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Physiological and Molecular Response of *Arabidopsis thaliana* (L.) to Nanoparticle Cerium and Indium Oxide Exposure

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Supporting Information

ABSTRACT: The effects of cerium oxide (CeO_2) and indium oxide (In_2O_3) nanoparticles (NPs) exposure on *Arabidopsis thaliana* (L.) Heynh. were investigated. After inoculation in half strength MS medium amended with 0−2000 ppm CeO_2 and In_2O_3 NPs for 25 days, both physiological and molecular responses were evaluated. Exposure at 250 ppm CeO_2 NPs significantly increased plant biomass, but at 500−2000 ppm, plant growth was decreased by up to 85% in a dose-dependent fashion. At 1000 and 2000 ppm CeO_2 NPs, chlorophyll production was reduced by nearly 60% and 85%, respectively, and anthocyanin production was increased 3−5-fold. Malondialdehyde (MDA) production, a measure of lipid peroxidation, was unaffected by exposure to 250−500 ppm CeO_2 NPs, but at 1000 ppm, MDA formation was increased by 2.5-fold. Exposure to 25−2000 ppm In_2O_3 NPs had no effect on *A. thaliana* biomass and only minor effects (15%) on root elongation. Total chlorophyll and MDA production were unaffected by In_2O_3 NPs exposure. Molecular response to NP exposure as measured by qPCR showed that both types of elements altered the expression of genes central to the stress response such as the sulfur assimilation and glutathione (GSH) biosynthesis pathway, a series of genes known to be significant in the detoxification of metal toxicity in plants. Interestingly, In_2O_3 NPs exposure resulted in a 3.8−4.6-fold increase in glutathione synthase (GS) transcript production, whereas CeO_2 NPs yielded only a 2-fold increase. It seems likely that the significantly greater gene regulation response upon In_2O_3 NPs exposure was directly related to the decreased phytotoxicity relative to CeO_2 treatment. The use of NP rare earth oxide elements has increased dramatically, yet knowledge on fate and toxicity has lagged behind. To our knowledge, this is the first report evaluating both physiological and molecular plant response from exposure to these important nanoparticles.

KEYWORDS: *Arabidopsis thaliana, CeO_2 and In_2O_3 NPs, Stress response, Gene expression, Anthocyanin, Lipid peroxidation, Sulfur assimilation pathway*

INTRODUCTION

The use of nanotechnology in industries such as medicine, energy, cosmetics, and agriculture has increased rapidly, and as such, concerns over the risk of nanomaterial exposure to the environment and to human health have been frequently raised.[1,2] It is now widely recognized that there is insufficient understanding of the fate and effects of nanoparticles in soil and plant systems. Although nanoparticle (NP) toxicity is still an area of active investigation, several studies have demonstrated negative effects on biota from NP exposure, including genotoxicity[3] and cytotoxicity[4] to microorganisms (*Escherichia coli, Bacillus subtilis*, and *Pseudomonas fluorescens*),[5,6] plants (*Cucurbita pepo* L., *Solanum lycopersicum* L., and *Zea mays* L.)[7−9] and animals (Zebrafish: *Danio rerio*).[10,11] Moreover, the potential hazards of nanoparticles to human health have been discussed in assays using human cells.[4,12] Given these findings, it is clear that a full and mechanistic understanding of the risks

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posed by nanoparticles in the environment, including bioaccumulation through the food chain, is necessary to adequately protect human and environmental health.

Rare earth elements (REEs) such as cerium and indium are known to possess useful and unique magnetic, catalytic, and optic properties.[13] For example, cerium oxide nanoparticles (CeO_2 NPs) are widely used in polishing, fuel cells, cosmetic additives, and industrial products.[14,15] However, mechanistic toxicity data regarding NP REEs metal oxides is scarce. Previous studies have suggested that CeO_2 NPs could cause cytotoxicity to microorganisms. It has been reported that CeO_2 NPs exposure at 230 ppm for 3 h decreased the survival rate of *E. coli* colony-forming units (CFU) to almost 0%.[16] Similar findings were reported by Pelletier et al.,[17] who showed that *E. coli* growth curves were negatively impacted by different sizes of CeO_2 NPs. García et al.[18] observed that CeO_2 NPs as low as 0.3 ppm were toxic to typical heterotrophic organisms and ammonia-oxidizing bacteria important to wastewater treatment operations and also caused significantly more bacterial deaths than similar exposures of Au NPs and TiO_2 NPs. Others have demonstrated that REEs nanoparticles can cause toxicity to other organisms such as *Caenorhabditis elegans*,[19] Zebrafish,[20] and *Daphnia magna*.[21] Notably, reactive oxygen species (ROS) production was significantly elevated in *C. elegans* at concentrations as low as 0.01721 ppm (100 nM) CeO_2 NPs.[19]

Plants are critical to both ecosystem function and to the human food supply; however, information on the interactions of REEs oxide NPs with these organisms is rather limited. Ma et al.[13] measured the effect of several REEs oxide NPs on the root elongation of a number of plants and species- and particle-specific effects on plant growth. Oxides of Gd, La, and Yb proved to be quite phytotoxic, whereas NP CeO_2 had negative effects on only one of seven species tested. Similarly, it has been reported that maize plants exposed to CeO_2 NP failed to translocate the NP.[22] A number of additional reports have addressed the effects of CeO_2 NPs on plant species such as *Medicago sativa* L., *Cucumis sativus* L., and *S. lycopersicum.* In general, results showed that CeO_2 NPs may accumulate in some plant species as exposure concentrations increase; however, traditional measures of phytotoxicity were largely unaffected by particle exposure.[3,23−25] Interestingly, Lopez-Moreno et al.[25] noted that NP CeO_2 negatively impacted the germination of four crops but also generally increased root and shoot elongation of seedlings of the same species. In another study, Lopez-Moreno et al.[3] described the NP-specific genotoxic effects in the form of DNA damage and mutations in soybean upon exposure to CeO_2 NPs as measured by random amplified polymorphic DNA assays. More recently, Zhao et al.[26] reported that ROS production caused by CeO_2 NPs exposure in *Z. mays* induced catalase and ascorbate peroxidase, both of which are related to stress defense. Notably, no reports were found in which the phytotoxicity of NP In_2O_3 was investigated. To achieve the necessary comprehensive understanding of plant−NP interactions, including overall response, effects, and accumulation under REEs oxide NPs exposure, the underlying biochemical and molecular mechanisms must be evaluated.

In the present study, *A. thaliana* was used as the model plant to investigate the effects of CeO_2 NPs and In_2O_3 NPs exposure. Representative parameters such as biomass, root length, chlorophyll and anthocyanin content, lipid peroxidation, and elemental content were measured to understand the plant's defense and response to abiotic stress caused by the REEs oxide NPs. Additionally, the regulation of antioxidant and stress-related gene transcripts was analyzed using quantitative reverse transcription PCR (qRT-PCR). These findings provide important information regarding plant detoxification pathways for NPs at both physiological and molecular levels and also have implications for determining the risk of REEs oxide NPs in consumer products.

MATERIALS AND METHODS

Experimental Design. Both CeO_2 (99.97%, 10−30 nm) and In_2O_3 NPs (99.995%, 20−70 nm) were purchased from US Research Nanomaterials, Inc. (Houston, TX). Different concentrations of CeO_2 and In_2O_3 NPs were twice dispersed by ultrasonic treatment (Ultrasonic Cleanser FS30, 100W, 42 kHz, Fisher Scientific, Atlanta, GA) in deionized H_2O for 30 min and were maintained in the dark at room temperature overnight as described in Hui and Xing.[27] Autoclaved half-strength Murashige and Skoog semisolid medium (2.22 g MS Basal medium with vitamins, 20 g sucrose in 1 L deionized H_2O, pH 5.7; *Phyto*Technology Laboratories, Shawnee Mission, KS)[28] supplemented with 8 g phytoblend agar (Caisson Laboratories, UT), was thoroughly mixed with different NPs- or NPs-free solutions (only half strength MS media) and then used as the substrate onto which Columbia wild type of *Arabidopsis thaliana* (L.) Heynh. seeds were inoculated.

A. thaliana seeds were sterilized by 70% (v/v) of ethanol for 5 min and then were soaked in 30% (v/v) of Clorox for 30 min. The seeds were then washed five times with autoclaved deionized H_2O.[29] Twenty-five sterilized seeds were placed on each Petri dish; there were four replicate dishes for each NPs concentration. Seeds were stratified at 4 °C for 24 h prior to transfer to a controlled environment cabinet (Percival Scientific, Perry, IA) with 16 h light and 8 h dark at 22 and 18 °C, respectively. Petri dishes were incubated vertically after germination to facilitate shoot and root growth for an additional 25 days. At harvest, shoot biomass and root length measurements were taken from each replicate dish.

Lipid Peroxidation Measurement. Lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay.[30] Malondialdehyde (MDA), which forms during fatty acid degradation and is indicative of lipid peroxidation, was determined as a function of REEs oxide NPs treatment. Specifically, 200 mg of plant tissues (shoots, roots) were homogenized in 4 mL of 0.1% (w/v) of trichloroacetic acid (TCA). The extracts were centrifuged at 7448*g* for 15 min at room temperature, and then 1 mL of supernatant was pipetted into mixture solution containing 2 mL of 20% (w/v) TCA and 2 mL of 0.5% (w/v) thiobarbituric acid (TBA). After heating at 95 °C for 30 min and cooling on ice, absorbance of supernatant was read at 532 and 600 nm by UV−vis spectrophotometer (Agilent 8453, Santa Clara, CA). The final MDA concentration was calculated based on Lambert−Beer's equation (extinction coefficient of MDA is 155 mM/cm).

Anthocyanin Measurement. Anthocyanin is an antioxidant as well as a stress response pigment produced by plants as a defense against ROS damage.[31] Harvested *A. thaliana* tissues (50 mg) were ground in liquid nitrogen and then mixed with 1 mL of 1% (v/v) HCl in methanol prior to incubation in the dark at 4 °C overnight. After adding 500 μL of chloroform and 500 μL of deionized H_2O to the extracts, the samples were vortexed and centrifuged at 13,000*g* for 2 min at 4 °C. The absorbance of the supernatant was measured at 530 and 657 nm by UV−vis spectrophotometer (Agilent 8453). The final anthocyanin concentration was determined by using $A_{530}-1/4\ A_{657}$.[32]

Chlorophyll Measurement. Chlorophyll content was determined modifying the method of Lichtenthaler.[33] Briefly, 50 mg of fresh leaves were cut into pieces (less than 1 cm) and added to 10 mL of 95% (v/v) ethanol to extract chlorophyll. All samples were incubated in the dark for 3 d, and the absorbance of supernatant was measured at 664.2 and 648.6 nm by UV−vis spectrophotometer (Spectronic Genesis 2, ThermoFisher Scientific, West Palm Beach, FL). Total chlorophyll were determined by Chla = $13.36A_{664.2} - 5.19A_{648.6}$, Chlb = $27.43A_{648.6} - 8.12A_{664.2}$, and total chlorophyll = Chla + Chlb.

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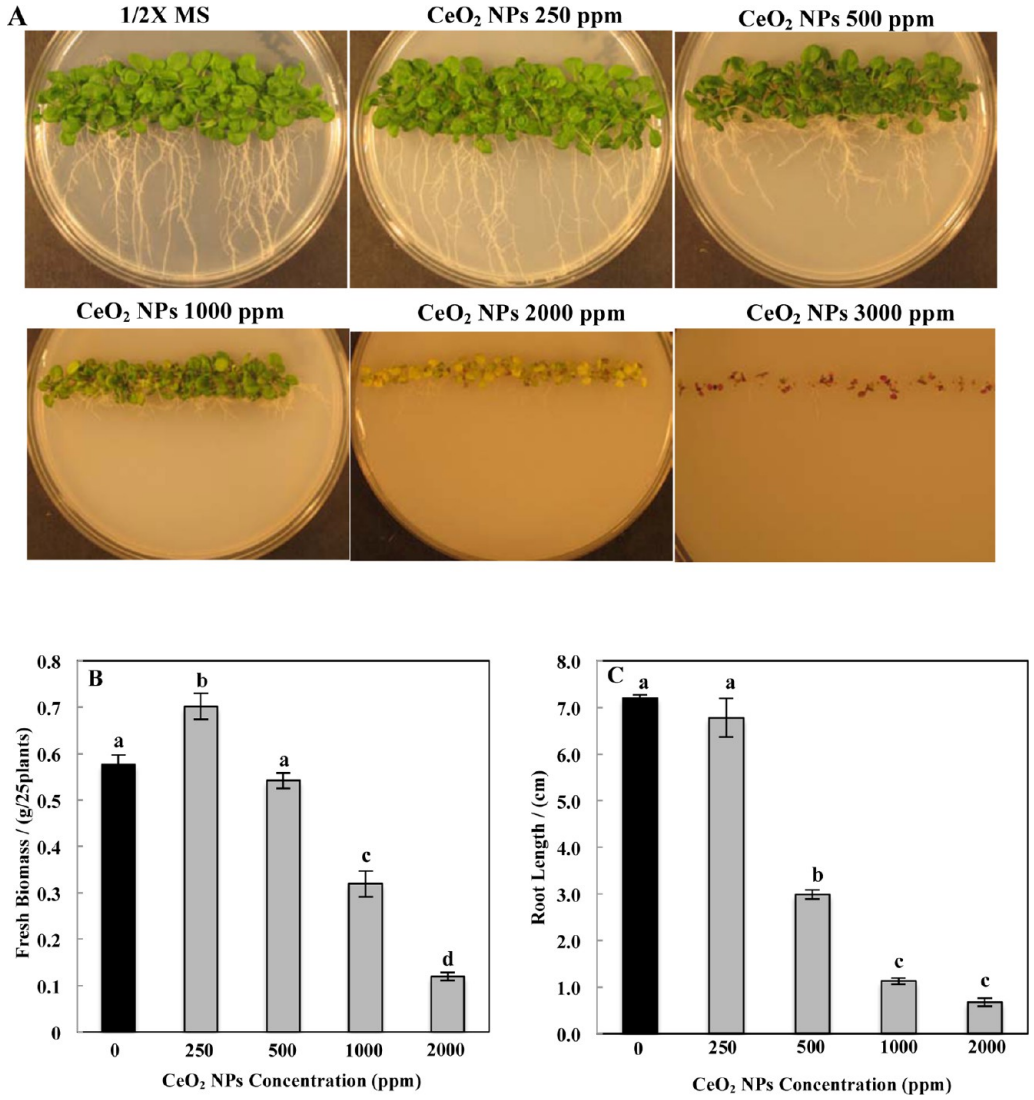

Figure 1. *Arabidopsis thaliana* treated with different concentrations of CeO_2 NPs. (A) Images of *A. thaliana* exposed to different nominated concentrations of CeO_2 NPs. (B) Fresh biomass of *A. thaliana* including roots and shoots. (C) Root length of *A. thaliana*. The means are averaged from four replicates. Error bars correspond to standard error of mean. Values of fresh biomass or roots length followed by different letters are significantly different at $p < 0.01$.

Metal Uptake Measurement. For elemental uptake analysis, *A. thaliana* plants were grown in liquid culture containing half-strength MS with and without NPs. On the solid MS medium, plants roots can grow only on the medium surface and hence could cause uneven exposure to NPs. Therefore, in order to expose the roots uniformly and directly to NPs, plants were grown in liquid culture. For this assay, *A. thaliana* plants were grown following the method described in Dixit and Dhankher.[34] Briefly, 45 seeds were germinated on a nylon mesh placed on half-strength MS agar plates (with 1% sucrose), and seeds were stratified at 4 °C for 24 h prior to transfer to a controlled environment cabinet (Percival Scientific, Perry, IA) with 16 h light and 8 h dark at 22 and 18 °C, respectively. The 14 days old seedlings along with the supporting mesh were transferred on a 2 cm long piece of 50 mL Nalgene plastic tube support placed in the magenta boxes containing half-strength MS liquid medium and allowed to acclimatize for additional period of 10 days under growth conditions as described above. Fresh MS liquid medium was used to replace the old one after five days. Once shoots and roots were fully developed, the plants were exposed to new medium mixed with CeO_2 NPs and In_2O_3 NPs for 96 h and then tissues harvested for metal uptake measurement. All samples were washed by DI water before being oven dried at 75 °C for 48 h. Oven-dried shoot and root tissues were digested in concentrated HNO_3 at 115 °C on a hot block for 1 h. The digests were diluted and analyzed by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500ce, Santa Clara, CA) for cerium and indium content as described in De La Torre-Roche et al.[35]

Regulation of Gene Expression by qRT-PCR. Changes in the expression of gene transcripts related to antioxidant defense and stress were analyzed by qPT-PCR in *A. thaliana* plants exposed to CeO_2 and In_2O_3 NPs. Total plant tissue was homogenized in liquid nitrogen and kept under −80 °C for RNA isolation. RNeasy plant mini kits (Qiagen, Germantown, MD) were used to isolate total RNA from *A. thaliana* according to the manufacturer instructions. The RNA concentration was quantified by NanoDrop spectrophotometry (ThermoScientific, West Palm Beach, FL). One microgram of total RNA was used for reverse transcription using Thermo Scientific Verso cDNA Synthesis Kit (ThermoScientific, West Palm Beach, FL) for first strand cDNA synthesis and was again quantified by NanoDrop spectrophotometry. All gene-specific primers used for quantitative real time PCR analysis were designed using the PrimerQuest (Integrated DNA Technologies, Coralville, IA). A complete list of primer sequences is provided in Table 1 of the Supporting Information. For specificity, primers were designed from the C-terminal nonconserved regions to give a product size of 100−150 bp. Finally, 200 ng/μL cDNA was used as template to

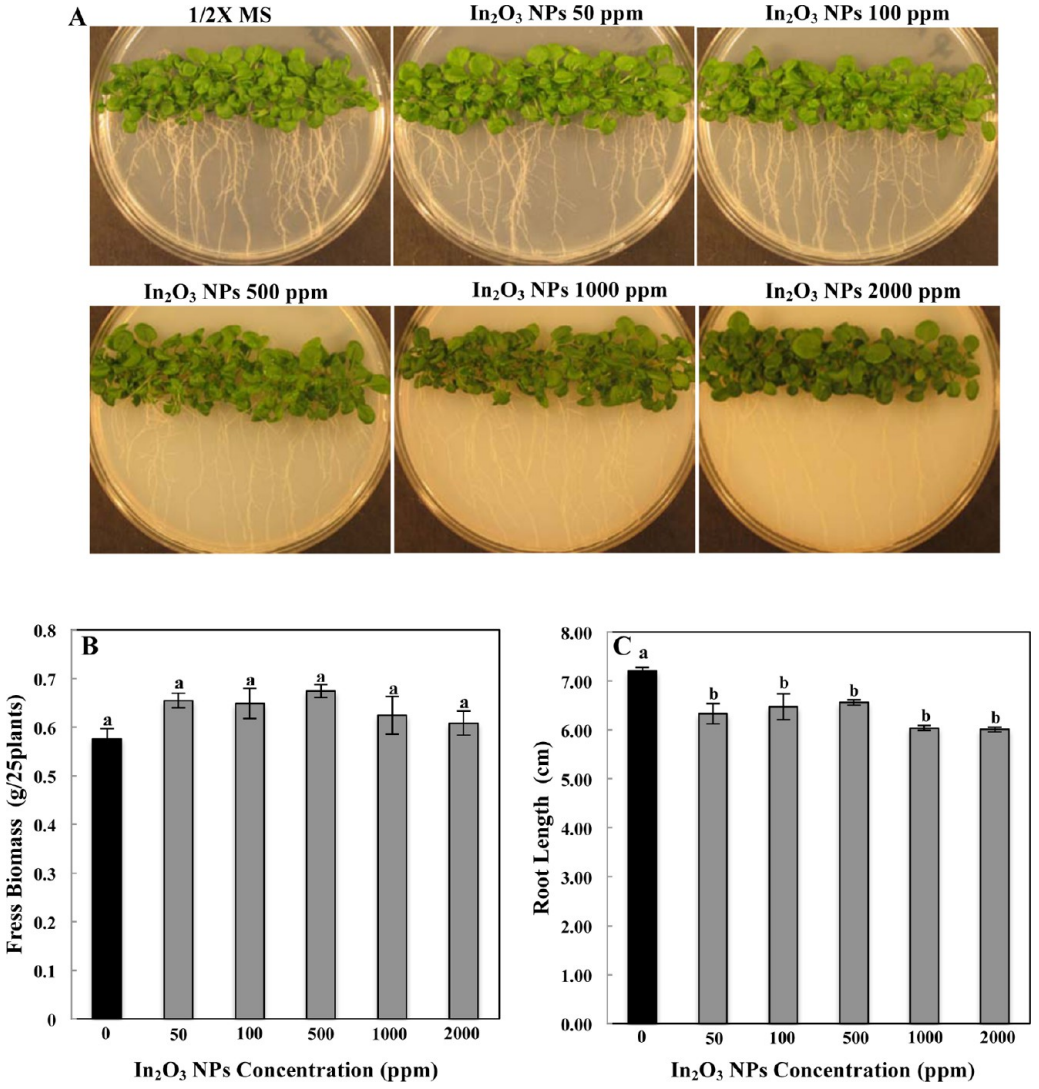

Figure 2. *Arabidopsis thaliana* treated with different concentrations of In_2O_3 NPs. (A) Images of *A. thaliana* exposed to different nominated concentrations of In_2O_3 NPs. (B) Fresh biomass of *A. thaliana* including roots and shoots. (C) Root length of *A. thaliana*. The means are averaged from four replicates. Error bars correspond to standard error of mean. Values of fresh biomass or roots length followed by different letters are significantly different at $p < 0.01$.

run qRT-PCR according to the manufacturer instructions for Mastercycler^R ep realplex (Eppendorf AG, Hamburg, Germany) with Absolute Blue qPCR SYBR Green Mix (Thermo Fisher Scientific, Surrey, U.K.). The qRT-PCR amplification program was 95 °C for 15 min; 95 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min, repeating 40 cycles; 95 °C for 15 s, 55 °C for 15 s, melting curve for 20 min; and 95 °C for 15s. The total volume from the qRT-PCR was 20 μL, and *A. thaliana actin 2* was used as a housekeeping gene for normalization. Relative quantity ($2^{-\Delta\Delta Ct}$ method) was then used to calculate relative gene expression level.[36]

Statistical Analysis. At harvest, replicate shoot and root tissues were separated and analyzed in quadruplicate for the various assays described above. Standard error of the mean were calculated and represented in all figures. Elemental content is expressed on a dry weight basis. One-way ANOVA followed by least significant difference (LSD) multiple comparison test ($p < 0.01$ and $p < 0.05$) was used to determine all differences of statistical significance among treatments.

RESULTS AND DISCUSSION

Growth Analysis of *A. thaliana* Exposed to CeO_2 and In_2O_3 NPs. For both CeO_2 and In_2O_3 NPs, there is no published evidence of any appropriate or biologically relevant concentration that cause toxicity in *A. thaliana* plants. Some of the published studies using maize, tomato, cucumber, and alfalfa plants used 0−4000 ppm of CeO_2 NPs;[24,25] however, for In_2O_3 NPs, there is no information available regarding its toxicity to plants. Therefore, in order to find the appropriate concentrations of these NPs that may cause toxicity to *A. thaliana*, we used a wide range of CeO_2 and In_2O_3 NPs concentrations ranging from 0 to 3000 ppm. The growth of *A. thaliana* in the presence of 0 to 3000 ppm CeO_2 NP is shown in Figure 1; visible signs of phytotoxicity are evident at concentrations as low as 500 ppm (Figure 1A). At 250 ppm of CeO_2 NPs, the total plant biomass was significantly increased ($p < 0.01$), although root length was unaffected (Figure 1B and C). At 500 ppm of CeO_2 NPs, fresh biomass was not significantly affected by exposure, but the average root length was reduced by nearly 60%. In addition, exposure to CeO_2 at concentrations higher than 500 ppm yielded a classic dose−response effect, with higher concentrations resulting in biomass reductions of 75−90%. Morphologically, at concentrations of 500 ppm CeO_2 NPs and higher, *A. thaliana* roots were stunted and failed to penetrate the MS medium; chlorosis of the leaves

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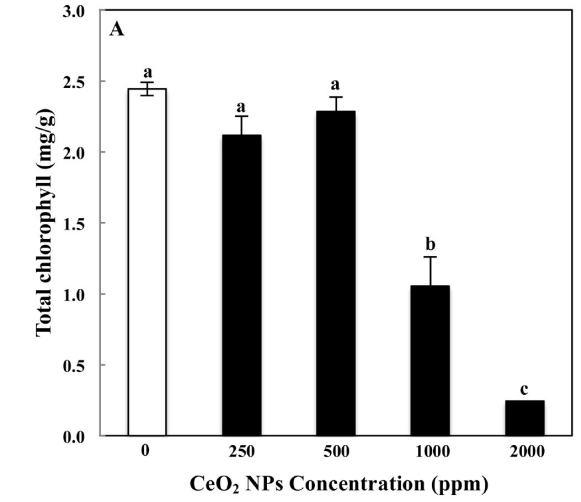
was also evident. At 3000 ppm, the plants did not survive exposure, and biomass could not be determined. These findings deviate from some published work with this nanoparticle. Zhang et al.[24] exposed cucumber to CeO_2 NPs at 2000 ppm and indicated that shoot and root biomass were unaffected by the nanoparticle. Similarly, Ma et al.[13] observed that CeO_2 NPs had no impact on root elongation of six out of seven plant species. Alternatively, our findings are in line with those of García et al.[37] where 640 ppm of CeO_2 NPs almost completely inhibited the germination of cucumber, lettuce, and tomato. At concentrations of 64 ppm, phytotoxicity was significantly reduced but for cucumber, the inhibition rate was still 90%. From the literature and based on our findings, it is clear that the phytotoxicity will not only be species-specific but at low concentrations growth enhancement may occur under some circumstances.

The effect of In_2O_3 NPs exposure on *A. thaliana* is shown in Figure 2. Because little is known about In_2O_3 phytotoxicity, a broader concentration range for exposure was employed as compared to CeO_2. Notably, no visible signs of phytotoxicity were evident at exposure concentrations up to 2000 ppm (Figure 2A). In terms of fresh biomass, NPs exposure resulted in significantly enhanced growth at 50 and 500 ppm and had no effect at the other concentrations (Figure 2B). However, root length was slightly but significantly reduced at all exposure concentrations (Figure 2C). On average, root length was reduced by 10−20% by In_2O_3 exposure, but this effect clearly had no impact on overall plant mass. To our knowledge, this is the first report of NP In_2O_3 effects on plant growth. It is clear that under similar exposure conditions, CeO_2 NPs seem to exert much greater phytotoxicity than does In_2O_3. This difference in phytotoxicity exerted by these REEs oxide NPs could be due to the differential mobility of NPs into plant cells and tissues under the conditions used for plant growth on solid half-strength MS media in the Petri dishes.

Chlorophyll Content of *A. thaliana* Plants Exposed to CeO_2 and In_2O_3 NPs. The chlorophyll content of *A. thaliana* exposed to CeO_2 and In_2O_3 NPs is shown in Figure 3. As anticipated from the images in Figure 1A, total chlorophyll content decreased significantly at higher concentrations of CeO_2 NPs exposure. Chlorophyll amounts at 250 ppm and 500 ppm NPs treatments were unaffected by particle exposure. This is particularly interesting at 500 ppm, where although root elongation was significantly inhibited, shoot biomass and chlorophyll were equivalent to the control plants. At the 1000 ppm and 2000 ppm, total chlorophyll content was reduced by 58% and 89%, respectively, relative to the control plants. Although the mechanism of phytotoxicity remains unresolved, it is clear that such a loss in photosynthetic potential would clearly compromise overall plant growth and vigor. A decrease in chlorophyll content upon exposure to metal nanoparticles has been reported by Jiang et al.,[38] Shi et al.,[39] and Oukarroum et al.[40] Interestingly, In_2O_3 NPs treatment, even at levels of 2000 ppm, had no effect on the chlorophyll content of *A. thaliana* tissues (Figure 2B). The total chlorophyll of control plants was approximately 2.4 mg/g, with the In_2O_3 NPs-exposed plants having values ranging from 2.2 to 2.5 mg/g. Notably, the data on chlorophyll content is in good agreement with the biomass results; CeO_2 NPs clearly exert significantly greater phytotoxicity on *A. thaliana* than does NP In_2O_3.

Effect of CeO_2 and In_2O_3 NPs on Membrane Integrity. Lipid peroxidation, which can be indirectly measured by MDA



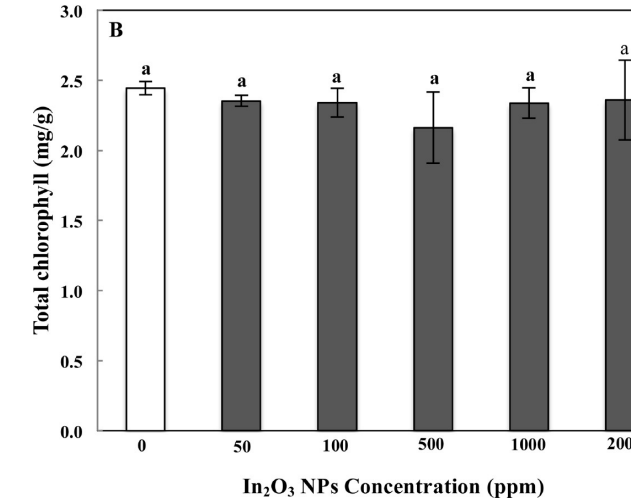


Figure 3. Total chlorophyll of *Arabidopsis thaliana* treated with different concentrations of CeO_2 (A) and In_2O_3 (B) NPs. The means are averaged from four replicates of *Arabidopsis* leaves. The error bars correspond to standard error of mean. Values of total chlorophyll followed by different letters are significantly different at $p < 0.01$.

formation, is an indicative of abiotic stress, such as that of induced metal toxicity. Cell membrane damage generally results from reactive oxygen species (ROS) production, which then damage phospholipids. The formation of MDA in *A. thaliana* as a function of CeO_2 In_2O_3 NPs exposure is shown in Figure 4. CeO_2 NPs at 250 and 500 ppm had no impact on MDA production, but at an exposure of 1000 ppm, the MDA levels were 4-fold higher than that observed in the control plants (Figure 4A, significant at $p \leq 0.01$). Metal and metal oxide NPs have been known to induce dose-dependent increase in lipid peroxidation in a number of plant species.[9,35] However, our findings are in contradiction with Zhao et al.,[26] which showed the activation of plant defense response and but subsequent lack of membrane damage by exposure to CeO_2 NPs. Interestingly, exposure to In_2O_3 NPs at concentrations up to 1000 ppm had no effect on *A. thaliana* lipid peroxidation (Figure 4B). MDA production in control plants was approximately 0.37 μM, and NPs-exposed plants had MDA levels of 0.37−0.42 μM. The lack of membrane damage upon In_2O_3 exposure suggests that either this particular NP does not induce ROS formation even at high exposure concentrations or

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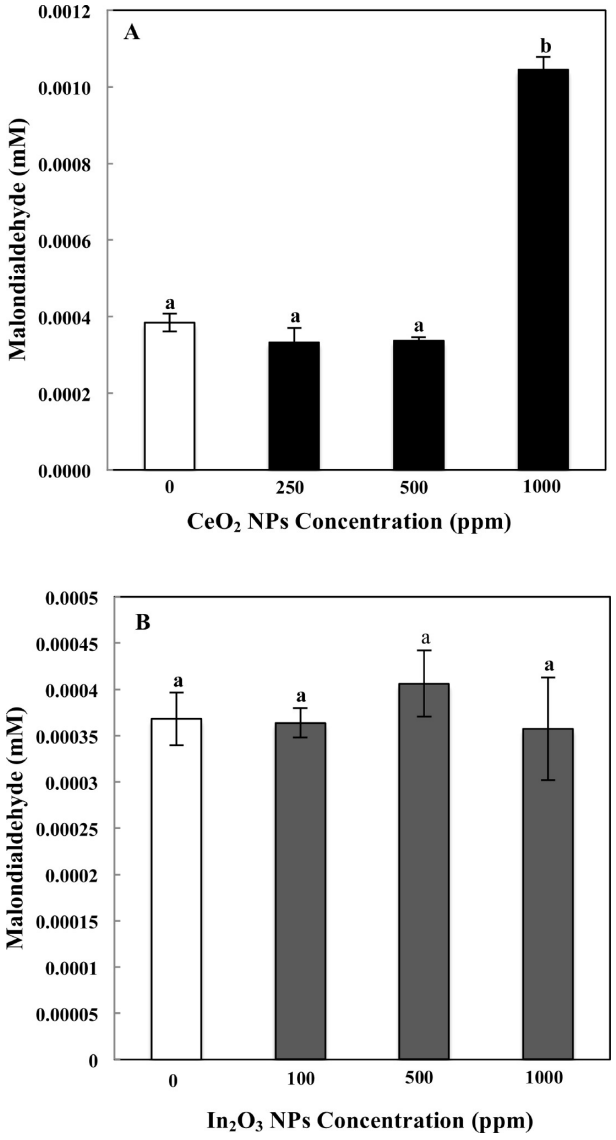

Figure 4. Lipid peroxidation of *Arabidopsis thaliana* treated with different concentrations of CeO_2 (A) and In_2O_3 (B) NPs. The means are averaged from four replicates of *A. thaliana*. The error bars correspond to standard error of mean. Values of malondialdehyde followed by different letters are significantly different at $p < 0.01$.

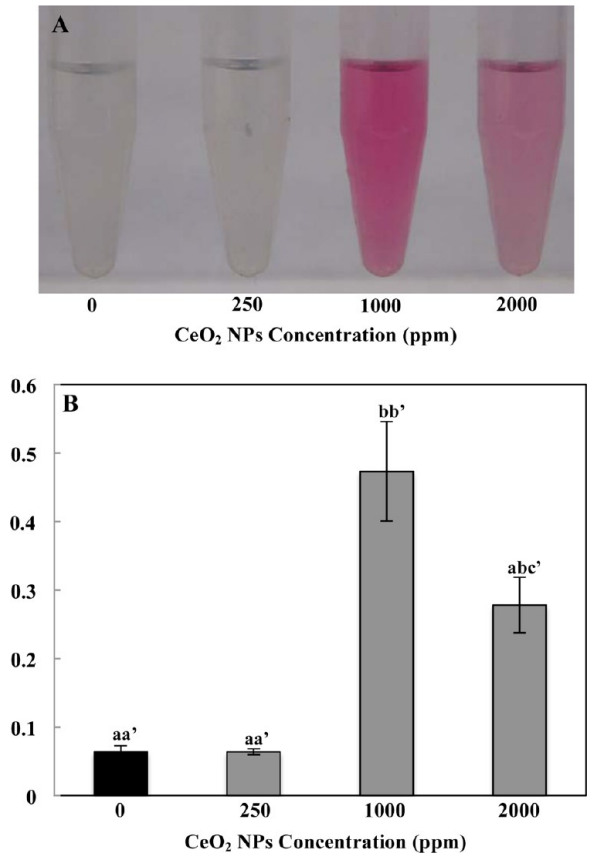

Figure 5. Quantification of anthocyanin in the leaves of *Arabidopsis thaliana* treated with different concentrations of CeO_2 NPs. (A) Image of anthocyanin color in *Arabidopsis* leaves increased as exposure doses of CeO_2 NPs increased. (B) Quantification of anthocyanin under different concentrations of CeO_2 NPs treatment. The means are averaged from four replicates of anthocyanin. Error bars correspond to standard error of mean. Values of anthocyanin followed by different letters are highly significant differences at $p < 0.01$. Values followed by different letters with apostrophe (′) are significant differences at $p < 0.05$.

perhaps that the plant's detoxification pathways are sufficient to address and remedy the induced stress.

Anthocyanin Production in *A. thaliana* Plants Treated with CeO_2 and In_2O_3 NPs. Because of the significant membrane damage likely resulting from ROS production and associated toxicity of CeO_2 NPs exposure, the anthocyanin content of exposed tissues was determined (Figure 5). Significant anthocyanin production as evident by extract pigmentation in *A. thaliana* was observed at the 1000 and 2000 ppm exposure levels (Figure 5A); no pigment production was evident at 250 ppm treatment. Quantitation of anthocyanin production confirms the results of Figure 5a; the 250 ppm exposure level had no impact on anthocyanin production (Figure 5B). However, exposure at 1000 ppm of CeO_2 NPs resulted in significantly greater ($p \leq 0.01$) anthocyanin content, but the effect was somewhat reduced but still significant ($p \leq 0.05$) at the 2000 ppm level. Interestingly, anthocyanin levels were not significantly affected by In_2O_3 NPs exposure.

Under abiotic stresses such as that of metal toxicity, salinity, and drought, ROS overproduction can damage important biological molecules such as lipids, protein, and DNA in plants.[41] Plant species have a number of enzymatic and nonenzymatic antioxidant pathways that can be simultaneously activated to defend against oxidative damage.[41,42] Anthocyanin, a type of flavonoid located in vacuole system,[43] is one of most common nonenzymatic antioxidants and operates as a superoxide radical scavenger, hydrogen donor, and metal chelator.[44] In our work, 1000 ppm CeO_2 NPs treatment clearly promoted anthocyanin production, although at higher doses the antioxidant concentration started to decrease. Similar results were reported by Kumar et al.,[45] where anthocyanin produced in Ceylon spinach (*Talinum triangulare* L.) was significantly increased by Pb exposure at concentrations up to 1 mM. The biosynthesis of anthocyanin in plants is catalyzed by phenylalanine ammonium-lyase (PAL) through a phenyl-propanoid pathway.[46] It is possible that highly stressful conditions caused by 2000 ppm CeO_2 NPs may induce the production of high levels of H_2O_2, which could inhibit the PAL activity and thus anthocyanin biosynthesis becomes somewhat disrupted. Further investigations are exploring this question. In our study, anthocyanin production was unaffected by In_2O_3

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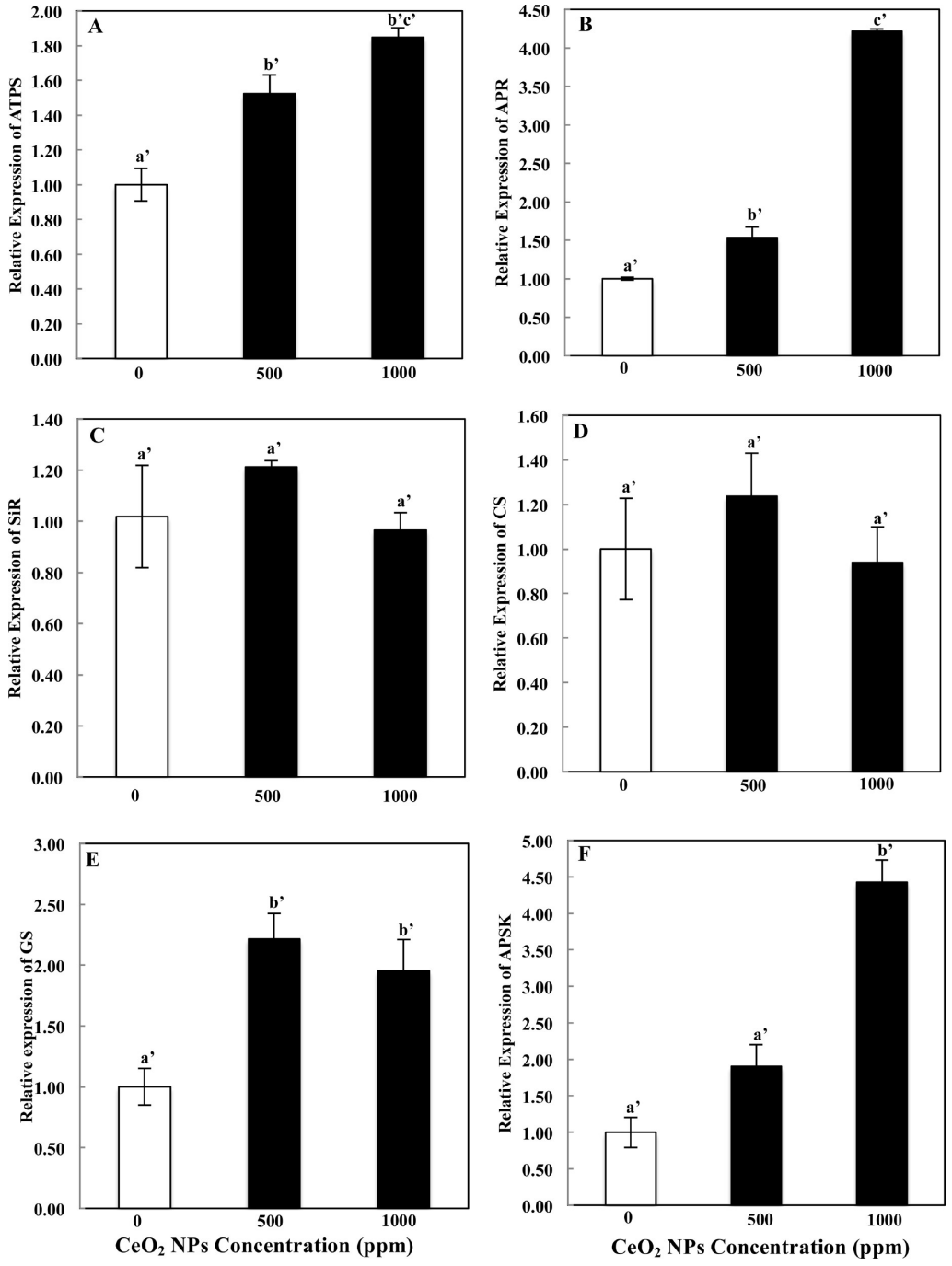

Figure 6. Relative expression of antioxidant related genes in response to CeO_2 NPs in *A. thaliana*. (A) Induction of sulfate adenylyltransferase (ATPS), (B) induction of adenosine-5′-phosphosulfate reductase (APSR), (C) sulfite reductase (SiR), (D) induction of cysteine synthase (CS), (E) induction of glutathione synthase (GS), and (F) induction of adenosine-5′-phosphosulfate kinase (APSK). Error bars correspond to standard error of mean. Relative expression values of each gene followed by different letters with apostrophe (′) are significant differences at $p < 0.05$.

NPs. However, given the observed lack of effect with biomass, chlorophyll, and lipid peroxidation, these findings are not particularly surprising.

Analysis of Cerium and Indium Uptake in *A. thaliana*. Root and shoot tissues of *A. thaliana* plants exposed to 0 or 1000 ppm CeO_2 or In_2O_3 NPs were digested and analyzed by ICP-MS. Control plants had measurable amounts of both elements. Shoot levels of both Ce and In were 0.009 (±0.002) ppm. Root concentrations were 0.068 (±0.005) and 0.043 (±0.015) ppm. The average Ce concentration in exposed shoots and roots were 121 (±30.8) and 686 (±171) ppm, respectively; shoot and root In levels were 88.2 (±24.6) and 1100 (±426) ppm, respectively. There was significant replicate variability in the element content of exposed plant tissues; values in parentheses represent standard error of the mean. This reason for this variability is not known but may have been a function of the growing conditions and the inability to differentiate surface contamination from elemental uptake in both shoot and root tissues. However, given the physiological and molecular response (described below) to exposure, it is clear that significant metal uptake had occurred. Reports on CeO_2 accumulation by plants seem to vary with species.

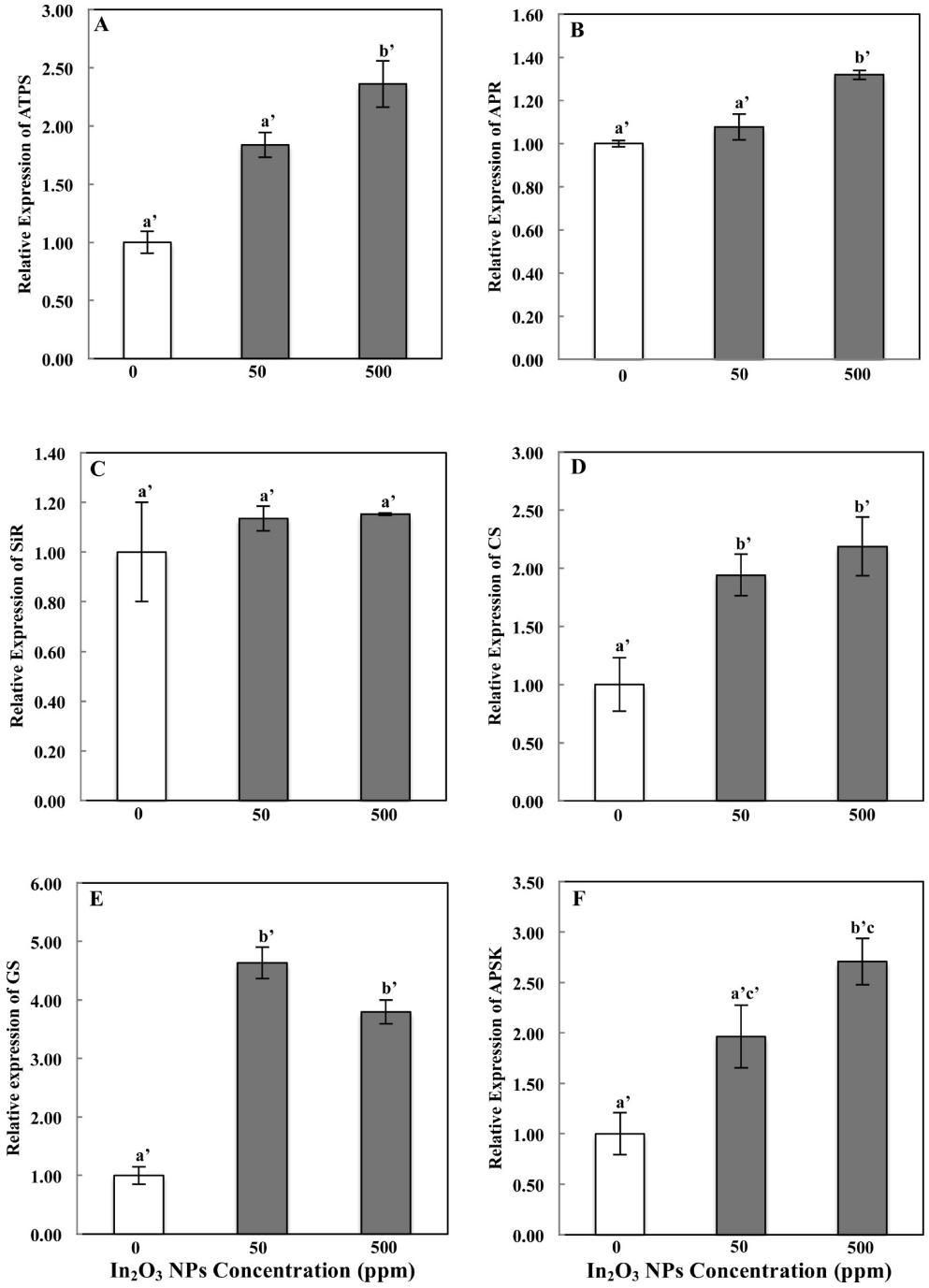

Figure 7. Relative expression of antioxidant related genes in response to In_2O_3 NPs in *A. thaliana*. (A) Induction of sulfate adenylyltransferase (ATPS), (B) induction of adenosine-5′-phosphosulfate reductase (APSR), (C) induction of sulfite reductase (SiR), (D) induction of cysteine synthase (CS), (E) induction of glutathione synthase (GS), and (F) induction of adenosine-5′-phosphosulfate kinase (APSK). Error bars correspond to standard error of mean. Relative expression values of each gene followed by different letters with apostrophe (′) are significant differences at $p < 0.05$.

Birbaum et al.[22] observed no measured translocation in maize, whereas plants such as alfalfa, cucumber, and tomato have been shown to accumulate the element.[3,23,24] To our knowledge, this is the first report of potential In_2O_3 NPs uptake and translocation by plants.

Effect of CeO_2 and In_2O_3 NPs on Antioxidant and Stress-Related Gene Expression. To evaluate antioxidant and stress-related gene expression under 500 or 1000 ppm CeO_2 NPs exposure, *A. thaliana* transcript expression was quantified with qRT-PCR (Figure 6). GSH is the major antioxidant molecule in cells[47] and has been shown to protect plants from oxidative stress caused by toxic metals and other abiotic stressors.[48,49] Therefore, our efforts were focused on the regulation of genes involved in the sulfur assimilation and the glutathione metabolic pathway in response to CeO_2 and In_2O_3 NPs exposure. The relative level of sulfate adenylyltransferase (ATPS) gene expression was significantly enhanced by both concentrations of CeO_2 NPs (Figure 6A). Adenosine-5′-phosphosulfate reductase (APR) can convert adenosine-5′-phosphosulfate (APS) to sulfite, and then sulfite reductase

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(SiR) reduces sulfite to sulfide, which is a precursor of cysteine.[50,51] Figure 6B and C show the relative expression of APR and SiR as a function of CeO_2 NPs exposure. APR expression at 1000 ppm of CeO_2 NPs treatment was 4-fold higher than untreated control plants, whereas the relative expression of SiR at the same treatment level remained unchanged. Similarly, cysteine synthase (CS) expression level also remained at the same level as the untreated control (Figure 6D). Whereas, glutathione synthase (GS) expression, which is critical for synthesis of GSH from its precursor γ-glutamyle cysteine, was more than doubled in *A. thaliana* exposed to 500 and 1000 ppm CeO_2 NPs (Figure 6E). In plants, adenosine phosphosulfate kinase (APSK) plays a crucial role in a secondary metabolic pathway of sulfate assimilation.[47] As shown in Figure 6F, treatment with CeO_2 NPs significantly increases APSK expression; at 1000 ppm of CeO_2 NPs, the gene was up-regulated by more than 4-fold over untreated plants.

The impact of In_2O_3 NPs exposure on these same genes is shown in Figure 7, and notably, the pattern of induction is somewhat similar to that observed with CeO_2 NPs. For example, the relative level of ATPS gene expression, which controls APS synthesis by catalyzing sulfate, was significantly increased by exposure to In_2O_3 NPs at both 50 and 500 ppm (Figure 7A). Also, the upregulation of genes (APR and SiR) involved in sulfite and sulfide biosynthesis was similar to CeO_2 NP exposure (Figure 7B and C). Conversely, the glutathione metabolic pathway was significantly induced, with more than a 4-fold increase in GS transcript expression at 50 ppm of In_2O_3 NPs treatment (Figure 7E). For CeO_2 NPs treatment, exposure to 500 ppm NP only doubled GS expression. Moreover, as precursor of glutathione, CS was also up regulated to a much higher extent upon In_2O_3 NPs treatment (Figure 7D). It is presumed that significantly greater activation of the glutathione pathway (CS, GS) upon In_2O_3 NPs exposure is responsible for general lack of phytotoxicity (biomass, chlorophyll, anthocyanin, and lipid peroxidation) observed with treated *A. thaliana*. The phenomenon of metal ion detoxification in plants through the glutathione metabolic pathway has been demonstrated previously.[49,53,54] In the field of nanoparticle phytotoxicity, others have recently begun to focus on tissue glutathione levels as an important parameter of study.[55]

Sulfur is a required cellular nutrient and is necessary for the biosynthesis of several important macromolecules.[56,57] After adenosine phosphosulfate (APS) formation under the catalysis of ATPS, subsequent sulfate assimilation can occur by two pathways in plants.[52,58] The primary pathway is that APS can be reduced to sulfite by sulfite reductase (SiR) and then channeled into cysteine synthesis, which is a precursor to biosynthesize glutathione. The secondary sulfated metabolic pathway is controlled by APSK, which plays an important role in plant growth and viability.[52,58] The genes involved in both of sulfated metabolic pathways were induced under the stressful conditions caused by CeO_2 and In_2O_3 NPs exposure. The pathway involved in glutathione metabolism is part of the primary sulfur assimilation pathway. Figures 6 and 7 display that transcripts related to antioxidants were induced by both REEs oxide NPs, suggesting an enhancement of plant defense to oxidative stresses through the glutathione pathway. Paulose et al.[59] reported gene expression of Abyssinian mustard (*Crambe abyssinica* Hochst. ex Fries) in response to metalloid arsenate exposure and demonstrated that transcripts related to sulfated metabolism (SiR, ATPS) and glutathione synthase (GS) were induced under this abiotic stress. Similarly, Zulfiqar et al.[60] noted that genes regulating the sulfur assimilation and glutathione biosynthesis were upregulated in *C. abyssinica* upon exposure to chromium.

Clearly, the literature on REEs metal oxide NP interactions with plants is under developed. Given the widespread and increasing use of this class of nanoparticles and the potential ecological and human health impacts through food chain contamination, it is clear that significant research into this area is necessary. This is the first report demonstrating differential regulatory response through altered expression of glutathione and sulfated metabolic pathways in response to REEs oxide NPs exposure. Future work should focus on parallel evaluation of both physiological and molecular mechanisms in plants as this approach facilitates a mechanistic understanding necessary for meaningful exposure and risk assessment.

■ ASSOCIATED CONTENT

Supporting Information

Complete list of primer sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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