Supplementary Information

Interaction of CuO Nanoparticles with Plant Cells: Internalization, Oxidative stress, Electron Transport Chain Disruption, and Toxicogenomic Responses

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Materials and methods

Reactive oxygen species determination

The hydrogen peroxide (H_2O_2) was detected based on reacting with ammonium molybdate to produce a peroxomolybdic acid complex. H_2O_2 content was performed using a hydrogen peroxide assay kit (A064, Jiancheng, Nanjing, China). The coefficient of variation (CV) was 1.7%. Absorbance was determined at 405 nm by a microplate reader (Thermo, USA).

Hydroxyl radical ($OH\cdot$) was determined using a hydroxyl free radial assay kit (A018, CV=1.4%, Jiancheng, Nanjing, China) based on the reaction of H_2O_2 with Fenton reaction. A colouration reaction was generated following the addition of the electron acceptor and Griess reagent. The absorbance was determined using a microplate reader (Thermo, USA) at 550 nm.

Superoxide anion ($O_2{\cdot}^-$) was determined by inhibition and produce4 produce superoxide anion assay kit (A052, CV=1.7%, Jiancheng, Nanjing, China). $O_2{\cdot}^-$ was produced by the reaction of xanthine and xanthine oxidase. The solution was colored after adding the electron acceptor and Griess reagent.

Antioxidant enzyme activity, lipid peroxidation and lactate dehydrogenase (LDH) determination

Catalase (CAT) was determined based on its ability to scavenge H_2O_2 according to the method of the catalase assay kit (A007-1-1, CV=1.7%, Jiancheng, Nanjing, China). CAT could catalyze H_2O_2, and the reaction could be ended by adding ammonium molybdate into the solution. The surplus H_2O_2 interacted with ammonium molybdate to produce a peroxomolybdic acid complex. The absorbance was determined at 405 nm by a microplate reader (Thermo, USA). One unit of CAT activity was defied as the amount of enzyme to 1 μmol H_2O_2 per second. SOD activity was examined by a superoxide dismutase assay kit (A001-3, CV=1.2%, Jiancheng, Nanjing, China), and expressed as U/mg protein.

Malondialdehyde (MDA) can react with thiobarbituric acid (TBA) to form TBA-reactive products. Lipid peroxidation in plant cells was assayed as the concentration of TBA-reactive products at 535 nm, equated with MDA by the level of malondialdehyde

(MDA) which was measured by using the lipid peroxidation MDA assay kit (S0131, Beyotime Biotechnology Institute, Jiangsu, China) following the manufacturer's protocol.

Lactate dehydrogenase (LDH) was performed by using LDH Cytotoxicity Assay Kit (C0016, Beyotime Institute of Biotechnology, Jiangsu, China). Cells were centrifuged (123 ×g, 10 min) after incubation with CuO NPs (12 mg/L), CuO BPs (12 mg/L) or Cu^{2+} (0.8 mg/L) for 4 h. The release of LDH in the supernatants was determined by detecting the absorbance at 490 nm using a microplate reader (Thermo, USA).

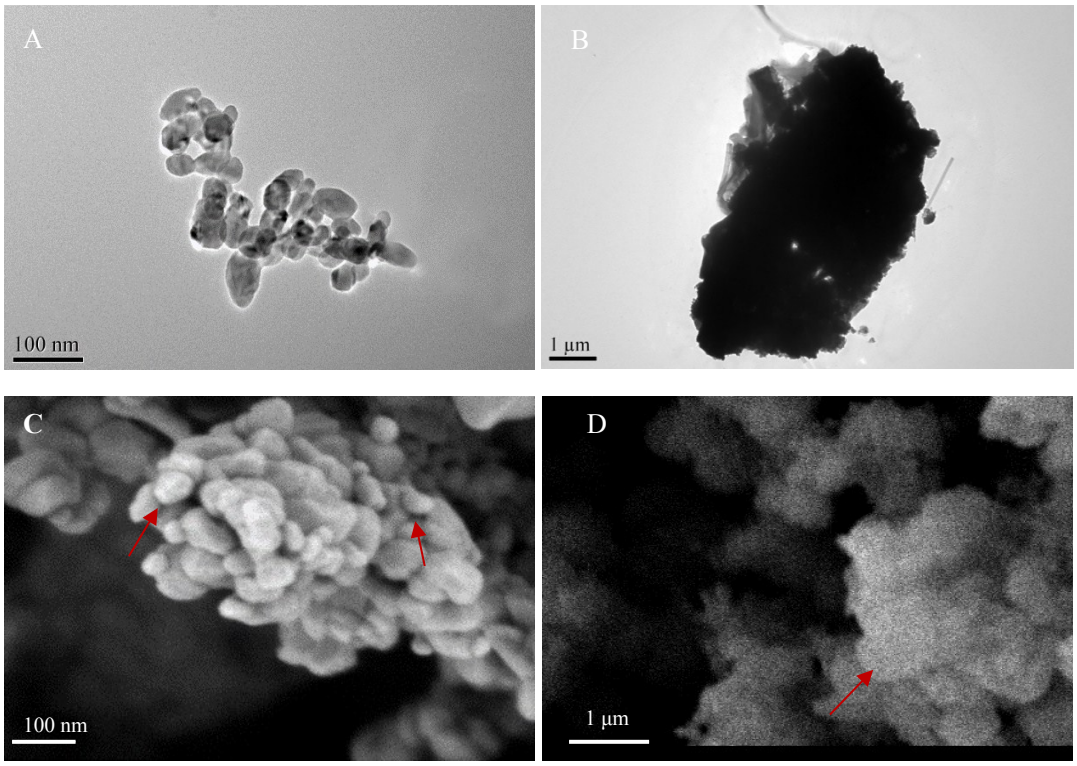


Figure S1. TEM and SEM images of CuO NPs and BPs. (A) and (B) were the TEM images of CuO NPs and BPs, respectively. (C) and (D) were the SEM images of CuO NPs and BPs, respectively.

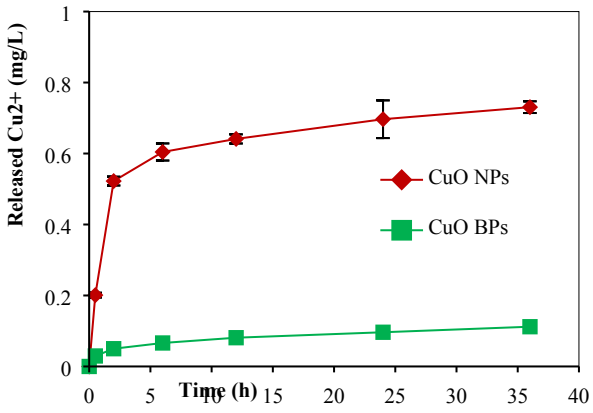


Figure S2. The dissolution of CuO NPs and CuO BPs as a function of incubation times (0-36 h) in the modified MS medium (n=3). The concentrations of CuO NPs and BPs were both 12 mg/L.

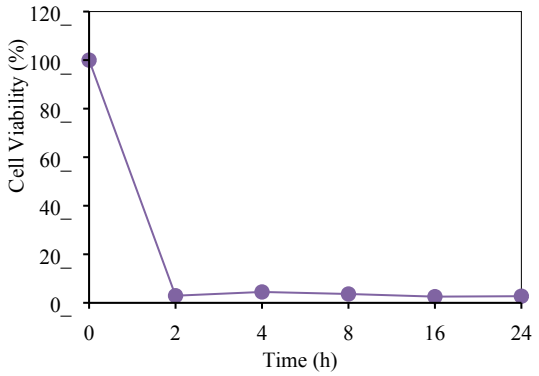


Figure S3. Cell viability after exposure to Cu^{2+} ions (9.6 mg/L, equal Cu content of CuO NPs at 12 mg/L) as a function of exposure time (0-24 h) (n=3).

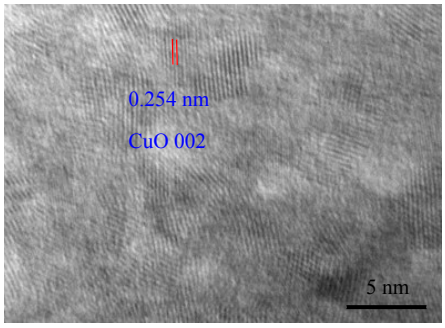


Figure S4. High resolution TEM (HRTEM) image of intracellular CuO NPs after exposure to CuO NPs (12 mg/L) for 12 h. This HRTEM image was the enlarged view of the region as marked with blue box in Figure 2B.

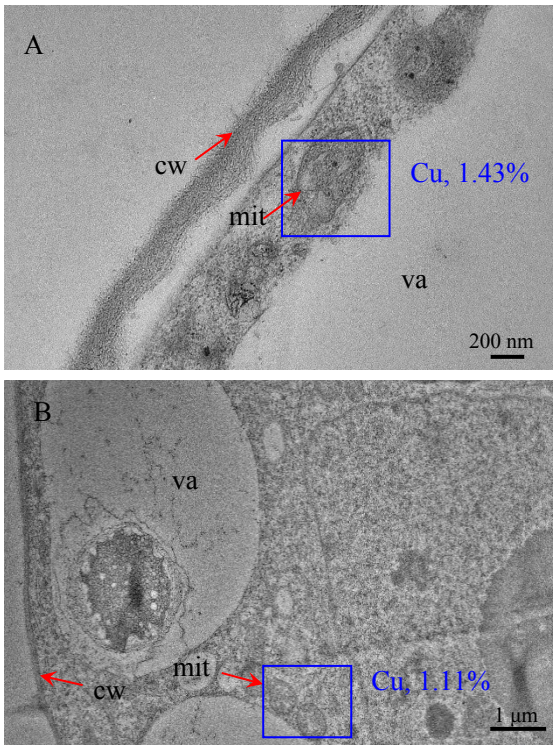
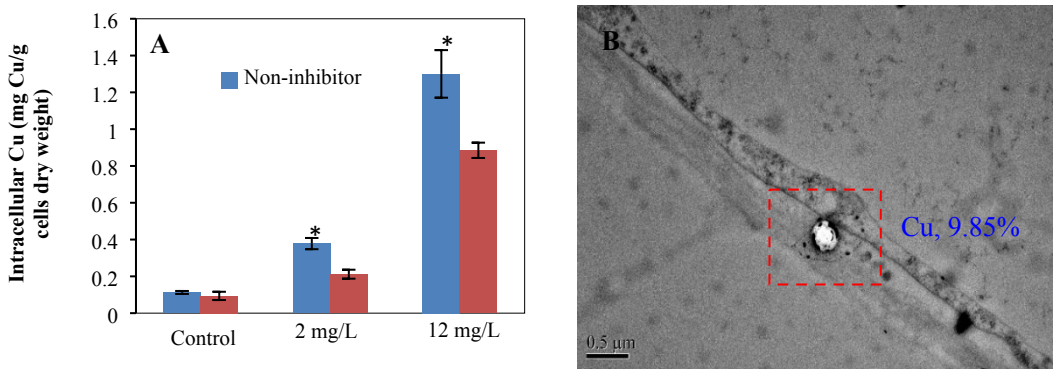
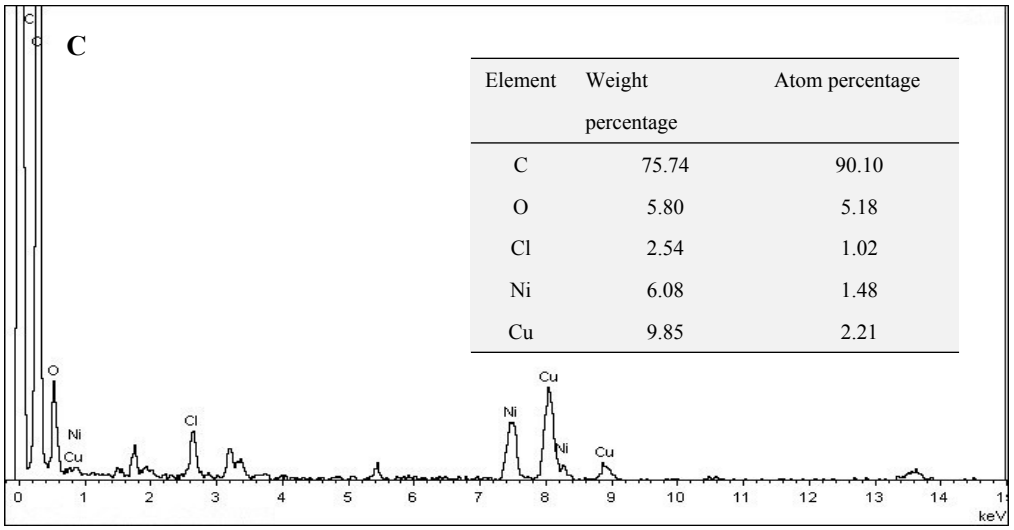


Figure S5. TEM images of BY-2 cells after exposure to CuO BPs (12 mg/L) (A) and Cu^{2+} (0.8 mg/L) (B) for 12 h. In the two panels, Cu contents marked with blue boxes were analyzed with EDS, and the weight percentages of Cu content were shown along with the blue boxes. cw: cell wall; va: vacuole; mit: mitochondria.





Element	Weight percentage	Atom percentage
C	75.74	90.10
O	5.80	5.18
Cl	2.54	1.02
Ni	6.08	1.48
Cu	9.85	2.21

Figure S6. Internalization of CuO NPs by BY-2 cells. (A): Intracellular Cu content with/without the pre-treatment of an endocytosis inhibitor (wortmannin 33 μM). After pre-treatment for 30 min, the cells were exposed to CuO NPs (2, 12 mg/L) for 12 h before Cu determination. (B): TEM image of plant cell after exposure to CuO NPs (12 mg/L, 12 h). (C): The elemental composition of vesicle in panel B (as marked with red dashed box) was analyzed with EDS. The insert in panel (C) listed the weight percentage and atom percentage of the detected elements. In panel (A), "*" indicates significant differences between the "Non-inhibitor" and "Inhibitor" treatments ($p < 0.05$, LSD, n=3).

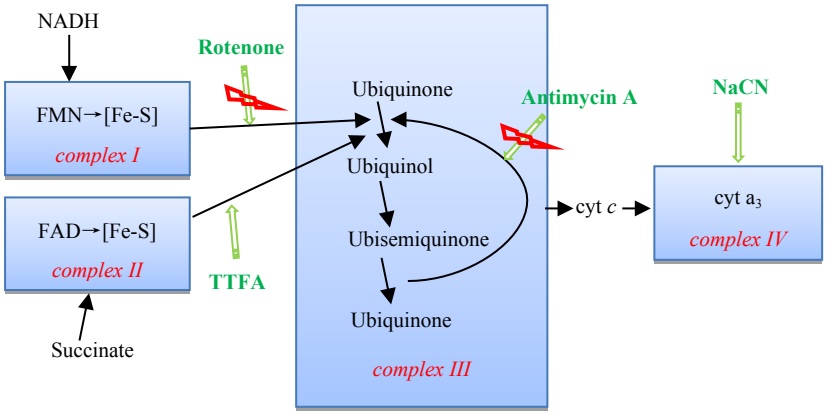


Figure S7. Schematic diagram of mitochondrial electron transport chain and the target sites of four inhibitors (rotenone, TTFA, antimycin A and NaCN) in mitochondria. Rotenone could block the transfer of electrons from NADH to ubiquinone. Antimycin A can block electron transfer from ubisemiquinone to ubiquinol. TTFA inhibits electron transfer from succinate to ubiquinone. NaCN inhibits the oxidation of cyt a_3 by O_2, which could decrease oxygen production. As indicated in the figure, CuO NPs blocked the electron transport from NADH to ubiquinone, and ubisemiquinone to ubiquinol. Cyt: cytochrome; TTFA: thenoyltrifluoroacetone; NaCN: sodium cyanide.

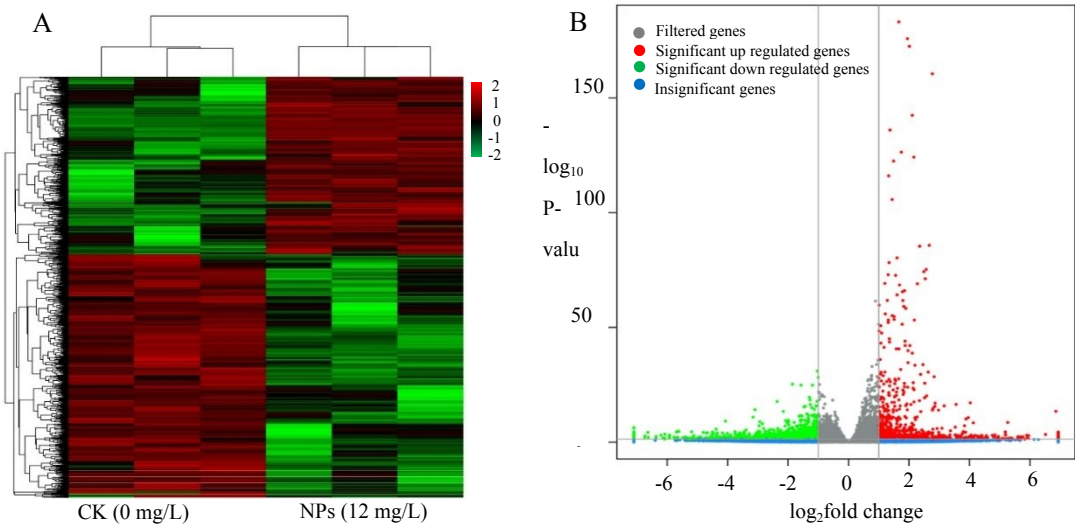


Figure S8. Hierarchical cluster (A) and volcano plot (B) for differential expression analysis of BY-2 cells after CuO NPs (0, 12 mg/L) exposure for 4 h. In panels A and B, red and green represent significantly up-, and down-regulated genes, respectively (P-value < 0.05, fold change > 2). In panel B, gray represents filtered genes, blue represents insignificantly expressed genes.

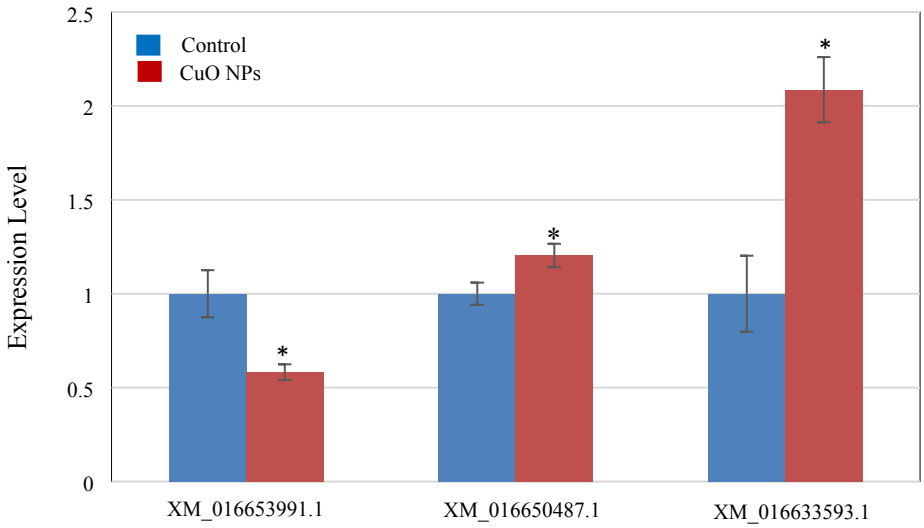


Figure S9. Expression level of the three selected genes as analyzed using qRT-PCR. The plant cells were exposed with CuO NPs (12 mg/L) for 12h. "*" represents significant difference at $p < 0.05$.

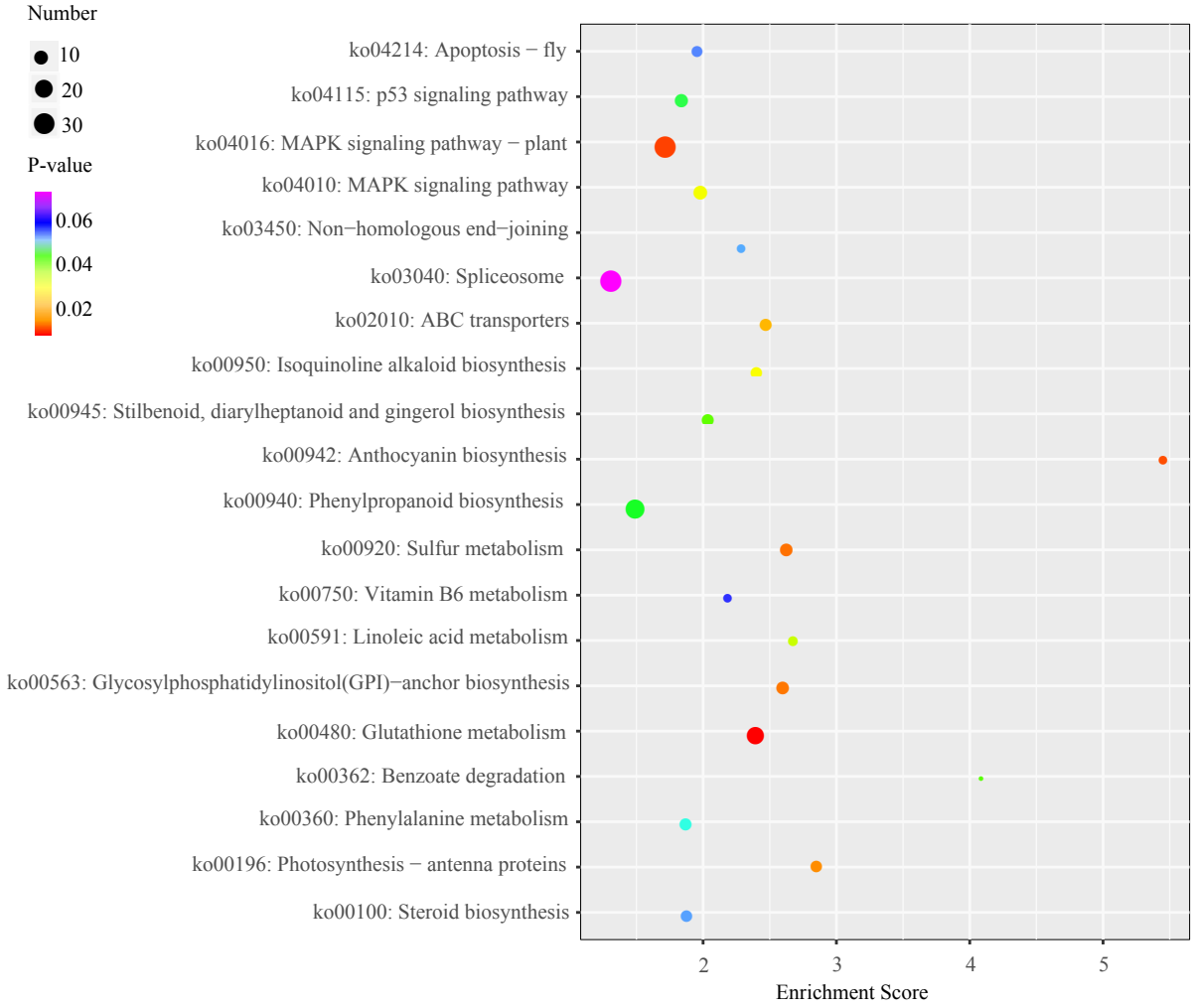


Figure S10. Bubble chart of the top 20 KEGG pathways. The left Y-axis indicate the KEGG pathways, and the X-axis indicate the respective enrichment scores. A high P-value is represented by purple, and low P-value is represented by red.

142 Table S1. The components of MS and modified MS medium (1/2 MS medium) for tobacco BY-2

143 cell culturing.

	Component	MS medium content (mg/L)	Modified MS medium (mg/L)
Macronutrients	NH_4NO_3	1650	875
	KH_2PO_4	225	112.5
	KNO_3	1900	975
	$CaCl_2·2H_2O$	440	220
	$MgSO_4·7H_2O$	370	185
Micronutrients	KI	0.83	0.415
	H_3BO_3	6.2	3.1
	$MnSO_4·4H_2O$	22.3	11.15
	$ZnSO_4·7H_2O$	8.6	4.3
	$Na_2MoO_4·2H_2O$	0.25	0.125
	$CuSO_4·5H_2O$	0.025	0.0125
	$CoCl_2·6H_2O$	0.025	0.0125
Iron Source	$EDTANa_2·2H_2O$	37.3	18.65
	$FeSO_4·7H_2O$	27.8	13.9
Vitamins and Carbon Source	Myo-inositol	100	50
	Glycine	2	1
	Thiamine-HCl	1	0.5
	Pyridoxine-HCl	0.5	0.25
	Nicotinic acid	0.5	0.25
	Sucrose	30000	15000

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Table S2. Results on RNA quality assessment of cells with and without CuO NPs (12 mg/L) exposure. The exposure time was 4 h.

Number	RNA concentration（μg/μL）	A260/280[a]	Volume (μL)	Total yield (μg)	RIN
CK1	0.7071	2.05	50	35.36	9.8
CK2	0.4972	1.99	50	24.86	10.0
CK3	0.6190	2.06	50	30.95	9.7
NP1	0.6894	2.01	50	34.47	10.0
NP2	0.5794	1.98	50	28.97	10.0
NP3	0.7641	2.03	50	38.21	10.0

[a]: A260/280 (the ratio of absorbance at 260 and 280 nm) is used to assess the purity of RNA. A ratio range at 1.8-2.1 is accepted as “purity” for RNA. “RIN (RNA Integrity Number) ≥7” was used for subsequent gene analysis.

Table S3. The primer sequences of the three selected genes during qRT-PCR, and the expression level comparison of the three genes between qRT-PCR and RNA-Seq results.

Transcript -ID (*N. tabacum*)	Forward primer	Reverse primer	P-value	Log_2fold change (qRT-PCR)	Log_2fold change (RNA-Seq)
XM_016653991.1	CACTGCTCCACGCTAACA	ATCATTAACTGCGTCAAGGC	1.98E-02	-0.78	-1.06
XM_016650487.1	TGGAGCAGACATTGCGAA	GATTGTGGTACTCTCACTCCT	1.49E-02	0.26	1.00
XM_016633593.1	AACCTCATTCTTCACTCGTTC	AGACCTTGCCGGACAATA	2.2E-03	1.06	2.01

Table S4. Characterization of CuO NPs and BPs

Particles	Purity (%)	Surface area (m^2/g)	Size from manufacturer (nm)	Size from TEM (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)
CuO NPs	>99.9	12.01	<50	30-40	-11.8[a]/-29.9[b]	238.4[a]/161.3[b]
CuO BPs	>99.9	0.51	5000	1500-2500	-14.5[a]/-15.3[b]	2403[a]/1377[b]

[a] Measured in 1/2 MS medium, the concentrations of CuO NPs and BPs were 12 mg/L.
[b] Measured in ultrapure water, the concentrations of CuO NPs and BPs were 12 mg/L.

Table S5. Information of RNA-Seq mapping results for the unexposed and CuO NPs-treated plant cells.

Sample	Software	CK	CK	CK	CuO NPs	CuO NPs	CuO NPs
Total reads (M)	Tophat	5.49	5.57	4.89	5.30	6.43	5.17
	Bowtie2	5.49	5.57	4.89	5.17	5.30	6.43
Total mapped reads (M)	Tophat	4.82 (87.9%)	4.96 (88.9%)	4.35 (89.0%)	4.72 (88.9%)	5.70 (88.6%)	4.59 (88.9%)
	Bowtie2	4.21 (76.8%)	4.31 (77.4%)	3.83 (78.3%)	4.07 (78.7%)	4.15 (78.2%)	4.95 (76.9%)
Reads mapped in proper pairs (M)	Tophat	4.18 (76.2%)	4.33 (77.8%)	3.77 (77.2%)	4.05 (76.3%)	4.83 (75.1%)	3.97 (76.9%)
	Bowtie2	3.91 (71.2%)	4.03 (72.3%)	3.59 (73.4%)	3.79 (73.3%)	3.87 (73.0%)	4.61 (71.8%)

Table S6. The top 10 GO terms in the category of biological process, cellular component and molecular function for the up-regulated genes in BY-2 cells after exposure to CuO NPs (12 mg/L) for 4 h.

GO ID	GO Term	Category	Enrichment score	P-value
GO:0032957	inositol trisphosphate metabolic process	Biological process	25.28	3.46E-06
GO:0042542	response to hydrogen peroxide	Biological process	10.11	4.05E-05
GO:0009408	response to heat	Biological process	5.55	9.92E-05
GO:0009693	ethylene biosynthetic process	Biological process	11.38	1.19E-04
GO:0010311	lateral root formation	Biological process	9.48	2.5E-04
GO:0009734	auxin-activated signaling pathway	Biological process	3.82	4.92E-04
GO:0006970	response to osmotic stress	Biological process	4.97	1.22E-03
GO:0010200	response to chitin	Biological process	4.53	1.86E-03
GO:0008152	metabolic process	Biological process	4.53	1.86E-03
GO:0009611	response to wounding	Biological process	3.76	2.14E-03
GO:0009705	plant-type vacuole membrane	Cellular component	5.42	8.25E-04
GO:0019005	SCF ubiquitin ligase complex	Cellular component	6.89	8.8E-04
GO:0005634	nucleus	Cellular component	1.45	1.78E-03
GO:0005737	cytoplasm	Cellular component	1.49	0.021
GO:0005802	trans-Golgi network	Cellular component	1.59	0.120
GO:0005794	Golgi apparatus	Cellular component	1.39	0.132
GO:0005768	endosome	Cellular component	1.52	0.136
GO:0005739	mitochondrion	Cellular component	1.27	0.157
GO:0005887	integral component of plasma membrane	Cellular component	1.32	0.192
GO:0048046	apoplast	Cellular component	1.22	0.230
GO:0016758	transferase activity, transferring hexosyl groups	Molecular function	14.22	4.66E-05
GO:0016491	oxidoreductase activity	Molecular function	3.79	1.02E-03
GO:0004674	protein serine/threonine kinase activity	Molecular function	2.08	6.66E-03
GO:0043565	sequence-specific DNA binding	Molecular function	2.23	0.013

GO:0005524	ATP binding	Molecular function	1.41	0.021
GO:0042802	identical protein binding	Molecular function	2.77	0.023
GO:0030170	pyridoxal phosphate binding	Molecular function	2.71	0.025
GO:0042803	protein homodimerization activity	Molecular function	2.42	0.035
GO:0044212	transcription regulatory region DNA binding	Molecular function	2.32	0.040
GO:0003700	transcription factor activity, sequence-specific DNA binding	Molecular function	1.44	0.057

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Table S7. The top 10 GO terms in the category of biological process, cellular component and molecular function for the down-regulated genes in BY-2 cells after exposure to CuO NPs (12 mg/L) for 4 h.

GO ID	GO Term	Category	Enrichment score	P-value
GO:0009624	response to nematode	Biological process	6.61	9.66E-05
GO:0009873	ethylene-activated signaling pathway	Biological process	4.06	1.53E-04
GO:0009809	lignin biosynthetic process	Biological process	6.32	3.85E-04
GO:0006952	defense response	Biological process	2.31	2.17E-03
GO:0042744	hydrogen peroxide catabolic process	Biological process	3.45	5.98E-03
GO:0007165	signal transduction	Biological process	3.48	0.010
GO:0006979	response to oxidative stress	Biological process	2.44	0.011
GO:0048364	root development	Biological process	2.71	0.016
GO:0006970	response to osmotic stress	Biological process	2.80	0.022
GO:0009611	response to wounding	Biological process	2.25	0.032
GO:0031966	mitochondrial membrane	Cellular component	5.17	2.54E-03
GO:0005576	extracellular region	Cellular component	1.73	0.019
GO:0005856	cytoskeleton	Cellular component	2.37	0.038
GO:0009579	thylakoid	Cellular component	1.90	0.074
GO:0005743	mitochondrial inner membrane	Cellular component	1.71	0.099
GO:0005739	mitochondrion	Cellular component	1.30	0.116
GO:0031969	chloroplast membrane	Cellular component	1.55	0.128
GO:0016021	integral component of membrane	Cellular component	1.13	0.155
GO:0005886	plasma membrane	Cellular component	1.12	0.231
GO:0009536	plastid	Cellular component	1.13	0.275
GO:0042409	caffeoyl-CoA O-methyltransferase activity	Molecular function	24.38	3.1E-06
GO:0052689	carboxylic ester hydrolase activity	Molecular function	7.42	6.3E-04
GO:0004674	protein serine/threonine kinase activity	Molecular function	1.84	0.011
GO:0003700	transcription factor activity	Molecular function	1.58	0.012
GO:0043565	sequence-specific DNA binding	Molecular function	1.91	0.025

GO:0016887	ATPase activity	Molecular function	2.34	0.039
GO:0004601	peroxidase activity	Molecular function	2.13	0.052
GO:0004842	ubiquitin-protein transferase activity	Molecular function	1.83	0.056
GO:0005215	transporter activity	Molecular function	2.06	0.058
GO:0044212	transcription regulatory region DNA binding	Molecular function	1.74	0.094

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171 Table S8. The genes in the GO terms associated with mitochondria.

GO ID	GO Term	P-value	Transcript ID (*N.tabacum*)	Gene ID (*N. tabacum*)	Log_2fold changes	Sequence matching rate	BLAST e value	Transcript ID (*Arabidopsis*)	Gene Name (*Arabidops*
GO: 0031966	mitochondrial membrane	0.018	XM_016655108.1	LOC107827888	-3.85	76.54	1E-153	AT1G34065.1	SAMC2
			XM_016658402.1	LOC107830762	-3.98	69.2	6E-57	AT3G17611.2	ATRBL14
			XM_016660535.1	LOC107832675	-1.10	70.81	2E-77	AT3G28580.1	NN
GO: 0005743	mitochondrial inner membrane	0.088	XM_016634074.1	LOC107809440	-1.48	69.97	3E-66	AT5G19630.1	NN
			XM_016645539.1	LOC107819429	-1.42	76.49	2E-72	AT1G02410.1	COX11
			XM_016659246.1	LOC107831479	+/0	—	—	—	NN
GO: 0005739	mitochondrion	0.075	XM_016580765.1	LOC107762408	-1.13	70.12	3E-125	AT3G25210.1	NN
			XM_016625037.1	LOC107801659	-2.72	72.52	0	AT5G22800.1	EMB86
			XM_016637446.1	LOC107812378	-1.43	64.95	5E-29	AT1G43980.1	PCMP-E58
			XM_016641052.1	LOC107815466	-1.58	74.85	0	AT4G17740.2	CTPA2
			XM_016641384.1	LOC107815753	-4.62	70.73	0	AT5G14220.1	PPOX2
			XM_016645539.1	LOC107819429	-1.42	76.49	2E-72	AT1G02410.1	COX11
			XM_016653798.1	LOC107826771	-1.83	70.11	4E-136	AT1G71060.1	NN
			XM_016653991.1	LOC107826946	-1.06	79.56	1E-171	AT3G27380.2	SDH2-1
			XM_016655108.1	LOC107827888	-3.85	76.54	1E-153	AT1G34065.1	SAMC2
			XM_016655916.1	LOC107828574	-2.45	67.42	6E-167	AT1G53600.1	PCMP-E63
			XM_016656504.1	LOC107829073	-1.80	—	—	—	NN
			XM_016658711.1	LOC107830996	-1.30	80	5E-09	AT1G50270.1	PCMP-E42
			XM_016659246.1	LOC107831479	+/0	—	—	—	NN
			XM_016659608.1	LOC107831816	-1.11	72.38	0	AT5G57480.1	NN
			XM_016660535.1	LOC107832675	-1.10	70.81	2E-77	AT3G28580.1	NN
GO: 0070469	respiratory chain	0.1026	XM_016634074.1	LOC107809440	-1.48	69.97	3E-66	AT5G19630.1	NN
GO: 0005749	mitochondrial respiratory chain complex II, succinate dehydrogenase complex	0.003	XM_016653991.1	LOC107826946	-1.06	79.56	1E-171	AT3G27380.2	SDH2-1

	(ubiquinone)								
GO: 0008137	NADH dehydrogenase (ubiquinone) activity	0.043	XM_016634074.1	LOC107809440	-1.48	69.97	3E-66	AT5G19630.1	NN
GO: 0010275	NAD(P)H dehydrogenase complex assembly	0.0009	XM_016637993.1	LOC107812824	-2.88	72.92	4E-24	AT5G39210.1	CRR7
GO: 0034551	mitochondrial respiratory chain complex III assembly	0.0003	XM_016659608.1	LOC107831816	-1.11	72.38	0	AT5G57480.1	NN
GO: 0051287	NAD binding	0.0003	XM_016660215.1	LOC107832374	-1.06	75.36	0	AT1G79750.1	NADP-ME
GO: 0070469	respiratory chain	0.1026	XM_016634074.1	LOC107809440	-1.48	69.97	3E-66	AT5G19630.1	NN
GO: 0005739	mitochondrion	0.157	XM_016580880.1	LOC107762521	1.06	79.53	0	AT3G12580.1	MED37C
			XM_016596624.1	LOC107776706	0/+	71.25	9E-113	AT4G25290.1	NN
			XM_016612125.1	LOC107790219	1.79	66.77	2E-64	AT5G38710.1	POX2
			XM_016628836.1	LOC107804891	1.46	73.42	0	AT3G46100.1	ATHRS1
			XM_016633593.1	LOC107809014	2.01	80.03	0	AT1G15690.1	AVP1
			XM_016634294.1	LOC107809633	2.63	—	—	—	NN
			XM_016640170.1	LOC107814719	2.02	—	—	—	NN
			XM_016641568.1	LOC107815875	2.21	67.95	2E-118	AT1G07590.1	NN
			XM_016653973.1	LOC107826927	2.21	68.61	1E-78	AT4G27940.1	MTM1
			XM_016655266.1	LOC107828015	1.18	72.96	0	AT2G38400.1	AGT3
			XM_016660604.1	LOC107832733	5.59	65.2	4E-58	AT5G57250.1	NN
GO: 0045041	protein import into mitochondrial intermembrane space	0.0001	XM_016650487.1	LOC107823778	1.00	71.38	0	AT2G29080.1	FTSH3

"—" indicated that this gene cannot match to that of *Arabidopsis*. Sequence matching rate of BY-2 cells in this work was matched with that of *Arabidopsis*.

"NN" in the last column indicates "No Name" of this gene was found from the genome of *Arabidopsis* (http://www.arabidopsis.org).

"+/0" means the gene only expressed in the unexposed cells, and this gene in the CuO NPs-treated cells was down-regulated.

"0/+" means the gene only expressed in the CuO NPs-treated cells, and this gene was up-regulated because it was not expressed in the unexposed cells.

Table S9. The genes that belong to glutathione metabolism in KEGG pathway analysis.

Transcript ID (*N. tabacum*)	Gene ID (*N. tabacum*)	Log_2fold change	Sequence matching rate	BLAST e value	Transcript ID (*Arabidopsis*)	Gene Name (*Arabidopsis*)
XM_016578004.1	LOC107759987	1.31	70.77	3.00E-77	AT5G02780.1	GSTL1
XM_016578005.1	LOC107759987	1.12	70.77	3.00E-77	AT5G02780.1	GSTL1
XM_016589828.1	LOC107770509	1.77	71.56	1.00E-83	AT5G02790.1	GSTL3
XM_016599675.1	LOC107779283	1.12	67.77	9.00E-39	AT1G02920.1	GSTF7
XM_016600186.1	LOC107779712	-3.70	69.9	3.00E-26	AT2G29450.1	GSTU5
XM_016600476.1	LOC107779974	1.42	70.39	2.00E-82	AT4G32320.1	APX6
XM_016603912.1	LOC107782951	1.66	73.42	3.00E-108	AT1G78380.1	GSTU19
XM_016603942.1	LOC107782987	1.48	72.98	9.00E-39	AT2G29420.1	GSTU7
XM_016605205.1	LOC107784134	-1.09	66.14	1.00E-44	AT4G39640.2	GGT1
XM_016611777.1	LOC107789895	1.74	66.67	1.00E-24	AT3G09270.1	GSTU8
XM_016617453.1	LOC107794892	-1.57	77.55	0	AT5G35790.1	APG1
XM_016617761.1	LOC107795177	1.20	81.25	1.00E-31	AT1G02920.1	GSTF7
XM_016621022.1	LOC107798067	1.53	68.9	3.00E-26	AT2G29420.1	GSTU7
XM_016624113.1	LOC107800866	1.60	69.9	3.00E-26	AT2G29450.1	GSTU5
XM_016641574.1	LOC107815922	-1.39	—	—	—	NN
XM_016650661.1	LOC107823949	1.37	72.86	3.00E-102	AT1G78380.1	GSTU19
XM_016659023.1	LOC107831268	1.16	67	1.00E-24	AT3G09270.1	GSTU8
XM_016660621.1	LOC107832748	1.01	71.22	0	AT5G27380.1	GSH2
XM_016640514.1	LOC107815009	-1.63	69.53	1.00E-16	AT2G29420.1	GSTU7

"—" indicated that this gene cannot match to that of *Arabidopsis*. Sequence matching rate of BY-2 cells in this work was matched with that of *Arabidopsis*.

"NN" in the last column indicates "No Name" of this gene was found from the genome of *Arabidopsis* (http://www.arabidopsis.org).