Supplementary Information

Molecular mechanism of silver nanoparticles inhibiting primary root growth of *Oryza sativa* L.

Jiajia Yang, Ruihua Chen, Huan Zhang, Ruonan Wang, Zhifeng Zhang*

School of Life Science, Shanxi Normal University, Taiyuan, 030031, China

*Corresponding author. Fax: (86) 0351-2051196. E-mail:zzfsx2012@126.com.

1. Measurement of Zeta potential, hydrodynamic diameter, and Ag^+ Release

The zeta potential and hydrodynamic diameter of AgNPs were measured using a Malvern Zetasizer Nano-ZS90 instrument (Malvern, USA). The UV-Vis absorption spectra of AgNPs suspensions were recorded with a Shimadzu UV-29100 spectrophotometer (Tokyo, Japan) using a quartz cuvette with a 1 cm optical path. The silver content in AgNPs and the silver ion release rate were determined by inductively coupled plasma mass spectrometry (ICP-MS, model NexION 300, PerkinElmer, USA). For analysis, AgNPs samples were digested with concentrated nitric acid and hydrogen peroxide. AgNPs were dispersed in a series of 20% Hoagland nutrient solution at a concentration of 10 $mg \cdot L^{-1}$. After intervals of 24 hours, the nutrient solutions were ultrafiltered using ultrafiltration tubes, and the concentration of Ag^+ ions in the filtrate was measured.

2. Transcriptome analysis

2.1 RNA extraction and quantification

The rice seedlings were separately treated with 0, 5.0, 10.0 mg L^{-1} AgNPs@PEI for 3-day-old. The primary roots were sampled, washed with ultrapure water three times, immediately put into a centrifuge tube, and frozen with liquid nitrogen for 15min. The total RNA of root tip cells was extracted with pre-cooled TRIzol (Invitrogen, Carlsbad, USA) reagent according to the instructions. RNA quantification was evaluated using the RNA NanDrop 2000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.2 Library construction and transcriptome sequencing

The isolated total RNA was processed for preparing an mRNA sequencing library using a TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Subsequently, the libraries were paired-end sequenced using an Illumina NovaSeq platform at Personal Biotechnology Co., Ltd. (Shanghai, China). Briefly, the quality and size of the libraries were detected using an Agilent 2100 bioanalyzer DNA kit (Agilent Technologies).

The raw reads were trimmed and quality controlled by FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads that contained adapters and low-quality sequences were removed from the raw data to obtain clean RNASeq data. (https://cutadapt.readthedocs.io/en/stable/). The Q20 and Q30 were evaluated. Differentially expressed genes analysis was performed using HTSeq. The results of gene expression (FPKM) were

calculated as centered $\log_{10}$ FPKM and used to analyze transcript expression levels.

2.3 Differential expression gene analysis and enrichment analysis.

The differentially expressed genes (DEGs) between the three samples were identified using the DESeq2 package, and a *P*-value (padj) < 0.05 and $|\log_2$ (fold change)| > 1 were set as the cut-off for identifying DEGs. For each expression profile, GOseq software was used to perform gene ontology (GO) enrichment analyses (http://geneontology.org/). Furthermore, the GOseq R package was also used to perform enrichment on differentially expressed genes. The GO term with corrected P-values less than 0.05 is recommended. The statistical enrichment of differential expression genes was tested using KOBAS software in KEGG (Kyoto Encyclopedia of Genes and Genome) pathways.

3. Metabolite profiling analysis

3.1 Metabolite extraction

Metabolite profiling was performed using a non-targeted metabolome method by Personal Biotechnology Co. Ltd. (Shanghai, China). Metabolite profiling was performed using primary roots of rice seedlings treated with 0, 5.0, and 10.0 mg L^{-1} AgNPs@PEI for 3 days. Approximately 0.2 g (±1%) of the primary roots were rapidly frozen in liquid nitrogen for subsequent analysis. After the sample was gradually thawed at 4 °C, a precooled methanol/acetonitrile/water solution (2:2:2, v/v) was added and subjected to ultrasonication for 30 minutes at 4 °C, followed by incubation at -20 °C for 10 min. Then, the samples were centrifuged at 14,000g for 20 min at 4˚C and the filtered supernatant was dried under vacuum. The samples were redissolved with 100μL acetonitrile/water (1:1, v/v) and extracted by centrifugation before LC-MS/MS analysis.

3.2 LC-MS/MS analysis

Chromatographic separation was accomplished in UHPLC system (Agilent 1290 Infinity LC) equipped with a Waters ACQUITY UPLC BEH Amide column (130Å, 1.7 μm, 2.1 mm × 100 mm). Mass spectrometry was performed using AB Triple TOF 6600 mass spectrometers (AB SCIEX) after the sample was separated by UHPLC. Samples were inserted into quality control (QC) samples in queue mode to monitor and evaluate the stability of the system and the reliability of the experimental data. Each sample was operated in both positive and negative ion modes by the electrospray ionization (ESI) source parameters.

The analysis conditions were as follows: column temperature, 25˚C; injection volume, 2 μL; flow rate, 0.5 mL/min. The mobile phases were water (25 mM Ammonium acetate and 25 mM

ammonia) (phase A) and acetonitrile (phase B). The gradient program was as follow: 95% B, 0-0.5 min; B varies linearly from 95% to 65%, 0.5-7 min; B changes linearly from 65% to 40%, 7-8 min; B maintained at 40%, 8-9 min; B changes linearly from 40% to 95%, 9-9.1 min; B maintained at 95%, 9.1-12 min.

The MS conditions were as follows: Ion Source Gas1 (Gas1):60, Ion Source Gas2 (Gas2):60, Curtain gas(CUR):30, source temperature: 600℃, IonSapary Voltage Floating (ISVF)±5500 V(positive and negative mode); TOF MS scan m/z range: 60-1000 Da, product ion scan m/z range:25-1000 Da, TOF MS scan accumulation time 0.20 s/spectra, product ion scan accumulation time 0.05 s/spectra.

3.3 Analysis of metabolomics data

After normalized to total peak intensity, the peak area data for all metabolites were further analyzed by R package (ropls), where it was subjected to multivariate data analysis, including unsupervised principal component analysis (PCA), supervised partial least squares discriminant analysis (PLS-DA), The metabolites with PLS-DA VIP > 1 and $P < 0.05$ were selected as the differentially expressed metabolites (DEMs). The metabolites of functional primary root detected were annotated into the KEGG (https://www.kegg.jp/) Database to reveal the functions and interactions of metabolites.

4. Determination of MDA content of 3-day-old primary roots in rice

MDA concentration was determined as follows: 200 mg of 3-day-old primary roots from both the CK and AgNPs-treated groups with different concentrations were homogenized in an appropriate amount of 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4000 g for 15 min. Then, 2 ml of the supernatant was mixed with 2 ml of 0.67% (w/v) thiobarbituric acid (TBA), heated in a boiling water bath for 20 min, and immediately cooled. The absorbance of the solution was measured at 532 nm and 600 nm using a Shimadzu UV-29100 UV/Vis spectrophotometer (Tokyo, Japan). The MDA concentration was calculated using an extinction coefficient of 155 mM/cm. TCA and TBA were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China).

5. Determination of H_2O_2 content of 3-day-old primary roots in rice

The H_2O_2 content was determined according to the method of Jana and Choudhuri. Briefly, a 0.5 g sample of roots was homogenized with phosphate buffer (pH 6.5,100 mM) in an ice bath. The

homogenate was centrifuged at 6000 g for 20 min at 4°C. Subsequently, the supernatant was collected, and its volume was adjusted to 3 mL. Then, 1 mL of 0.1% titanium tetrachloride with 20% H_2SO_4 (v/v) was added to 3 mL of the solution, and the mixture was centrifuged at 6000 g for 15 min at 4 °C. The absorbance of the supernatant was measured at 410 nm.

Table S1

Table S1 Statistics of data by RNA-Seq

Sample	Raw Reads	Raw Bases	Q20 (%)	Q30 (%)	Clean Reads	Multiple Mapped	Exon (%)	Intergenic (%)
CK1-1	47849010	7177351500	97.84	94.36	42382936	2011251 (5.18%)	96.3	5.13
CK1-2	43966184	6594927600	97.93	94.6	37990138	1493603 (4.26%)	96.6	4.97
CK1-3	44480832	6672124800	97.85	94.4	39143490	1534023 (4.22%)	96.09	5.13
AgNPs1-1	41448390	6217258500	97.71	93.95	37259420	1527801 (4.40%)	96.77	5.35
AgNPs1-2	47042100	7056315000	97.66	93.85	43025230	1779816 (4.42%)	96.81	5.44
AgNPs1-3	48099822	7214973300	97.93	94.49	43215856	1775948 (4.37%)	97.2	5.26
AgNPs2-1	42394018	6359102700	97.82	94.21	38264842	1605713 (4.46%)	96.79	5.17
AgNPs2-2	42513302	6376995300	97.82	94.28	38307358	1605978 (4.46%)	96.97	5.08
AgNPs2-3	38989010	5848351500	97.95	94.62	34899706	1411924 (4.32%)	96.94	4.99

Table S2

Table S2 Composition of the Hoagland nutrient solution used for the primary root

No.	Nutrient element	Concentration (g L^{-1})
1	$Ca(NO_3)_2 \cdot 4H_2O$	94.5
2	KNO_3	60.7
3	$NH_4H_2PO_4$	11.5
4	$MgSO_4 \cdot 7H_2O$	49.3
5	$FeSO_4 \cdot 7H_2O$	5.57
6	HBO_3	2.86
7	$MnCl_2 \cdot 4H_2O$	1.81
8	$ZnSO_4 \cdot 7H_2O$	0.22
9	$CuSO_4 \cdot 5H_2O$	0.08
10	$(NH_4)_2MoO_4 \cdot 4H_2O$	0.02
11	Na·EDTA	7.45

Fig. S1

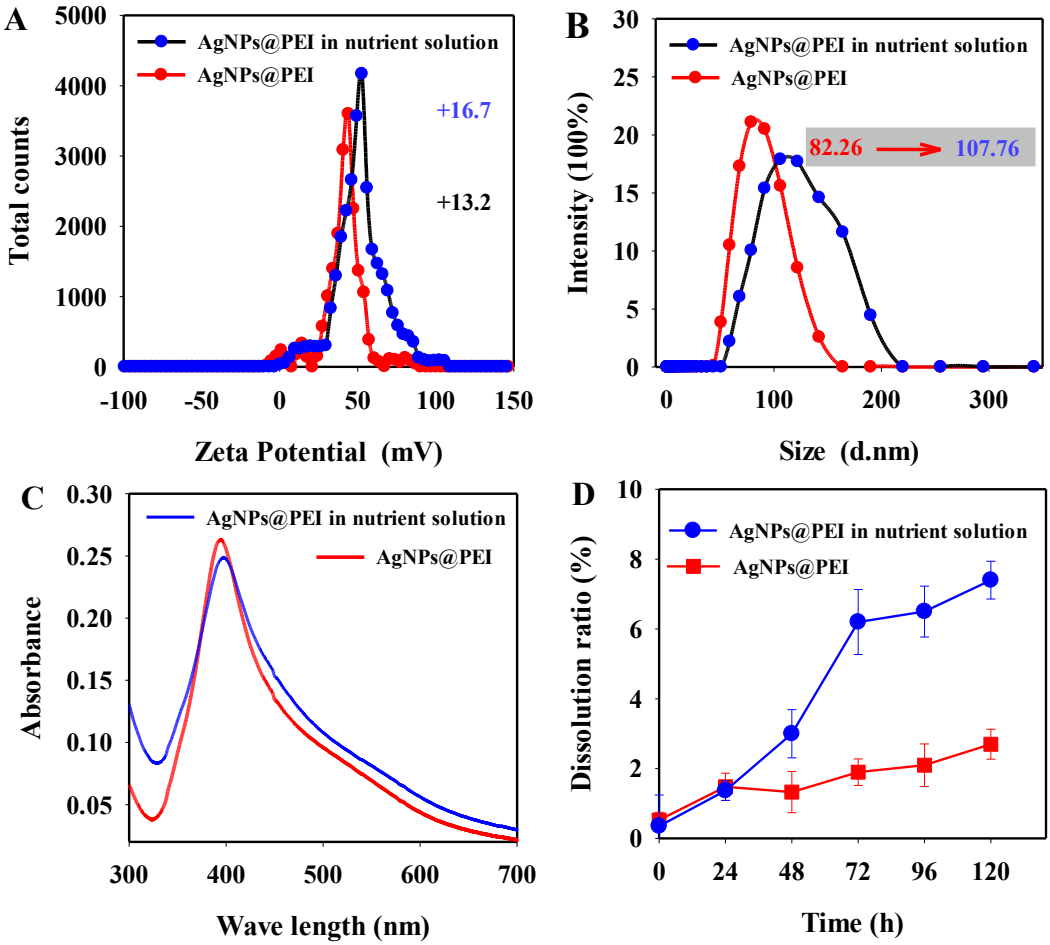

Fig. S1. Comparative analysis of AgNPs@PEI property changes in distilled water versus 20% nutrient solution over 3 days. (A) Zeta potential. (B) Hydrodynamic diameter. (C) UV-Vis absorption spectra. (D) Ag^{+} dissolution dynamics. All measurements, except Ag^{+} release tests performed at 10 mg L^{-1}, were conducted at an AgNPs concentration of 50 mg L^{-1}.

Fig. S2

Fig. S2 Changes of primary root length of rice under AgNPs@PEI exposure.

Fig. S3

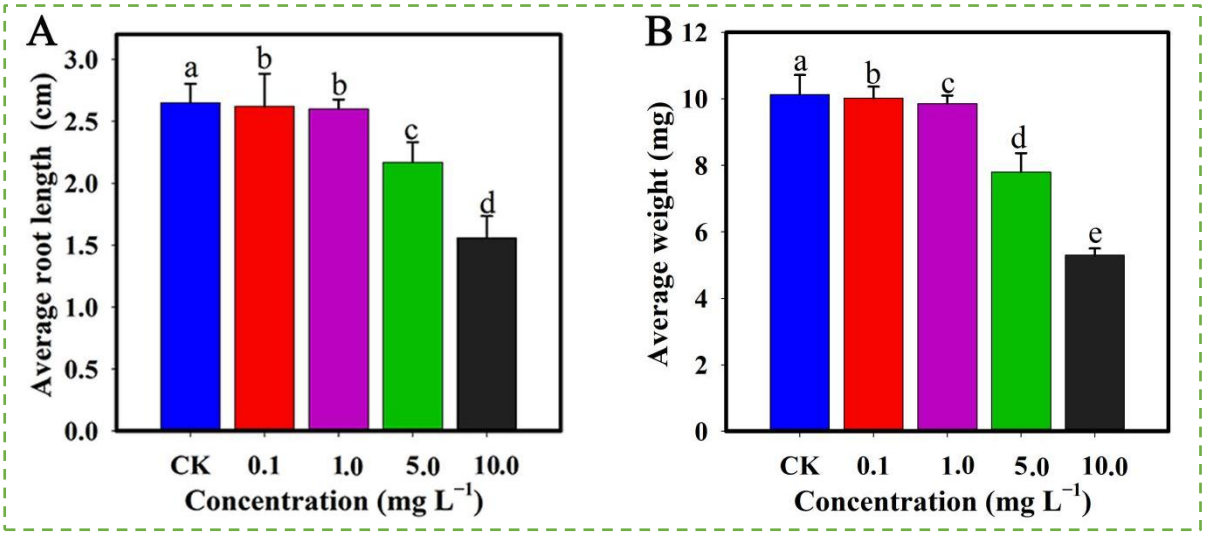

Fig. S3 (A)Changes of primary root length of rice under AgNPs@PEI exposure; (B) Changes of primary root dry weight of rice under AgNPs@PEI exposure.

Fig. S4

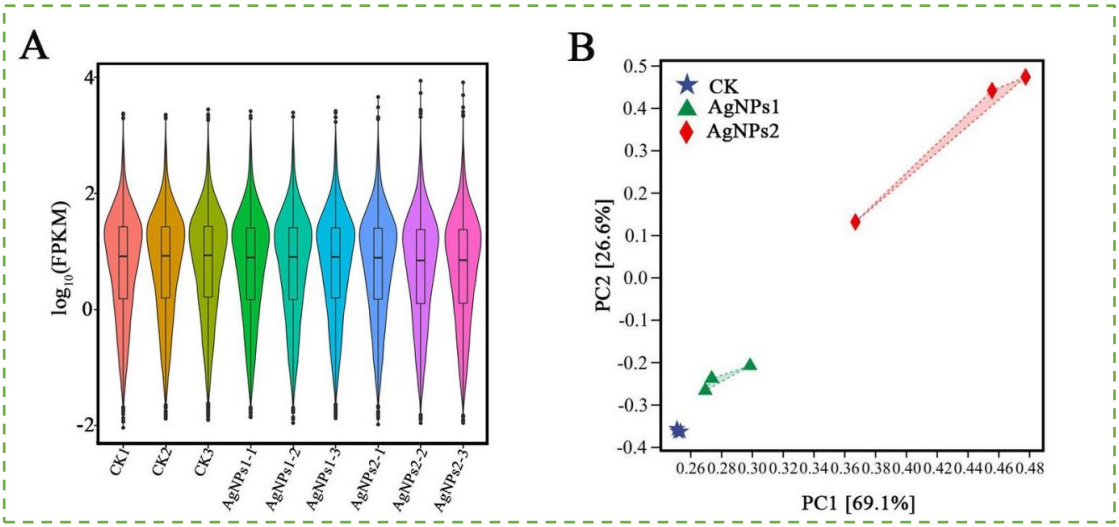

Fig. S4 (A) Violin plot graph; (B) PCA score plot graph (AgNPs1: 5.0 mg L^{-1}; AgNPs2: 10.0 mg L^{-1}).

Fig. S5

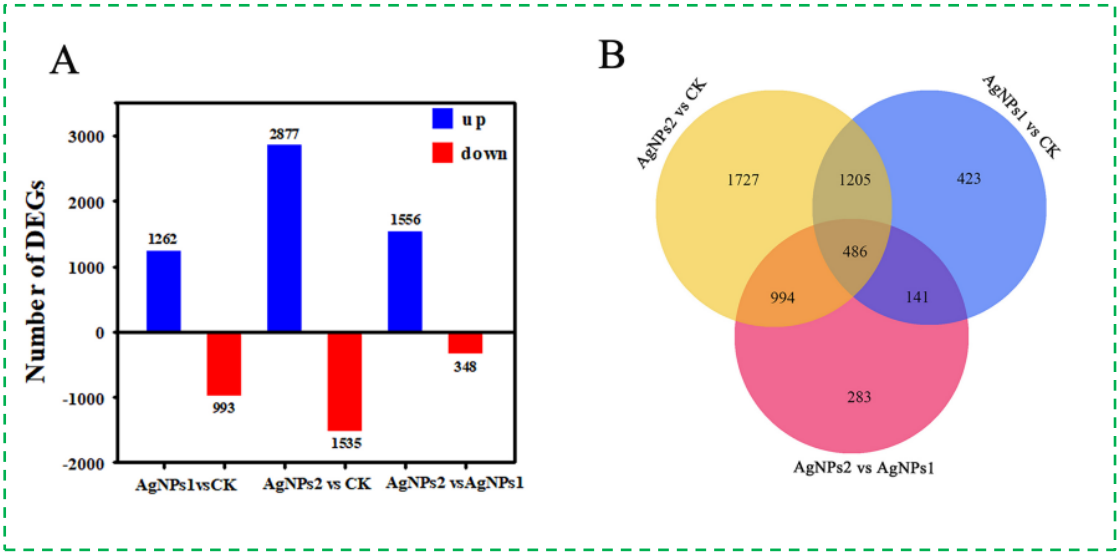

Fig. S5 (A) Numbers of differentially expressed genes derived among the three comparisons; (B) Venn diagram analysis.

Fig. S6

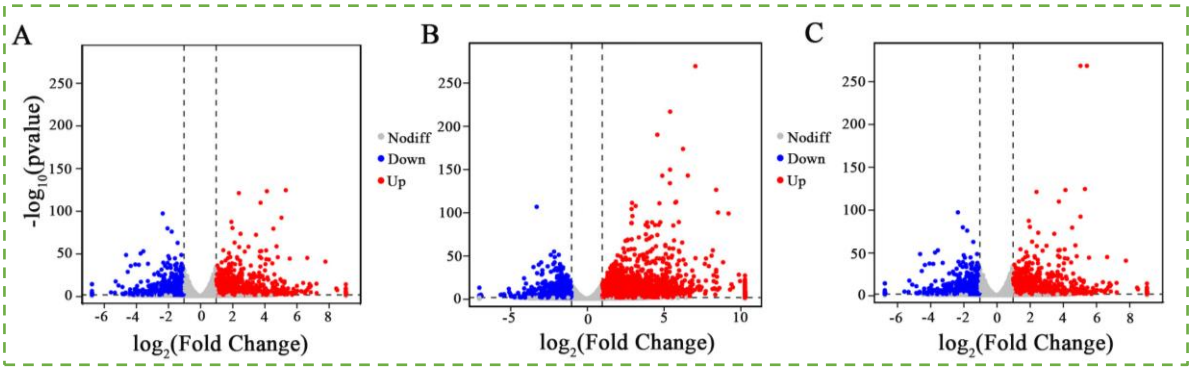

Fig. S6 The volcano plot graph of (A)AgNPs1 vs CK; (B) AgNPs2 vs CK; (C) AgNPs2 vs AgNPs1(AgNPs1: 5.0 mg L^{-1}; AgNPs2: 10.0 mg L^{-1}). red represents significantly upregulated genes, blue represents significantly downregulated genes, and gray represents not significantly expressed genes.

Fig. S7

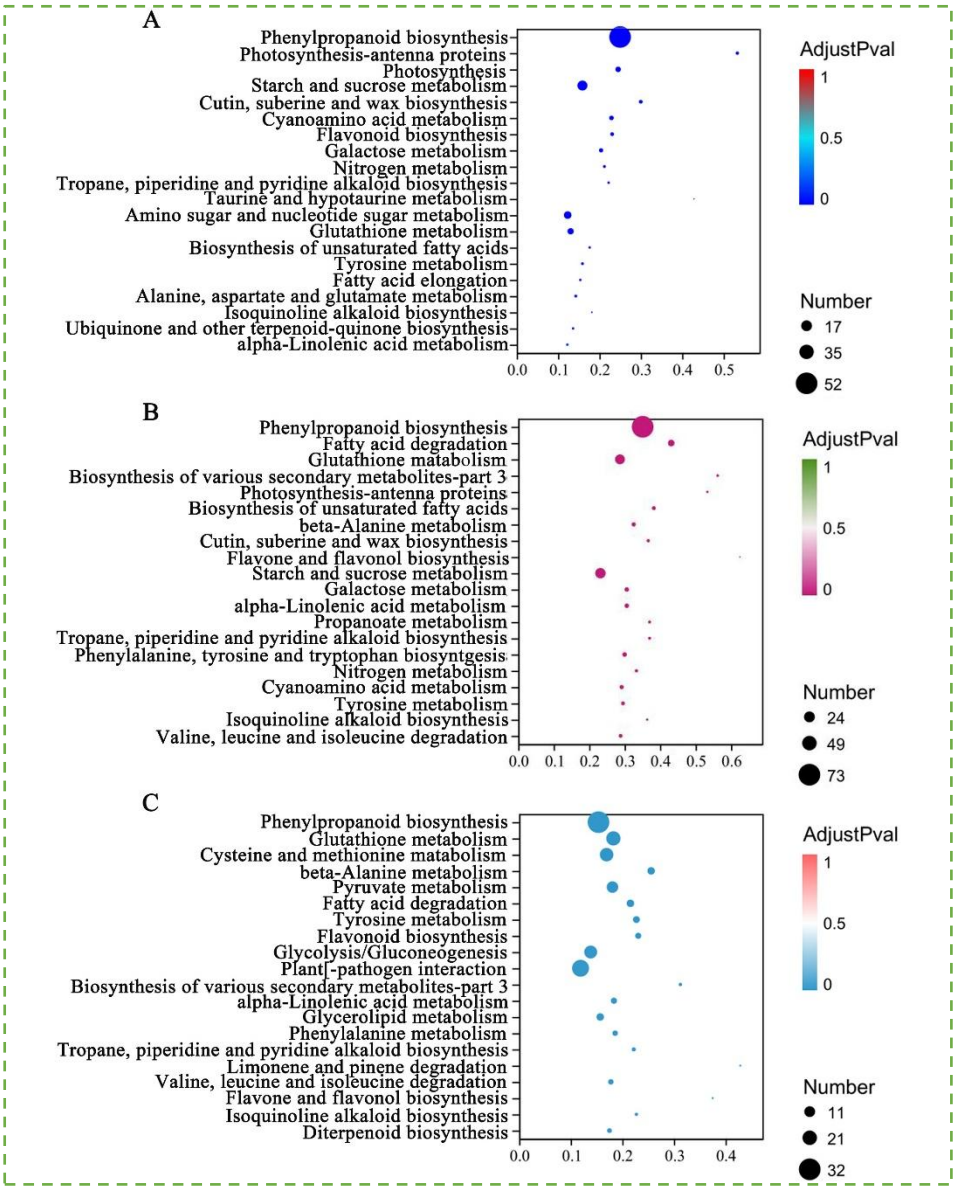

Fig. S7 KEGG pathway analysis of DEGs specifically regulated under AgNPs exposure in primary root for 3 days, (A): AgNPs2 vs CK, (B): AgNPs1 vs CK, (C): AgNPs2 vs AgNPs1 (CK:control; AgNPs1: 5 mg L^{-1}; AgNPs 2: 10 mg L^{-1}).

Fig. S8

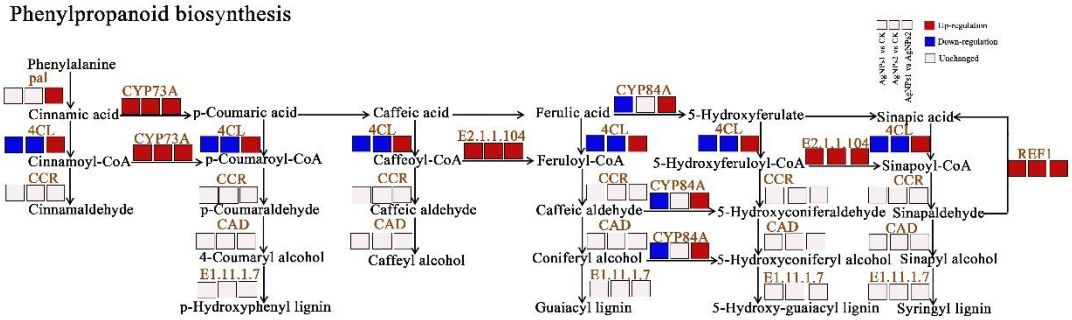

Fig. S8 Schematic diagram of phenylpropane biosynthesis

Fig. S9

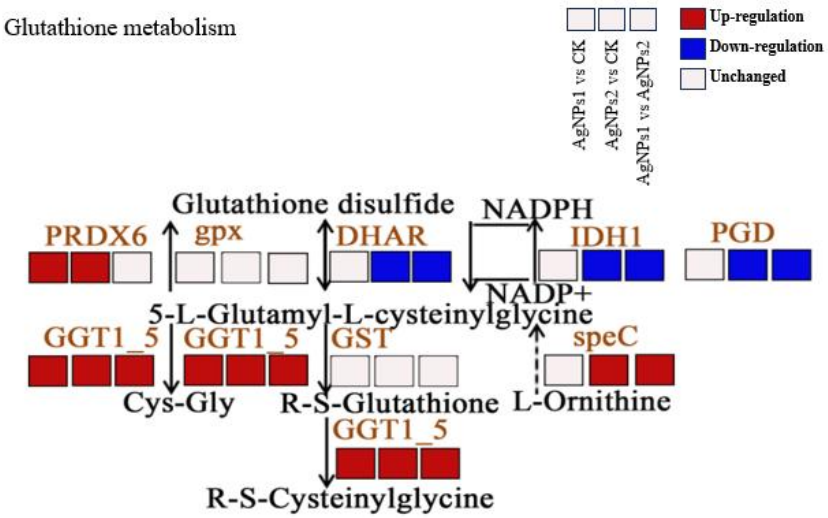

Fig. S9 Schematic diagram of glutathione metabolism

Fig. S10

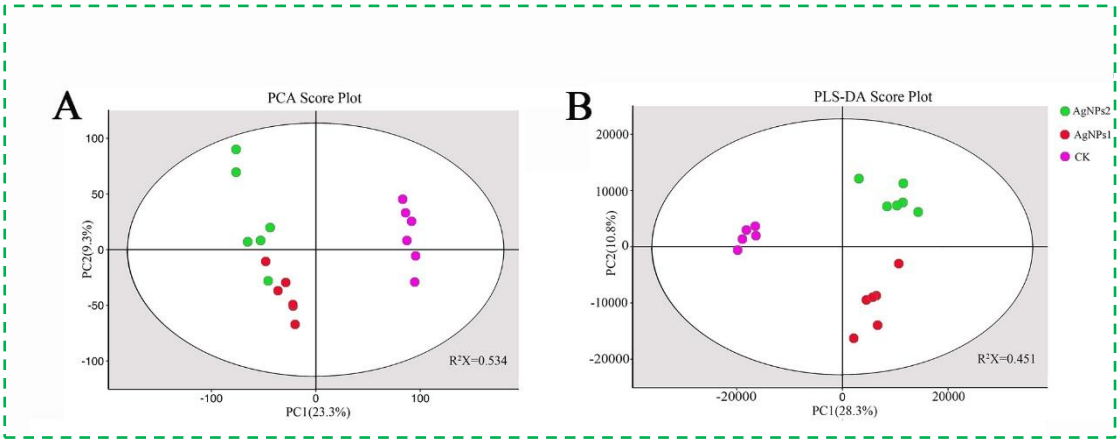

Fig. S10 (A) PCA and (B) PLS-DA score plots of all detected metabolites under AgNPs exposure in primary root for 3 days.

Fig. S11

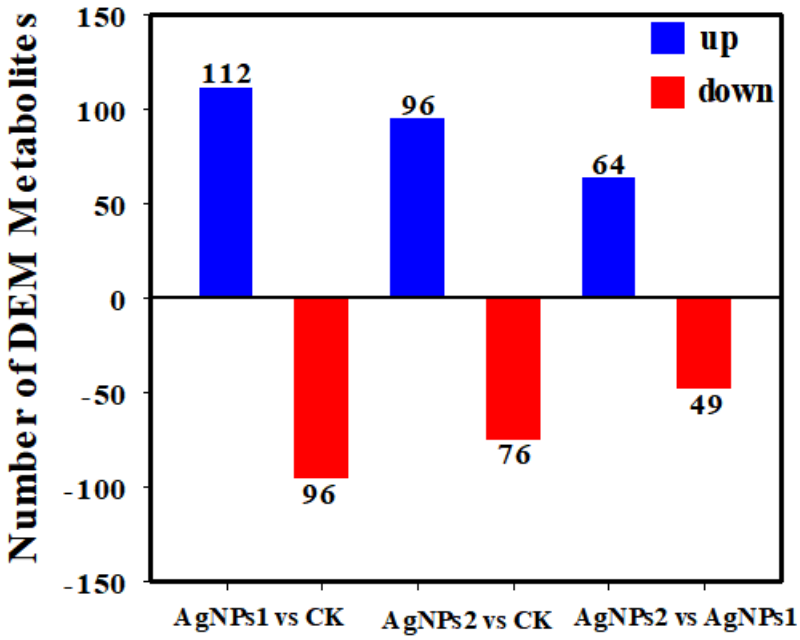

Fig. S11 Numbers of differentially expressed metabolites (DEM) derived among the three comparisons (AgNPs1: 5.0 mg L^{-1}; AgNPs2: 10.0 mg L^{-1}).

Fig. S12

Fig. S12 Heatmap analysis of DEMs. Note: The color scale indicated the relative levels of metabolites in roots, red indicates upregulated metabolites, while blue indicates downregulated metabolites (AgNPs1: 5.0 mg L^{-1}; AgNPs2: 10.0 mg L^{-1}).

Fig. S13

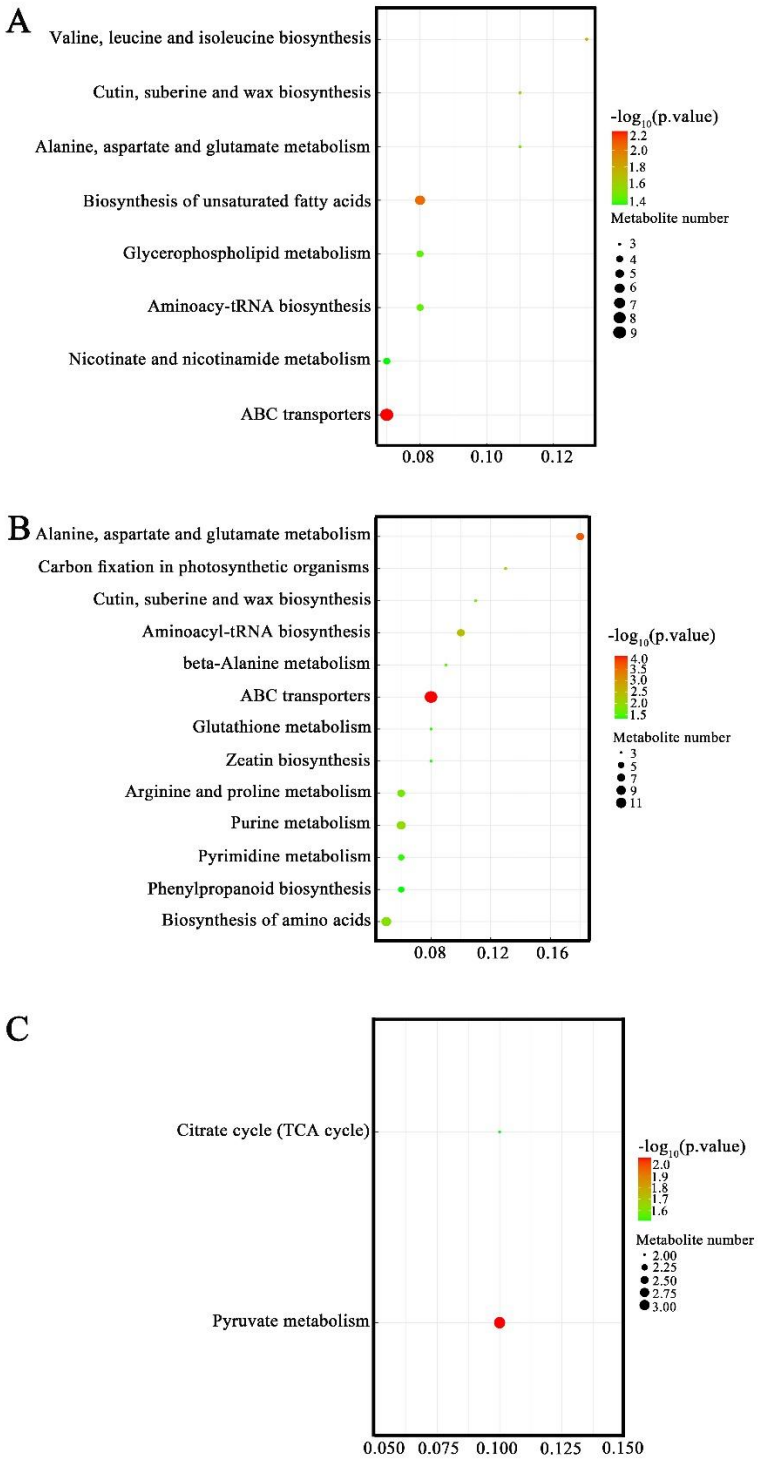

Fig. S13 The bubble plot of significant metabolic pathways generated from LC-MS/MS metabolomics.