Oxidative stress-induced toxicity of CuO nanoparticles and related toxicogenomic responses in *Arabidopsis thaliana*

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Abstract

Microarray analysis of toxicogenomic effects of CuO NPs on *Arabidopsis thaliana* was conducted. *Arabidopsis* growth was significantly inhibited by CuO NPs (10 and 20 mg/L). CuO NPs (10 and 20 mg/L) caused significant root damage after short-time (0-2 h) exposure while their corresponding Cu²⁺ ions (0.80 and 1.35 mg/L) did not show any root damage. After longer exposure times (1 and 2 days), Cu²⁺ ions induced obvious root damage, indicating that released Cu²⁺ ions from CuO NPs contributed partial toxicity during CuO NPs exposure. After CuO NPs (10 mg/L) exposure for 2 h, reactive oxygen species (ROS) generation in root tips was much higher than that in the corresponding Cu²⁺ ions (0.8 mg/L) treatment. The gene ontology categories identified from microarray analysis showed that CuO NPs (10 mg/L) caused 1658 differentially expressed genes (p<0.01, fold change>3). Of these, 1035 and 623 genes were up-regulated and down-regulated, respectively. 47 genes among all the up-regulated genes were response to oxidative stress, in which 19 genes were also related to “response to abiotic stimulus” and 12 genes were involved in the phenylpropanoid biosynthesis of the KEGG metabolic pathway. The expression of all the selected genes (*RHL41*, *MSRB7*, *BCB*, *PRXCA*, and *MC8*) measured using quantitative RT-PCR was consistent with the microarray analysis. CuO NPs contributed much stronger up-regulation of oxidative stress-related genes than the corresponding Cu²⁺ ions.

Capsule abstract

CuO nanoparticles contributed much stronger up-regulation of oxidative stress-related genes than the corresponding Cu²⁺ ions, thus causing growth inhibition of *Arabidopsis*.

Keywords: CuO nanoparticles; Phytotoxicity; Oxidative stress; Microarray; Gene expression

1. Introduction

With increasing production and application of CuO nanoparticles (NPs), the environmental concentration of CuO NPs will continue to rise (Bondarenko et al., 2013; Liu and Cohen, 2014), which raises the need for a comprehensive toxicological risk assessment. Currently, the toxicological data on various types of organisms are reported, and CuO NPs toxicity is higher in comparison with other NPs such as TiO₂ and ZnO (Vicario-Parés et al., 2014). Oxidative stress induced by CuO NPs or their released Cu²⁺ ions is commonly recognized as a major mechanism for CuO NPs toxicity. Fahmy and Cormier (2009) found that CuO NPs induced oxidative stress and cytotoxicity in airway epithelial cells (HEp-2), and Cu²⁺ ions dissolved from CuO NPs (both in the cell and in the culture media) did not significantly induce the cytotoxic effect or the oxidative damage. On the contrary, the released Cu²⁺ ions from CuO NPs played a key role in triggering reactive oxygen species (ROS) generation and DNA damage in bacteria (*Escherichia coli*) while the particulate CuO were not involved in these stress responses (Bondarenko et al., 2012). In our previous study, both CuO NPs and the released Cu²⁺ contributed to the total ROS generation in A549 human lung cancer cells (Wang et al., 2012a). The role of CuO NPs dissolution on oxidative stress in organisms may be influenced by the medium composition, exposure time, initial CuO NPs concentration and the tolerance of the test organisms. For plants, their tolerance of CuO NPs-induced oxidative stress is still unclear, and the dominant source

(CuO NPs or the released Cu^{2+}) of ROS generation after CuO NPs exposure will be investigated in this work.

Recent studies have highlighted that changes in gene expression upon exposure to environmental contaminants (e.g., heavy metals) can be detected earlier than any changes in physiology and/or morphology of plants (Hou et al., 2015). The obtained gene expression data may yield a better understanding of the molecular mechanism of toxicants. Microarray analysis is recognized as a sensitive approach for the early detection of potential toxicity of contaminants, and an effective tool for identifying the oxidative stress-related genes (Hou et al., 2015; Van Aerle et al., 2013). For nanotoxicological investigation, the microarray technology has been applied to detect changes in gene expression in human cells (Li et al., 2014), zebrafish embryos (Van Aerle et al., 2013), and bacteria (*Escherichia coli*) (Cui et al., 2012). For plants, microarray has been applied to detect changes in gene expression in plants exposed to and CdS quantum dots (QDs) (Marmioli et al., 2014; 2015) and Ag NPs (Kaveh et al., 2013). Marmioli et al. (2014) reported that sensitivity of *Arabidopsis thaliana* to CdS QDs was not due to the released Cd^{2+} ions according to differential responses of *Arabidopsis* genes. On the contrary, the investigation on Ag NPs revealed that differentially expressed genes and stress induced by Ag NPs originated from both the particulate Ag NPs and released Ag^+ ions (Kaveh et al., 2013). For CuO NPs, higher toxicity was reported than Ag NPs due to their distinct capabilities of intracellular uptake and dissolution (Cronholm et al., 2013). However, the toxicogenomic response to CuO NPs including both particulate CuO NPs and released Cu^{2+} ions has received little attention. In addition, the oxidative stress-related gene expression can be extensively investigated in conjunction with the

ROS determination upon CuO NPs exposure based on microarray analysis. In the present work, *Arabidopsis* was selected as a model plant. The overall objective of this study is to provide new insights into the role of particulate CuO NPs and their released Cu²⁺ ions in phytotoxicity and transcriptional response of plants upon CuO NPs exposure.

2. Materials and Methods

2.1. Culture of Arabidopsis seedlings

Seeds of *Arabidopsis* ecotypes (Bay-0) were provided by Agricultural University of Hebei, China. The *Arabidopsis* seeds were plated on 1/2 Murashige and Skoog (MS) agar medium (pH, 5.8-6.0) (Murashige and Skoog, 1962) and stratified in the dark at 4 °C for 2 days. After stratification, the seeds were cultured under light irradiation (16-h light 8-h dark, ~100 µmol/m²/s) at 22 °C for 5 days. The seedlings were then transferred to 1/4 dicotyledonous nutrient medium under 60% relative humidity and a 16 h photoperiod (approximately 100 µmol/m²/s) for further culture. The composition of full dicotyledonous nutrient solution is shown in Table S1.

2.2. CuO nanoparticles

CuO NPs were obtained from Beijing Nachen S&T Ltd. The morphology of CuO NPs was observed using transmission electron microscopy (TEM, JEM-2100, JEOL, Japan). The dissolution of CuO NPs was investigated following the approach of our previous study (Zhao et al., 2013). Briefly, CuO NPs (final concentration, 10 and 20 mg/L) were suspended in 1/4 dicotyledonous nutrient medium with the assistance of sonication (100 W, 40 kHz) for 30 min in a sonication bath (KQ-500DB, Kunshan ultrasonic instrument Co., China). The CuO NPs suspensions were shaken (150 rpm) for different periods of time (0-8 days), and then centrifuged

(9,280×g, 20 min). The supernatants were filtered by a glass membrane (0.22 μm). The Cu concentrations of the filtrates were determined using graphite furnace atomic absorption spectrophotometry (Solaar M6, Thermo, Boston, USA).

2.3. Growth inhibition experiments

Nine-day old *Arabidopsis* seedlings were exposed to CuO NPs suspensions (10, 20 mg/L) which were prepared in 1/4 dicotyledonous nutrient medium. After exposure for 7 days, the biomass such as shoot fresh weigh, root fresh weight and root length of *Arabidopsis* was measured. In addition, to further investigate the short-term damage of roots after CuO NPs exposure, the roots were exposed in CuO NPs suspensions (10, 20 mg/L) for 0-2 days and then washed with deionized water three times. The wiped roots were stained in 10 g/L Trypan blue solution for 5 min. The stained roots were rinsed again with deionized water to remove additional Trypan blue. Finally, the root tips (1-2 cm) were cut off from roots using sterile scissors, and viewed under a light microscope (Olympus, DP72). In consideration of the possible toxicity of dissolved Cu²⁺ from CuO NPs, Cu²⁺ (0.80 and 1.35 mg/L) treatments in which Cu²⁺ ions were supplied from CuSO₄·5H₂O were set up in comparison with CuO NPs (10 and 20 mg/L) treatments according to the dissolution test as described above.

2.4. Oxidative stress detection

Reactive oxygen species (ROS) generation could be accumulated much earlier in root than obvious biomass decrease of *Arabidopsis* plant. Therefore, *Arabidopsis* roots were exposed to CuO NPs for a short time (0-2 h) for ROS detection. Briefly, 9-day old *Arabidopsis* seedlings were treated with CuO NPs or Cu²⁺ ions for 5 min, 30 min, 1 h and 2 h. The treated seedlings

were washed and incubated in 10 μ mol/L 2',7'-dichlorofluorescein diacetate (H₂DCFDA) solution for 30 min. The H₂DCFDA were prepared in phosphate buffered saline (PBS) (10 mM, pH 7.4). The stained seedlings were quickly washed with PBS for three times. The root tips (1 cm) were then excised from *Arabidopsis* seedlings with sterile scissors, and immediately observed using a confocal microscope (Fluoview-1000, Olympus). The excitation and emission wavelengths were 514 nm and 500-530 nm, respectively. Thirty root tips were examined for each treatment. The fluorescence intensity (FI) of the product of H₂DCFDA and ROS was quantified, and the oxidative stress was evaluated as follows (Wang et al., 2011): Relative ROS level (%) = $\frac{FI_{\text{Exposure}}}{FI_{\text{Control}}} \times 100\%$.

2.5. RNA extraction and microarray analysis

To obtain enough biomass for RNA extraction, 15-day old *Arabidopsis* plants were selected for microarray analysis. Briefly, *Arabidopsis* plants were exposed to 10 mg/L CuO NPs for 2 h. The roots of *Arabidopsis* were then washed with deionized water, and immediately frozen in liquid N₂. Total RNA was extracted from the seedling roots using TRIZOL Reagent (Life technologies, US) following the manufacturer's instructions and purified by RNeasy micro kit (QIAGEN, Germany). GeneChip 3'IVT Express Kit (Affymetrix, US) was used to obtain biotin-labeled cRNA from the total RNA. Array hybridization of the obtained cRNA was performed using GeneChip[®] Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA) (Zhang et al., 2012). The hybridized array (Affymetrix *Arabidopsis* ATH1 Genome Array) was used for the microarray analysis at Bohao Biotechnology Company (Shanghai, China). Each treatment was run in three biological replicates. The heat map clustering was designed from the

differentially expressed genes (fold change >3, p<0.01) with R statistical platform and gplots package. The functional analysis of the differentially expressed genes (fold change>3, p<0.01) was based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>). The up-regulated genes in GO categories (p<0.05, gene number >4) were further classified using the database for annotation, visualization and integrated discovery (DAVID) (<https://david.ncifcrf.gov/>) (Huang et al., 2009a,b). Functional enrichment analysis was performed using the functional annotation clustering tool in DAVID (Classification Stringency, High).

2.6. Quantitative real-time PCR

Microarray results for the CuO NPs exposure experiments were verified using quantitative real-time PCR (RT-PCR) (Allanach et al., 2008). The 15-day old *Arabidopsis* plants were firstly treated with CuO NPs (10 mg/L) or Cu²⁺ (0.8 mg/L) for 0.5, 1 and 2 h. Total RNA in the treated roots was extracted using RNA extraction kit (Invitrogen), and the extracted RNA samples were analyzed using the SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus) kit (Takara Bio). Five genes (RHL41, MSRB7, MC8, PRXCA and BCB) related to oxidative stress were quantified under the Applied Biosystems 7300 real-time PCR system. Actin2 was included as a reference gene. The primers of *Actin2*, *RHL41*, *MSRB7*, *MC8*, *PRXCA* and *BCB* were shown in Table S2. The relative expression amount of target genes was obtained using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{\text{gene,treatment}} - Ct_{\text{actin,treatment}}) - (Ct_{\text{gene,control}} - Ct_{\text{actin,control}})$ (Livak and Schmittgen, 2001). Each treatment was run in three biological replicates and four technical replicates.

2.7. Statistical Analysis

All the experiments were run in triplicates or more, and the data were presented with mean $\pm$ standard deviation. Data were analyzed by one-way ANOVA with a LSD test using SPSS Statistics 20.0. We used $p < 0.05$ for the statistical significance.

3. Results and Discussion

3.1. Effect of CuO NPs on the growth of *Arabidopsis* seedlings

The size of individual CuO NPs was ranged 30-50 nm as indicated from the TEM image (Fig. S1). The dissolution test showed that CuO NPs released Cu^{2+} ions in the medium (Fig. S2). The equilibrium was reached at 48 h, and 0.80 and 1.35 mg/L Cu^{2+} ions could be released from 10 and 20 mg/L CuO NPs, respectively. As shown in Fig. 1A, CuO NPs highly inhibited the growth of *Arabidopsis*. The growth parameters such as fresh weights of root and shoot, and root length were also measured (Fig. 1B-1D). Clearly, fresh weight (both root and shoot) and root length of *Arabidopsis* were significantly reduced by CuO NPs ($p < 0.05$). However, the difference between 10 mg/L and 20 mg/L CuO NPs was not significant ($p > 0.05$).

To recognize the effect of CuO NPs dissolution on *Arabidopsis* growth inhibition, a Cu^{2+} treatment was included in the root damage test (Fig. 2). The un-exposed roots did not show any damage during 30 min-2 day incubation based on Trypan blue staining test (data not shown) while root tips began to be damaged after exposure to CuO NPs (both 10 and 20 mg/L) for 30 min as indicated by the blue color on the root surface. The CuO NPs-induced damage was the highest at 1-day, and was relieved after 2-day exposure. The corresponding Cu^{2+} ions (0.8 and 1.35 mg/L) did not cause visible root damage up to the exposure time of 2 h, indicating that CuO NPs-induced root damage was mainly caused by NPs themselves within short-time (0-2 h)

exposure. During longer exposure times (1 and 2 days), Cu^{2+} ions (0.8 and 1.35 mg/L) induced obvious root damage, indicating that the released Cu^{2+} ions from CuO NPs contributed partial toxicity during CuO NPs exposure. It should be noted that the dissolution of CuO NPs may continue to proceed in the presence of plants. Therefore, the contribution of Cu^{2+} ions on root damage may be underestimated. The NPs-induced root damage could be explained as follows: (1) CuO NPs were taken up by the root of rice (Peng et al., 2015) and maize (Wang et al., 2012b) plants in the form of particulate CuO. During attachment and uptake, cell wall and other cell structure could be physically damaged as indicated by the previous findings on rice root (Wang et al., 2015) and tobacco plant cell (Liu et al., 2013); (2) CuO NPs attached onto root surface and/or entered root cells and dissolved locally, therefore leading to high Cu ion concentration around and in the roots; and/or (3) CuO NPs induced cellular oxidative stress of root cells, thus causing direct damage. The last explanation will be investigated in the next section.

3.2. CuO-NPs induced ROS generation

The distribution of ROS (green color) on CuO NPs-treated root tips was observed using confocal microscopy (Fig. 3A). ROS generation was triggered after 5-min exposure to CuO NPs (10 mg/L) in comparison with the untreated roots. The generation maintained a high level during all the exposure times (0.5-2 h). The corresponding Cu^{2+} (0.8 mg/L) could also induce the generation of ROS, however, the intensities were much lower for all the exposure times. The quantified data on ROS generation was shown in Fig. 3B. For all the exposure times, ROS generation in CuO NPs treatment was significantly higher (2-5 times) than the Cu^{2+} and control treatments ($p < 0.05$), clearly suggesting that oxidative stress for the CuO NPs treatment was

mainly caused by the particulate CuO rather than its released Cu²⁺ ions. In addition, the highest oxidative stress occurred at 30 min. After that, the oxidative stress was slightly mitigated, possibly because of the defense of the antioxidant system (Rico et al., 2013).

3.3. Gene ontology categories identified from microarray analysis

Changes in gene expression associated with toxicants could be observed earlier than physiological or morphological responses (Hou et al., 2015). Therefore, microarray was employed to early detect the gene expression of *Arabidopsis* after CuO NPs exposure. Statistical analysis revealed that CuO NPs (10 mg/L) caused 1658 differentially expressed genes (fold change>3). Of these, 1035 and 623 genes were up-regulated (Table S3) and down-regulated (Table S4), respectively. To visualize the differential gene expression of the CuO NPs-treated roots, the most significant genes (p<0.01, fold change>3) were subjected to the hierarchical clustering analysis (Fig. S3).

The up-regulated genes in GO categories (p<0.05, gene number>4) were further classified using the database for annotation, visualization and integrated discovery (DAVID). The GO category “response to stimulus” (Genes per Category, 32%) was the highest over-represented term in biological processes (Fig. 4A). This finding is in agreement with the study of Kaveh et al. (2013), in which the same category was identified when *Arabidopsis* was exposed to both Ag NPs and Ag⁺. In another study, three genes (*AT4G18450*, *PBP1* and *ERF1*) related to “response to stimulus” were also differentially expressed in the roots of tomato plant after exposure to heavy metal Cr (Hou et al., 2015). “Response to organic substance” (13%), and “biopolymer modification” (12%) were the other two most representative categories of biological processes.

The most represented category of cellular components was membrane (26%) while that of molecular functions were “transferase activity” (19%), and “cation binding” (18%) (Fig. 4B).

On the other hand, 14 biological processes, 3 cellular components, and 9 molecular functions were revealed from the significantly down-regulated genes ($p < 0.05$, gene number > 4) after CuO NPs exposure (Fig. S4). The major categories (Genes per Category $> 10\%$) of biological process, cellular component, and molecular functions were metabolic process (45%), membrane (27%), and transferase activity (20%) and oxidoreductase activity (10%), respectively. The complete list of up- and down-regulated genes by CuO NPs is provided in Table S5 and Table S6, respectively.

3.4. Oxidative stress-responsive Genes

Biological process “response to oxidative stress” was clearly identified (Table S5-1). This indicated that *Arabidopsis* roots highly suffered from oxidative stress when exposed to CuO NPs, which is consistent with our finding on ROS generation in Fig. 3. Thus, 47 up-regulated genes related to “response to oxidative stress” were identified and listed in Table 1.

Of all the 47 up-regulated oxidative stress responsive genes, 19 genes were also related to “response to abiotic stimulus” as shown in Table 1 (indicating with “*”). In these 19 genes, *PRXCA* (Peroxidase 33, *PRX33*) is the effector protein in ROS signaling pathways. The up-regulation of *PRXCA* could promote the generation of H_2O_2 , thus resulting in the oxidative burst in apoplast (Ali et al., 2005). Arginine decarboxylase (*ADC1* and *ADC2*) is the rate-limiting enzyme of catalyzing the biosynthesis of polyamine, and excessive polyamine can promote the generation of nitric oxide (NO) (Alcázar et al., 2010). NO concentration above 10^{-6} M could

253 potentially impair photosynthetic electron transport in peas (Gould et al., 2003). The
 254 up-regulation of *ADC1* and *ADC2* indicated that NO may participate in the ROS generation and
 255 accumulation in *Arabidopsis*. In addition, NO generation was enhanced when calcium binding
 256 protein *TCH2* was activated because *TCH2* activation was a requirement for NO induction
 257 (Matika and Loake, 2014). The rapid synthesis of NO and accumulation of H₂O₂ and other ROS
 258 could trigger a programmed cell death (PCD) process, thus leading to death of plant cells (Wang et
 259 al., 2013). Moreover, the programmed cell death proteins *MC8* and *BAG6* were reported to be
 260 induced under oxidative stress (He et al., 2008; Kang et al., 2006), which supports our finding
 261 that *MC8* and *BAG6* in the *Arabidopsis* root were up-regulated (Table 1). Meanwhile, the defense
 262 responses in *Arabidopsis* would be activated by CuO NPs-induced abiotic stress in *Arabidopsis*.
 263 *DREB2A* (dehydration-responsive element-binding protein 2A) responds to cold, high-salt and
 264 dehydration (Sakuma et al., 2006). CuO NPs may cause damage to *Arabidopsis* in common with
 265 those stresses. The up-regulated expression of *DREB2A* was also observed when *Arabidopsis* was
 266 exposed to Cd and denaturation (Suzuki et al., 2002). Moreover, zinc finger proteins (*STZ* and
 267 *RHL41*) have been shown to play important roles in the defense responses of plants to oxidative
 268 and abiotic stresses. The expression of these two genes was related to the tolerance of drought,
 269 salinity, and oxidative stress (Rizhsky et al., 2004). While Asmuss et al. (2010) reported that zinc
 270 finger structures might be sensitive targets for metals like Cd (II), Cu (II), Co (II) and Ni (II).
 271 Other genes such as *BCB* (blue copper binding protein), *AT4G12400* (putative stress-inducible
 272 protein), *MPK3* (mitogen-activated protein kinase 3), and five heat shock proteins (*HSP101*,
 273 *HSP70T-2*, *HSP70*, *HSP17.6A*, and *AT1G54050*) as listed in Table 1 could also participate in the

responses of *Arabidopsis* (Hanagata et al., 2011; Nakagami et al., 2005; Suzuki et al., 2002). These genes were also observed to be up-regulated after exposure to CuO NPs in the present work (Table 1).

Twelve genes were involved in the phenylpropanoid biosynthesis of the KEGG metabolic pathway (Table S7), including 2 phenylalanine ammonia-lyases and 10 peroxidases. The phenylpropanoid biosynthesis pathway serves as a starting point for the production of many compounds, such as flavonoids, coumarins, and lignans (Fraser and Chapple, 2011). These antioxidative compounds are able to directly eliminate excessive ROS in plants (Korkina, 2007). Thus, the activation of phenylpropanoid biosynthesis pathway may be able to resist oxidation and maintain membrane integrity. Similar finding has been observed in the wild recretohalophyte *Reaumuria trigyna* when suffering salt stress (Dang et al., 2013).

3.5. Quantitative assessment of the expression level of oxidative stress-related genes by RT-PCR

To validate the microarray results and identify the effect of dissolved Cu^{2+} on the expression of oxidative stress-related genes, five genes related to oxidative stress were further investigated using quantitative RT-PCR. Clearly, all these five genes (*RHL41*, *MSRB7*, *BCB*, *PRXCA*, and *MC8*) were up-regulated after exposure to CuO NPs for 2 h (Fig. 5), which is consistent with the microarray analysis in Table 1. For the Cu^{2+} treatment, *RHL41*, *MSRB7* and *MC8* were generally down-regulated at all the test exposure times (0.5, 1, and 2 h). The expression of *BCB* after exposed to Cu^{2+} for 0.5-2 h was not significantly different from the un-exposed control ($p < 0.05$). *PRXCA* was significantly up-regulated after exposed to Cu^{2+} at all the exposure times ($p < 0.05$). However, at a given exposure time, the fold change for the Cu^{2+} treatment (1.6-3.3) was much

lower than that observed in the CuO NPs treatment (3.1-26.3). All these RT-PCR data on CuO NPs and Cu²⁺ treatments indicated that CuO NPs themselves contributed most of ROS generation and oxidative stress, rather than their released Cu²⁺, which is highly consistent with the confocal microscopy data (Fig. 3).

The expression level of these 5 genes as a function of exposure times (0-2 h) in *Arabidopsis* roots were further analyzed. As shown in Fig. 5A, the expression of *RHL41* (*Zat12*) reached the highest value after exposure to CuO NPs (10 mg/L) for 0.5 h, and then decreased subsequently, which is consistent with the ROS generation as function of exposure times (Fig. 3B). It is reported that *Zat12* played a key role in reactive oxygen signaling and abiotic stress tolerance in *Arabidopsis*, which could be resulted from the activation of antioxidant defense genes (Davletova et al., 2005; Xie et al., 2012). Actually, the activation of *Zat12* expression could occur in response to heat, salinity, drought, or wounding (Davletova et al., 2005). In another study, Cu²⁺ (10 μM) and Cd²⁺ (50 μM) also resulted in significant up-regulation of *RHL41* in *Arabidopsis* roots upon 2-h exposure (Weber et al., 2006). *MSRA7* is able to reduce methionine sulfoxide and restore methionine, thus acting as part of defense system against oxidative stress by eliminating ROS in plants. CuO NPs significantly increased the expression level of *MSRB7* in *Arabidopsis* root, indicating that the defense system was activated after exposure. Li et al. (2012) reported that the accumulation of H₂O₂ was increased in *MSRB7*-knockdown *Arabidopsis* while reduced in *MSRB7*-overexpressing *Arabidopsis* under environmental stresses (e.g., methyl viologen exposure), which is in agreement with our finding that ROS generation was obviously decreased after CuO NPs exposure (Fig. 3B) when the *MSRB7* had the highest expression level after

exposure to CuO NPs for 2 h (Fig. 5B). For both *BCB* and *PRXCA* (*PRX33*), the expression after CuO NPs exposure for 0.5 h was increased weakly, and then increased significantly after exposed for 2 h (Fig. 5C-5D). The *BCB* protein which is commonly located on a cell membrane could be induced by oxidative stress. The *BCB* gene family is related to various aspects of cell metabolism including lignification of the cell wall fraction. Lignin formation in response to oxidative stress is one of the protective mechanisms from damaging membranes (Ezaki et al., 2005). *PRXCA* (*PRX33*) as a type of cell wall peroxidase participates in apoplastic H₂O₂ production in *Arabidopsis* (O'Brien et al., 2012). Moreover, it was observed that *PRXCA* as well as *PRX34*, and *RBOHD* were positively regulated by H₂O₂, indicating the feed-forward increases in H₂O₂ production from the basal state during stress (O'Brien et al., 2012), which could be a possible reason for the obvious increase of *PRXCA* after CuO NPs exposure for 2 h. For *MC8*, the significant expression started from 2 h (Fig. 5E), which was later than other four genes (*RHL41*, *MSRB7*, *BCB*, and *PRXCA*). The programmed cell death protein *MC8* is involved in promoting programmed cell death in response to various stresses such as H₂O₂, and UV light. It was speculated that the accumulation of ROS such as H₂O₂ after CuO NPs for 2 h failed to be eliminated by the defense system, thus causing the activation of programmed cell death proteins such as *MC8*.

4. Conclusions

In this study, we observed significant growth inhibition of *Arabidopsis* after exposure to CuO NPs (10 and 20 mg/L). Root cells of *Arabidopsis* were severely damaged after CuO NPs (10 and 20 mg/L) exposure for 2 h while their corresponding dissolved Cu²⁺ ions (0.80 and 1.35 mg/L)

337 did not show any root damage. After longer CuO NPs exposure (1-2 days), the released Cu²⁺ ions
338 from CuO NPs contributed partial toxicity. Confocal microscopy investigation showed that ROS
339 accumulation induced by CuO NPs originated from both the particulate NPs and the released Cu²⁺
340 ions. Global gene expression analysis showed that 1658 genes in *Arabidopsis* root were
341 differentially expressed after CuO NPs exposure for 2 h, in which 1035 and 623 genes were
342 up-regulated and down-regulated, respectively. We further analyzed all the 47 up-regulated genes
343 that were response to oxidative stress. The quantitative RT-PCR results of five selected genes
344 (*RHL41*, *MSRB7*, *BCB*, *PRXCA*, and *MC8*) were in agreement with the microarray analysis. In
345 addition, it is revealed that CuO NPs contributed much stronger up-regulation of oxidative
346 stress-related genes than the corresponding Cu²⁺ ions. All these data clearly showed that the
347 oxidative stress in *Arabidopsis* was mainly caused by the CuO NPs themselves. This finding is
348 helpful for better understanding the transcriptional response of higher plants upon CuO NPs
349 exposure.

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Figures and Tables

Fig. 1. Effect of CuO NPs on the growth of *Arabidopsis* seedlings (9-day old) after 7-day CuO NPs (10, 20 mg/L) exposure. (A) Images of *Arabidopsis* seedlings after CuO NPs exposure; (B), (C), (D): Fresh weight, root fresh weight, and root length of *Arabidopsis* seedlings after CuO NPs exposure. In panels B-D, different letters indicate significant difference among different concentrations of CuO NPs ($p < 0.05$, LSD).

10 mg/L CuO NPs 20 mg/L CuO NPs 0.8 mg/L Cu²⁺ 1.35 mg/L Cu²⁺

Fig. 2. Damage of root tips of *Arabidopsis* (9-day old) after exposure to CuO NPs and Cu²⁺ ions for 0-2 days. The root tips were dyed with trypan blue before observation. The blue color on the surface of roots indicated the damaged cells on root tips. The scale bar of each image was 200 μ m.

Fig. 3. ROS production in root tips of *Arabidopsis* seedlings (9-day old) after the exposure of CuO NPs (10 mg/L) and Cu²⁺ (0.8 mg/L). (A): Confocal microscopy images of root tips at different exposure times (5 min-2 h). The brilliant green color (indicating with red arrow) on roots showed the accumulation of ROS; (B): Relative ROS generation in roots observed from fluorescence intensities of confocal microscopy images in panel A. Different letters in panel B indicate significant difference among the un-exposed control, CuO NPs and Cu²⁺ groups (n=11, LSD, p<0.05).

Fig. 4. Major GO functional categories ($p < 0.05$, gene number > 4) for the up-regulated genes (1035 genes) pre-identified in *Arabidopsis* roots after CuO NPs exposure. *Arabidopsis* roots (15-day old) were exposed to CuO NPs (10 mg/L) for 2 h before analysis. The GO functional categories were identified from DAVID database. (A) Biological Processes; (B) Cellular Components (red columns) and Molecular Functions (blue columns).

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Fig. 5. Relative expression levels of five genes in *Arabidopsis* roots (15-day old) after exposure to CuO NPs (10 mg/L) or the corresponding Cu²⁺ concentration (0.8 mg/L) for 0-2 h. The five genes were *RHL41* (A), *MSRB7* (B), *BCB* (C), *PRXCA* (D), and *MC8* (E). Real time-PCR was performed for gene expression analysis. Gene expression values were normalized to the expression amount of Actin2. For CuO NPs or Cu²⁺, significant differences between the non-exposure group (0 h) and the exposure groups are shown by asterisks (n=3, p < 0.05).

557 **Table 1.** List of 47 up-regulated genes that belonged to “response to oxidative stress” category
558 (p<0.05, gene number>4) after CuO NPs exposure. The roots of *Arabidopsis* (15-day old) were
559 exposed to 10 mg/L CuO NPs for 2 h before further analysis.

Probe ID	Fold change	Symbol	Locus identifier	Gene name
253161_at	253.27	<i>SEN1</i>	AT4G35770	Senescence-associated protein DIN1
246099_at *	90.33	<i>BCB</i>	AT5G20230	Blue copper protein
260804_at	54.04	<i>AT1G78410</i>	AT1G78410	VQ motif-containing protein
249459_at	52.47	<i>AT5G39580</i>	AT5G39580	Peroxidase 62
259479_at	45.22	<i>AT1G19020</i>	AT1G19020	Hypothetical protein
250351_at *	38.36	<i>HSP17.6A</i>	AT5G12030	Heat shock protein 17.6A
266299_at	31.78	<i>GSTU5</i>	AT2G29450	Glutathione S-transferase 103-1A
260248_at *	27.91	<i>HSP101</i>	AT1G74310	Heat shock protein 101
256245_at *	26.86	<i>HSP70</i>	AT3G12580	Heat shock protein 70-4
254241_at	26.04	<i>CRK11</i>	AT4G23190	Cysteine-rich receptor-like protein kinase 11
261475_at #	22.39	<i>AT1G14550</i>	AT1G14550	Peroxidase 5
247655_at *	20.44	<i>RHL41</i>	AT5G59820	C2H2-type zinc finger protein ZAT12
261474_at #	19.78	<i>AT1G14540</i>	AT1G14540	Peroxidase 4
259410_at	19.71	<i>AT1G13340</i>	AT1G13340	Regulator of Vps4 activity in the MVB pathway protein
261648_at *	18.56	<i>STZ</i>	AT1G27730	Zinc finger protein STZ/ZAT10
250646_at #	17.10	<i>PA2</i>	AT5G06720	Peroxidase 53
252291_s_at *,#	15.61	<i>PRXCA</i>	AT3G49110	Peroxidase 33
267357_at	13.91	<i>HSPRO2</i>	AT2G40000	HS1 PRO-1 2-like protein
256337_at	12.36	<i>AT1G72060</i>	AT1G72060	Serine-type endopeptidase inhibitor
249467_at	11.95	<i>NAC6</i>	AT5G39610	NAC domain containing protein 6
264660_at	8.79	<i>HEMA2</i>	AT1G09940	Glutamyl-tRNA reductase 2
251984_at #	8.72	<i>PAL2</i>	AT3G53260	Phenylalanine ammonia-lyase 2
254432_at	8.53	<i>AT4G20830</i>	AT4G20830	Reticuline oxidase-like protein
246228_at #	7.98	<i>AT4G36430</i>	AT4G36430	Peroxidase 49
253203_at *	7.36	<i>ADC2</i>	AT4G34710	Arginine decarboxylase 2
252906_at	7.06	<i>GGT1</i>	AT4G39640	Gamma-glutamyl transpeptidase 1
255111_at #	6.60	<i>AT4G08780</i>	AT4G08780	Peroxidase 38
263150_at *	6.49	<i>AT1G54050</i>	AT1G54050	HSP20-like chaperone
262731_at *	6.32	<i>MC8</i>	AT1G16420	Metacaspase 8
254839_at *	6.13	<i>AT4G12400</i>	AT4G12400	Putative stress-inducible protein
252592_at *	6.02	<i>MPK3</i>	AT3G45640	Extracellular signal-regulated kinase 1/2
253099_s_at #	5.59	<i>AT4G37520</i>	AT4G37520	Peroxidase 50
247327_at #	5.50	<i>AT5G64120</i>	AT5G64120	Peroxidase 71
257670_at	5.23	<i>AT3G20340</i>	AT3G20340	Hypothetical protein
257315_at	5.04	<i>ERD5</i>	AT3G30775	Proline dehydrogenase 1
250702_at #	4.99	<i>AT5G06730</i>	AT5G06730	Peroxidase 54

254784_at *	4.90	<i>NUDT7</i>	AT4G12720	Nudix hydrolase 7
265675_at *	4.45	<i>HSP70T-2</i>	AT2G32120	Heat-shock protein 70T-2
262832_s_at	4.24	<i>PCR2</i>	AT1G14870	Cadmium resistance protein 2
263845_at #	4.12	<i>PAL1</i>	AT2G37040	Phenylalanine ammonia-lyase 1
250781_at *	3.70	<i>DREB2A</i>	AT5G05410	Dehydration-responsive element-binding protein 2A
263063_s_at #	3.67	<i>AT2G18140</i>	AT2G18140	Peroxidase 14
254385_s_at	3.51	<i>MSRB7</i>	AT4G21830	Peptide methionine sulfoxide reductase B7
266590_at *	3.38	<i>BAG6</i>	AT2G46240	BCL-2-associated athanogene 6
251091_at *	3.15	<i>RSR4</i>	AT5G01410	Pyridoxal biosynthesis protein PDX1.3
249583_at *	3.13	<i>TCH2</i>	AT5G37770	Calcium-binding protein CML24
263241_at *	3.08	<i>ADC1</i>	AT2G16500	Arginine decarboxylase 1

560 Note: “*” indicates that this probe ID is also related to “response to abiotic stimulus”;
561 “#” indicates that this probe ID is also related to the phenylpropanoid biosynthesis of the KEGG
562 (Kyoto Encyclopedia of Genes and Genomes) metabolic pathways.
563