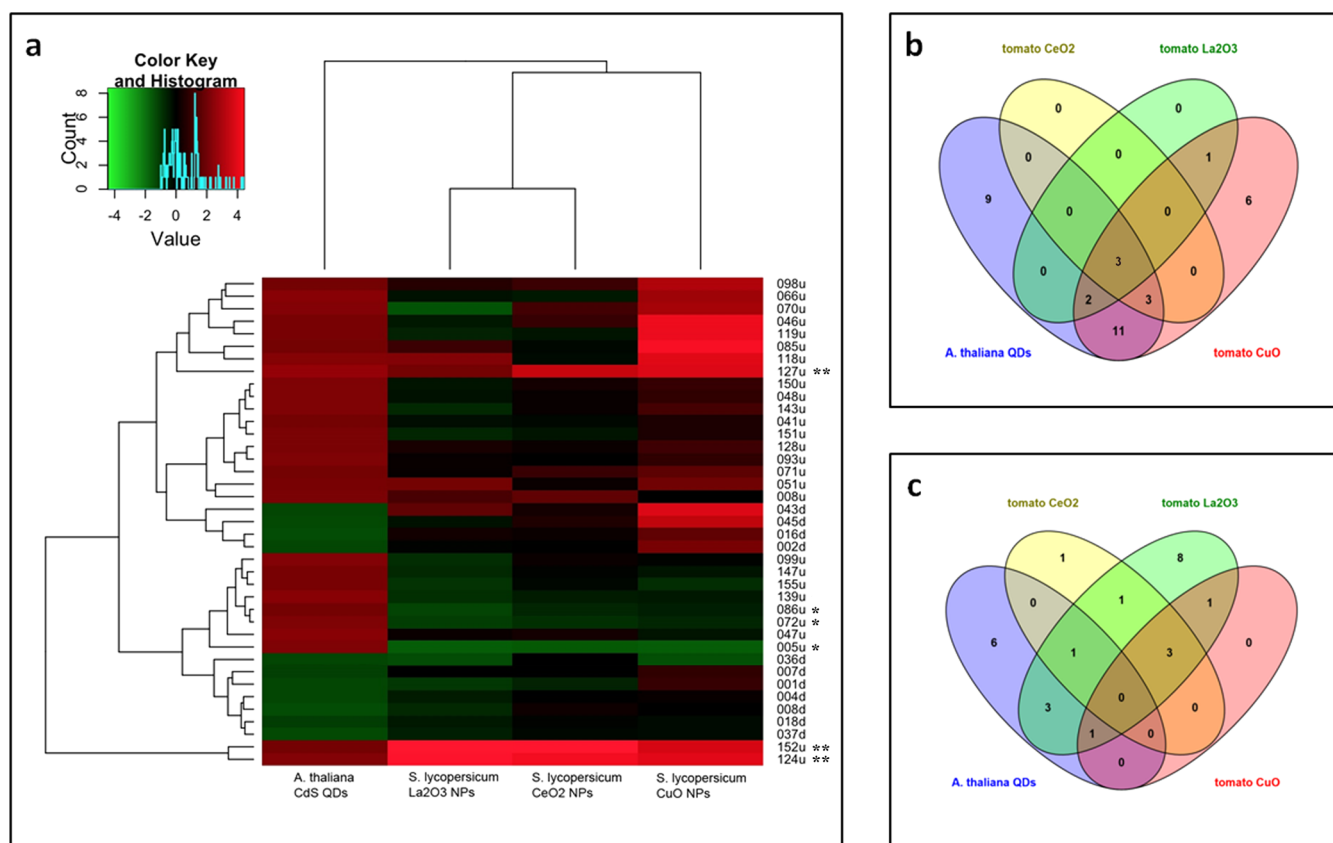
**Figure 2.** Comparison between *A. thaliana* and *C. pepo*. Heatmap (a) and Venn diagrams of the genes up-regulated (b) and down-regulated (c) of *A. thaliana* (Marmioli et al., 2014) compared with *C. pepo* treated with 500 mg L<sup>-1</sup> of CuO NPs, 500 mg L<sup>-1</sup> La<sub>2</sub>O<sub>3</sub> NPs, or 500 mg L<sup>-1</sup> CeO<sub>2</sub> 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*.

354 zucchini, La content in bulk and NPs treatments were  
355 statistically equivalent, but ion treatment resulted in lower La  
356 content. The Cu root content was 941 and 447 mg kg<sup>-1</sup> for  
357 CuSO<sub>4</sub> and CuO (bulk) treatment, respectively. Levels of La  
358 after NP exposure were significantly higher. It was noted that  
359 La levels in tomato shoots exceeded those in zucchini, and this  
360 correlates with the observed physiological effects. With Ce in  
361 ionic and NPs form, this resulted in a greater translocation to  
362 the shoots than with bulk CeO<sub>2</sub> and correlated with observed  
363 toxicity. The literature supports these findings, where the  
364 phytotoxicity of La<sub>2</sub>O<sub>3</sub> NPs is greater than CeO<sub>2</sub> NPs, likely  
365 caused by the higher level of dissolution and La<sup>3+</sup> release that  
366 occurred in the plant (cucumber).<sup>30,31</sup> The lower dissolution  
367 rate of CeO<sub>2</sub> NPs correlates with the decreased level of  
368 translocation to the leaves and with the lower impact on plant  
369 growth and ROS generation.<sup>31</sup> Translocation of Cu after NP  
370 exposure was 3-fold higher than in bulk and ion treatments, in  
371 partial agreement with reports for alfalfa roots and shoots<sup>21</sup> and  
372 for maize.<sup>32</sup>

### 373 Gene-Expression Analysis of the ENM Treatments.

374 Gene-expression analysis was conducted to identify up- and  
375 down-regulated genes for the two plant species treated with the  
376 three metals. Table S10 shows the genes of interest and the  
377 relevant literature for reference. From the 204 *A. thaliana* genes  
378 reported in Marmioli et al. (2014),<sup>18</sup> 71 were isolated as  
379 zucchini orthologs, but only 46 were found both in zucchini  
380 and in tomatoes. Of those, a total of 38 orthologue genes were  
381 used for the study.

382 Expression analyses performed with RT-qPCR for treated  
383 and untreated zucchini are shown in Figure 2, along with results  
384 from Marmioli et al. (2014)<sup>18</sup> for *A. thaliana* exposed to CdS  
385 QDs (Figure 2). Heatmaps (Figure 2a) and Venn diagrams  
386 (Figure 2b,c) showed a trend of general down-regulation upon  
387 exposure to La<sub>2</sub>O<sub>3</sub> NPs or CuO NPs (500 mg L<sup>-1</sup>) relative to  
388 untreated controls. Treatment with CeO<sub>2</sub> NPs produced few  
389 changes in the level of expression of most of the analyzed  
390 genes. It is also interesting that the expression profile for the  
391 treatments with the two lanthanides, La<sub>2</sub>O<sub>3</sub> and CeO<sub>2</sub>, were  
392 quite different. In fact, the response to La<sub>2</sub>O<sub>3</sub> and CuO NP  
393 treatments were actually quite similar (Figure 2a), confirming  
394 results obtained in the physiological analyses (Figure 1 and  
395 Tables S2–S4). Of the 38 genes analyzed, 7 were  
396 simultaneously expressed (either up- or down-regulated) in  
397 zucchini across the CuO, La<sub>2</sub>O<sub>3</sub>, and CeO<sub>2</sub> NPs treatments  
398 (Figure 2). When we take into consideration the results from  
399 the *A. thaliana* treatments from Marmioli et al. (2014),<sup>18</sup> only  
400 one gene was simultaneously expressed and modulated (up-  
401 regulated in this case) across all treatments (Figure 2b). Of the  
402 seven genes in common, one, 026u (*GTP2*), was down-  
403 regulated and encodes for a glucose 6-phosphate transporter 2  
404 located in the chloroplast and in the vacuolar membrane.  
405 Another common down-regulated gene was 048u (*SNRK2-9*),  
406 which encodes a member of SNF1-related serine and threonine  
407 protein kinases that are calcium- and calmodulin-dependent  
408 and is involved in osmotic stress response. A third down-  
409 regulated gene was 051u (*PLP2*), which encodes for a lipid acyl



**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* (Marmioli et al., 2014) compared with *S. lycopersicum* treated with 500 mg L<sup>-1</sup> of CuO NPs, 500 mg L<sup>-1</sup> La<sub>2</sub>O<sub>3</sub> NPs, or 500 mg L<sup>-1</sup> CeO<sub>2</sub> 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*.

410 hydrolase with wide substrate specificity. This gene plays a role  
411 in cell death and contributes to resistance against viral infection  
412 and cadmium toxicity. The gene 150u (*RPS12A*) is a  
413 chloroplast gene that encodes a ribosomal protein S12. Gene  
414 152u (*ORF31*) encodes for an electron carrier located in  
415 chloroplast endomembrane system and in the cytochrome b6f  
416 complex. In literature, *ORF31* was reported as highly responsive  
417 under high-level H<sub>2</sub>O<sub>2</sub> conditions. The gene was coexpressed  
418 with *ZAT12*, a zinc-finger protein involved in abiotic stress  
419 response, such as heat and oxidative stress. These findings not  
420 only underlie the central role of the chloroplast in cellular  
421 response to ROS and abiotic stress (Figure S6) but also the  
422 putative role of *ORF31* as a biomarker of abiotic stress  
423 response, such as during ENM treatment. Gene 155u (*PSBN*)  
424 encodes a photosystem II (PSII) low-molecular-weight protein  
425 that is located on thylakoid membrane. Several chloroplast  
426 genes are among those commonly down-regulated by NP  
427 exposure. The only gene up-regulated in all four the treatments  
428 (including *A. thaliana*) was 005u (*BIP3*), which encodes for  
429 Heat Shock Protein 70 (HSP70) with ATP-binding function.  
430 This gene is involved in protein folding, as well as response to  
431 heat stress and pollen-tube growth, and is located in the lumen  
432 of the endoplasmic reticulum. In *A. thaliana*, *BIP3* is also  
433 involved in transcriptional regulation as a part of the mediator  
434 complex. As shown in the gene network reported in Figure S7,  
435 this gene also physically interacts with BZIP28, a putative  
436 membrane-tethered transcriptional factor that is up-regulated in  
437 response to heat. *BIP3* gene product also physically interacts

with ERDJ3A and ERDJ3B, two DNAJ domain proteins strictly  
438 related to the modulation of heat stress response and pollen  
439 germination. *BIP3* is also coexpressed in *A. thaliana* with *PNP-*  
440 *A* (Plant Natriuretic Peptide A), which was reported in  
441 Marmioli et al. (2014)<sup>18</sup> as one of the more differentially  
442 expressed genes in *A. thaliana* CdS QDs resistant mutant  
443 *atnp01* (Figure S7). PNPs are a class of systemically mobile  
444 molecules that may function as a component of plant defense  
445 response and systemic acquired resistance (SAR). 446

Results from the RT-qPCR analysis of treated and untreated  
447 tomato are shown in Figure 3, along with results from  
448 Marmioli et al. (2014)<sup>18</sup> for *A. thaliana* exposed to CdS QDs.  
449 Different from the response observed in *C. pepo*, treatments  
450 with the lanthanide CeO<sub>2</sub> and La<sub>2</sub>O<sub>3</sub> NPs gave quite similar  
451 results in terms of differential gene expression. Tomato  
452 response to CuO NPs produced a more consistent pattern of  
453 up-regulation. For tomatoes, a total of six genes were up-  
454 down-regulated with the CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, and CuO NPs  
455 treatments; three of these genes (Figure 2) were also up-  
456 regulated in *A. thaliana* from Marmioli et al. (2014).<sup>18</sup> The  
457 first down-regulated gene was 072u (*SKS13*), which encodes  
458 for an endomembrane system protein involved in oxidor-  
459 eductase activity and copper-ion binding; *SKS13* was down-  
460 regulated in all Cu treatments (NPs, bulk, and ion), suggesting  
461 that it may not be specifically involved in response to ENM  
462 exposure. A second down-regulated gene was 086u  
463 (*At3g59845*), which encodes a cytosolic Zn<sup>2+</sup> ion binding  
464 dehydrogenase family protein known to be involved in the  
465

466 response to oxidative stress. Similar to zucchini, 005u (*BIP3*)  
467 was commonly down-regulated in tomatoes across the three  
468 treatments, suggesting a possible central role in response to  
469 ENM exposure. Regarding genes commonly overexpressed in  
470 tomatoes and *A. thaliana*, the first one is 152u (*ORF31*), which  
471 was also up-regulated in zucchini exposures. A second gene  
472 commonly up-regulated in tomatoes and *A. thaliana* was 124u  
473 (*PRRS*), which codes for a pseudoresponse regulator whose  
474 mutation affects circadian-associated biological events, acting as  
475 a transcriptional repressor of *CCA1* and *LHY* (001d). As  
476 observed in *A. thaliana* (Figure S8), 001d was down-regulated  
477 in tomatoes upon exposure to CeO<sub>2</sub> and La<sub>2</sub>O<sub>3</sub> NPs (Figure  
478 3a). *LHY* (Late Elongated Hypocotyl) and *CCA1* (Circadian  
479 Clock Associated1) belong to the MYB-transcription-factor  
480 family and are involved in the regulation of circadian rhythm  
481 and oxidative stress response in plants. The importance of this  
482 finding is reinforced by the activity of *At1g13880* (*ELM2*), a  
483 gene found to be a key element in the tolerance and resistance  
484 of the Cds QDs of *A. thaliana* mutant *atnp01*; this gene is also  
485 a putative MYB-transcription factor, highlighting the central  
486 role of this transcriptional-regulator family in response to  
487 ENMs. The gene 127u (*GGCT2;1*) was up-regulated in  
488 tomatoes across all exposures (as well as in *A. thaliana*); this  
489 gene encodes a  $\gamma$ -glutamyl cyclotransferase 2;1 (*GGCT2;1*)  
490 that belongs to the ChaC-like family protein and is involved in  
491 Cd<sup>2+</sup> and Pb<sup>2+</sup> response. It is interesting to observe that some  
492 of the genes differentially modulated in the two species (*GTP2*,  
493 *PLP2*, and *BIP3* and *SNRK2-9*, *LHY*, and *GGCT2;1*,  
494 respectively) were reported in literature as coexpressed in *A.*  
495 *thaliana* under hypoxia and bacterial infection conditions  
496 (Table S10).

497 Comparison of the results for tomato and zucchini samples  
498 exposed to CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, and CuO NPs treatments with that of  
499 *A. thaliana* exposed to Cds quantum dots shows that in spite of  
500 a large phylogenetic distance, the general response of tomatoes  
501 is more similar to *A. thaliana* than it is with zucchini plants  
502 (Figure S9). In both zucchini and tomatoes, treatment with  
503 CeO<sub>2</sub> NPs caused only small changes in gene expression, and  
504 this agrees with the physiological responses, which showed that  
505 CeO<sub>2</sub> NPs were significantly less toxic than La<sub>2</sub>O<sub>3</sub> and CuO  
506 NPs. These findings agree with soil-based studies showing  
507 greater La<sub>2</sub>O<sub>3</sub> phytotoxicity and La accumulation in exposed  
508 plants.<sup>26</sup> Interestingly, La<sub>2</sub>O<sub>3</sub> had different effects on the two  
509 plant species at both the physiological and the genetic level.  
510 These findings correlate with published data on response to  
511 ENM exposure, both with regard to species-specific response  
512 (one material, many species) and with different nanomaterials  
513 on a single species.<sup>9,15,33</sup> With regard to CuO NPs, the different  
514 effects observed in tomato and zucchini plants indicate two  
515 different response pathways to the same exposure: tomatoes  
516 respond with a general up-regulation of genes mainly involved  
517 in copper and cadmium response (similar to what was seen in  
518 *A. thaliana* upon treatment with Cds QDs), whereas the  
519 zucchini response showed a general down-regulation of nearly  
520 all genes studied.

521 Considering the three plant species (zucchini, tomato, *A.*  
522 *thaliana*) and different ENM exposures, only two genes were  
523 consistently modulated, although in opposite ways (according  
524 to *A. thaliana* results): *BIP3* (005u) and *ORF31* (152u) (Figure  
525 S9SI). Their biological interactions, as described in SI Figure  
526 S6–S7, point out how the two genes are involved in abiotic  
527 stress response (not only to heat and oxidative stress but  
528 potentially also to ENMs exposure; Table S10) and chloroplast

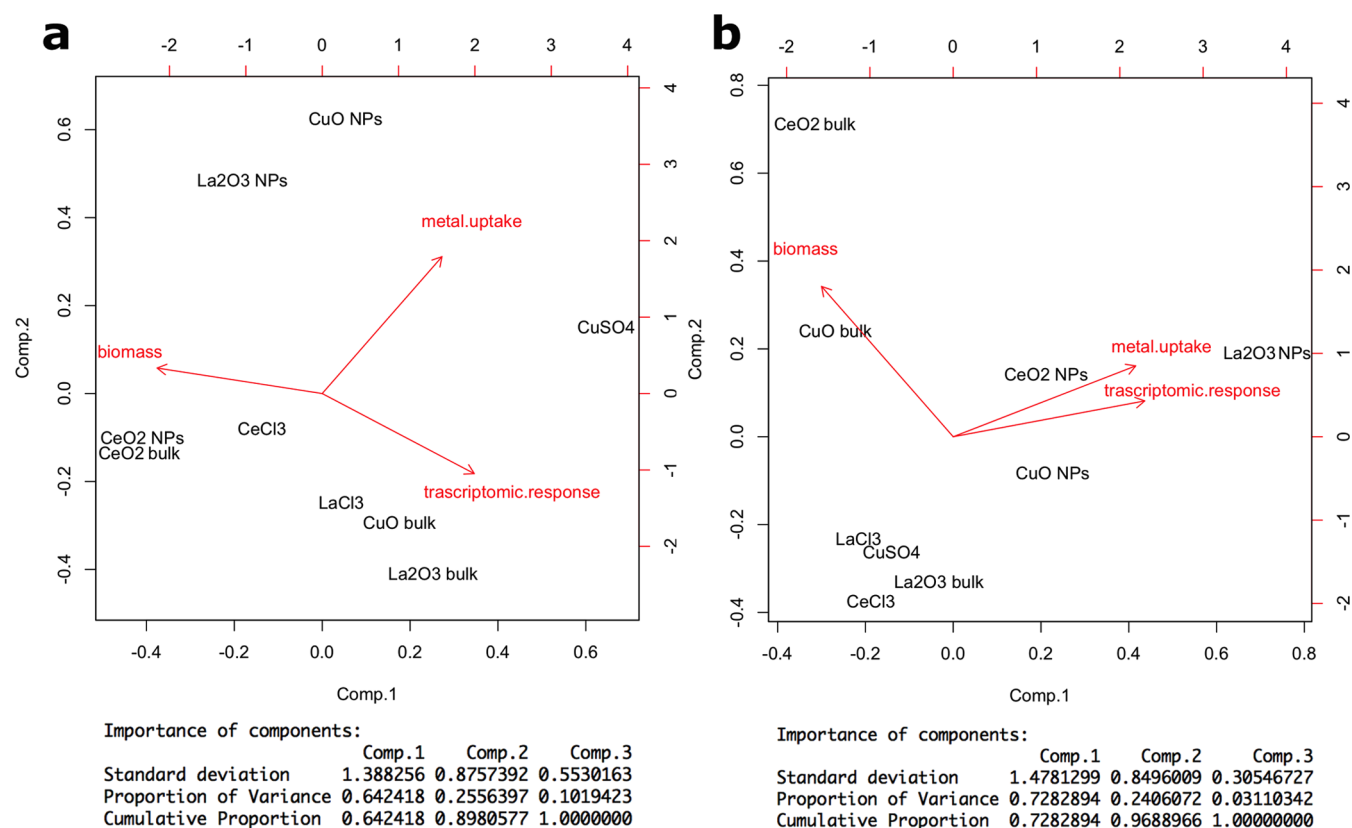
529 functionality and appear to play a central role in ENMs toxicity  
530 and response;<sup>34,35</sup> these genes can be considered potential  
531 biomarkers for ENM exposure. Principal component analysis  
532 (PCA) performed on zucchini and tomato in different  
533 treatments (Figure S10) showed the major components of  
534 variability to be related to a “plant-specific” response (response  
535 of zucchini and tomato) and to a “nanospecific” response  
536 (gene-expression level related to the treatment); these  
537 represent 80.8% of the total variance observed. Again, among  
538 the genes analyzed, 005u (*BIP3*), 152u (*ORF31*), and 124u  
539 (*PRRS*) were the most responsive out of the 38 genes analyzed  
540 in both species, representing the major determiners of the  
541 variance observed.

542 **Comparison with Bulk and Ion.** RT-qPCR on the  
543 putative biomarkers isolated from the NP exposure (Figures  
544 2 and 3) was tested on the two species upon exposure to bulk  
545 CuO, La<sub>2</sub>O<sub>3</sub>, and CeO<sub>2</sub>, as well as to the corresponding metal  
546 salts. Of the seven simultaneously expressed genes in zucchini  
547 (Figure 2), the three NP treatments all group separately from  
548 the corresponding bulk and ionic exposures. This ENMs  
549 difference is clearly greatest for NP CuO, followed by La<sub>2</sub>O<sub>3</sub>,  
550 suggesting that at least for these seven genes, much of the  
551 effects observed with NP exposure are indeed “nanospecific”  
552 (Figure S11). For CeO<sub>2</sub>, the bulk and ion transcriptomic  
553 response groups fairly closely to NP, indicating that for these  
554 selected genes, much of the differential response shown in  
555 Figure 2 is traceable to Ce exposure in general and therefore  
556 not size-specific. In addition, *BIP3* (005u) and *ORF31* (152u)  
557 were the only two genes in which the expression level was  
558 significantly different across all genes for the NP exposure  
559 (Figure S11). There is a potential for 005u and 152u to serve  
560 as candidate biomarker genes for NP exposure in zucchini and  
561 possibly other related plants.

562 The scenario was different when we considered the  
563 transcriptomic response of tomato in the presence of NP,  
564 bulk, and ion treatments (Figure S12). As with zucchini, CuO  
565 NPs caused a response separate from the two other forms of  
566 Cu, suggesting that much of the change (at least in these seven  
567 genes) with the exposure to ENMs is size-specific and likely  
568 mechanistically distinct from the Cu ion. Unlike zucchini, the  
569 two lanthanides behave differently, with bulk and NP La<sub>2</sub>O<sub>3</sub>  
570 grouping more closely together and CeO<sub>2</sub> having a more  
571 significant “nanospecific” effect on transcription. Specifically,  
572 *BIP3* (005u), *SKS13* (072u), and 086u (Zn<sup>2+</sup>-ion-binding  
573 dehydrogenase) were consistently down-regulated across all the  
574 treatments. 152u (*ORF31*) was up-regulated in tomatoes upon  
575 exposure to NPs CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, and CuO but also in the  
576 presence of bulk CuO, CuSO<sub>4</sub>, and LaCl<sub>3</sub>. The overall picture  
577 observed in tomatoes seems to be more complex than in  
578 zucchini and will require further analyses and subsequent  
579 additional study to be fully understood, including exposures at  
580 lower NPs concentrations and in soil-based systems over longer  
581 periods of time.

582 **“Nanospecific” Response.** This study elucidates the  
583 involvement of some of the molecular mechanisms of response  
584 triggered in plants upon ENM exposure (response to abiotic  
585 stress), also highlighting the primary role of the chloroplast.  
586 The results showed different responses between zucchini and  
587 tomatoes upon exposure to NP CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, and CuO, both at  
588 the physiological and molecular level. In addition, the toxicity of  
589 CeO<sub>2</sub> NPs was lower (Figure 1), partially because of low  
590 translocation to the leaf. Conversely, La<sub>2</sub>O<sub>3</sub> NPs produced  
591 greater toxicity, especially in tomatoes, and this correlates with





**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.

592 the high uptake and translocation to the shoots (Figure 1,  
 593 Table S1). CuO NPs response at physiological level was similar  
 594 to that of La<sub>2</sub>O<sub>3</sub> NPs, whereas the transcriptomic response  
 595 differed on the basis of species. Exposure to equivalent bulk and  
 596 ionic materials confirmed the general fact that NPs are  
 597 accumulated to a greater extent but that equivalent metal  
 598 ions (salts) often exert the greatest toxicity. It is important to  
 599 point out that in the targeted transcriptional analysis, the effect  
 600 from NP exposure differs from that of the equivalent bulk and  
 601 ion materials. After analyzing the overall effects observed in the  
 602 experiments performed (biomass, metal uptake, and gene  
 603 expression), we determined that the degree of the “nanobased”  
 604 specificity was represented as 89% and 96% of the total variance  
 605 observed in zucchini and tomatoes, respectively (Figure 4).  
 606 This nanoresponse does depend on the specific particle type  
 607 and element (nano, bulk, or ion form) but may indicate a  
 608 unique mechanism of toxicity for the ENMs. The high  
 609 variability in response based both on particle type and plant  
 610 species (Figures 4 and S10) supports the contention of Aslani  
 611 et al. (2014),<sup>33</sup> who recommends assessing NP exposure,  
 612 effects, and risk on a case-by-case basis. The current work  
 613 shows some consistent molecular responses related to oxidative  
 614 stress and points out the potential role of the chloroplast in the  
 615 ENMs response<sup>34,35</sup> (Figures S7, S11, and S12) in both zucchini  
 616 and tomatoes (partially). Further investigations are underway  
 617 to verify if 005u and 152u are true molecular biomarkers of  
 618 exposure and effects in a wide range of plant species, with the  
 619 objective of developing new and effective strategies to assess  
 620 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.<sup>36</sup> 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<sup>37</sup> and  
 their environmental implications.<sup>38</sup> 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<sup>39</sup> or for plants  
 to facilitate heavy metal phytoremediation.<sup>40</sup> 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 un-  
 known.<sup>41,42</sup> Moreover, this work demonstrates that the  
 application of high-throughput methodologies such as tran-  
 scriptomics, but also (potentially) proteomics<sup>43</sup> and metab-  
 olomics,<sup>44</sup> coupled with analytical and physiological data can  
 bridge the gap between genotype and phenotype regarding  
 plant response to ENM exposure.

## 647 ■ ASSOCIATED CONTENT

## 648 ● Supporting Information

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

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

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## 672 Notes

673 The authors declare no competing financial interest.

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## 683 ■ REFERENCES

684 (1) Bumbudsanpharoke, N.; Ko, S. Nano-food packaging: an  
685 overview of market, migration research, and safety regulations. *J.*  
686 *Food Sci.* **2015**, *80* (5), 910–23.  
687 (2) Holden, P. A.; Nisbet, R. M.; Lenihan, H. S.; Miller, R. J.; Cherr,  
688 G. N.; Schimel, J. P.; Gardea-Torresdey, J. L. Ecological nano-  
689 toxicology: integrating nanomaterial hazard considerations across the  
690 subcellular, population, community, and ecosystems levels. *Acc. Chem.*  
691 *Res.* **2013**, *46* (3), 813–822.  
692 (3) Holden, P. A.; Klaessig, F.; Turco, R. F.; Priester, J. H.; Rico, C.  
693 M.; Avila-Arias, H.; Mortimer, M.; Pacpaco, K.; Gardea-Torresdey, J.  
694 L. Evaluation of exposure concentrations used in assessing  
695 manufactured nanomaterial environmental hazards: are they relevant?  
696 *Environ. Sci. Technol.* **2014**, *48* (18), 10541–10551.  
697 (4) Petersen, E. J.; Henry, T. B.; Zhao, J.; MacCuspie, R. I.;  
698 Kirschling, T. L.; Dobrovolskaia, M. A.; Hackley, V.; Xing, B.; White, J.  
699 C. Identification and avoidance of potential artifacts and misinter-  
700 pretations in nanomaterial ecotoxicity measurements. *Environ. Sci.*  
701 *Technol.* **2014**, *48*, 4226–46.  
702 (5) Hawthorne, J.; De la Torre Roche, R.; Xing, B.; Newman, L. A.;  
703 Ma, X.; Majumdar, S.; Gardea-Torresdey, J.; White, J. C. Particle-size  
704 dependent accumulation and trophic transfer of cerium oxide through  
705 a terrestrial food chain. *Environ. Sci. Technol.* **2014**, *48*, 13102–13109.  
706 (6) Conway, J. R.; Hanna, S. K.; Lenihan, H. S.; Keller, A. A. Effects  
707 and implications of trophic transfer and accumulation of CeO<sub>2</sub>

nanoparticles in a marine mussel. *Environ. Sci. Technol.* **2014**, *48* (3),  
1517–1524. 708

(7) Gardea-Torresdey, J. L.; Rico, C. M.; White, J. C. Trophic  
transfer, transformation, and impact of engineered nanomaterials in  
terrestrial environments. *Environ. Sci. Technol.* **2014**, *48*, 2526–2540. 712

(8) Schwab, F.; Zhai, G.; Kern, M.; Turner, A.; Schnoor, J. L.;  
Wiesner, M. R. Barriers, pathways and processes for uptake,  
translocation and accumulation of nanomaterials in plants – Critical  
review. *Nanotoxicology* **2016**, *10* (3), 257–78. 716

(9) Ma, C.; White, J. C.; Dhankher, O. P.; Xing, B. Metal-based  
nanotoxicity and detoxification pathways in higher plants. *Environ. Sci.*  
*Technol.* **2015**, *49*, 7109–7122. 719

(10) Silins, I.; Högberg, J. Combined Toxic Exposures and Human  
Health: Biomarkers of Exposure and Effect. *Int. J. Environ. Res. Public*  
*Health* **2011**, *8* (3), 629–647. 722

(11) Brain, R. A.; Cedergreen, N. Biomarkers in aquatic plants:  
selection and utility. *Rev. Environ. Contam Toxicol.* **2009**, *198*, 49–109. 724

(12) Schudoma, C.; Steinfath, M.; Sprenger, H.; van Dongen, J. T.;  
Hincha, D.; Zuther, E.; Geigenberger, P.; Kopka, J.; Köhl, K.; Walther,  
D. Conducting Molecular Biomarker Discovery Studies in Plants. In  
*High-Throughput Phenotyping in Plants: Methods and Protocols*;  
Normanly, J., Ed.; 2012, 918, pp 127–150; 10.1007/978-1-61779-  
995-2\_10 730

(13) Tsuji, J. S.; Maynard, A. D.; Howard, P. C.; James, J. T.; Lam,  
C.; Warheit, D. B.; Santamaria, A. B. Research strategies for safety  
evaluation of nanomaterials, part IV: risk assessment of nanoparticles.  
*Toxicol. Sci.* **2005**, *89*, 42–50. 734

(14) Chen, S.; Chen, B.; Fath, B. D. Ecological risk assessment on the  
system scale: A review of state-of-the-art models and future  
perspectives. *Ecol. Modell.* **2013**, *250*, 25–33. 737

(15) Landa, P.; Vankova, R.; Androva, J.; Hodek, J.; Marsik, P.;  
Storchova, H.; White, J. C.; Vanek, T. Nanoparticle-specific changes in  
*Arabidopsis thaliana* gene expression after exposure to ZnO, TiO<sub>2</sub>, and  
fullerene soot. *J. Hazard. Mater.* **2012**, *241*, 55–62. 741

(16) Landa, P.; Prerostova, S.; Petrova, S.; Knirsch, V.; Vankova, R.;  
Vanek, T. The transcriptomic response of *Arabidopsis thaliana* to zinc  
oxide: a comparison of the impact of nanoparticles, bulk, and ionic  
zinc. *Environ. Sci. Technol.* **2015**, *49* (24), 14537–14545. 745

(17) Kaveh, R.; Li, Y.-S.; Ranjbar, S.; Tehrani, R.; Brueck, C. L.; Van  
Aken, B. Changes in *Arabidopsis thaliana* gene expression in response  
to silver nanoparticles and silver ions. *Environ. Sci. Technol.* **2013**, *47*,  
10637–10644. 749

(18) Marmiroli, M.; Pagano, L.; Savo Sardaro, M. L.; Villani, M.;  
Marmiroli, N. Genome-wide approach in *Arabidopsis thaliana* to assess  
the toxicity of cadmium sulfide quantum dots. *Environ. Sci. Technol.*  
*Environ. Sci. Technol.* **2014**, *48*, 5902–5909. 753

(19) Anjum, N. A.; Adam, V.; Kizek, R.; Duarte, A. C.; Pereira, E.;  
Iqbal, M.; Lukatkin, A. S.; Ahmad, I. Nanoscale copper in the soil-plant  
system-toxicity and underlying potential mechanisms. *Environ. Res.*  
**2015**, *138*, 306–325. 757

(20) Dimkpa, C. O.; McLean, J. E.; Latta, D. E.; Manangon, E.; Britt,  
D. W.; Johnson, W. P.; Boyanov, M. I.; Anderson, A. J. CuO and ZnO  
nanoparticles: phytotoxicity, metal speciation, and induction of  
oxidative stress in sand-grown wheat. *J. Nanopart. Res.* **2012**, *14*,  
1125–39. 762

(21) Hong, J.; Rico, C. M.; Zhao, L.; Adeleye, A. S.; Keller, A. A.;  
Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Toxic effects of copper-  
based nanoparticles or compounds to lettuce (*Lactuca sativa*) and  
alfalfa (*Medicago sativa*). *Environ. Sci.: Processes Impacts* **2015**, *17*, 177. 766

(22) Dahle, J. T.; Arai, Y. Environmental geochemistry of cerium:  
applications and toxicology of cerium oxide nanoparticles. *Int. J.*  
*Environ. Res. Public Health* **2015**, *12*, 1253–1278. 769

(23) Ma, C.; Chhikara, S.; Xing, B.; Musante, C.; White, J. C.;  
Dhankher, O. P. Physiological and molecular response of *Arabidopsis*  
*thaliana* (L.) to nanoparticle cerium and indium oxide exposure. *ACS*  
*Sustainable Chem. Eng.* **2013**, *1*, 768–778. 773

(24) Majumdar, S.; Almeida, I. C.; Arigi, E. A.; Choi, H.;  
VerBerkmoes, N. C.; Trujillo-Reyes, J.; Flores-Margez, J. P.; White,  
J. C.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Environmental 776

- 777 Effects of Nanocerium on Seed Production of Common Bean (*Phaseolus*  
778 *vulgaris*): A Proteomic Analysis. *Environ. Sci. Technol.* **2015**, *49* (22),  
779 13283–13293.
- 780 (25) Ma, Y.; He, X.; Zhang, P.; Zhang, Z.; Guo, Z.; Tai, R.; Xu, Z.;  
781 Zhang, L.; Ding, Y.; Zhao, Y.; Chai, Z. Phytotoxicity and  
782 biotransformation of La<sub>2</sub>O<sub>3</sub> nanoparticles in a terrestrial plant  
783 cucumber (*Cucumis sativus*). *Nanotoxicology* **2011**, *5* (4), 743–53.
- 784 (26) De la Torre Roche, R.; Servin, A.; Hawthorne, J.; Xing, B.;  
785 Newman, L. A.; Ma, X.; Chen, G.; White, J. C. Terrestrial trophic  
786 transfer of bulk and nanoparticle La<sub>2</sub>O<sub>3</sub> does not depend on particle  
787 size. *Environ. Sci. Technol.* **2015**, *49* (19), 11866–11874.
- 788 (27) The Tomato Genome Consortium. The tomato genome  
789 sequence provides insights into fleshy fruit evolution. *Nature* **2012**,  
790 *485*, 635–641.
- 791 (28) Esteras, C.; Gomez, P.; Monforte, A. J.; Blanca, J.; Vicente-  
792 Dolera, N.; Roig, C.; Nuez, F.; Pico, B. High-throughput SNP  
793 genotyping in Cucurbita pepo for map construction and quantitative  
794 trait loci mapping. *BMC Genomics* **2012**, *13*, 80.
- 795 (29) Dimkpa, C. O.; McLean, J. E.; Martineau, N.; Britt, D. W.;  
796 Haverkamp, R.; Anderson, A. J. Silver nanoparticles disrupt wheat  
797 (*Triticum aestivum* L.) growth in a sand matrix. *Environ. Sci. Technol.*  
798 **2013**, *47*, 1082–1090.
- 799 (30) Ma, Y.; Zhang, P.; Zhang, Z.; He, X.; Li, Y.; Zhang, J.; Zheng, L.;  
800 Chu, S.; Yang, K.; Zhao, Y.; Chai, Z. Origin of the different  
801 phytotoxicity and biotransformation of cerium and lanthanum oxide  
802 nanoparticles in cucumber. *Nanotoxicology* **2015**, *9* (2), 262–70.
- 803 (31) Cornelis, G.; Ryan, B.; McLaughlin, M. J.; Kirby, J. K.; Beak, D.;  
804 Chittleborough, D. Solubility and batch retention of CeO<sub>2</sub> nano-  
805 particles in soils. *Environ. Sci. Technol.* **2011**, *45* (7), 2777–82.
- 806 (32) Wang, Z.; Xie, X.; Zhao, J.; Liu, X.; Feng, W.; White, J. C.; Xing,  
807 B. Xylem- and phloem-based transport of CuO nanoparticles in maize  
808 (*Zea mays* L.). *Environ. Sci. Technol.* **2012**, *46*, 4434–4441.
- 809 (33) Aslani, F.; Bagheri, S.; Muhd Julkapli, N.; Juraimi, A. S.;  
810 Hashemi, F. S. G.; Baghdadi, A. Effects of engineered nanomaterials on  
811 plants growth: an overview. *Sci. World J.* **2014**, *2014*, 641759.
- 812 (34) Van Nhan, L.; Ma, C.; Rui, Y.; Liu, S.; Li, X.; Xing, B.; Liu, L.  
813 Phytotoxic mechanism of nanoparticles: destruction of chloroplasts  
814 and vascular bundles and alteration of nutrient absorption. *Sci. Rep.*  
815 **2015**, *5*, 11618.
- 816 (35) Wang, Z.; Xu, L.; Zhao, J.; Wang, X.; White, J. C.; Xing, B. CuO  
817 Nanoparticle interaction with *Arabidopsis thaliana*: toxicity, parent-  
818 progeny transfer and gene expression. *Environ. Sci. Technol.* **2016**, *50*,  
819 6008.
- 820 (36) Cramer, G. R.; Urano, K.; Delrot, S.; Pezzotti, M.; Shinozaki, K.  
821 Effects of abiotic stress on plants: a systems biology perspective. *BMC*  
822 *Plant Biol.* **2011**, *11*, 163.
- 823 (37) Szakal, C.; Roberts, S. M.; Westerhoff, P.; Bartholomaeus, A.;  
824 Buck, N.; Illuminato, I.; Canady, R.; Rogers, M. Measurement of  
825 nanomaterials in foods: integrative consideration of challenges and  
826 future prospects. *ACS Nano* **2014**, *8* (4), 3128–3135.
- 827 (38) White, J. C.; Xing, B. Environmental Nanotoxicology. *Environ.*  
828 *Sci. Technol.* **2016**, *50*, 5423–5423.
- 829 (39) Rawat, N.; Neelam, K.; Tiwari, V. K.; Dhaliwal, H. S.  
830 Biofortification of cereals to overcome hidden hunger. *Plant Breed.*  
831 **2013**, *132*, 437–445.
- 832 (40) Halimaa, P.; Lin, Y. F.; Ahonen, V. H.; Blande, D.; Clemens, S.;  
833 Gyenesei, A.; Häikiö, E.; Kärenlampi, S. O.; Laiho, A.; Aarts, M. G.;  
834 Pursiheimo, J. P.; Schat, H.; Schmidt, H.; Tuomainen, M. H.;  
835 Tervahauta, A. I. Gene expression differences between *Noccaea*  
836 *caerulescens* ecotypes help to identify candidate genes for metal  
837 phytoremediation. *Environ. Sci. Technol.* **2014**, *48* (6), 3344–3353.
- 838 (41) Tong, T.; Wilke, C. M.; Wu, J.; Binh, C. T. T.; Kelly, J. J.;  
839 Gaillard, J. F.; Gray, K. A. Combined toxicity of nano-ZnO and nano-  
840 TiO<sub>2</sub>: from single- to multinanomaterial systems. *Environ. Sci. Technol.*  
841 **2015**, *49* (13), 8113–8123.
- 842 (42) Servin, A. D.; White, J. C. Nanotechnology in agriculture: next  
843 steps for understanding engineered nanoparticle exposure and risk.  
844 *NanoImpact.* **2016**, *1*, 9.
- (43) Marmiroli, M.; Imperiale, D.; Pagano, L.; Villani, M.; Zappettini, 845  
A.; Marmiroli, N. The proteomic response of *Arabidopsis thaliana* to 846  
cadmium sulfide quantum dots, and its correlation with the 847  
transcriptomic response. *Front. Plant Sci.* **2015**, *16* (6), 1104. 848
- (44) Zhao, L.; Huang, Y.; Hu, J.; Zhou, H.; Adeleye, A. S.; Keller, A. 849  
A. <sup>1</sup>H NMR and GC-MS Based Metabolomics Reveal Defense and 850  
Detoxification Mechanism of Cucumber Plant under Nano-Cu Stress. 851  
*Environ. Sci. Technol.* **2016**, *50* (4), 2000–2010. 852