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Molecular and physiological responses to titanium dioxide and cerium oxide nanoparticles in *Arabidopsis*

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Abstract

Changes in tissue transcriptomes and productivity of *Arabidopsis thaliana* were investigated during exposure of plants to two widely used engineered metal oxide nanoparticles, titanium dioxide (nano-titanium) and cerium dioxide (nano-cerium). Microarray analyses confirmed that exposure to either nanoparticle altered the transcriptomes of rosette leaves and roots, with comparatively larger numbers of differentially expressed genes (DEGs) found under nano-titanium exposure. Nano-titanium induced more DEGs in rosette leaves, whereas roots had more DEGs under nano-ceria exposure. MapMan analyses indicated that while nano-titanium up-regulated overall metabolism in both tissues, metabolic processes under nano-ceria remained mostly unchanged. Gene enrichment analysis indicated that both nanoparticles mainly enriched ontology groups such as responses to stress (abiotic and biotic), and defense responses (pathogens), and responses to endogenous stimuli (hormones). Nano-titanium specifically induced genes associated with photosynthesis, whereas nano-ceria induced expression of genes related to activating transcription factors, most notably those belonging to the ethylene responsive element binding protein family. Interestingly, there were also increased numbers of rosette leaves and plant biomass under nano-ceria exposure, but not under nano-titanium. Other transcriptomic responses did not clearly relate to responses observed at the organism level. This may be due to functional and genomic redundancy in *Arabidopsis*, which may mask expression of morphological changes, despite discernable responses at the transcriptome level. Additionally, transcriptomic changes often relate with transgenerational phenotypic development, hence it may be productive to direct further experimental work to integrate high-throughput genomic results with longer-term changes in subsequent generations.

Keywords

Arabidopsis thaliana; Transcriptome; Growth; Nano-ceria; Nano-titanium; Engineered Nanoparticles

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INTRODUCTION

Nanotechnology continues to be a rapidly developing industry, which relies on manipulating physical and chemical properties of matter at the atomic and molecular scales [1]. The technology has prompted innovation in diverse fields, including food, agriculture, health, transport, defense, energy, electronics, and communications [2]. Creating engineered nanoparticles (ENPs) is a major focus that includes use of diverse starting materials, e.g., metals, metal oxides, nonmetals, carbon, polymers, and lipids [3]. Thus production and uses of ENPs are expected to increase, and consequently, so will their impact on the environment, with the latter being relatively much less understood [4]. The metal oxide group of ENPs is considered one of the most important, indicated by their extensive and diverse applications; among them cosmetics, sunscreens, textiles, self-cleaning coatings, water-treatment agents, solar batteries, and automobile catalytic converters [5]. Among metal oxide ENPs, titanium dioxide nanoparticles (nano-titania) are expected to reach the highest production levels, between 7800 and 38000 tons per year, and production of cerium oxide nanoparticles (nano-ceria) will rank third with estimated rates rising to 35 – 700 tons per year [6]. While nano-titania mainly is discharged via surface run off, and in sewerage sent to wastewater treatment plants [7], release of nano-ceria to the environment primarily is attributed to combustion exhaust from diesel motors, along with releases associated with their usage in polishing and coatings, and biomedical applications [8].

The same properties that make ENPs uniquely suited to diverse technological applications also may elicit unanticipated responses among the great diversity of endpoints considered in the fields of human and ecological toxicology. For example, nano-titania can bioaccumulate [9] potentially becoming toxic to aquatic species [10], plants [11], and humans [12, 13]. Similarly, nano-ceria may be toxic to algae [14], plants [15, 16], earthworms [17], and humans [18, 19]. There are few studies [20–25] that have evaluated changes in terrestrial plant transcriptomes under exposures to nanoparticles. However, connections between transcriptomic responses and phenotypic development in exposed plants are starting to be established. As ENP-induced regulatory mechanisms in plants are not well understood, we explored this area in the present study using an integrated approach focusing on changes in the transcriptome and effects on plant growth.

Previously, we reported phenotypic and genomic responses under exposure to nano-titania and nano-ceria in *Arabidopsis* germinants [26]. There we found affected genes annotated for groups responsive to abiotic (oxidative stress, salt stress, water transport) and biotic (respiratory burst as a defense against pathogens) stimuli, and which regulate metabolism (DNA metabolism, hormone metabolism, tetrapyrrole synthesis, and photosynthesis). We also demonstrated that both ENPs increased percentages of germinating seeds, early development of hypocotyls and cotyledons, and numbers of leaves.

In the present study, we further examined if exposure to these two ENPs, which have different physico-chemical properties, would produce similar transcriptomic and physiological changes over the plant lifecycle compared to our earlier research with *Arabidopsis* germinants. The goals of the research were threefold, to: 1) compare responses

to exposure to the two ENPs to determine whether similar modes of action appear to occur for the two particles throughout plant development; 2) evaluate responses in both roots and shoots to determine if one tissue is more susceptible or responsive to ENP exposure; and 3) compare changes in gene expression with phenological development to determine the degree to which ENP exposure results in larger-scale developmental shifts. To address these goals, we followed plant growth and development by measuring numbers of primary rosette leaves, diameters of primary rosette leaves, aboveground biomass, numbers of siliques and silique dry weight. Also, we examined transcriptomic responses in two tissues, i) rosette leaves and ii) roots. Gathering such information would provide better insight into the applicability of using molecular changes in young plants as assessment tools to determine risk and potential ecotoxicological implications of releases of metal ENPs.

MATERIALS AND METHODS

Nanoparticle suspension preparation and characterization

Noncoated nano-titania (P25, Evonik Degussa), and noncoated nano-ceria (NM-212, Mercator), with primary particle sizes (diameter) of 21nm and 33nm respectively, were used for all the experiments in this study. The methods used to prepare and characterize the ENP suspensions have been described in our recent studies [26–28], and these optimized methods (as described in this section) are retained in all our subsequent and ongoing studies in order to maintain the consistency. Suspensions were prepared at 0 (control) and 500 mg ENPs L⁻¹ using 0.1 M KCl and millipore water, for nano-titania and nano-ceria, respectively, to promote stability and improve measurability of the zeta-potential. Suspensions were indirectly sonicated for 30 min. This indirect sonication method was performed using a Misonix S4000 digital sonicator (QSonica), with a 14-cm-diameter probe [cup horn] contained in a chilled reservoir in which a suspension-containing vessel is submerged. Nanoparticles were added to the diluent and sonicated at 50% power (~ 100 W) for 30 min. The pH was adjusted to 5.4 using an Accumet XL15 meter (Fisher Scientific), in conjunction with zeta-potential measurements using ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments), to ensure suspension consistency between batches of prepared suspensions used for the exposures. Mean physical particle sizes in stock suspensions and diluted suspensions were similar to (nano-titania) or smaller than (nano-ceria) sizes indicated by the manufacturer. In all cases, particle sizes ranged from approximately 10 nm to a small cohort exceeding 50 nm.

Preparation of pots, seed germination, nanoparticle exposures, and plant care

Arabidopsis thaliana wild-type ecotype Columbia (Col-0) seeds (Lehle Seeds) were surface-sterilized with 70 % (v/v) ethanol, further sterilized in 50 % (v/v) household bleach, and then washed four times with sterile water. Seeds were stratified (4°C for 8 h) and then placed on the surface of *Arabidopsis* potting media (PM-15–13, Lehle Seeds) contained in pots (5.7 cm diameter; 5.7 cm height). Pots were kept in a growth chamber for seed germination and plant growth (22°C, light 100 µmol m⁻² s⁻¹, photoperiod 16 h/8 h light/dark).

Plants for phenotypic observations ($n = 35$ to 40) and microarray analyses ($n = 3$) were exposed by watering from above with control solution or the ENP treatment suspensions

twice during the seed germination stage (Day 0–4) and for a third time during the primary rosette stage (Day 17).

Phenotype observations

Plant growth was assessed repeatedly during development by taking the following measurements a) number of rosette leaves (Days 25 and 29 for nano-titania; 24 and 31 for nano-ceria), b) diameter of rosette leaves (Days 28, 32, 36, 40, 44, 48, 50 and 54 for nano-titania; 26, 31, 35, 38, 42, 45, 49, and 51 for nano-ceria), c) plant height (Days 32, 36, 40, 44, 48, 50 and 54 for nano-titania; 26, 31, 35, 38, 42, 45 and 49 for nano-ceria), d) number of siliques per plant, e) dry weight of siliques, and f) final aboveground biomass. The number of rosette leaves from the basal rosette of each plant were counted. Rosette diameters were measured as the maximum distance from the leaf-tip to leaf-tip across the basal rosette. Plant height was measured to the top of the live tissue for each plant. Siliques (from all branches of the plant) were counted at the time of the harvest. The weight of siliques (10 siliques per plant), and aboveground biomass were determined after air drying samples at 60°C for 6 days. Glassine envelopes and coin envelopes (paper #7) were used for silique and aboveground biomass samples respectively. Sample weights were determined by subtracting the weights of the envelopes. The two-tailed *t*-test for unequal variances was used to identify statistically significance differences ($p < 0.05$) between ENP treatments and their respective controls.

Molecular techniques

Microarray Procedures and Data Analyses.—Effects of exposure to the ENPs on gene expression in rosette leaves and roots were confirmed by microarray and quantitative real-time polymerase chain reaction (qRT-PCR) analyses as per our earlier work [22]. Total RNA was extracted from ~200 mg per sample of 29-day old plant tissues ($n = 3$) using the RNeasy RT (Molecular Research Center Inc.) followed by DNase treatment. The extraction was purified on RNeasy mini spin columns (Qiagen, Inc.). RNA quality and integrity was assessed by using an Agilent RNA 6000 Nano Assay kit and a 2100 Agilent Bioanalyzer (Agilent Technologies, Inc.). RNA was prepared for the Affymetrix *Arabidopsis* whole genome oligonucleotide GeneChip arrays (ATH1) according to the manufacturer's protocol. The ATH1 microarray contains 22,746 probe sets representing all predicted genes. Considering redundant sequences, the number of genes using available annotation tools is estimated to be 25,000 to 30,000 [29]. Among genes determined to be statistically different between control and respective ENP treatment, we limited further analyses to those exhibiting at least a 2-fold change in expression (i.e., either up-regulated or down-regulated).

Biotin-labeled cRNA was produced from 15 µg total RNA using an Affymetrix “one-way” labeling kit. Total cRNA was quantified using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies). Quality was evaluated after cRNA fragmentation on a 2100 Bioanalyzer. Following overnight hybridization (45°C) to Affymetrix ATH1 GeneChips in an Affymetrix Model 640 GeneChip hybridization oven, arrays were washed and stained using an Affymetrix 450 fluidics station as per the manufacturer and then scanned on an Affymetrix Model 3000 scanner. Raw scanning data files (Affymetrix.cel) were obtained using Affymetrix GeneChip Operating Software, v1.4, and then files were analyzed by

Bioconductor SimpleAffy to assess data quality [30]. Data were quantile normalized and gene calls were generated using a robust multichip average algorithm [31]. Next, ArrayStar, v5.0 (DNASTar, Inc.) was used to generate gene expression results and calculate p values (moderated t test). The complete microarray data sets discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) repository and are accessible through GEO series accession number GSE80461 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=uhwxogyaltcrfod&acc=GSE80461>).

Differentially expressed genes (DEGs; $p < 0.05$, 2-fold) were evaluated for relevance to canonical pathways and biological functions using MapMan software [32]. The list of DEGs obtained was imported into the MapMan gene ontology program to organize them into various functional categories and pathways ("Bins" and "sub-Bins"). Differential fold-change values were displayed via a false color code in diagrams. Transcripts that increase and decrease are shown in red and blue respectively.

A Cytoscape (version 3.1.1) plugin, the Biological Networks Gene Ontology tool (BiNGO 3.0.2) was used to identify overrepresented gene ontology (GO) terms [33]. GO consists of three hierarchically structured vocabularies that describe gene products in terms of their associated biological processes, molecular functions and cellular components. In this study, we present the over-representation (enrichment) of these GO terms within these three structured hierarchies. For this, we used up- and down-regulated DEGs to retrieve the functional profile of these gene sets. This enrichment of GO terms was performed using a hypergeometric test with corrected p -values at significance levels of $p < 5E-7$ and $p < 0.05$, after applying the Benjamini and Hochberg FDR correction [34].

Quantitative real-time PCR procedures and data analyses.—Quantitative real-time PCR was used to validate expression fold-changes for 12 representative genes that were identified via microarray as significantly up- or down-regulated, essentially as in [22]. Expression levels of up- and down-regulated genes were quantified in rosette leaves and roots. Primers for an endogenous reference gene, *ef1A1* [26] and regulated genes, were designed from mRNA sequences in GeneBank using PrimerSelect (DNASTar, Inc.) and Primer3 Plus [35] (Supplemental Data, Table S1). Oligonucleotides were produced by Eurofins MWG Operon, Inc. There were two technical qPCR replicates (25 μ L) for each standard (created by dilution series of cloned target sequence), unknown (cDNA from treated or control sample) and no template control. All qPCR thermal profiles contained these steps: activation for 15 min at 95°C; 40 cycles of [15 s at 95°C, 30 s at annealing temperature (Supplemental Data, Table S1), 30 s at 72°C]; 1 min at 95°C, followed by a ramp from the annealing temperature up to 95°C. Absolute quantity and amplicon dissociation data for qPCRs were collected on a Stratagene Mx3005P workstation (Agilent Technologies, Inc.).

Transcript quantities of regulated genes were normalized by the endogenous reference gene. Normalized quantities of regulated gene transcripts from treated samples were then calibrated to those from control samples to produce gene expression fold-change ratios. Standard errors (SE) of fold-change among biological replicates were also calculated.

RESULTS

Phenotype analysis

Rosette leaves were counted on days 25 and 29 for nano-titania, and on days 24 and 31 for nano-ceria. While nano-titania did not have any significant effect on rosette leaf numbers on either day (Figure 1A), under nano-ceria numbers of rosette leaves were significantly increased on both days (Figure 1B; day 24: $p = 0.023$, day 31: $p = 0.0001$). With the exception of day 35, when rosette leaf diameters in plants exposed to nano-ceria treatment were significantly higher than the control values (Figure 1D; $p = 0.029$), there was no significant difference in the diameters of leaves between ENP treatments and their respective controls (Figures 1C, D). Neither ENP significantly affected plant height (Figures 1E, F). Above-ground dry biomass was unaffected by nano-titania exposure (Figure 1G), whereas it was significantly higher under nano-ceria exposure (Figure 1H; $p = 0.044$). Neither ENP significantly affected the number of siliques per plant (Figures 1I, J) or silique weight (Figures 1K, L).

Microarray analysis

Overall, regardless of tissue (rosette leaves and roots), less than 10% and 5% of genes in *A. thaliana* were significantly regulated by exposure to nano-titania and nano-ceria, respectively (Table 1). In general more genes were affected under nano-titania than under nano-ceria exposure (Table 1). More specifically, under nano-titania, over 2-times more DEGs (2-fold) were found in rosette leaves compared with respective values under nano-ceria, and in roots the comparable increase between ENPs was greater than 13-times. Generally more up-regulated than down-regulated genes were recorded for all ENP-tissue comparisons except for genes expressed in rosette leaves under nano-ceria. Fewer genes were affected by either ENP as the fold-change threshold was increased, reaching only a very few at 8 fold-change. The decreases in numbers of DEGs as the fold-change threshold was increased were more visible in tissues treated with nano-ceria where at 8 fold-change no DEGs were recorded in rosette leaves and very few were found in roots. Of all the 2 fold-change DEGs, 207 transcripts were common between tissue types under nano-titania (Supplemental Data, Table S2), whereas only 9 DEGs were common under nano-ceria (Supplemental Data, Table S3). Microarray data for all 2 fold-change DEGs are listed in the supplementary data (Supplemental Data, Tables S4-S7).

Exposure to nano-titania

We found 1789 (1136 up-regulated, 653 down-regulated) and 1764 (966 up-regulated, 799 down-regulated) DEGs in rosette leaves and roots, respectively, under nano-titania exposure regime (Table 1). In rosette leaves, the top 50 DEGs (38 up-regulated and 12 down-regulated) mediate a variety of biological processes (Supplemental Data, Table S8). Notable up-regulated genes encode for triterpenoid biosynthesis, indole glucosinolate metabolism, iron transport, tryptophan catabolism, and oxidation-reduction reactions. Notable down-regulated transcripts annotate for transcription, response to ethylene stimulus, and response to jasmonic acid stimulus. Transcripts expressing greater up-regulation (8-fold) generally relate to cell wall processes, and comparably down-regulated transcripts annotate for transcription activity.

In roots, top 50 DEGs (37 up-regulated and 13 down-regulated) annotate for responses to oxidative stress, photosynthesis, chloroplast organization and metal ion transport (Supplemental Data, Table S9). Notable up-regulated processes annotate for harvesting light, photosynthesis, response to jasmonic acid, responses to oxidative stress, and carbohydrate transport. Notable down-regulated DEGs encode for metal ion transport, protein phosphorylation and responses to auxin stimulus. Up-regulated transcripts with fold-change 8 involve rRNA processing, responding to light and stress, and comparably down-regulated DEGs annotate for metal ion transport, root development, and nitrate transport.

MapMan Analysis

The metabolism overview map revealed many up-regulated genes in both tissues under nano-titania influence photosynthesis, cell wall proteins and energy metabolism (Figure 2). The biotic stress overview map showed nano-titania up-regulated genes associated with the cell wall (synthesis, modification, degradation), abiotic stress (heat, salt, wounding), and various hormonal signaling pathways and mitogen-activated protein kinases (MAPK) (Supplemental Data, Figure S1). Additional MapMan analysis can be found in the Supplemental Results.

Over-representation of Gene Ontology (GO) Terms

In leaves, we found significant over-representation of GO terms among up-regulated genes (Figure 3A), which were more numerous compared with representation among down-regulated genes (Figure 3B). Significantly over-represented GO terms for up-regulated transcripts included those for metabolic processes such as photosynthesis, growth, carbohydrate metabolism, and organization of cellular components. Also enriched were terms associated with responses to biotic and abiotic stress. .

Significantly over-represented GO terms for up-regulated transcripts in roots included those related to response to stress, response to biotic and abiotic stimulus, and carbohydrate metabolism (Figure 3C). Down-regulated transcripts enriched biological process terms related to transport and secondary metabolism (Figure 3D). Additional over-representation of GO terms can be found in the Supplementary Results.

Exposure to nano-ceria

We found 130 (56 up-regulated, 74 down-regulated) and 754 (391 up-regulated, 363 down-regulated) DEGs in rosette leaves and roots, respectively, that responded to nano-ceria (Table 1). In rosette leaves, the top 50 DEGs (18 up-regulated and 32 down-regulated) mediate a variety of biological processes (Supplemental Data, Table S10). Notable up-regulated genes affect transcription, aging, regulation of hydrogen peroxide metabolism and the cell cycle. Notable down-regulated genes were those that respond to the auxin stimulus and cell wall modifications.

In roots, the top 50 DEGs (20 were up-regulated and 30 down-regulated) mediate variety of biological process (Supplemental Data, Table S11) such as regulation of transcription, phenylpropanoid biosynthesis, seed maturation, and response to gibberellin stimulus being notable among up-regulated genes, and cell wall organization, syncytium formation, cell

signaling, cell cycle, and polysaccharide catabolic process being notable among down-regulated genes.

MapMan Analysis

The metabolism overview map revealed only 17 genes within identified pathways in rosette leaves, and most of them were down-regulated transcripts annotating processes such as cell wall degradation, light reactions and the Calvin cycle (Figure 4A). In contrast to rosette leaves, roots exposed to nano-ceria showed 153 genes visible in the pathways (Figure 4B), with most of these transcripts mapping to cell wall protein, carbohydrate, lipid, and secondary metabolism. Biotic stress overview map (Supplemental Data, Figure S3) showed nano-ceria mostly down-regulated genes in rosette leaves associated with the biotic and abiotic stress, auxin and brassinosteroid hormonal signaling plus secondary metabolism. Whereas in roots nano-ceria up-regulated genes associated with biotic and abiotic stress, mitogen-activated protein kinase (MAPK) signaling.. MapMan's regulation overview (Supplemental Data, Figure S4) revealed that except for the EBP transcription factors, and signaling associated with sugar and nutrient physiology, the regulatory components are down-regulated in rosette leaves, whereas in roots there was a general down-regulation of regulatory components.

Over-representation of Gene Ontology (GO) Terms

Up-regulated genes in rosette leaves enriched responses to stress, endogenous stimulus (ethylene stimulus), and biotic stimulus (UV-B light and photosynthetic acclimation) (Figure 5A). Down-regulated transcripts mostly enriched biological processes related to stimuli such as stress, defense and endogenous stimulus (hormones, especially auxin) (Figure 5B).

In roots, nano-ceria up-regulated transcripts enriched processes similar to those observed in leaves (response to stress and stimuli) as well as transport processes, specifically protein transport to the chloroplast (Figure 5C). Down-regulated transcripts enriched carbohydrate metabolism, growth, response to endogenous and biotic stimuli (Figure 5D).

Validation of microarray results

The expressions of twelve genes found to be significantly regulated via microarray analyses were validated by qPCR; three up-regulated and three down-regulated genes each for roots and rosette leaves. Expression of each gene was normalized to the expression of the *ef1A1* endogenous reference gene. The magnitude and direction of fold-changes determined by qPCR were similar to those from microarrays. The linear correlations (R^2) for qPCR and microarray data from regulated genes in rosette leaves and roots were 0.9194 and 0.8704 respectively (Supplemental Data, Figures S9A & B).

DISCUSSION

Differential Responses to nano-ceria and nano-titania

Nano-titania and nano-ceria altered the transcriptomes of *A. thaliana* plants in unique ways, suggesting that the modes of action of the particles may be different. Overall, the transcriptomes in both rosette leaves and roots were less affected by exposure to nano-ceria

than to nano-titania. Compared with nano-ceria, nano-titania significantly changed more transcripts in both tissues. In rosette leaves, nano-ceria exposure resulted in 130 DEGs (2-fold), which is 13 times less than the comparable number found under exposure to nano-titania. For roots, although we identified 754 DEGs under nano-ceria, this response is 2-times less than the comparable number of DEGs found under nano-titania.

Unlike under nano-titania, exposure to nano-ceria did not up-regulate overall metabolism (Figures 4A & 4B). Rather, it was overwhelmingly down-regulated in both tissues under nano-ceria exposure. The very few up-regulated transcripts associated with metabolic pathways were observed only in the root tissues, mapped to lipid metabolism (phospholipid synthesis), minor carbohydrate metabolism, and secondary metabolism (terpenoids). Phospholipids and terpenoids are compounds involved in adaptive responses to stress and defense [36, 37] as has been documented previously for *Arabidopsis* [38]. This response to stress was further confirmed in the MapMan biotic stress overview (Supplemental Data, Figure S3) where under nano-ceria exposure roots showed slight up-regulation of genes associated with these stress pathways; none of these pathways were up-regulated in rosette leaves under nano-ceria exposure. Interestingly, the MapMan regulation overview (Supplemental Data, Figure S4) on nano-ceria exposed leaves showed up-regulation of 10 transcription factors, out of which 7 belong to the AP2 (APETALA2)/EREBP (ethylene-responsive element binding proteins) family of plant transcription factors, which plays an important role in reproductive and vegetative development of *Arabidopsis* [39] (Licausi et al. 2013; Mizoi et al. 2012). Also, the regulation overview map revealed up-regulation of nonsymbiotic hemoglobin gene, AHb1 in nano-ceria exposed leaves. AHb1 is induced during low oxygen conditions and under high nitric oxide (NO) levels, and is known to modulate growth and development [40, 41].

Differential Responses in Rosette Leaves and Roots

Although the transcriptomes generally were distinct between rosette leaves and roots, there were transcripts affected by ENP exposure common to both tissues. Within these common transcripts, however, 47 were found with opposite regulation between tissues when exposed to nano-titania (Supplemental Data, Table S12) while only 5 transcripts had opposite regulation when exposed to nano-ceria (Supplemental Data, Table S13). The fact that opposite regulation was more prevalent in nano-titania tissues again suggests that the two ENPs have distinct effects on plant gene expression, and underscores the complexity of plant responses to metal oxide ENP exposure.

Nano-titania exposure in rosette leaves led to up-regulation of metabolism (Figure 2; Supplemental Data, Tables S8, S9). The ENP is known to increase photosynthesis in *Arabidopsis* by promoting light absorption of chloroplasts by increasing light harvesting complex II [42]. MapMan analysis of leaf responses revealed a robust up-regulation of transcripts associated with light reactions, photorespiration, Calvin cycle and tetrapyrrole synthesis (Figure 2A), whereas in roots an overall down-regulation of these processes occurred (Fig 2B). Similar to other studies [26, 43], in the present study nano-titania up-regulated genes in both tissues associated with stress (Supplemental Data, Figure S1), including genes responsive for biotic and abiotic stress. Most abiotic stress genes annotate

for heat, cold, salt stress and drought, while biotic stress genes are associated with cell wall biogenesis and degradation.

Secondary metabolism, which is part of the defense response to biotic stress, mostly was up-regulated in rosette leaves, and down-regulated in roots. MapMan analyses indicated that most of the down-regulated genes associated with secondary metabolism in roots affect the nitrogen-containing secondary compounds biosynthesis (NSCB) group, which is important in plant defense [44]. However, in general, the enrichment pattern under nano-titania in roots was similar to that in leaves which is based primarily on common effects on metabolic processes associated with growth and development.

MapMan pathway analysis revealed contrasting responses between tissue types concerning hormone metabolism (Supplemental Data, Figure S2). Collectively, up-regulation of brassinosteroids (BRs) in rosette leaves and ABA in roots suggest that nano-titania modulates signaling of hormones essential for plant development. There was up-regulation of brassinosteroid hormone metabolism in leaves (Figure S2A), whereas in roots there was up-regulation of abscisic acid (ABA) hormone metabolism (Figure S2B). ABA plays a critical role in lateral root development, whose plasticity is dependent on developmental and environmental signals [45]. BRs are essential for normal growth and development in *Arabidopsis*, and their homeostasis is maintained through feedback mechanisms affecting expression of multiple genes [46]. BRs regulate growth through a protein complex that includes leucine rich repeat (LRR) kinases [47]. Interestingly, the MapMan analysis in the present study confirmed this positive correlation between brassinosteroid hormone signaling and receptor kinases. Specifically, up-regulation of BRs in rosette leaves correlated with up-regulation of LRR receptor kinases, and down-regulation of BRs in roots correlated with down-regulation of LRR receptor kinases.

The MapMan pathway and gene enrichment analyses indicated that nano-titania activates stress response mechanisms, up-regulating growth and metabolism in *Arabidopsis*. The responses were more pronounced in rosette leaves than in roots. Gene enrichment analysis with BiNGO on nano-titania up-regulated transcripts in rosette leaves revealed significant over-representation of GO terms that include various interconnected metabolic processes, cellular processes, protein metabolism and responses to stress. For metabolic processes, enrichment was noted in carbohydrate metabolism, lipid metabolism, and photosynthesis. For cellular processes, enrichment occurred in photosynthesis, cell differentiation and cell growth. Finally, enrichments under nano-titania were found for processes related to catalysis reactions involving kinases, and at the whole cell level for functions involving the cytoplasm, plasma membrane and cell wall. Again collectively, as for BRs indicated above, responses in rosette leaves suggest that nano-titania exposure affects transcriptomic categories associated with growth and metabolism.

Nano-titania also down-regulated transcripts in leaves, in particular, those that enrich autophagy and microtubule binding processes. Autophagy has been suggested as a mechanism of nanoparticle toxicity [48]. It has been found in human cells under nano-titania [49], however, comparable literature concerning plant cells is lacking. The autophagy pathway has been associated with binding of microtubule associated proteins (MAPs) to

microtubules [50], and is consistent with the down-regulated genes associated with microtubule binding found in the present study. The responses found under nano-titania of down-regulated genes associated with autophagy and microtubule binding may be of minor importance in rosette leaves, since it appears that the primary response to nano-titania is up-regulation of processes related to growth and development.

Even though there was overall down-regulation of metabolism in both tissues, nano-ceria exposure did alter the transcriptomes in *Arabidopsis* leaves and roots differently. It is noteworthy that in leaves, nano-ceria increased transcriptional activity of the AP2/EREBP family genes including *DREB1B/CBF1*, *DREB1C/CBF2*, *DREB1D/CBF4* and *DREB2A* that are induced by other abiotic stressors to affect plant development [51, 52]. In addition, also in leaves, there was enrichment of gene ontologies associated with transcription.

Gene Expression in Relation to Changes in Growth

Although nano-titania up-regulated a suite of metabolic processes associated with growth and development, many effects were subtle and largely not measureable at the whole plant level (Figures 1 A, C, E, G, I, K). The lack of significant growth differences between the control and particle treatments suggests that the effect of exposure to nano-titania may be relatively benign over the long term. We postulate that this minor impact may be due either to redundant physiological processes or to compensatory mechanisms in *A. thaliana*. Other studies [53–57] have shown few large-scale changes in plants under nano-ceria exposure. In *Arabidopsis*, changes in the transcriptome have been shown to correlate with early phenotypic responses such as defense against pathogens and inhibition of root hair development [58]. In addition to possible functional and genomic redundancy, redundancy in signaling networks in *Arabidopsis* may prevent morphological changes from emerging even when significant changes occur in gene regulation [59–61].

In contrast to responses to nano-titania, exposure to nano-ceria induced certain transcripts in rosette leaves that are consistent with the different phenotypic responses observed between particle treatments. Analysis of up-regulated DEGs in leaves under nano-ceria identified enriched functional categories associated with responses to chemical and hormone stimulus, and acclimation of photosynthesis. At the molecular processes level there was significant enrichment associated with transcriptional activation, and when considered with the up-regulation observed of the AP2/EREBP transcription family protein genes, this suite of responses may explain the higher number of rosette leaves and above-ground dry weight biomass in nano-ceria exposed plants (Figures 1B & H). There were also non-significant trends of increased rosette diameter (Figure 1D) and decreased plant height (Figure 1E) relative to controls until day 42. The connections between nano-ceria exposure, regulation of AP2/EREBP transcription factors and *Arabidopsis* development will be highlighted in a follow-on paper.

In previous studies where various plant species were exposed to nano-ceria, minor to no effects on plant growth and development were found [62–64]. One study [38] focusing on effects of nominal concentrations (500 and 1000 ppm) of nano-ceria on *Arabidopsis* biomass and root length revealed an initial dose-dependent increase in biomass followed by a decrease in biomass. The same study found a dose-dependent decrease in root length. In the

present study, plants were investigated under different growth and particle exposure conditions, with a single nominal concentration (500 mg ENPs L⁻¹), in order to compare the responses at the level of transcriptome with those at the level of plant growth.

To our knowledge, the present study is the first comprehensive examination of effects of exposure to nano-ceria on *Arabidopsis* throughout its lifecycle, done by assessing molecular biology and phenological parameters. In this study, nano-ceria was found to affect only some growth and development parameters during a single generation of growth. Longer-term assessments are needed for nano-ceria, nano-titania and other ENPs, concerning fitness across sequential generations. Extensive long-term studies, including transgenerational (epigenetic) studies, may be required to confirm that exposure to nano-titania does not affect important plant developmental processes and the ability to compete for resources. Only few studies have documented epigenetic effects of nanoparticles in animal models [65, 66]. Also in plants, only handful of studies have evaluated epigenetic changes as a result of nanoparticle exposure. A study on tomato seedlings [67] investigating transgenerational effects of nano-ceria exposure, found that the second generation seedlings treated with nano-ceria were smaller, weaker, and had extensive root hair development. Epigenetic processes such as histone modification and DNA methylation can have significant impact on the stress responses in plants [68], and as such have been used to quantify epigenetic changes. Single-walled carbon nanotubes were found to accelerate seminal growth, but inhibit primary root hair growth in maize plants [69]. The study also found global deacetylation of histone H3, thus providing a link between the plant phenotype and epigenetic changes. Another study [70] found changes in the methylation pattern of DNA (hypermethylation) in root cells of *Allium cepa* when exposed to multi-walled carbon nanotubes. Plants being sessile are constantly exposed to various environmental stressors, and thus have to continuously adjust their responses to these external stimuli. At non-genotoxic concentrations, the observed responses (phenotypic and genomic) may tend to be inconclusive, and thus genetic and epigenetic changes may play major roles in stress response and adaptability. Stress-induced epigenetic changes and the prospective transgenerational inheritance are of great interest from an ecotoxicology point of view [71], and this would assume even more significance with nanoparticle-induced stress, given the non-conventional toxicity profiles of nanoparticles. Thus, more research studies are warranted to evaluate the epigenetic processes in relation to changes in gene expression, as well as sensitive phenotypic endpoints..

CONCLUSIONS

Nano-titania and nano-ceria, which are structurally and functionally different metallic oxide ENPs, induced distinct transcriptomic responses in *Arabidopsis* rosette leaves and roots. Nano-titania altered the transcriptome in leaves and roots to a greater magnitude than did nano-ceria. The number of genes, the affected metabolic processes and the enriched gene ontologies were of significantly higher magnitude in nano-titania, for either tissue. While nano-titania up-regulated transcripts related to photosynthesis, and carbohydrate, lipid and secondary metabolic pathways in leaves and roots, there was an overall down-regulation (with the exception of up-regulation in minor carbohydrate metabolism in roots) of these processes in both leaves and roots under the nano-ceria exposure. While defense responses were up-regulated under nano-titania, they were comparatively down-regulated under nano-

ceria. In this study, the nanoparticle induced changes at the transcriptome level did not entirely correlate with the chosen phenotypic endpoints, with the exception of an observed increase in the number of rosette leaves following the nano-ceria exposure. In our earlier study[26], nano-titania and nano-ceria induced transcriptomic responses correlated with the phenotypic responses associated with germination and growth. Several inferences can be made from these observations. First, the nanoparticle induced transcriptional signatures at an early growth stage (germinant) of plant underlie the observed phenotypes -enhanced germination and growth. Second, as the seedling develops in to a mature plant, functional and genomic redundancies, as discussed earlier, may have prevented morphological changes from emerging even when significant changes occur at the transcriptional level. Third, more sensitive growth and developmental endpoints, especially at the physiological level (e.g., photosynthetic activity, chlorophyll content and stomatal index) may provide better alternatives to the endpoints used in the present study. Fourth, quantification of epigenetic changes associated with nanoparticle exposure, and thereby potential transgenerational effects may provide further insights in understanding and correlating the observed transcriptomic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multi-panel bar charts showing growth and development characteristics of *Arabidopsis*. Phenotypic development of *Arabidopsis thaliana* exposed to nanoparticles (A: nano-titania, B: nano-ceria) number of rosette leaves, (C, D, respectively as before for nanoparticles) diameter of rosette leaves, (E, F, respectively as before) plant height, (G, H, respectively as before) plant biomass, (I, J, respectively as before) number of siliques, and (K, L, respectively as before) silique weight. Significant differences within each panel are indicated with different letters at the top of bars (t -test, $p < 0.05$, mean $\pm$ SE). Nano-ceria control:

sterilized millipore water; Nano-titania control: 0.1m M KCl; ENP treatments: 500 mg ENPs L⁻¹

Figure 2. Metabolic overview map upon nano-titania exposure.

Metabolic pathways associated with the transcriptional changes affecting *Arabidopsis thaliana* upon nano-titania exposure. Overview of the expression changes related to metabolic pathways observed in *Arabidopsis thaliana* plants in the (A) rosette leaves and (B) roots using MapMan software. The represented spots are only for genes showing a significant ($P < 0.05$, 2-fold) change in expression between the treatment and the untreated control that were attributed to the respective bins by MapMan. Up- and down-regulated genes are indicated in red and blue colors respectively.

Figure 3. Overrepresented GO-categories (Biological Processes) in *Arabidopsis thaliana* rosette leaves and roots upon nano-titania exposure.

The network graphs show BiNGO visualization of the overrepresented GO terms (Biological Processes) for the A) Up-regulated genes, and B) Down-regulated genes in rosette leaves, and C) Up-regulated genes, and D) Down-regulated genes in roots, in *Arabidopsis thaliana* upon nano-titania exposure. The color represents the significance of over-representation of a particular category. Significantly overrepresented categories are colored yellow ($P = 0.05$) to orange ($P = 5 \times 10^{-7}$). P values are Benjamini and Hochberg corrected. Uncolored nodes are not overrepresented, but they may be the parents of the overrepresented terms.

Figure 4. Metabolic overview map upon nano-ceria exposure.

Metabolic pathways associated with the transcriptional changes affecting *Arabidopsis thaliana* upon nano-ceria exposure. Overview of the expression changes related to metabolic pathways observed in *Arabidopsis thaliana* plants in the (A) rosette leaves and (B) roots using MapMan software. The represented spots are only for genes showing a significant ($P \leq 0.05$, 2-fold) change in expression between the treatment and the untreated control that were attributed to the respective bins by MapMan. Up- and down-regulated genes are indicated in red and blue colors respectively.

Figure 5. Overrepresented GO-categories (Biological Processes) in *Arabidopsis thaliana* rosette leaves and roots upon nano-ceria exposure.

The network graphs show BiNGO visualization of the overrepresented GO terms (Biological Processes) for the A) Up-regulated genes, and B) Down-regulated genes in rosette leaves, and C) Up-regulated genes, and D) Down-regulated genes in roots, in *Arabidopsis thaliana* upon nano-ceria exposure. The color represents the significance of over-representation of a particular category. Significantly overrepresented categories are colored yellow ($P = 0.05$) to

orange ($P = 5 \times 10^{-7}$). P values are Benjamini and Hochberg corrected. Uncolored nodes are not overrepresented, but they may be the parents of the overrepresented terms.

Table 1.

Number of differentially expressed genes in *Arabidopsis* leaves and roots exposed to nano-titania and nano-ceria sorted by significance and fold change

Sort	Rosette Leaves		Roots	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Nano-titania				
Significance, fold change	2			
<i>p</i> 0.01	569	275	438	326
<i>p</i> 0.05	1136	653	966	798
<i>p</i> 0.1	1365	831	1269	1007
Fold change, <i>p</i>	0.05			
Fold change 2	1136	652	966	798
Fold change 4	167	90	324	134
Fold change 8	20	18	108	28
Nano-ceria				
Significance, fold change	2			
<i>p</i> 0.01	11	17	121	152
<i>p</i> 0.05	56	74	391	363
<i>p</i> 0.1	111	110	574	492
Fold change, <i>p</i>	0.05			
Fold change 2	56	74	391	363
Fold change 4	11	3	53	85
Fold change 8	0	0	4	20

Table 2.

The significantly expressed transcripts that were found common in *Arabidopsis* roots and leaves, and oppositely regulated, as a result of nano-ceria exposure

Locus	Fold Change		Gene Ontology Process
	Rosette Leaves	Roots	
At1g24530	2.28	−2.83	unknown
At1g56150	2.35	−3.35	response to auxin stimulus
At2g45080	2.43	−2.17	cell cycle, cell division, regulation of cell cycle
At5g45630	6.32	−2.86	unknown
At5g51990	4.88	−2.55	regulation of DNA-dependent transcription, response to stress, abscisic acid mediated signaling pathway