# Transcriptome analysis reveals that multiple metabolic pathways operate in *Zea mays* roots subjected to graphene

**Zhiwen Chen** ( b1301031@cau.edu.cn )
Institute of Plant Physiology and Ecology Shanghai Institutes for Biological Sciences
https://orcid.org/0000-0002-0268-5942

**Jianguo Zhao**
Shanxi Datong University

**Jie Song**
Shanxi Datong University

**Shenghua Han**
Shanxi Datong University

**Yaqin Du**
Shanxi Datong University

**Yuying Qiao**
Shanxi Datong University

**Zehui Liu**
Shanxi Datong University

**Jun Qiao**
Shanxi Datong University

**Weijia Li**
Shanxi Datong University

**Jingwei Li**
Shanxi Datong University

**Haiyan Wang**
Shanxi Datong University

**Baoyan Xing**
Shanxi Datong University

**Qiliang Pan**
Shanxi Datong University

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# Abstract

**Background:** To explore the effects and molecular mechanism of graphene on the growth and development of *Zea mays* L., the seeds were randomly divided into the control and experimental groups in this study, the roots of *Zea mays* L. seedlings were watered by different concentrations (0~100 mg/L) graphene.

**Results:** By evaluating the root growth indices of maize, 50 mg/L graphene increased significantly the total root length, root volume, the number of root tips and root forks of maize seedlings compared with the control group. The contents of nitrogen and potassium in the soil around the roots were elevated after the treatment of 50 mg/L graphene. Then, we compared the transcriptome changes of *Zea mays* roots in response to 50 mg/L graphene treatment. Transcriptional factor regulation, plant hormone signal transduction, nitrogen and potassium metabolism as well as secondary metabolism in maize roots subjected to graphene showed significant up-regulated expressions, all of which might be related to mechanisms underlying graphene response. Based on qPCR validations, we proposed several candidate genes that might be responded to the graphene treatment in maize roots.

**Conclusion:** The transcriptional profiles presented here provide a foundation for deciphering the mechanism between the graphene and maize roots interaction.


# Background

Nanomaterials have been widely used in the fields of electronics, machinery, energy and biomedicine [1–4]. Researchers showed that nanomaterials could improve seed germination rate and promote plant growth [5, 6]. Nanomaterials mainly include metals, metal oxides, polymers and carbon nanoparticles, among which carbon nanomaterials have attracted extensive attention due to their unique chemical properties as well as low toxicity [6]. Graphene is a member of the carbon nanomaterials family, with the most promising engineered nanomaterials for its huge surface area, unparalleled mechanical property, electrical and thermal conductivity [7]. Graphene contains a large surface area, which carrying a variety of functional groups of oxygen-containing, including carboxyl, hydroxyl, and carbonyl groups to endow graphene higher water dispersity [8]. A myriad of studies proved that graphene both had positive and adverse effects on the growth and development processes in plants.

As to the positive effects of graphene on promoting the growth and development processes in plants, studies were mainly focused on the following aspects: firstly, graphene can promote the germination of plant seeds. Zhang et al. found that graphene-treated tomato seeds germinated much faster than control seeds, because of graphene penetrating seed husks to facilitate water uptake [9]. He et al. found that graphene could act as a water transporter in promoting germination of spinach seeds in soil [10]. In addition, hydrated graphene ribbon could promote aged seed germination of wheat [11]. Secondly, graphene could promote plant growth and development. Liu et al. found that graphene significantly increased the number of lateral roots and fresh weight of rice seedlings [12]. Zhang et al. found that

graphene could regulate the synthesis of gibberellic acid in tomatoes and promote the roots growth of tomato seedlings [9]. Chakravarty et al. found that graphene significantly promoted the growth of roots, stems, leaves, flowers and fruits of cilantro and garlic, leading to elevated yield [13]. Anjum et al. found that graphene could improve the growth status of *Vicia faba* and promote the roots growth of *Vicia faba* [14]. Low concentration of sulfonated graphene could scavenge ROS in roots, altered root morphology and improve health state of maize seedlings [15]. Graphene activated the reproductive system of *Gossypium hirsutum* and *Catharanthus roseus* as well as reduced the toxic effects caused by drought and salt stresses [16]. Thirdly, recent researches indicated that graphene has the potential to act as a carrier to slowly release the fertilizer's effect and improve the utilization efficiency of nutrients [17–19]. Finally, graphene could inhibit the growth of pathogen of plants. For instance, He et al. found that graphene as an antibacterial agent could extend the vase life of cut flowers [20]. Wang et al. found that graphene presented antifungal effect for controlling *Fusarium graminearum* of wheat [21].

Apart from the positive effects, adverse effects on plants were also reported for concerning about the potential risk of graphene [22]. Current study reported that graphene has toxic effects on *E. coli* [23] and plants, such as inhibiting seedling growth in cabbage and tomatoes, inducing reactive oxygen species (ROS) production and cell death, and altering plant morphology [24]. High concentrations of graphene also inhibit the growth of rice, ultimately reducing its biomass [12]. Reduced graphene induced cytotoxicity and inhibits photosynthetic performance of the green alga [25]. Graphene also induced cell death in Arabidopsis thaliana [26, 27], cadmium uptake, growth inhibition photosynthesis inhibition, and nutritional disorder in wheat seedlings [28, 29], cadmium uptake, pH alteration, iron overload and oxidative damage in rice [30, 31], roots growth inhibition and oxidative stress in apple [32].

In general, it seems that positive effects of graphene on plants were at low concentrations while most studies of adverse effects were conducted at high concentrations graphene to study the acute toxicity in plant [15, 23, 32]. In rice plants, graphene absorbed into leaves could be converted into $CO_2$ and released through mineralization [33], indication of the environmental fate of graphene in soil-plant system. The current progresses of researches were almost focused on the physiological and phenotypic data of plants, and has not been dissected from the level of molecular or gene evidence. This study explored the effects and molecular mechanism of graphene on the root growth and development of *Zea mays* L., we performed transcriptome expression profiles to clarify the effects of graphene on the roots of *Zea mays* seedling.

# Results

# Characterization of graphene

The ultraviolet-visible absorption spectrogram (Fig. 1A) shows a strong absorption peak at 270 nm, which is a typical peak of graphene. Raman spectroscopy is a nondestructive and sensitive method of analysis of graphene and grapheme derivatives [34, 35]. Raman spectroscopy (Fig. 1B) shows that G peak appears near 1576 $cm^{-1}$, which is generated by the stretching and far-moving of sp2 hybridized atoms in

carbon rings or long chains, representing the ordered sp2 bond structure. Peak D appeared near 1348 $cm^{-1}$, which was related to sp3 hybrid structure, representing defects and amorphous structure at the edge of graphene. A wide 2D peak appeared near 2707 $cm^{-1}$, indicating that the number of graphene layers prepared was within 10 layers. High-resolution scanning electron microscopy (Fig. 1C) and transmission electron microscopy (Fig. 1D) showed that the graphene presented a transparent sheet structure with slightly wrinkled and undulate surface. The number of graphene layers prepared was 3–5 layers as observed under transmission electron microscopy.

# Exogenous Graphene Promoted The Growth And Development Of Maize

Six concentration gradients (0, 20, 25, 33, 50 and 100 mg/L) of graphene were used to treat maize seedling roots and irrigated through the roots. At the seedling stage, the phenotypes of maize plants were observed (Fig. 2). As shown in the Fig. 2, the graphene concentration of 50.00 mg/L could promote the growth of maize plants. Subsequently, we measured multiple physiological indexes to analyze the effect of graphene on the growth or development of maize plants.

# Exogenous graphene enhanced *Zea mays* root development

Root architecture of maize seedlings under six concentration gradients of graphene (0, 20, 25, 33, 50 and 100 mg/L) was investigated. The root growth and development of maize seedlings under graphene treatments were promoted, especially for the 50 mg/L graphene concentration (Fig. 3A). Root architecture, including total root length, total project area, total surface area, root volume, the number of root tips and root forks were measured (Fig. 3B-G). Compared to the control group, 25, 50 and 100 mg/L graphene increased the total root length (Fig. 3B), root volume (Fig. 3E), the number of root tips (Fig. 3F) and root forks (Fig. 3G) of maize seedlings. Total project area (Fig. 3C) and total surface area (Fig. 3D) were not affected by any of graphene treatment. 33 mg/L graphene promoted the number of root tips (Fig. 3F), but inhibited the development of total length (Fig. 3B). Among them, The maize seedlings treated with 50 mg/L graphene displayed significant higher values than CK (Fig. 3). Based on the above assay results, the 50 mg/L graphene concentration was used for the subsequent experiments and analyses.

# Transcriptome Sequencing

To gain insights into how graphene might induce a promoting effect leading to enhanced root development, a comparative transcriptome analysis was performed. We collected root tissue samples from maize seedlings treated with 50 mg/L graphene (X100) and used untreated seedlings as the corresponding controls (CK). All samples were used for transcriptome sequencing with three biological replicates (Additional file 1: Table S1). In total, 170.02 million raw reads were obtained for the CK libraries

(CK-1, CK-2 and CK-3), and 166.69 million raw reads were obtained for the X100 libraries (X100-1, X100-2 and X100-3). After removing adapter and low-quality sequences along with contaminated reads, 24.15 Gb and 23.92 Gb high-quality clean bases remained from the CK and X100 libraries, respectively (Table S1). Using the *Z. mays* genome of B73 [36], the number of total clean reads was 43.67–60.13 million for the CK libraries (79.65–87.30% mapped rate, and 77.66–85.09% unique mapped rate) and 49.56–59.40 million for the X100 libraries (77.83–84.46% mapped rate, and 75.66–82.18% unique mapped rate) (Additional file 2: Table S2).

# Genes that are differentially expressed in the root samples of *Zea mays* in response to graphene treatment

We firstly calculated the Pearson correlation coefficient (PCC) of all the genes, and generated a heatmap plot showing changes in gene expression (as shown in Fig. 4A). The correlation coefficients of the three biological replicates were greater than 0.90, indicating that the RNA-seq data were reliable for further analysis. Based on principal component analysis of six samples, the transcriptional response observed in *Zea mays* roots exposed to 50.00 mg/L graphene was due to the graphene treatment, and varieties with graphene treatment exhibited two levels of gene expression (as shown in Fig. 4B).

We were particularly interested in identifying transcripts that were differentially expressed in the root sample in response to graphene treatment, as such transcripts may represent genes related to root development under graphene treatment. The expression value of each gene was calculated using FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) algorithm. A two-fold change and a *p*-value of less than 0.05 were set as the cutoffs to define genes with significant differential expression (Fig. 4C). We identified 962 differentially expressed genes, among which 792 were graphene-induced and 170 were graphene-repressed (Fig. 4D).

# Gene Enrichment Analysis For Differentially Expressed Genes

To investigate possible biological functions that determine the different responses of the maize plants to 50.00 mg/L graphene treatment, we used GOseq [37] to perform GO category enrichment analysis for differentially expressed genes. Figure 5 lists the results of the gene ontology (GO) analysis for differentially expressed genes after graphene treatment. GO terms associated with important biological processes, such as the cellular, metabolic, developmental, and immune system processes, biological regulation, response to stimulus and detoxification were enriched in maize exposed to graphene treatment. GO terms associated with important cellular component, such as cell, membrane and organelle parts were enriched. GO terms associated with important molecular function, such as catalytic activity, transporter activity, nucleic acid binding transcription factor activity, antioxidant activity and transcription factor activity were enriched.

DEGs were subjected to COG database [38] to classify the gene function and homology. Additional file 3: Figure S1 lists the results of the COG analysis for differentially expressed genes. Most DEGs were distributed on the orthologous groups of 1) secondary metabolites biosynthesis, transport and catabolism, 2) carbohydrate transport and metabolism, 3) amino acid transport and metabolism, 4) lipid transport and metabolism and 5) defense mechanisms.

DEGs were subjected to KEGG pathway analysis to identify the functional categorization. Additional file 4: Figure S2 lists the results of the KEGG analysis for differentially expressed genes. Most DEGs were categorized on the functional pathways of 1) metabolisms, including phenylpropanoid biosynthesis, glutathione metabolism, flavonoid biosynthesis, carbon metabolism, amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, terpenoid backbone biosynthesis, biosynthesis of amino acids, as well as starch and sucrose metabolism etc; 2) cellular process of peroxisome; 3) environmental information processing, including plant hormone signal transduction, ABC transporters, phosphatidylinositol signaling system, circadian rhythm in plant and plant-pathogen interaction. DEGs with up-regulation were assigned to 73 KEGG pathways, including phenylpropanoid biosynthesis, glutathione metabolism, flavonoid biosynthesis, and nitrogen metabolism (Fig. 6A). DEGs with down-regulation were significantly enriched in 14 KEGG pathways, including amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism pathways as well as plant hormone signal transduction (Fig. 6B) etc,. The results revealed that graphene could affect the expression of maize root genes, showing majority of up-regulation genes. The enrichment analysis illustrated that graphene treatment had extensive and distinct effects on the life processes in maize.

# Transcription Factors Enriched In Maize Plants Exposed To Graphene

We found that the GO term “transcription factor activity, protein binding” was significantly enriched in maize roots subjected to graphene treatment (Fig. 5). Transcription factors are DNA-binding proteins that play a key role in gene transcription and expression that mediated many processes. Many transcription factors in the roots of maize responded to the graphene treatment, and the responses differed by up or down regulated expressions (Table 1). 44 maize transcription factor genes classified into 7 different families according to PlantTFDB [39], were differentially expressed under the graphene treatment, including ERF, WRKY, bHLH, MYB and MYB-like, NAC, AP2 as well as MADS-box. Among them, 32 transcription factor genes were up-regulated and 12 were down-regulated. The transcription factor genes activated in *Z. mays* roots in response to the graphene treatment mostly belonged to the MYB and MYB-like, WRKY, NAC and bHLH families, suggesting that these transcription factor genes might response to the graphene specifically in *Z. mays* root.

Many studies have proved that plant root development could be regulated by ERF [40], WRKY [41, 42], bHLH [43–45], MYB and MYB-like [46–49], NAC [50, 51], AP2 [52, 53] as well as MADS-box [54, 55] TF genes. After exposure to graphene treatment of *Z. mays* roots, there were three up and one down-

regulated ethylene-responsive (ERF) transcription factor genes (Fig. 7A), eight up and one down-regulated WRKY TF genes (Fig. 7B), five up and one down-regulated bHLH TF genes (Fig. 7C), ten up and two down-regulated MYB and MYB-like TF genes (Fig. 7D), three up and four down-regulated NAC TF genes (Fig. 7E), three up and one down-regulated AP2 TF genes (Fig. 7F) as well as two down-regulated MADS-box TF genes (Fig. 7G). As proved above, the total root length, root volume, the number of root tips and root forks (Fig. 3) of maize seedlings were increased after 50 mg/L graphene treatment. These improved root phenotypes might be affected by these differentially expressed transcription factor genes. Therefore, these TFs were considered the candidate graphene-responsive genes, and might be the internal causes in promoting the development of roots in *Z. mays*.

# Plant Hormone Signaling Pathways

Plant hormones, including auxin, cytokinin (CK), gibberellin (GA), abscisic acid (ABA), ethylene, brassinosteroid (BR), jasmonate (JA), salicylic acid (SA), and strigolactone (SL) play critical roles in the plant's processes of growth, development and adapting to the external changing environments [56–59]. We identified differentially expressed genes related to nine hormone signal transduction pathways. An overview of gene expression patterns under graphene treatment in maize roots is shown in Fig. 8. Four auxin-responsive genes, such as gene-LOC100281448 (IAA9), gene-LOC103642166 (auxin response factor 11) and gene-PIN5c (auxin efflux carrier PIN5c) were differentially expressed in maize under graphene conditions, indicating the existence of crosstalk between graphene and auxin signaling. Therefore, auxin modulates the plant's response to graphene by altering the expression of genes involved in root growth regulation.

The cytokinin (CK) signaling pathway plays an important role in the plant's growth regulation. Four genes associated with the cytokinin signaling pathway showed significantly differentially expression under graphene treatment (Fig. 8). The expression of gene-cko1 (cytokinin oxidase1), gene-LOC100280143 (cytokinin-N-glucosyltransferase 1) and gene-LOC100282611 (cytokinin-O-glucosyltransferase 2) was increased. We found that five genes associated with the GA pathway were also up-regulated expressed, including gene-LOC100283080 (Gibberellin 20 oxidase 2), gene-LOC100283652 (gibberellin receptor GID1L2) and gene-gar1 (gibberellin responsive 1). JA and SA play important roles in plant defense response. The expression of six JA-related genes, such as gene-LOC100283794 (Jasmonate-induced protein), gene-LOC103629478 (jasmonate O-methyltransferase) and gene-LOC100273620 (Jasmonate-regulated gene 21), and one SA-related gene (salicylic acid-binding protein 2) were up-regulated in the roots of maize subjected to graphene. Two gene involved in brassinosteroid (BR) and strigolactone (SL) signal transduction respectively were induced in response to graphene, suggesting the roles of graphene-responsive hormones are emerging to function. Together, these results demonstrated that hormones might form a complex regulatory network related to the graphene response in roots.

# Nitrogen And Potassium Metabolism

We also identified eight nitrogen and potassium metabolism genes that were differentially expressed (Fig. 9). All the five nitrogen metabolism genes were up-regulated (Fig. 9A) and three of them (gene-GLN6, gene-nrt2 and gene-nrt2.2) were validated by qRT-PCR analysis (Fig. 9B). These genes were annotated into the glutamine synthetase root isozyme 1 and nitrate transporter, which were involved in the nitrogen transmembrane transport and root development. In view of this, we measured the N content in the soil around the maize seedling roots, and the N content in 50.00 mg/L graphene was significantly increased, up to 1.64 times (Fig. 9C).

In addition, the expressions of three potassium metabolism genes (gene-HAK20, gene-HAK21 and gene-kup1) were up-regulated by RNA-seq (Fig. 9D) and qRT-PCR data (Fig. 9E). These genes were involved in the potassium ion transmembrane transport and uptake. We also measured the K content in the soil around the maize seedling roots, and the K content in 50.00 mg/L graphene was increased by 1.33 times (Fig. 9F). The results indicated that the soil fertility, such as the content of N and K, could be elevated after irrigating the graphene, which further promote the growth and development of maize seedling roots.

# Qrt-pcr Identification Of Degs In Response To Graphene

Furthermore, to validate the RNA-seq results, 20 DEGs were screened for qRT-PCR validation, including 14 transcription factor genes, three nitrogen metabolism and three potassium metabolism genes. We analyzed the expression of these genes using quantitative real-time PCR (qRT-PCR) analysis and compared the results with the RNA-seq data (Table 2). These transcripts had similar expression patterns in the qRT-PCR and RNA-seq experiments and the correlation coefficient between the two sets of data was 0.7783 (Table 2).

## Discussion

Recently, the carbon nanomaterials researches had been focused on the applications in agriculture and forestry [6, 12, 60–62]. Studies have shown that graphene carbon nanotubes could affect the growth of maize root, promote the growth of seminal root, and had no effect on the growth of primary root, but will restrain the growth of root hair [60]. Liu et al. [12] proved that graphene could promote rice seed germination, affect root development and other physiological indicators. Graphene could interact with plants through root irrigation or leaf spraying. Graphene-induced of plant growth occurs in low concentrations but showed inhibition effects on plant growth in high concentrations. However, the mechanism of graphene's interaction with plants has not been thoroughly elucidated. In this study, we studied the transcriptomic responses of maize roots after the exposure to the 50 mg/L low concentration graphene solution.

# Identification of candidate graphene-related genes in*Z. mays*

Using the RNA-seq technology, we successfully identified 962 differentially expressed genes in the roots of *Zea mays* subjected to graphene treatment. After exposure to graphene, the number of up-regulated DEGs was more than the down-regulated. Using GO and KEGG pathway analysis, we designed a transcriptome map of the maize seedling roots exposed to graphene. GO analysis showed that the GO terms “transporter activity”, “transcription factor activity” as well as “response to stimulus and detoxification” were significantly enriched. KEGG pathway analysis indicated that DEGs of graphene treatment were involved in metabolisms and plant hormone signal transduction. Therefore, the maize root may has specific mechanism to respond graphene effect, such as the transcription factors, detoxification, metabolism, and hormone signals.

# Graphene-related transcription factor genes in*Z. mays*

Plant responses to graphene may be related to the transcription factor genes. For example, several WRKY genes were significantly induced by graphene in the roots of maize. WRKY transcription factors are one of the largest families of transcriptional regulators in plants and WRKY proteins often act as activators or repressors to modulate important plant processes [63]. Li et al., [41] showed that WRKY genes were involved in the root elongation in *Arabidopsis Hypoxia*. Wang et al., [42] reported a WRKY transcription factor gene that could affect adventitious root formation in *Catalpa Scop*.

MYB and MYB-like TF families are involved in controlling trichomes development and root hair formation [46–49]. In addition, the R2R3 MYBs, the basic helix-loop-helix (bHLH) factors, and the WD40 repeat (WDR) protein, plays a crucial role in trichome development. These three groups of TFs form a trimeric activator complex, MYB-bHLH-WDR (MBW) that positively regulates the expression of downstream targets, which, in turn, induces trichome formation [64].

Studies also showed that bHLH transcription factor genes were involved in the root hair and meristem development in plants [43–45], and NAC transcription factor genes regulated lateral root development in potato [50] and enhances root length in wheat [51]. In our study, we identified some differentially expressed WRKY, MYB and MYB-like, bHLH as well as NAC transcription factor genes in response to the graphene treatment. It is possible to speculate that graphene could stimulate maize root growth by up-regulating these transcription factor gene expressions.

# A potential graphene response regulatory network in*Z. mays*

Based on our analysis, we propose a model for the mechanism underlying graphene effect on the root growth of maize (Fig. 10). When the roots of maize seedlings grown in soil are exposed to gaphene, the N and K contents of soil would be elevated because of the cation-π interactions in graphene, which lead to the accumulation of cations on the graphene surface [65]. Thus, the transporters in the root (such as Nitrate transporter, nrt2.2; potassium transporter 5, HAK20) may recognize the corresponding signals and trigger the expression increase of transporter genes, and the change of phytohormones, including auxin,

cytokinin and gibberellin, and so on. These hormones might affect the downstream transcription factors, such as WRKY, MYB, bHLH and NAC, which regulate the expression of target genes at the transcriptional level to promote the root elongation or hair growth. The graphene responses also involve detoxification and carbohydrate metabolism.

## Conclusions

In this study, six low concentrations graphene were used to irrigate maize seedling roots to identify the optimal working solution in promoting the growth and development of *Zea mays*. Then, we used RNA-seq analysis to generate a transcription map of genes expressed in the roots of *Zea mays* in response to graphene treatment. Our results presented important insights into the molecular mechanisms that govern the response of *Zea mays* to graphene. The genes that were differentially expressed in maize root after graphene treatment are potential candidates for better utilize the graphene from the perspective of molecular biology. These results laid a theoretical foundation for the subsequent research on the molecular mechanism of the interaction between graphene and maize roots.

## Methods

# Graphene characterization

Graphene was obtained and generated from our own lab. The characteristics of graphene were analyzed by ultraviolet-visible absorption spectrogram and Raman spectroscopy (HORIBA, LabRAM HR Evolution). Raman spectra were obtained using Renishaw inVia™ Qontor with a 532 nm excitation laser. The morphology of graphene was examined using scanning electron microscopy (SEM, TESCAN MAIA 3 LMH) and transmission electron microscopy (TEM, TecnaiG2F20 S-TWIN TMP).

# Maize plants cultivation and graphene exposure treatment

Maize seeds with the identical size divided into six groups (30 seeds in each group) were germinated in potting soil in a growth chamber, and the resulting seedlings were maintained in a controlled environment at 28 °C day/20 °C night, with a 16-h light/8-h dark photoperiod. Graphene working solutions (0, 20, 25, 33, 50 and 100 mg/L) was diluted to terminal concentrations with deionized water. An aqueous solution of sodium hydroxide (0.1 M) was used to neutralize the working solutions to pH 6.3–6.5. Maize seeds and seedlings were irrigated with six graphene solution concentrations, respectively. The graphene working solutions were irrigated once a week from the beginning of sowing job. After germination, the maize seedlings cultivated in soil pots were watered with 1 L working solution with different concentrations of graphene per week. For the blank group, the irrigation solution was the same amount of distilled water, and the other treatments were consistent with the experimental group. After 30 days of exposure to graphene, the maize roots were thoroughly washed with deionized water, dried with

absorbent paper to remove the surface water, following promptly frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

# Root Architecture Analysis Of Maize Seedlings

The maize seedlings treated with six graphene concentrations were used for root architecture analysis. After 30 days of exposure to graphene, the maize roots were thoroughly washed with deionized water, then the roots were scanned using Epson Perfection V850 Pro (Seiko Epson Corp., Tokyo, Japan) at 600 dpi. Then scanned images were quantized by WinRHIZO (Version 4.0b, Regent Instruments Inc., Quebec, Canada) [66]. Root architecture, including total root length, total project area, total surface area, root volume, the number of root tips and root forks were measured.

# Rna Extraction, Libraries Construction And Sequencing

Total RNA of maize roots from the control and 50 mg/L graphene treatment groups were isolated. A total of 1 μg purified mRNA was selected for cDNA library construction using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer protocol. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for the hybridization. In order to select cDNA fragments of preferentially 300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina novaseq platform and 150 bp paired-end reads were generated. Three biological replicates were performed for both CK and graphene treatment groups.

# Rna-seq Data Quality Control And Reads Mapping

Raw data (raw reads) of fastq format were firstly processed through FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the

same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. These clean reads were then mapped to the *Zea mays* (assembly B73 RefGen_v4) reference genome [67] sequences using HISAT2 software [68, 69]. Only reads with perfect match or one mismatch were further analyzed to calculate the expression values. The clean data were deposited in the Genome Sequence Archive in the BIG Data Center of Sciences under accession code CRA002623.

# Quantification of gene expression levels and differentially expressed gene analysis

Gene expression levels were calculated by fragments per kilobase of transcript per million fragments mapped. The formula is shown as follow:

FPKM = {cDNA Fragments \over {Mapped Fragments(Millions) *Transcript Length(kb)}}.

Differential expression analysis of two groups was performed using the DESeq2 [70]. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *p* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Genes with an adjusted *p*-value < 0.01 and two-fold up and down change identified by DESeq2 [70] were assigned as differentially expressed. Multiple Experiment Viewer (MeV) [71] was used to display the gene expression patterns from the FPKM values.

# Differentially Expressed Gene Functional Annotation And Enrichment Analyses

Differentially expressed gene functions were annotated based on the following databases: Nr (NCBI non-redundant protein sequences, ftp://ftp.ncbi.nih.gov/blast/db/), Nt (NCBI non-redundant nucleotide sequences, ftp://ftp.ncbi.nih.gov/blast/db/), Pfam (The database of Homologous protein family, http://pfam.xfam.org/), COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/), Swiss-Prot (A manually annotated and reviewed protein sequence database, http://www.uniprot.org/), KO (KEGG Ortholog database, http://www.genome.jp/kegg/), as well as GO (Gene Ontology, http://www.geneontology.org/).

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution [37], which can adjust for gene length bias of DEGs. We used KOBAS [72] software to test the statistical enrichment of differential expression genes in KEGG pathways.

# Rna Extraction, Cdna Synthesis And Qrt-pcr Validation

Total RNAs of maize roots from the control and 50.00 mg/L graphene treatment groups were extracted using the RNAprep pure plant kit (TIANGEN, Shanghai, China) according to the manufacturer's protocol. The resulting RNAs were treated with DNase I prior to synthesizing cDNA with oligo (dT) primers and M-MLV Reverse Transcriptase (Invitrogen); these products were diluted 5-fold before use. For quantitative real-time PCR (qRT-PCR), Primer5 software was used to design gene-specific forward and reverse primers (Additional file 5: Table S3). Analyses were performed with SYBR-Green PCR Mastermix (TaKaRa) on a cycler (Mastercycler RealPlex; Eppendorf Ltd, Shanghai, China). The *Zea mays* GAPDH (*ZmGAPDH*) gene was used as the internal reference, and the relative amount of the amplified product was calculated following the 2-ΔΔCt method [73]. The relative expression levels were normalized by calibrating with the CK sample from roots. The root sample was washed by the DEPC sterile water three times before extracting the RNA.

# Measurement of N and K contents in soil around the maize root system

Soil nutrient analyzer was used to determine the contents of N and K around the root system of maize seedlings in the soil according to the manufacturer's protocol. The soil around the root system was sampled with the 5-point sampling method. The soil samples were thoroughly mixed and dried for 24 hours. After being sifted with a 1 mm sieve, the soil samples were used for further measurement, respectively.

# Statistical analysis

Each treatment was conducted in triplicate, and the results are presented as the means ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA). Significance of difference between means was determined by least significant difference (LSD) at the 0.05 probability level. Pairwise comparisons were used by Student's t test and differences were regarded as significant at $p < 0.05$. All statistical analyses were performed using SPSS 21 (Predictive Analytics Software statistics 21).

## List Of Abbreviations

DEGs: Differentially expressed genes; GO: Gene ontology; COG: Clusters of Orthologous Groups; FPKM: Fragments per kilobase of transcript per million mapped fragments; *Z. mays*: *Zea mays*; qRT-PCR: Quantitative real-time polymerase chain reaction

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Compliance and ethics

The authors declare that they have no competing interests.

### Availability of data and materials

All the data generated or analyzed during this study are included in this published article and its Additional files. The clean data were deposited in the Genome Sequence Archive in the BIG Data Center of Sciences under accession code CRA002623.

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

ZWC and JGZ conceived the project; ZWC and ZHL performed the experiment. ZWC, JS, SHH, YQD, YYQ, JQ, WJL, JWL, and HYW analyzed the data; BYX and QLP performed the SEM and TEM, Raman spectra analysis. ZWC and JGZ wrote and revised the manuscript. All authors have read and approved the manuscript.

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# Tables

**Table 1** Differentially expressed transcription factor (TF) genes between CK and graphene treatment of root samples in *Zea mays*.

| TF family | Gene Numbers | Up | Down |
|---|---|---|---|
| ERF | 4 | 3 | 1 |
| WRKY | 9 | 8 | 1 |
| bHLH | 6 | 5 | 1 |
| MYB and MYB-like | 12 | 10 | 2 |
| NAC | 7 | 3 | 4 |
| AP2 | 4 | 3 | 1 |
| MADS-box | 2 | 0 | 2 |
| total | 44 | 32 | 12 |

**Table 2** qRT-PCR validation of the RNA-seq results.

| Gene ID | Function annotations | RNA-seq | | qRT-PCR | | Regulation |
|---|---|---|---|---|---|---|
| | | Fold change | FDR | Fold change | *p*-value | |
| gene-EREB60 | AP2-EREBP transcription factor | 2.74 | 6.12E-04 | 2.28 | 2.16E-03 | up |
| gene-LOC541743 | Transcription factor MYB30 isoform X1 | 3.51 | 4.87E-03 | 5.58 | 2.67E-06 | up |
| gene-MYB64 | Transcription repressor MYB6 | 2.48 | 1.42E-06 | 5.32 | 3.12E-03 | up |
| gene-myb8 | Transcription factor MYB8 | 3.83 | 5.45E-12 | 6.26 | 2.66E-02 | up |
| gene-LOC103651266 | WRKY transcription factor 51 | 3.74 | 2.39E-05 | 2.18 | 1.89E-03 | up |
| gene-WRKY45 | WRKY DNA-binding domain superfamily protein | 0.37 | 3.91E-04 | 0.25 | 3.06E-04 | down |
| gene-LOC103628959 | MADS-box transcription factor 26 | 0.30 | 1.75E-04 | 0.38 | 8.46E-02 | down |
| gene-bHLH94 | bHLH DNA-binding domain superfamily protein | 2.33 | 1.88E-04 | 4.37 | 4.56E-03 | up |
| gene-LOC103631852 | Transcription factor EMB1444 | 2.22 | 4.93E-04 | 3.89 | 2.49E-05 | up |
| gene-LOC103633674 | NAC transcription factor 32 | 8.55 | 2.85E-04 | 6.26 | 1.80E-03 | up |
| gene-LOC103653847 | NAC domain-containing protein 7 | 0.20 | 6.75E-03 | 0.13 | 5.17E-03 | down |
| gene-LOC103625838 | Transcription factor JUNGBRUNNEN 1 | 2.46 | 3.63E-03 | 3.36 | 4.39E-02 | up |
| gene-LOC103630375 | Ethylene-responsive transcription factor ERF020 | 0.36 | 9.69E-03 | 0.29 | 3.68E-05 | down |
| gene-LOC103635988 | Ethylene-responsive transcription factor WRI1 | 2.86 | 2.45E-04 | 4.16 | 6.44E-03 | up |
| gene-GLN6 | Glutamine synthetase root isozyme 1 | 2.88 | 2.34E-07 | 2.65 | 3.32E-02 | up |
| gene-nrt2 | Nitrate transport 2 | 3.42 | 3.66E-03 | 3.56 | 1.26E-04 | up |
| gene-nrt2.2 | High affinity nitrate transporter | 3.14 | 7.32E-04 | 3.68 | 1.86E-03 | up |
| gene-HAK20 | Potassium transporter 5 | 3.00 | 8.11E-03 | 3.22 | 3.36E-03 | up |
| gene-HAK21 | Potassium transporter 21-like isoform X1 | 4.87 | 4.65E-15 | 5.56 | 3.72E-03 | up |
| gene-kup1 | Potassium ion uptake permease 1 | 3.06 | 9.09E-09 | 3.26 | 4.70E-03 | up |

# Additional Files

**Additional file 1: Table S1.** Characteristics of the RNA-sequencing data from six root samples of maize.

**Additional file 2: Table S2.** The mapping results of RNA-seq clean reads from six root samples using the *Z. mays* genome of B73.

**Additional file 3: Figure S1.** COG database to classify the differentially expressed genes function and homology.

**Additional file 4: Figure S2.** KEGG pathway analysis to identify the differentially expressed genes functional categorization.

**Additional file 5: Table S3.** List of forward and reverse primers used for qRT-PCR analyses.

# Figures

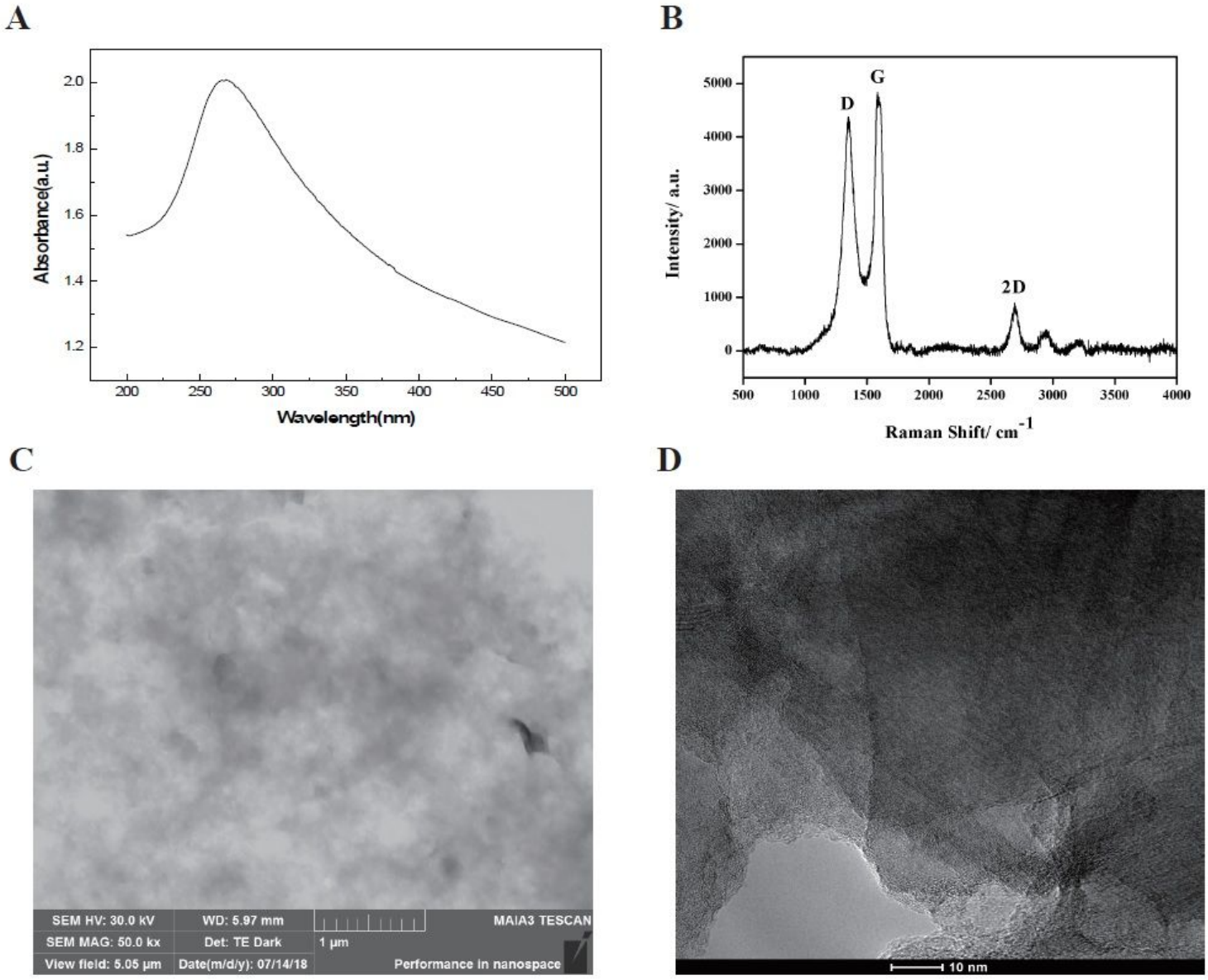



**Figure 1**

Characterization of Graphene. (A): Ultraviolet-visible absorption spectrogram, (B): Raman spectra, (C): TEM image, and (D): TEM image.

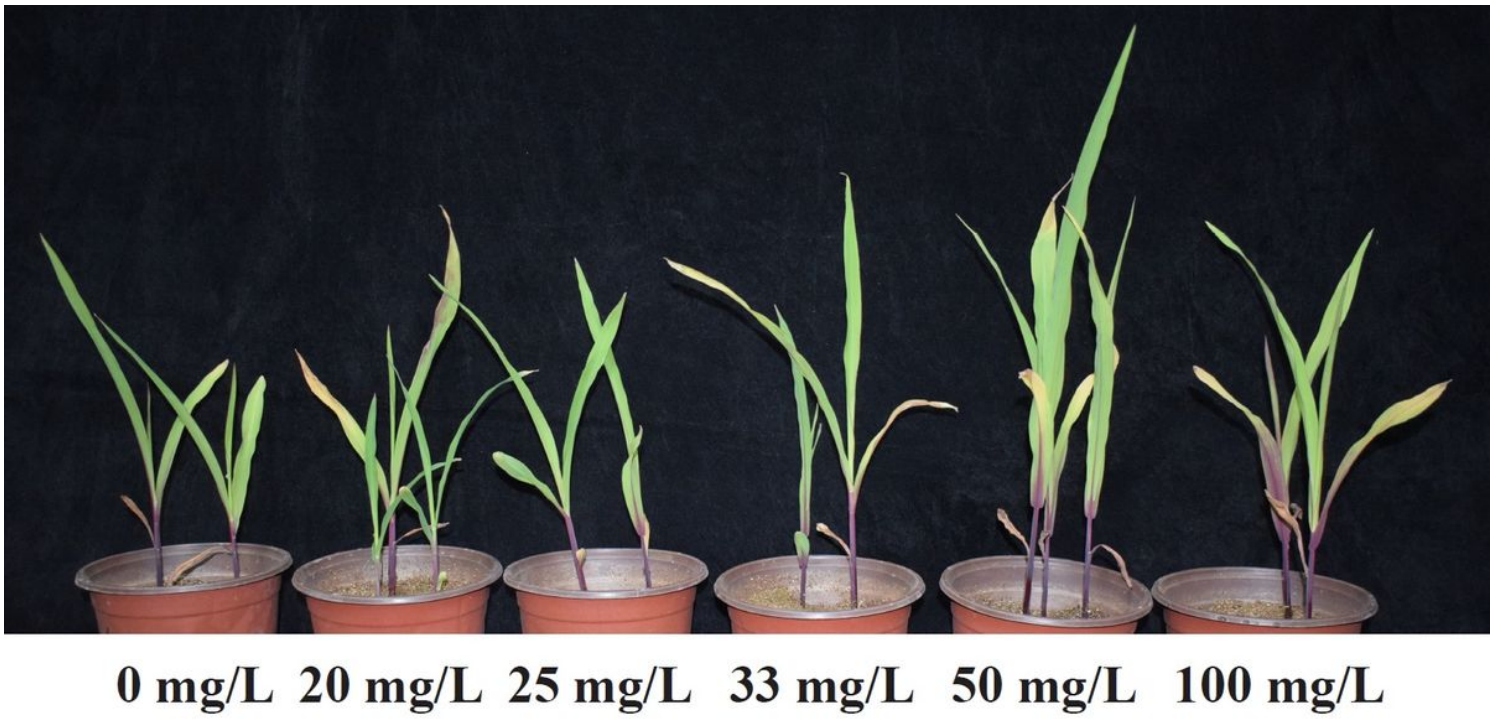
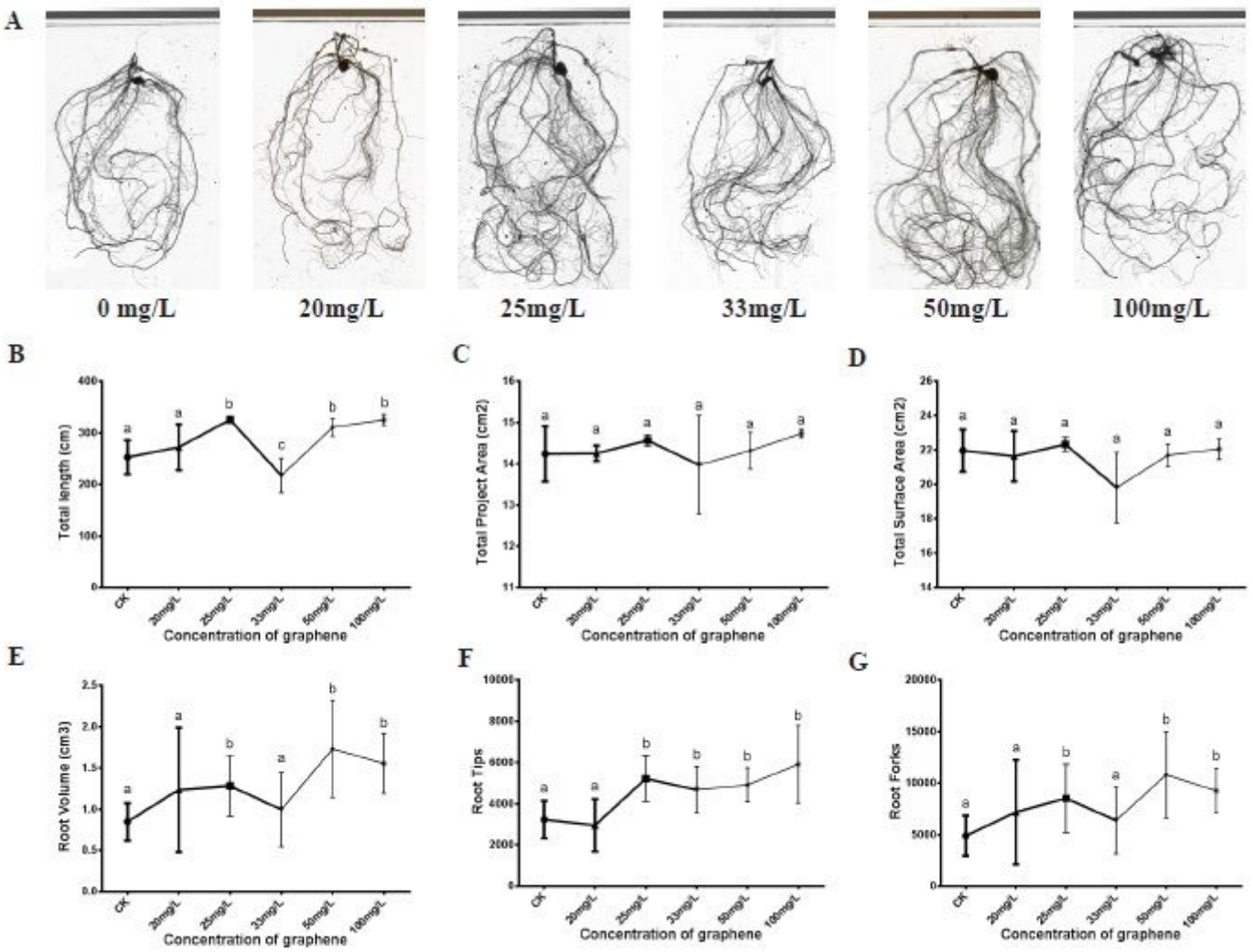


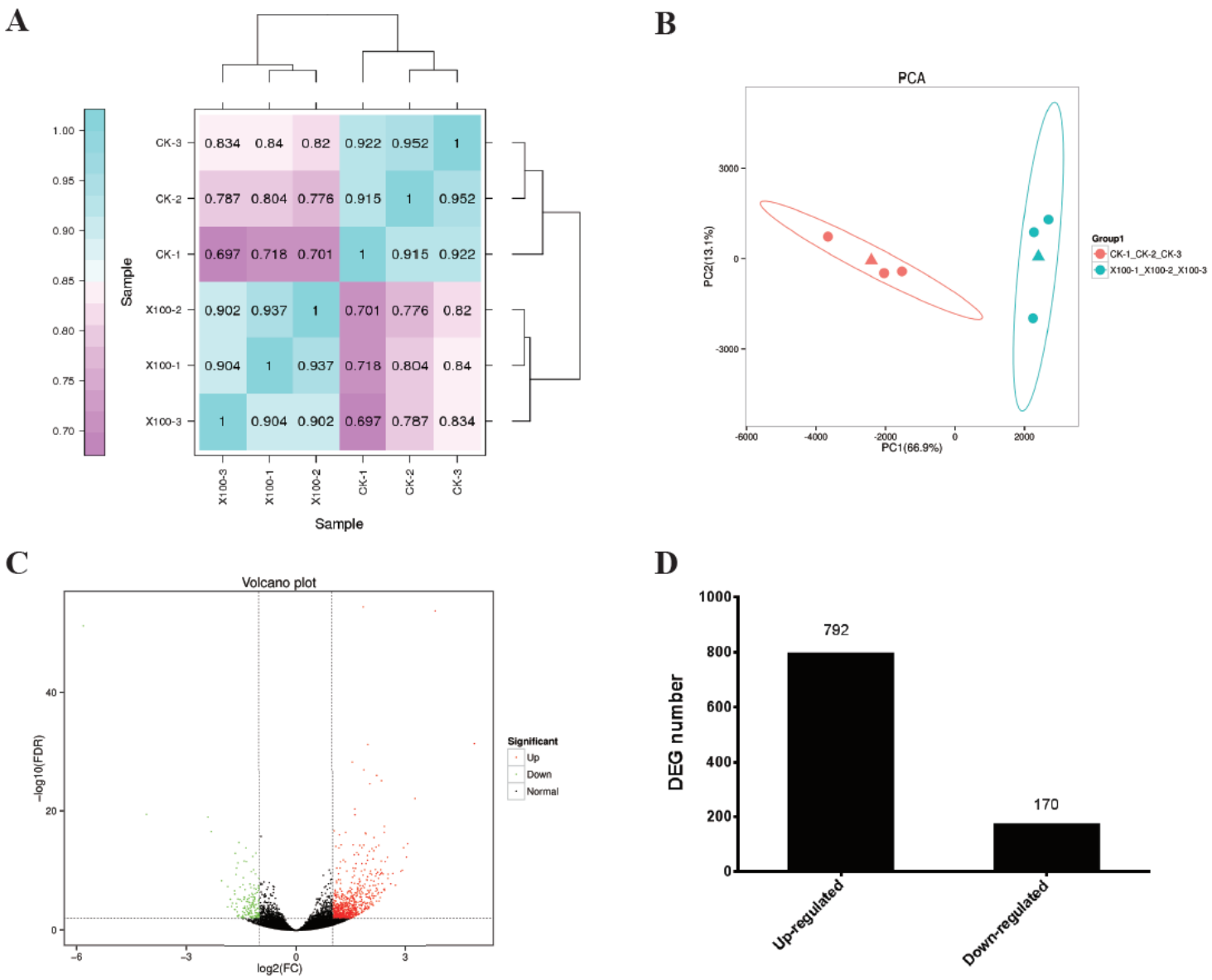

**Figure 2**

Phenotype of Zea mays seedlings with roots watering by six different concentrations of graphene.



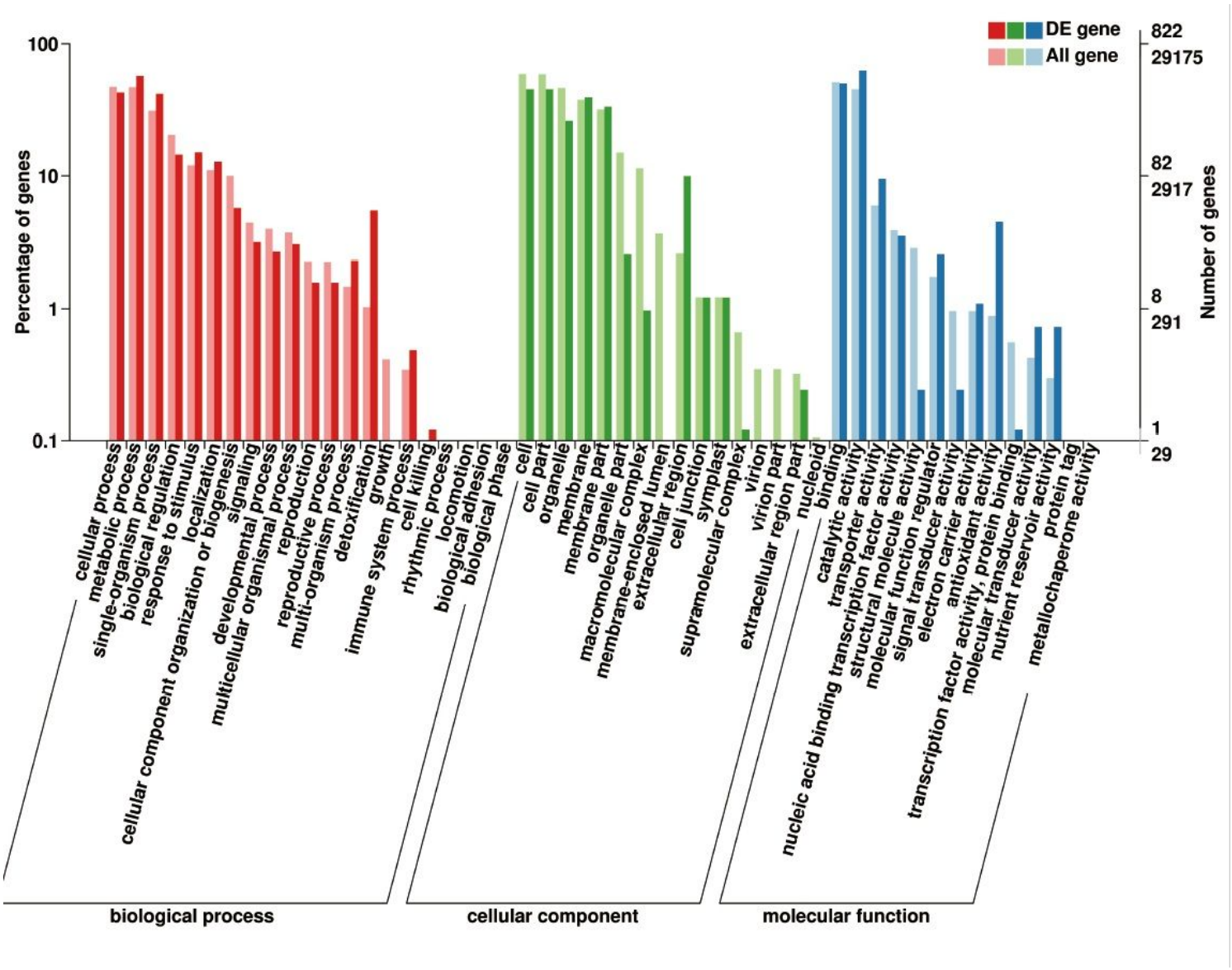

## Figure 3

Root architecture analysis of maize seedlings under six concentration of graphene (0, 20, 25, 33, 50 and 100 mg/L). (A): root morphology, (B): total root length, (C): total project area, (D): total surface area, (E): root volume, (F): the number of root tips, (G): the number of root forks.



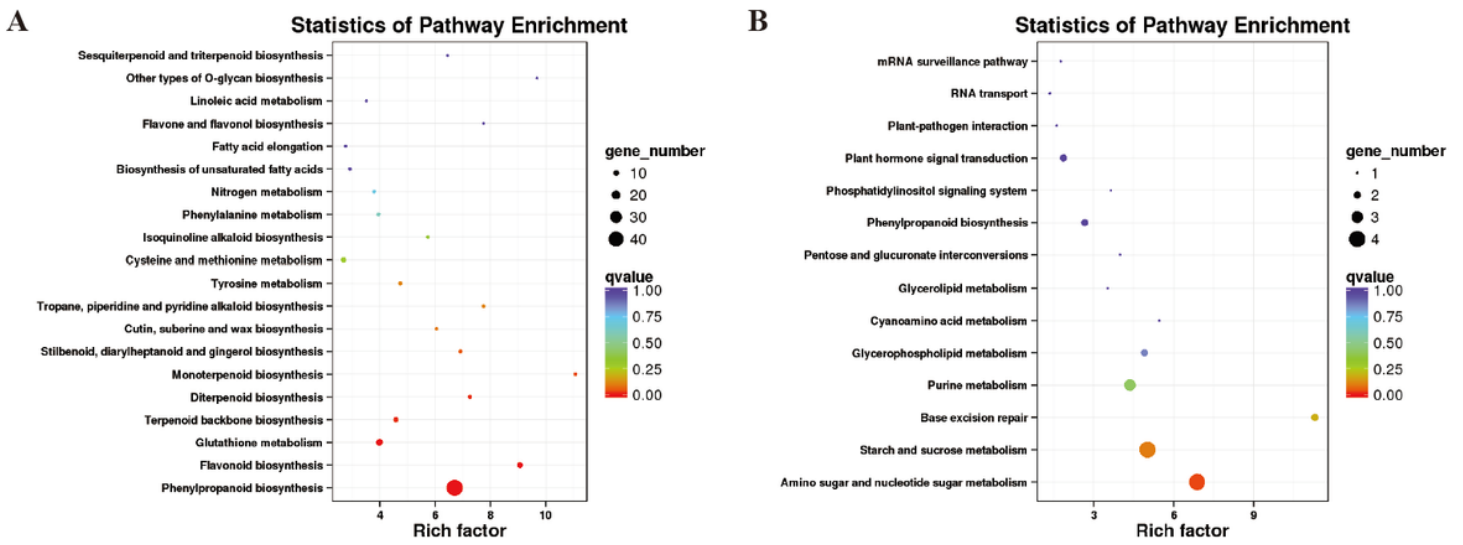

## Figure 4

Overview of maize root transcriptome response to 50 mg/L graphene (X100) and CK. (A) Pearson correlation coefficient (PCC) of all the genes between six samples. (B) Principal component analysis of all samples. Red and light blue colors represent the samples of CK and exposed to 50 mg/L graphene, respectively. (C) Volcano plot of differentially expressed genes. (D) The number of up and down-regulated expressed genes.



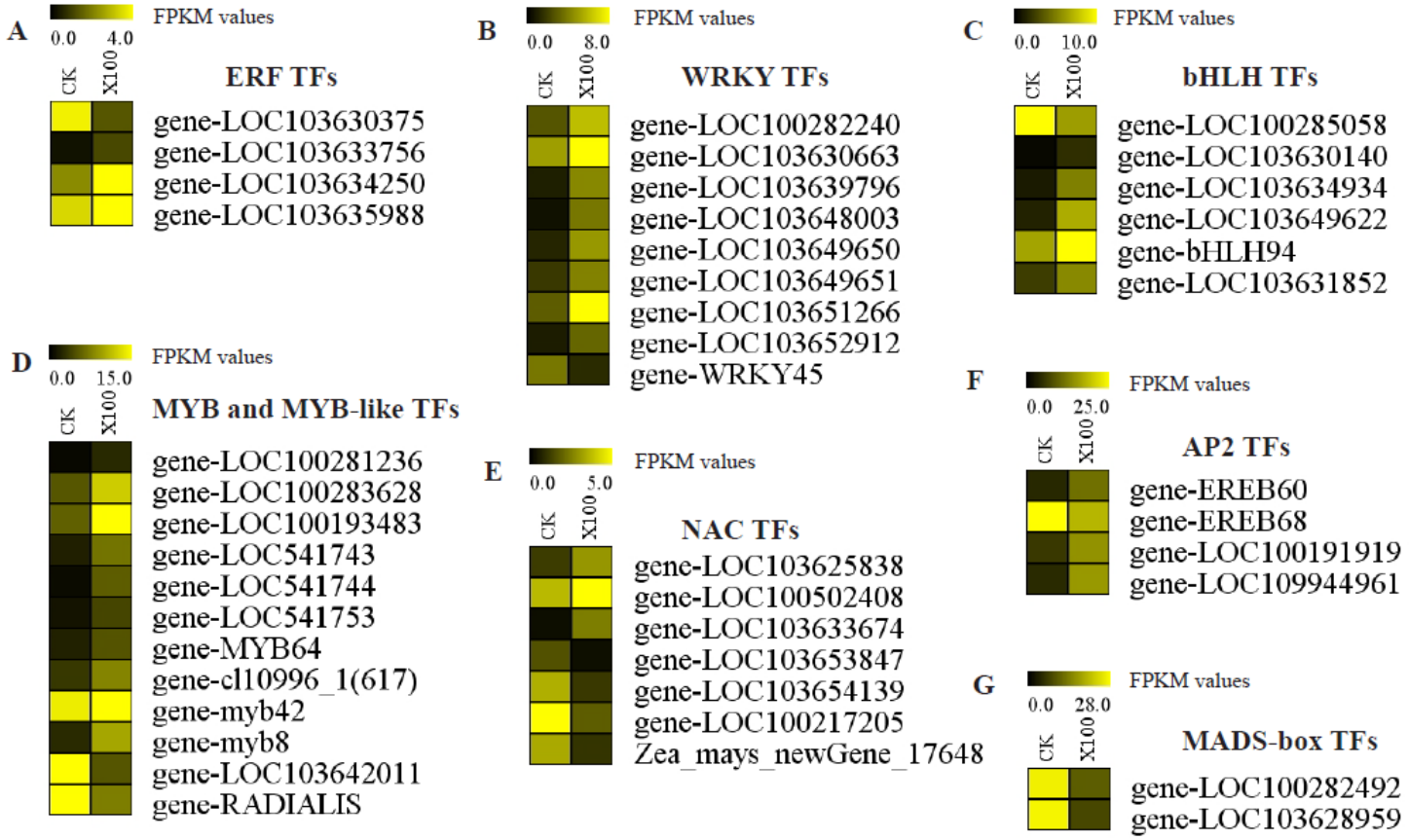

## Figure 5

Gene ontology (GO) enrichment analysis of differentially expressed genes exposure to graphene.




## Figure 6

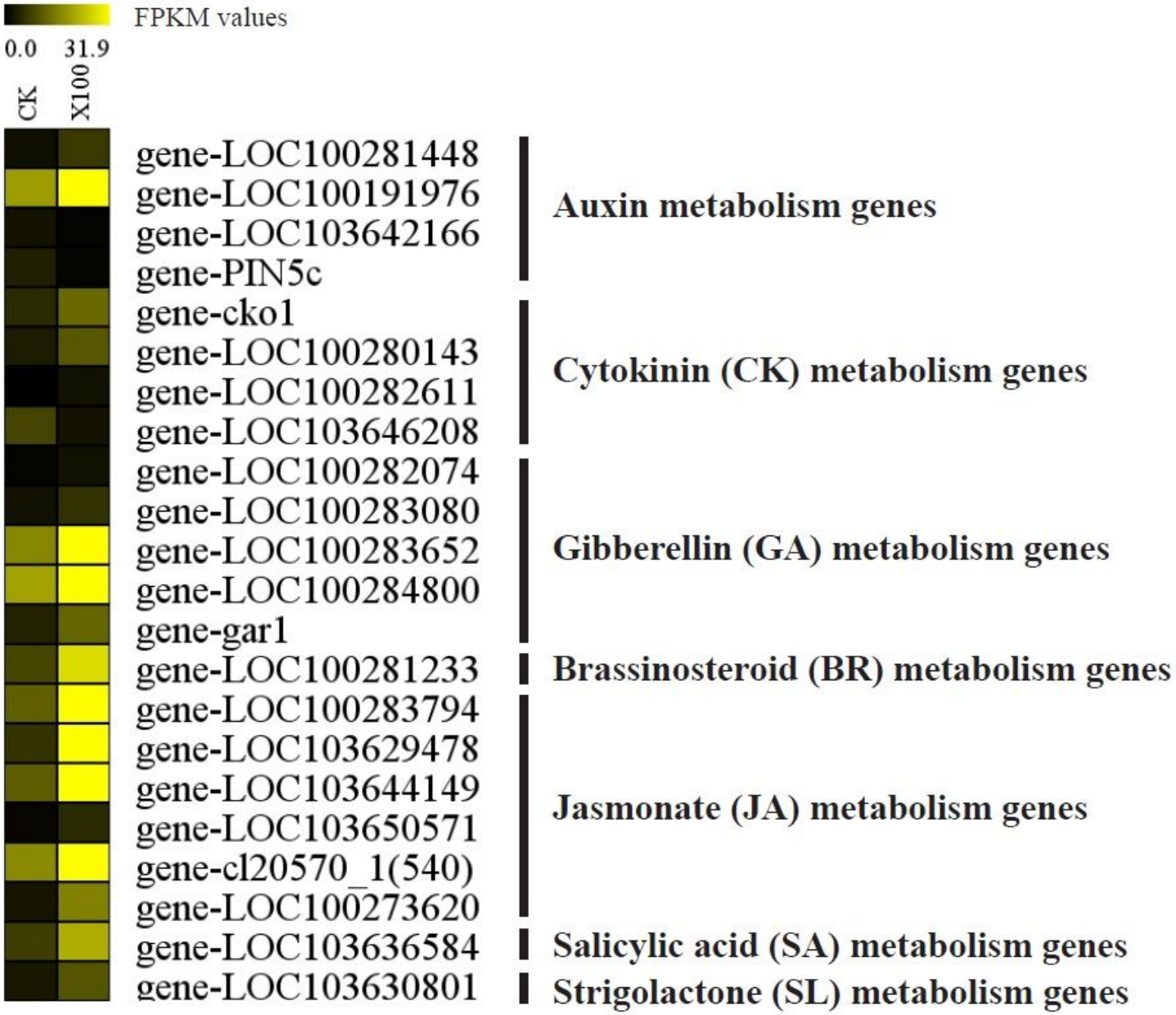
KEGG pathway analysis of differentially expressed genes enriched. (A) Top 20 pathways of significantly up- (left) and (B) 14 pathways of significantly down- (right) regulated genes enriched.




## Figure 7

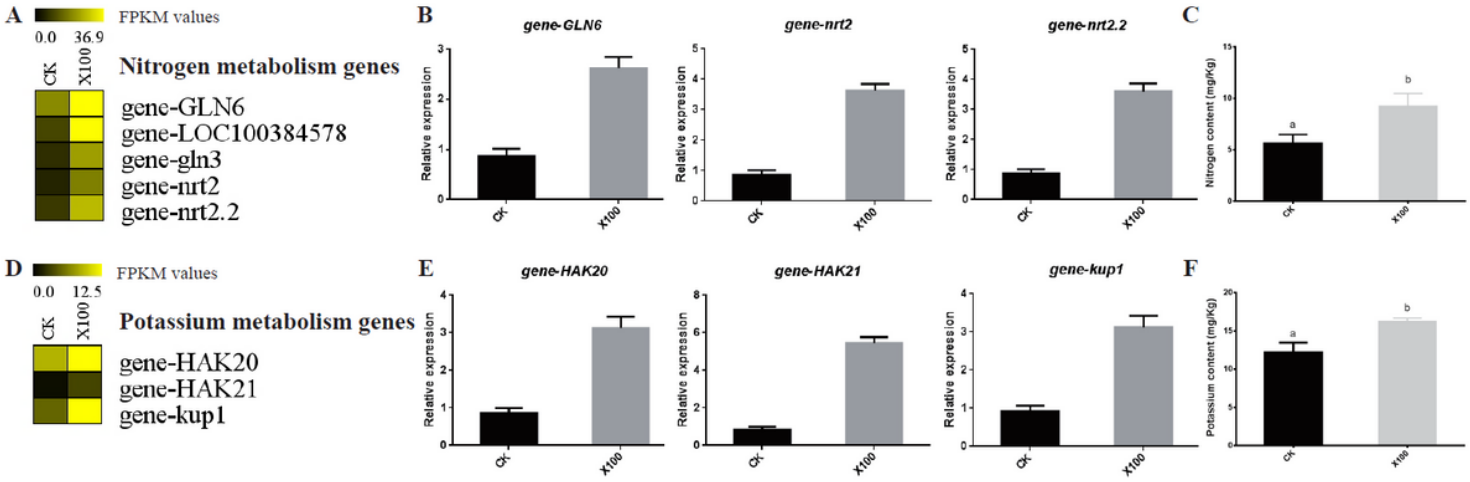
Differentially expressed transcription factor (TF) genes between CK and graphene treatment of root samples in Zea mays. The colored bars represent the FPKM values of the DEGs. (A): ERF, (B): WRKY, (C):

bHLH, (D): MYB and MYB-like, (E): NAC, (F): AP2, (G): MADS-box.




## Figure 8

Differentially expressed genes involved in hormones signaling pathways.




## Figure 9

Up-regulated expression of genes involved in nitrogen and potassium metabolism. (A): Nitrogen metabolism gene expression profile of RNA-seq data. (B): Validation of the nitrogen metabolism gene expression by qRT-RCR. (C): Content of nitrogen in the soil around the maize seedling roots of CK and 50 mg/L graphene treatments. Values are mean ± SE (n=5). $p<0.05$ by students T-test. (D): Potassium metabolism gene expression profile of RNA-seq data. (E): Validation of the potassium metabolism gene expression by qRT-RCR. (F) Content of potassium in the soil around the maize seedling roots of CK and 50 mg/L graphene treatments. Values are mean ± SE (n=5). $p<0.05$ by students T-test.

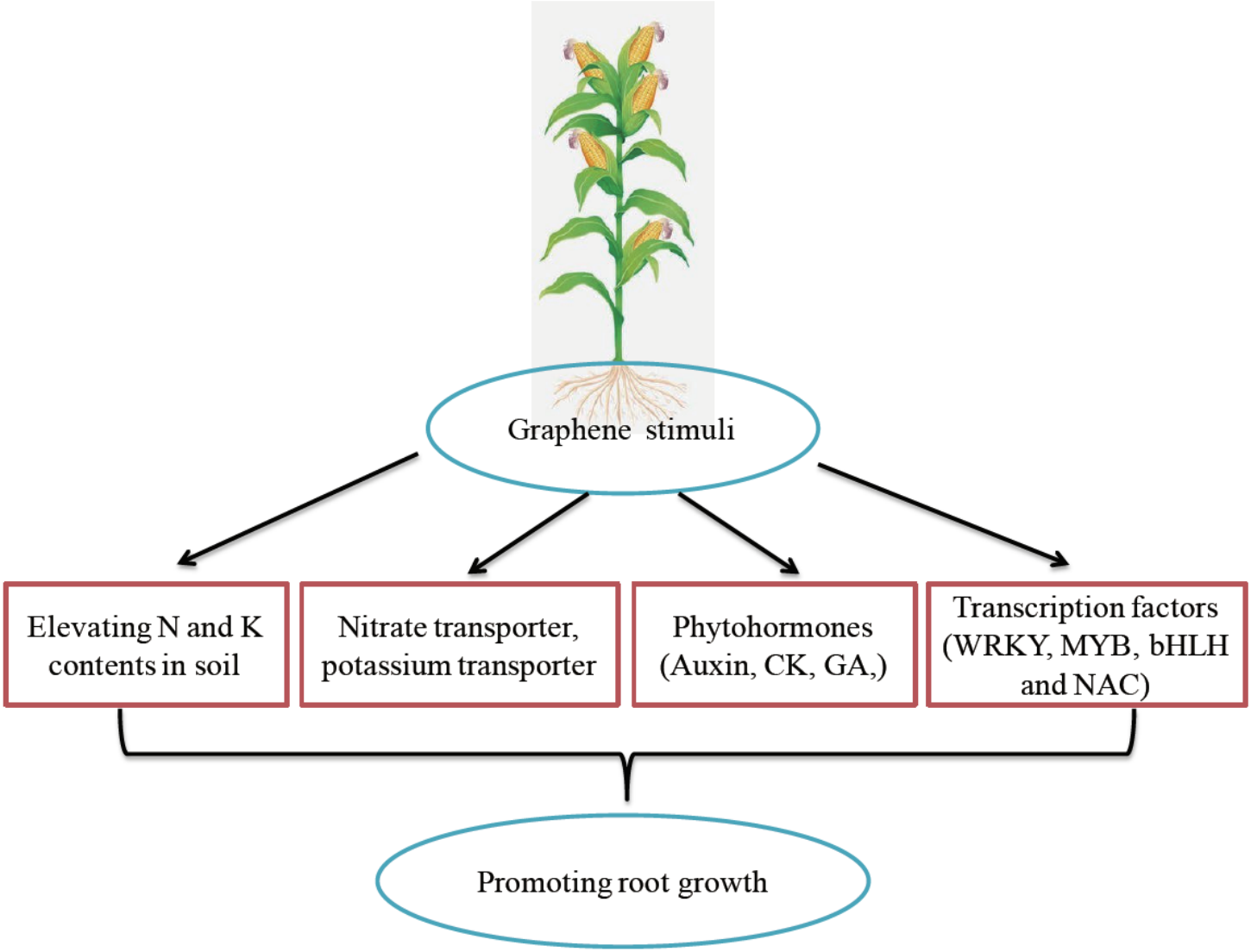



**Figure 10**

The putative model of the graphene in promoting maize roots development.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile4FigureS2.pdf
- Additionalfile2TableS2.docx
- Additionalfile5TableS3.docx
- Additionalfile1TableS1.docx
- Additionalfile3FigureS1.pdf