Understanding of Model Plant *Arabidopsis thaliana* Response to the Coexistence of BPA and TiO₂-NPs in low environmental concentration: A Comparative Proteomics Study

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Abstract

This work presents the most extensive proteomic description of *Arabidopsis thaliana* in the knowledge of its responses to BPA and TiO₂-NPs. Previous studies have reported that nanoparticles (NPs) and Bisphenol A (BPA) are toxic to the environment. However, the jointed toxicity is not yet well understood. This study was aimed to investigate the combined toxicity of BPA and TiO₂-NPs to plants. Model plant *Arabidopsis thaliana* was selected as the target plant. The seedlings were randomly separated into 5 groups and treated with BPA (1000, 100, 10 and 0 µg/kg) and TiO₂-NPs (100, 10, 1 and 0 mg/kg). The plant height, biomass and root length indicated no significant toxicity of low concentration of BPA and TiO₂-NPs to the growth. In the results of comparative proteomics, both positive and negative effects were observed in root growth, plant development and energy metabolism, *et.al*, according to GO and KEGG analysis.

Introduction

During the last decades, about 7.2 million dry tons of biosolids were generated in the U.S. per year, 55% of which (about 3.9 million dry tons) were applied to soil for nutrients recycles, soil health enhance or degraded lands restoration.¹ In European countries, the number is about 2.39 million dry tons biosolids generated per year.² Biosolids, mostly based on the urban wastewater sewage sludge, were applied as fertilizer providing abundant plant nutrients and organic matter to soils and will improve and stimulate plant growth.³ It was considered an important biological resource for sustainable agriculture, which is economical and beneficial. The only disadvantage of the application of biosolids in agriculture is their pollutant load, the contaminants of emerging concern (CECs), including heavy metals, organic compounds, pharmaceuticals, and nanoparticles. Questions have been raised about the coexistence of organic toxicants and nanoparticles in biosolids, such as bisphenol A (BPA)⁴ and titanium dioxide nanoparticles (TiO₂-NPs)⁵ which were at insignificant levels, could affect the plant growth and its bio-system.

According to recent researches on crop plants effects of BPA, this endocrine-disrupting chemical, once be absorbed by roots, can act as an estrogen in the plant system. BPA was reported toxicity to mung bean by inhibiting shoot and root development, decreasing chlorophyll content and stomata size, and also, photosynthetic activity appeared to decrease(although not statistically significant).⁶ In Wang, S., et al.'s study on the mechanism of BPA affects soybean growth. BPA can regulate the levels of single endogenous hormones and the ratios of growth and stress hormones in plant roots and showed a dose-dependent manner.⁷ Treatment with lower BPA concentration(1.5 mg•L⁻¹) improved soybean seedling growth, while in higher concentrations of BPA treatment(from 3 to 96 mg•L⁻¹), inhibitions of seedling growth were observed. Although nanoparticles naturally exist in the environment, the wide usage of TiO₂-NPs in manufacturing and commercial products could certainly enhance the TiO₂-NPs to reach agricultural soils.⁸ Especially the precise engineered nano-materials with high reactive and sensitive

capacity may exhibit toxicity to the environment.⁹ Movafeghi, A., et al., found TiO₂-NPs were uptake by the aquatic plant *Spirodela polyrrhiza* when exposed. Subsequently, all the plant growth parameters were significantly decreased, and changes in antioxidant enzyme activities were found, such as the significantly increased of superoxide dismutase.¹⁰

Nanoparticles with high surface reactivity and large surface area, have a high capability to amalgamate with other pollutants and transit into the organism, accompany with interactive effects, such as bioconcentration enhancement, endocrine disruption and developmental neurotoxicity.^{11,12} Experimentally analyzes of the effects of BPA and TiO₂-NPs combine toxicity to animals have been done. Zebrafish share a high gene similarity (about 87%) with human. Studies have demonstrated that interaction between BPA and TiO₂-NPs results in zebrafish sex endocrine disruption and reproductive impairment,¹¹ and gut microbiota dysbiosis.¹³

But what will happen when plants exposed to these two contaminants at the same time? How plants respond to these abiotic stresses. A comprehensive understanding of plant responses to the combine toxicity of TiO₂-NPs and BPA is required. But to the best of our knowledge, plant responses to this kind of stress have not been well studied. Our group was deeply interested in the proteome responding process of plant proteomics. As a target, the model plant, *Arabidopsis thaliana* was introduced in this study, because of its simple genome but rich genetic resources.¹⁴ We hereby provide new insights into the molecular mechanisms associated with response and tolerance to combine toxicity of BPA and TiO₂-NPs in the model plant *Arabidopsis thaliana* roots.

Materials And Methods

Plant culture and exposure

Analytical grade (> 98%) of BPA was obtained from Sigma-Aldrich (Missouri, US). Anatase TiO₂-NPs (15nm average particle size) and agar powder were purchased from Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc. (Massachusetts, US). Murashige & Skoog MS Medium were purchased from Research Products International Corp. (Illinois, US). Wild type *Arabidopsis thaliana* seeds were obtained from the Biotechnology Education Company, ED VOTEK (Washington DC, US).

In the plant growth experiment, the effects of BPA and TiO₂-NPs concentrations were investigated. Treatments (Table 1) of the two contaminants were set according to the previous report. It was predicted that BPA concentration in sludge application soil was 297 ng/g⁴ and TiO₂-NPs concentrations were reported 136 mg/kg in sewage treatment plant (STP) sludge.⁵ Thus in our experiment, to simulate low contaminants concentrations in real biosolids, BPA concentrations were simplified and ranged from 1000 to 10 µg/kg, while TiO₂-NPs concentrations from 100 to 1 mg/kg. To eliminate other uncertain influence parameters in biosolids, such as organic matter and soil colloid, all media in the treatment groups and control were supplemented with 1× MS medium and 0.6% (g/g) agar powder.¹⁵ Before each exposure, the

stock TiO₂-NPs suspensions were dispersed in Millipore water (MPW) and homogenized in an ultrasonic bath for 30 min.

Table 1
Experimental design of BPA and TiO₂-NPs concentrations in treatments and control

Groups	Concentration of contaminants	
	BPA	TiO ₂ -NPs
G1	1000 µg/kg	100 mg/kg
G2	100 µg/kg	10 mg/kg
G3	10 µg/kg	1 mg/kg
G4	0	10 mg/kg
G5	100 µg/kg	0
Control	0	0

Plant Growth Conditions And Harvest

For germination rate experiment, the *Arabidopsis thaliana* seeds were firstly vernalized at 4°C for 2 days. Then seeds were evenly sown in petri dishes with culture medium, 100 seeds per group. The germination rates were counted every day for seven days and primary root lengths were determined at the end of week 1 and week 2.

After germination, 30 *Arabidopsis thaliana* plants per group were transplanted into glass jars and put into an environmental growth chamber (Ohio, US) for five weeks. Parameters were set with 16 h/8 h day/night photoperiod, 25/22°C day/night temperature, and 65 ~ 70% relative humidity. Plants were regularly watered with 1/4 MS medium solution every two days. For the first week, each jar was watered 2 mL. The irrigation water volume was then gradually increased to 15 mL per jar until the 5th week due to the need for plant growth.

After five weeks of growth, *Arabidopsis thaliana* plants were considered mature. When harvest, plant height was measured and the average height was calculated, plant biomass in fresh weight was recorded and the average calculated too. Plants were then divided into three parts: roots, leaves and stems immediately stored at -20°C for further analyses. For plant growth condition please see SI Fig. 1.

All the research works were done in UTEP, Texas, US. The permission of collecting plant material has been obtained. The plant material collection complies with federal legislation and state law.

Total Protein Extraction

After harvest, the plant roots were carefully cleaned and immediately used to do the total protein extraction by using a total protein extraction kit (G-Biosciences, Missouri, US). Briefly, to lyse plant roots, every 1g of each sample was added 2 ml TPE Buffer-I and transfer into a grinder to grind until a homogeneous suspension was achieved. This Preparation was carried out on ice to avoid excess heating and protein degradation. The homogenate was transferred to a 15 ml tube and added 240µl TPE Buffer-II, vortex immediately for 30s, put in a boiling hot water bath for another 30s, and vortex again for 30s. Repeat this heating and vortexing process (30s each) until a clear solution was seen. After 10 min of incubation in a boiling water bath, the extracts tubes were centrifuged for 10 min at 16000 g, at -4°C. The supernatant was transferred to another tube and stored at -20°C for further analyses. The protein concentrations were quantified using a Thermo Pierce bicinchoninic acid (BCA) protein assay kit.

Proteomic Analysis Using 1d Lc-ms/ms

Each sample containing 600 µg of protein was reduced with 5 mM dithiothreitol (DTT) for 30 min at 55°C, followed with 10 mM iodoacetamide to alkylate the reduced thiol groups for 30 min in the dark. Thereafter, it was diluted 8-fold to the final concentration of 1 M urea/50 mM NH₄HCO₃. The proteins were then digested overnight at 37°C with sequencing grade trypsin (Sigma-Aldrich, Missouri, US). The protein digestion was quenched using trifluoro-acetic acid (TFA) to the final concentration of 0.05% TFA. The resulting tryptic peptides were then desalted using solid-phase extraction cartridges (Discovery DSC-18 SPE Tube) prior to 1D LC-MS/MS analysis.

Each group sample was run in triplicate separated by blanks, using a Dionex Ultimate 3000 RSLC nanosystem online coupled to a Q-Exactive (Thermo Fisher Scientific, US). Peptides were introduced to the analytical column (Acclaim PepMap RSLC, 75 µm×15 cm, nanoViper, C18, 2 µm, 100 Å) and separated using a 90 min gradient from 5 to 40% solvent B at a flow rate of 300 nL/min (solvent A: 0.1% formic acid 5% acetonitrile, solvent B: 0.1% formic acid 80% acetonitrile). The mass spectrometer (MS) was operated in a data-dependent mode. Full scan MS spectra were acquired at a mass resolution of 70000 (mass range 400–1600 m/z and AGC target value of 1E6) in the Orbitrap analyzer. Tandem mass spectra, of the 10 most abundant peaks from the preceding full scan MS spectra, were acquired via fragmentation using higher-energy collisional dissociation (HCD) at a mass resolution 17500 (AGC target value of 2E5, NCE 28%, isolation width 4 m/z). The dynamic exclusion time was set at 15 s. A polysiloxane ion, m/z 445.12003, was used as lock mass throughout.

Spectra Data Processing And Database Search

The resultant MS/MS spectra were searched against *Arabidopsis thaliana* UniProt sequence databases with 77369 entries (downloaded on 2019.10.09). In the database search, mass tolerances were set to 10 ppm and 0.02 Da for precursor and fragment ions, respectively, and up to two missed cleavages were permitted. Cysteine carbamidomethylation was included as a fixed modification. Oxidation of methionine and deamidation of asparagine and glutamine were allowed as variable modifications. Peptide and

protein identification was performed using PeptideProphet and ProteinProphet in the Trans-Proteomics Pipeline respectively, with the target false discovery rate (FDR) at 1%.^{16,17}

Protein quantification was performed using a label-free approach called spectral counting,¹⁸ which tallies the number of MS/MS spectra assigned to any peptide belonging to each protein.¹⁹ Spectral counting was performed using the Abacus tool,²⁰ since the algorithm provides an automated spectral counting with robust treatment of ambiguity due to shared peptides in homologous proteins. The resulting spectral count data was analyzed using a proteomics software, Scaffold (version 4.10.0),²¹ which performs model-based statistical analysis of differential protein expression. The spectral count data was transformed by log2 transformation and was centered by the median of the three controls in each protein. The software reports statistical significance scores (Fisher's Exact Test, $p < 0.05$) among treatments and control. To visualize the protein expression changes with varying of different group of treatments, a heat map of the spectral count data was drawn after performing hierarchical clustering with average linkage and Euclidean distance metric.

GO Functional, Pathway Enrichment and Protein Interaction Network Analysis

In order to comprehensively analyze the biological functions, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis were carried by the database of QuickGO (<https://www.ebi.ac.uk/QuickGO/>) and the UniProt (<https://www.uniprot.org/>). The GO Term analysis included the biological process, cellular component and the molecular function.

SRTING version 11.0 (<https://string-db.org/>).was used to analysis the protein interaction information of all significant differentially expressed protein, and their biological functions were also obtained. Then, the interaction network of these proteins was then visualized.

Statistical Analysis

All experimental data obtained were using at least 3 independent biological replicates. Significant differences for all statistical tests were evaluated at the level of $P \leq 0.05$ with one-way ANOVA test (LSD method). All data analyses were conducted using SPSS Statistics, Version 26.0.

Results And Discussion

Morphological and physiological response analysis

Plant morphological and physiological responses had been shown in SI Table 1.

To determine how BPA and TiO₂-NPs affects *Arabidopsis thaliana* seeds germinate. We first inspected the morphological responses every day in the first week. As shown in Fig. 1 panel A and B, seeds germinated on day 2 and generally came to the highest germination rate on day 7. From which, we can see G5 (with 100 µg/kg BPA and 0 TiO₂-NPs) had the lowest GR with 68.98%, while the highest germination rate

(87.97% significant, $p < 0.05$) were obtained in G1 (with 1000 $\mu\text{g/kg}$ BPA and 100 mg/kg TiO_2 -NPs). This indicated that the seed germination was affected by the coexistence of BPA and TiO_2 -NPs and it possibly helped seeds to germinate under low environmental concentration. We believed that it is the TiO_2 -NPs which really affected the increasing of germination rate. This positive effect of nanoscale TiO_2 on germination was also reported in other plants, such as soya bean.²²

When it came to roots growth, the coexistence of these two contaminants seemed not quite helpful. As shown in Fig. 1 panel C and D, We investigated the root growth on the first and second week and the response varied. With the medium concentration of BPA (G5, 100 $\mu\text{g/kg}$ BPA and 0 TiO_2 -NPs) or TiO_2 -NPs (G4, 0 BPA and 100 mg/kg TiO_2 -NPs) individually, plant roots grew longer and had more branch roots than the others. As in higher concentrations treatment, shorter primary roots and more branch roots were obtained.

After 5 weeks of cultivation, plant height and biomass were recorded. Significant height with a 49.2% higher compared to control was found in G3 (10 $\mu\text{g/kg}$ BPA and 1 mg/kg TiO_2 -NPs) and significant plant biomass with a 13.8% weightier comparing to control was found in G4 (with 0 BPA and 10 mg/kg TiO_2 -NPs). Other treatments were not significant, which suggested that it is hard to know from plant morphology outcome that whether the plant was poisoned or not by the low concentration BPA and TiO_2 -NPs in the environment. More insight and proteomic investigations were needed to be introduced in.

Comparative Proteomics

A total of 3985 proteins were identified and quantified across all the treatment groups and control against *Arabidopsis thaliana* UniProt sequence database, and among which, 134 proteins (SI Table 2) were found significant differentially expressed, as shown in the heat map in Fig. 2.

Venn diagrams in Fig. 3(a) segregate the significant differentially expressed proteins in different treatment groups compared to control identified using MS/MS analysis. Of the 134 proteins identified, there were 116 proteins in G1 compared to control (G1 v.s. Control), 38 proteins in G2 compared to control (G2 v.s. Control), and 12 proteins in G3 compared to control (G3 v.s. Control). This indicated that with the higher exposure concentration of the mixed contaminants, proteins that were involved in the responded process became more, which made sense that with the more strict abiotic stresses more damages occurred and plant needed to invoke more bioprocesses to deal with, as shown in the interaction networks in Fig. 4.

With the same contaminant concentrations, G2, G4 and G5, compared to control the number of significant differentially expressed proteins differed. There were 14 and 12 proteins identified significant differentially expressed in G4 and G5, responded to BPA and TiO_2 -NPs exposure, relatively. In G4, according to the GO analysis, 7 down-regulated proteins were found that responded to BPA. Such as, Glutathione S-transferase F3(GSTF3_ARATH) and Glutathione S-transferase F2 (GSTF2_ARATH) were 2 defense-related compounds during plant stress, which respond for binding a series of heterocyclic compounds and had a detoxification role against certain herbicides,^{23,24} Beta-glucosidase

23(BGL23_ARATH) may participate in the control of root colonization by *P.indica* by repressing defense responses such as BPA;²⁵ Jacalin-related lectin 33 (JAL33_ARATH) a sugar-binding protein, respond for carbohydrate binding;²⁶ Dehydrin ERD14 (ERD14_ARATH) a molecular chaperone, were also down-regulated. Another 7 proteins were found up-regulated (3 found in G4 only). Such as Chaperonin 60 subunit beta 2 (CPNB2_ARATH), a ATP binding protein, assisted protein folding that requires ATP hydrolysis;²⁷ Receptor for activated C kinase 1C (GPLPC_ARATH), involved in multiple hormone responses and developmental processes which positive regulation of protein phosphorylation; Sucrose synthase 4 (SUS4_ARATH) was a sucrose-cleaving enzyme that provided UDP-glucose and fructose for various metabolic pathways; Cell division control protein 48 homolog E (CD48E_ARATH), which probably functions in cell division and growth processes; Protein TIC110 (TI110_ARATH) involved in protein precursor import into chloroplasts.

In G5, according to the GO annotation, 1 in 7 down-regulated proteins was found that responded to TiO₂-NPs, Beta-glucosidase 23 (BGL23_ARATH), which was also found significant down-regulated in G4. Within the 7 up-regulated proteins, Chaperonin 60 subunit beta 2 (CPNB2_ARATH) also found significant up-regulated in G4; cell division control protein 48 homolog E (CD48E_ARATH) (a cell division and growth processes), Chaperone protein dnaJ 2 (DNAJ2_ARATH) (plays a continuous role in plant development), Germin-like protein subfamily 2 member 4 (GL24_ARATH) (participated in the regulation of root development). This indicated that in the response of BPA and TiO₂-NPs stress, plant primary root length would be shorter and more lateral roots were observed, which consistent with the previous result of plant roots growth.

In G2, besides the proteins that were mention in G4 and G5, there were another 26 proteins (13 up-regulated, 13 down-regulated) were found significant differentially expressed. The down-regulated proteins were, such as nitrile-specifier protein 1 (JAL28_ARATH, participated nitrile biosynthetic process²⁸), A0A384KEJ8_ARATH(a defense response protein, reactions, triggered in response to the presence of a foreign body or the occurrence of an injury, which result in restriction of damage to the organism attacked or prevention/recovery from the infection caused by the attack²⁹), Myrosinase 4 (BGL34_ARATH, for Hydrolyzes sinigrin), Protein sieve element occlusion B (SEOB_ARATH, for phloem development), Probable plastid-lipid-associated protein 1 (PAP1_ARATH, involved in light/cold stress), which suggest that the plant growth would have been affected under higher exposure of the contaminants and reflected in the plant heights and biomasses. But the negative morphology outcomes were not observed in our study (including G1, G2 and G3), which may indicate that the toxic effects of the co-existence of BPA and TiO₂-NPs were too low in this concentration. The up-regulated proteins were, such as Tubulin beta-6 chain (TBB6_ARATH, involved in microtubule cytoskeleton organization and microtubule-based process), Calmodulin-1 (CALM1_ARATH, metal-binding), Ras-related protein RABA2a (RAA2A_ARATH, intracellular vesicle trafficking and protein transport), Glyceraldehyde-3-phosphate dehydrogenase GAPCP2 (plays a specific role in glycolytic energy production), 3-oxoacyl-[acyl-carrier-protein] synthase I, (KASC1_ARATH, catalyzes the condensation reaction of fatty acid synthesis), which

suggested that plant stimulated the ability of resource transportation and energy metabolism to overcome the stress and maintain or somehow enhance their growth.

Interaction Networks

Interaction networks of significant proteins were shown in Fig. 4. The role of proteins at the core and the relationship between proteins can be easily observed. With the higher concentration exposure of BPA and TiO₂-NPs, the interaction networks became more complicated (Fig. 4 and SI Table 3, 4). The STRING analysis showed that, in the circumstance of BPA alone in G5, 2 KEGG pathways were found influenced. The metabolisms of protein processing in the endoplasmic reticulum and GTP hydrolysis were depressed. As a result, plant root growth was depressed. In the case of TiO₂-NPs alone in G4, 2 affected KEGG pathway were glutathione metabolism and starch and sucrose metabolism, which on the other hand enhance the plant growth at biomass and plant height (although it was not significant). As the co-existence of BPA and TiO₂-NPs and the applied concentration got higher, 17 pathways were got in G1, 5 pathways in G2, and 3 pathways in G3. The involved processes were various. The positive-regulated biological processes were, such as, regulation of protein phosphorylation, regulation of cell growth by extracellular stimulus, amino acid homeostasis, regulation of transcription and DNA-templated, regulation of cell proliferation, pollen tube growth and root hair elongation, *et.al*. The negative-regulated biological processes were, such as, regulation of cellular response to oxidative stress, ethylene-activated signaling pathway, photoinhibition, regulation of stomatal movement, membrane permeability, pollen tube growth and lateral root formation, *et.al*. As we can see, plants always got their own way to survive.

Conclusion

To understand the possible effects of applying biosolids in agriculture, it is important to analyze the possible contaminants that exist in biosolids, the behavior and toxicity of BPA and TiO₂-NPs in the plants, and the response of proteins in roots. Considering these aspects, both positive and negative effects of BPA and TiO₂-NPs were observed in the model plant, *Arabidopsis thaliana*. The results suggested that higher concentration of TiO₂-NPs improved the germination and branch roots growth, but no significant help to the growth when the plant has grown to maturity.

The inside view of the comparative proteomics analysis, the impact of biological processes of root growth, plant development and energy metabolism observed. Plants seem to have a pearl of wisdom in their system to maintain a balance. Thus, in our study, the practice of applying biosolids in agriculture, especially contained BPA and TiO₂-NPs, low concentrations were acceptable. It seemed to do no harm to plant growth. But further examination is needed such as generational observation to have a comprehensive understanding of the long-term impact of biosolids in agriculturing.

Declarations

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Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Plant response to different concentration of combine BPA and TiO₂-NPs. A, Dynamic germination rate counted every day in the first 7 days. B, Seeds germination rate counted at 7th day after sown. C Primary root length measured at day 7 and day 14 after seeds sown. D Image of roots at day 7 and day 14. E Average plant height measured at 5th week. F Total plant biomass of fresh weight determined when harvest at 5th week.

Figure 2

Heat map of significant differentially expressed proteins with hierarchical clustering analysis. The colors in the heat map indicate the relative abundance of proteins (fold changes compared to the average spectrum counts of each protein expressed in all groups). Proteins with the lightest green color indicated no expression in this treatment and the green to red colors indicated higher expressions.

Figure 3

Venn diagram of significant differentially expressed proteins identified. (a) All treatment groups compared to control, numbers in the control circle indicated the proteins that were not found in treatments, while numbers in the treatment circle indicated that proteins were only expressed in treatments when exposed to contaminants. And numbers in the overlap areas represented the up-regulate proteins(the left number) and down-regulated proteins (the right number) found in treatments. (b) G1 v.s. Control, G2 v.s. Control and G3 v.s. Control. (c) G2 v.s. Control, G4 v.s. Control and G5 v.s. Control.

Figure 4

Interaction networks of significant proteins. With the higher concentration exposure of BPA and TiO₂-NPs, the interaction networks became more and more complicated.

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