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Environmental significance

Global climate change (e.g., extreme drought, heat, flooding) threatens more than 90% of the land and results in losing half of the crop yield worldwide, leading to the increased difficulty in maintaining food security by 2050. Therefore, the demand for new technology to enhance crop tolerance against abiotic stresses is increasingly urgent. Herein, we report a solution to combat drought stress by employing advanced carbon dots (PNDs), which were rationally designed with concerted strategies of nitrogen doping and surface modification with polyacrylic acid. Such engineering process improved their ability to eliminate ROS and facilitate their penetration through plant cells. These features of the engineered nanomaterials enabled us to distinguish our work by identifying the dynamic molecular pathways responding to the stimulus by PNDs, and unravelling the sophisticated correlation network of the functioning genes and metabolites during combatting drought stress.

Physiological and molecular level understanding of advanced carbon dots to enhance maize drought tolerance: modulation of photosynthesis and signaling molecules

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ABSTRACT

Drought stress is posing severe threat to the global crop production. Herein, we report a solution to combat drought stress by employing advanced carbon dots, which are rationally designed with the concerted strategies of nitrogen doping and surface modification with polyacrylic acid, defined as PNDs. Doping carbon dots (CDs) with nitrogen (N) improves the ability to eliminate reactive oxygen species (ROS), and polyacrylic acid could facilitate the penetration of CDs through plant cells into chloroplasts. Under drought stress, foliar-applied PNDs (5 $mg \cdot L^{-1}$) could decrease ROS accumulation, substantially improve net photosynthesis rate (206.8%), and promote water uptake by increasing roots abscisic acid (ABA, 6.9%) and proline (Pro, 36.3%) in maize, demonstrating multiple positive functions. PNDs could recover maize growth under drought stress by modulating photosynthesis and signaling molecules. The results of dynamic monitoring showed that ABA and Pro were synthesized in maize leaves first, and then accumulated in roots through long-distance transport. Elevated level of ABA and Pro could promote aquaporin activity and maintain osmotic pressure in roots, thereby alleviating drought stress of maize. This work demonstrates PNDs will be promising alternatives for sustainable nano-agriculture in responding to global climate change and food security crisis.

KEYWORDS

Carbon dots; Drough; Reactive oxygen species; Photosynthesis; Plant signals

1. INTRODUCTION

Global climate change (e.g., drought, heat, flooding) could threaten more than 90% of the land, cause the loss of half of the crop yield worldwide and lead to the increasing difficulty in maintaining food security by 2050.[1] The adverse environmental conditions would cause various abiotic stresses to increase numerous reactive oxygen species (ROS) accumulation in plants, resulting in oxidative damage to inhibit plants physiological processes, especially photosynthesis.[2,3] Studies demonstrated CO_2 fixation and electron transport in photosystem would be limited under abiotic stress.[4] Chen *et al* reported D1 protein, one of the core proteins for repairing photosystem II (PSII) could be damaged during heat stress, due to ROS accumulation that reduced its de novo synthesis.[5] In addition, plant defense systems would be activated responding to various abiotic stresses.[6] Some key signaling compounds, such as jasmonic acid (JA), abscisic acid (ABA) and proline (Pro), contribute to this induced tolerance in whole plant.[7] JA can induce antioxidant reaction to decrease ROS accumulation.[8] ABA could regulate the opening of leaf stomata and the activity of aquaporin (AQP) to enhance plant drought tolerance.[9] Pro could increase plant water uptake in drought by controlling osmotic pressure.[10] Therefore, scavenging ROS, improving photosynthesis, and modulating plant signals would be an effective way to enhance plants tolerance against abiotic stresses.[3]

Recently, nano-enabled agriculture based on engineered nanomaterials (ENMs) shows potential in enhancing sustainable crop production and tolerance to abiotic stresses.[11] For example, Dimkpa *et al* reported that ZnO ENMs could alleviate drought effects in sorghum by improving nutrients uptake.[12] Also, single-walled carbon nanotubes improved germination and growth of *Hyoscyamus niger* during drought stress through increasing water uptake, activating plant defense system and reducing electrolyte leakage.[13] Recently, Chen *et al.* demonstrated that carbon-based ENMs (carbon dots, CDs) could promote tomato growth under drought stress through regulating photosynthesis, antioxidant system, osmotic adjustment, and soil microbial environment.[14] This is particularly intriguing, as CDs are with low cost, good biocompatibility,

high chemical inertness, and nontoxicity in food chain and human cells,[15] thus it may be a promising alternative for nano-enabled agricultural food production.[16] To amplify this, some emerging studies indicated CDs could act as artificial antenna to increase light harvesting ability, increase chlorophyll content and improve Rubisco activity, resulting in enhanced photosynthesis;[17] heteroatom-free CDs could act as proton donors for scavenging ROS in rice;[14] nitrogen doped CDs (N-CDs) exhibited an outstanding ROS scavenging activity aided by the enriched electron density.[18,19] Despite these successful attempts that demonstrated the isolated positive performances of CDs, it remains unknown whether the CDs, engineered ingeniously but feasibly, could veritably enhance plant tolerance against abiotic stresses through a combination of tools including scavenging ROS, improving photosynthesis, and inducing signaling molecules. There is also no systematic investigation on the molecular pathways of induced tolerance from CDs to elucidate the mechanism at the molecular level.

In this context, we designed a type of advanced CDs with negative charge and small size, which are key merits for the uptake and transport of ENMs in plants. Specifically, polyacrylic acid modified N-CDs (PNDs) may penetrate plant cells and exhibit high ROS scavenging activity.[23] To unveil PNDs' functioning mechanism, we selected drought as the source of abiotic stress, which is one of the strongest influencer on plant growth and can directly destroy the plant photosynthetic system via ROS accumulation and thereby reduce crop production.[20] Maize (*Zea mays* L.) was selected as a model plant, as it is a main crop for feeding humans and grows in Asia, America and Africa, which suffer from drought every year that can cause 20–30% yield loss.[21] With these judicious considerations and preparations, we investigated: 1) the effect of foliar-applied PNDs on maize growth under drought; 2) the modulation of induced tolerance by foliar-applied PNDs; and 3) the molecular pathways of PNDs to enhance maize drought tolerance.

2. MATERIALS AND METHODS

2.1 Synthesis and characterization of ENMs

N-CDs were synthesized via the hydrothermal method (Details in Supplementary text S1).[22] The negatively charged PNDs were prepared by adding polyacrylic acid (1 mL, 10%, Sigma Aldrich) into the N-CDs solution and heated at 80 °C for 4 h.[23] The transmission electron microscopy (TEM) images were obtained from a JEM–2100 electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 200 kV. Photoluminescence (PL) analysis was conducted with a F–7000 fluorescence spectrophotometer (Hitachi, Japan). X–ray photoelectron spectroscopy (XPS) spectra were obtained from a Thermo Kalpha X–ray photoelectron spectrometer (US). The Zeta-potential of ENMs was measured with a Malvern Zetasizer (ZEN3600, UK).

2.2 Maize cultivation under drought stress

Maize seeds (*Zea mays* L. No. 9 Shiyu) were bought form Liaoning East Asia Seed Industry Co., Ltd. Two hundred maize seeds were cultivated and germinated in a growth chamber for 3 days in dark. Then they were moved to the pots (1.5 kg soil per pot) and cultivated under natural light source (temperature 25 ℃, humidity 50%). The soil properties were pH of 7.1, organic matter of 18.5 $g \cdot kg^{-1}$, alkaline hydrolysis N of 90.2 $mg \cdot kg^{-1}$, available P of 15.4 $mg \cdot kg^{-1}$ and rapidly available K of 100.3 $mg \cdot kg^{-1}$. In this study, three seedlings were retained in each pot, then experimental groups (5 replicates) with spraying N-CDs (5 $mg \cdot L^{-1}$) and PNDs (1, 5, and 10 $mg \cdot L^{-1}$) under drought (5 mL per plant each time), and two control groups (non-drought and drought without exposure) were named Non-CK and CK respectively. Before spraying, the suspension of PNDs was dispersed ultrasonically for 30 min, thus the dosing of PNDs could be even. During the spraying process, each pot was evenly rotated to guarantee the same amount of PNDs sprayed on each seedling. All plants were watered daily and kept at 75% soil moisture (SM) for 20 days. After that, the pots were maintained under two watering conditions: drought-stressed (35% SM) and well-watered (75% SM).[24] Details of watering processes was shown in Supplementary text S2. After spraying for seven consecutive days (from the 26th day), the maize seedlings were harvested. The soil moisture sensor (TZS–IW, China) was used for

measuring water contents in all pots. Details of soil moisture parameters can be seen in Supplementary text S3. Root length, surface area, number of tips and volume were analyzed by a root scanner (Instruments Regent LA2400, Japan). The maximum fluorescence value (Fp), net photosynthesis rate (Pn), and transpiration rate (E) of maize seedlings were carried out by a FluorCam chlorophyll fluorescence imaging system & photosynthesis analyzer (PP Systems, TARGAS–1, China). The distribution of PNDs in maize leaf was observed by using a Nikon A1 laser confocal microscope (CLSM, Nikon, Japan) equipped with 405 nm laser for excitation. The element contents were determined using inductively coupled plasma mass spectrometry (ICP–MS) (ICAP–TQ, Thermo Fisher Scientific, Germany). The qRT–PCR analysis, Pro and ABA were determined according to previous reports.[25–28] The detailed measurements of ROS, Pro ABA, K^+/Na^+, ATP and NADPH can be found in the Supplementary text S4. The qRT–PCR analysis is described in Supplementary text S4 and the reference genes and primers are added in table S1.

2.3 Maize transcriptome sequencing process

The sampling time was 33 days old for maize seedling and the specific time period was the beginning of photosynthesis (9:00–11:00 am), which was consistent with our previous work.[24] Total RNA was isolated using the Trizol Reagent (Invitrogen Life Technologies), after the concentration, quality and integrity were determined with a NanoDrop spectrophotometer (Thermo Scientific). Three micrograms of RNA were collected as input material for the RNA sample preparations. Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, the Oligo(dT) magnetic beads are used to enrich the mRNA with polyA structure in the total RNA, and the RNA is interrupted to a fragment of about 300 bp in length by means of ion interruption. RNA was used as a template, 6-base random primers and reverse transcriptase were used to synthesize the first strand of cDNA, and the first strand cDNA was applied as a template to synthesize the second strand cDNA. After the library was constructed, PCR amplification was used to enrich the library

fragments, and then the library was selected according to the fragment size. The library size was 450 bp, and the quality of the library was then checked by the Agilent 2100 Bioanalyzer. The total concentration of the library and the effective concentration of the library were detected. After RNA extraction, purification, and library building of the samples, the second-generation sequencing technology (Next-Generation Sequencing, NGS) was used to perform paired-end (PE) sequencing on these libraries based on the Illumina sequencing platform (Personal Biotechnology Cp. Ltd., Shanghai). For each gene, expression level (6G) was measured by Fragments Per Kilobases Per Millionreads (FPKM). Also, HTSeq (0.9.1) statistics was conducted to compare the Read Count values on each gene as the original expression of the gene. FPKM was used to standardize the expression. DESeq (1.30.0) was used to analyze the genes of difference expression with screened conditions as follows: expression difference multiple |log2FoldChange| > 1, significant $p < 0.05$. Gene function annotation was conducted by comparison to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases.

2.4 HPLC–MS/MS analysis of metabolites

Fresh plant tissue (maize shoots) samples harvested were frozen with liquid nitrogen immediately. 100 mg of the plant samples were taken and ground evenly in liquid nitrogen, and placed in a 2 ml centrifuge tube to mix with 1.5 ml of the extract (methanol/water (80:20)). The sample was placed in an ice bath and sonicated for 30 min (35 kHz); then it was centrifuged at 4 °C and 12000 rpm for 15 min. The supernatant obtained was spin-dried with a rotary evaporation concentrator in vacuo (4 ℃), and obtained solids were reconstituted with 200 μL methanol acetonitrile water (4:4:2). The supernatant was taken to centrifuge (10 min at 4 ℃ and 12000 rpm) for high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS, Thermo Scientific, Germany) determination. 2-Chloro-L-phenylalanine was added in the preparation of extraction solution, which could be used as the standard for metabolite identification and quantification. A quality control (QC) sample was prepared by mixing

aliquots (150 μL per aliquot) of all samples to obtain a pooled sample. The control was methanol solution (80%). QC contains the material information of all samples to control and evaluate the stability and accuracy of the sample information collection by mass spectrometry.

2.5 Statistical analysis

All treatments were conducted at least in triplicate. Data were presented as a mean ± standard deviation. The data analysis of variance (ANOVA) was done by using Origin95_64 at $p < 0.05$. Principal component analyses (PCA) method was run on HPLC–MS/MS data via online resources (http://www.metaboanalyst.ca/) and detailed analysis methods can be seen the Supplementary text. The student t-test was conducted to determine the differences between groups. Correlation network (gene and metabolites related to pathways) was analyzed with SPSS version 17.0 software and visualized using Cytoscape (version 3.3.2). All elements performed in this network were considered to have significant correlation at $p \leq 0.05$ using SPSS version 17.0 software.

3. RESULTS AND DISCUSSION

3.1 Materials characterization and leaf distribution

The preparations of N-CDs and subsequent negatively charged N-CDs are shown in Figure 1a. After surface modification by polyacrylic acid, the PNDs showed a negative charge at –15.04 mV, distinct from the charge neutrality of normal N-CDs (Figure 1b). TEM image indicated N-CDs were less than 5 nm in size, with an average size of 2.8±0.4 nm (Figure 1c and S1), and the PNDs had good dispersion, and the same size distribution and clear lattice (0.21 nm) with N-CDs (Figure 1d, 1c and S1). Moreover, both N-CDs and PNDs exhibited blue fluorescence and the maximum emission peaks at 420 nm upon the excitation with 365 nm (Figure S2). There was no difference in the interior structure and chemical components between N-CDs and PNDs (Figure S3). Nevertheless, the surface charge of PNDs should have important influences on nanoparticle–plant interactions.[29] Lew *et al* demonstrated that ENMs with high negative or positive zeta potential could favor their transport in plants, especially adsorption to the

chloroplast lipid membrane.[30] The TEM and CLSM images proved the uptake and transport of PNDs in maize leaves after foliar spraying with PNDs (Figure 1e, S4 and S5), illustrating the PNDs were located around the chloroplasts. These results demonstrated that PNDs could enter chloroplasts in leaf cell through mesophyll cell walls and plasma membranes. Previous reports demonstrated that carbon-based ENMs could permeate the cuticle via polar aqueous pores or enter the leaf through stomatal pathway, then passed through the epidermis and mesophyll, finally be transported to chloroplasts through lipid bilayers.[23, 31, 32] Compared with neutral ENMs, negatively modified carbon ENMs could enter the chloroplast efficiently.[23] Therefore, prepared PNDs could transport into the chloroplast due to negative surface charge.

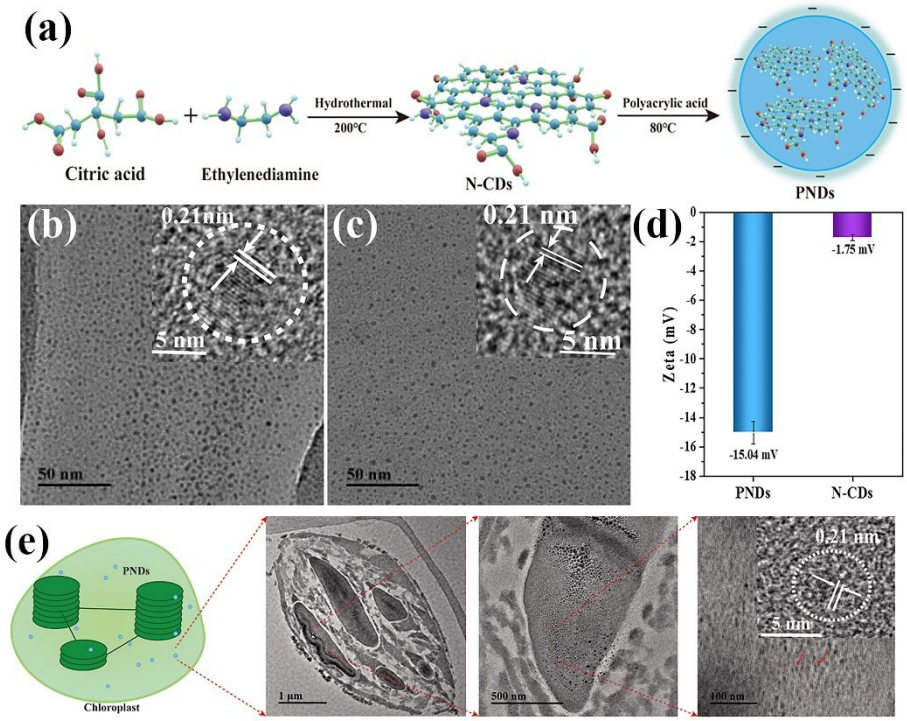

Figure 1. Synthesis and characteristics of ENMs. Illustrative diagram of preparation (a), TEM HTEM images (b, c) and ζ-potential (d) of N-CDs and PNDs; (e), TEM and HRTEM images of PNDs entering the chloroplast.

3.2 PNDs promoted maize growth and photosynthesis under drought

As shown in Figure 2a and Figure S6, the ROS also increased in the maize without any treatment (CK) under drought and the maize had slow growth. In contrast, the fluorescent signal of ROS was weak after foliar spray of N-CDs (5 mg·L^{-1}) and PNDs (1, 5 and 10 mg·L^{-1})

(Figure 2a). Similar to the most ENMs in agriculture,[33,16] PNDs showed the concentration dependence with foliar application. The 5 mg·L^{-1} of PNDs was the optimal dose. There was no significant difference in the fluorescent signal between the maize treated with PNDs (5 mg·L^{-1}) under drought stress and the maize grown in normal status (Non-CK) (Figure 2a). Moreover, the images of Fp demonstrated that foliar sprayed PNDs with the concentration of 5 mg·L^{-1} had the maximum Fp (12678.9) in maize leaves under drought stress (Figure 2b), which reflected the intensity of light reaction maize under drought.[26] In addition, with foliar spray of PNDs (1, 5 and 10 mg·L^{-1}), the growth of maize was clearly improved in comparison with CK, though both sets were under drought stress (Figure S6): the plant heights increased and the leaves were larger. PNDs (5 mg·L^{-1}) could increase fresh weight (shoots 52.0% and roots 102.7%), dry weight (shoots 222.7% and roots 283.3%), root length (160.2%), and root tips (95.6%) (Figure 2c, 2d and S7).

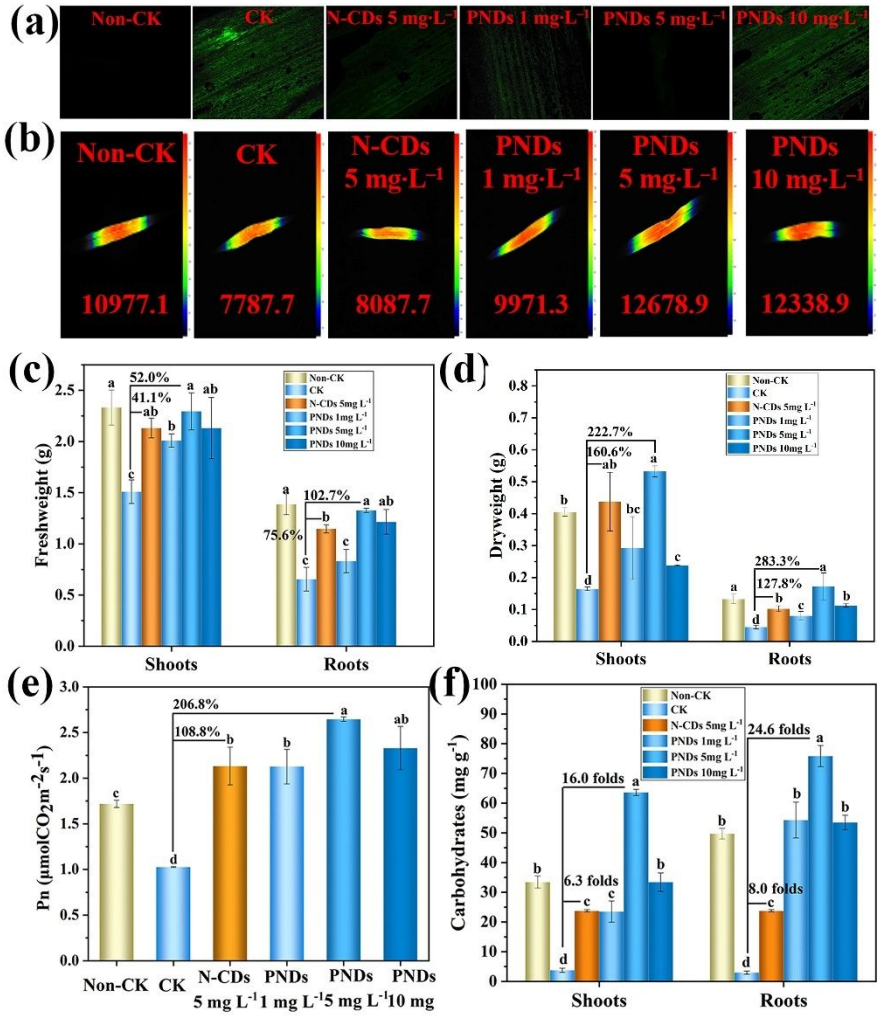

Figure 2. PNDs promoted maize growth under drought. (a) ROS generation was monitored by confocal imaging of DCF fluorescence in leaf with N-CDs, and different PNDs concentrations under 488 nm excitation; (b) comparison of changes in maximum fluorescence value (Fp); (c) fresh weight (FW), (d) dry weight (DW), (e) net photosynthesis rate (Pn) and (f) carbohydrates content of maize after foliar application of PNDs (0, 1, 5 and 10 mg·L^{-1}) and N-CDs (5 mg·L^{-1}) under drought.

Previous studies reported that the fluorescent CDs could improve the plant photosynthesis.[17] In this study, after spraying PNDs (1, 5 and 10 mg·L^{-1}), the increase in Pn of maize was correspondingly observed (Figure 2e), which increased the carbohydrate content in both roots and shoots (Figure 2f). Remarkably, the PNDs with the concentration of 5 mg·L^{-1} could increase the Pn level to 206.8%, and the carbohydrate content in shoots to 16.0-fold and in roots to 24.6-fold (Figure 2e and 2f). Foliar spray of PNDs also up-regulated the transport genes (*ZmSUT4*) to improve transportation of carbohydrate from maize shoots to roots (Figure S8), which promoted the roots growth (Figure S7).[34] Therefore, foliar sprayed PNDs could enhance

maize drought tolerance by scavenging ROS, improving Fp, enhancing photosynthesis, and promoting growth. Moreover, this promoted effect was better than that of N-CDs (Figure 2), since negatively charged ENMs could transport easily in plants.[29,30]

3.3 PNDs promoted water uptake by modulating Pro and ABA under drought stress

Herein, foliar sprayed PNDs were found to increase the production of ATP (35.3%) and NADPH (Nicotinamide Adenine Dinucleotide Phosphate) (26.6%) (Figure S9a and S9b), indicating more energy could be supplied for synthesizing Pro.[35, 36] Pro was an important signal for controlling osmotic pressure, and was synthesized in plant leaves.[10] The as-synthesized Pro could be transported from shoots to roots, and the *ZmBetProt* gene plays a key role in this process.[37] Figure 3a showed that its expression was up-regulated by 1.1-fold. These results demonstrated that more Pro was transported from leaves to roots after foliar spraying PNDs under drought stress, and the Pro content increased by 36.3% in root and decreased by 56.6% in leaves (Figure 3b and S9c). Pro can accumulate in roots and the ratio of K^+/Na^+ increased on abiotic stress.[38] In our study, we found the Na^+ content decreased by 53.6%, while the K^+ content increased by 77.0% in maize roots after treating with PNDs (Figure S10). Accordingly, the ratio of K^+ to Na^+ increased by 47.7% in root (Figure 3c), which indicated that osmotic pressure decreased. Compared to CK, the decrease in osmotic pressure induced by the application of PNDs can maintain cellular homeostasis, which increased the drought tolerance of maize.[38] Therefore, foliar sprayed PNDs could increase the drought tolerance of maize through modulating interaction of Pro and osmotic pressure.

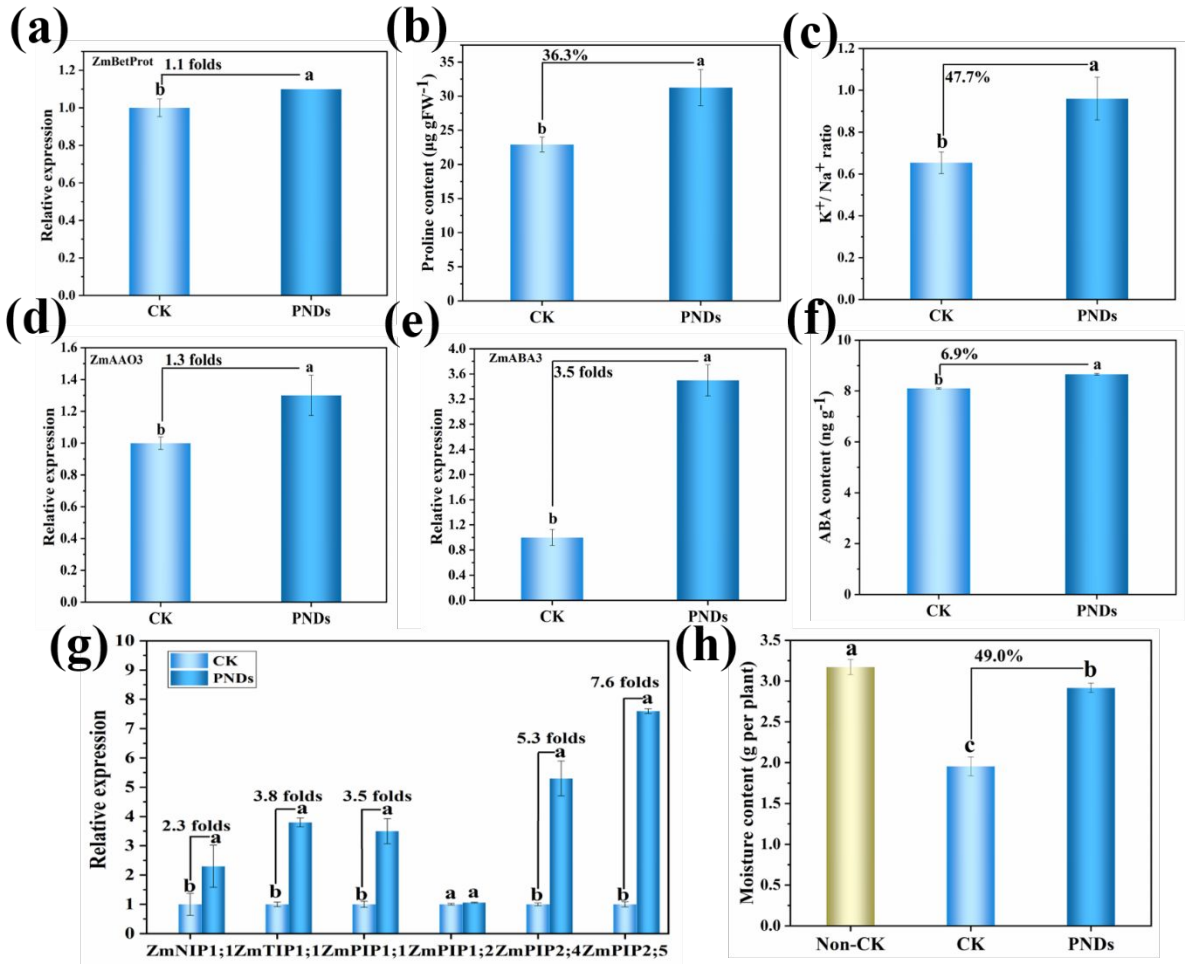

Figure 3. PNDs promoted water uptake by modulating Pro and ABA under drought stress after foliar spraying PNDs (5 mg·L^{-1}). (a), expression of *ZmBetProt* gene in shoots; (b) Pro content in roots; (c) K^+/Na^+ ratio in roots; (d) and (e) expression of *ZmAAO3* and *ZmABA3* gene in maize shoots; (f) ABA content in roots; (g) expression of AQP gene in maize roots; (h) the moisture content of maize.

ABA is another drought-responsive signal in plants, which played a central role in drought tolerance through modulating water uptake and evaporation.[20, 35] Herein, the up-regulated expression of the ABA synthesis key genes (*AAO3*, 1.3-fold and *ABA3*, 3.5-fold) was observed in shoots (Figure 3d and 3e). According to previous study, ABA synthesized in leaves can be transported to roots and accumulated in roots.[6] In figure 3f, the ABA content increased by 6.9% in roots after treating with PNDs. ABA could act as an intermediate factor during the regulation of AQP expression in response to abiotic stresses.[20] Herein, the major AQP genes (*ZmPIP1;1, ZmPIP1;2, ZmPIP2;4, ZmPIP2;5, ZmTIP1;1* and *ZmNIP1;1*), involved in maize water

transport were examined.[37] These AQP genes were overexpressed after foliar spraying PNDs (Figure 3g), compared with CK. AQP, as a central component of plant water system, is crucial for controlling the amount of water absorbed by plant cells.[39] Therefore, foliar sprayed PNDs increased the ABA content in roots, which in turn improved the activity of AQP, and promoted water uptake under drought stress. As discussed above, PNDs promoted water uptake by modulating Pro and ABA under drought stress (Figure 3), and the moisture content increased by 49.0% in maize (Figure 3h). Water uptake alleviated the drought stress and ensured the growth of crops. Correspondingly, the ABA content (Figure S11) in leaves decreased (18.2%) and the stomata opened (Figure S12 and S13), then E was increased by 157.3% (Figure S11) and Pn was improved (206.8%) (Figure 2e).

3.4 The dynamic process of enhancing maize drought tolerance by PNDs

Photosynthesis, Pro and ABA all could enhance maize drought tolerance, but which factor functioned first remained unknown. Thus, a series of dynamic experiments were designed to investigate the relationship between photosynthesis and signals. As shown in Figure 4a and Figure S14, compared with CK, Pn remained at higher level with PNDs, since the application of PNDs could eliminate ROS to protect photosystem.[3] Previous research reported that CDs could regulate the gene expression in maize.[40] Herein, the overexpression of ABA synthesis genes in the leaves were observed after spraying PNDs on the first day, as a result, ABA content increased (Figure 4b). The content of Pro in leaves also increased, because the increase of photosynthesis would provide NADPH and ATP for the energy in synthesis of Pro[35,36] (Figure 4b). Then, the transporter gene of them was upregulated, and the transport of ABA and Pro from leaves to roots had begun (Figure 4c). On the fifth day, the content of ABA and Pro in the roots started to increase, which could modulate AQP genes expression and osmotic pressure to promote water uptake in roots for plant drought tolerance (Figure 4d).[38.37] On the seventh day, the drought stress of plants was reduced, and signals were transmitted from the roots to the leaves (Figure 4e), decreasing the synthesis of ABA content in the leaves, opening stomata, and

increasing E (Figure 4f). Overall, these results demonstrate that foliar sprayed PNDs can firstly promote photosynthesis, induce the synthesis of signal substances (ABA and Pro) in the leaves, and then they were transported from the leaves to the roots (Figure 4 and S14). The ABA and Pro accumulated in roots can up-regulate AQP and cause low osmotic pressure.[37,38] Thus, water uptake of roots was promoted and drought stress was alleviated. The weakened drought signal was transmitted from the roots back to the leaves, reducing the ABA synthesis in the leaves, opening the stomata, increasing the E, and promoting growth (Figure S14 and S15).

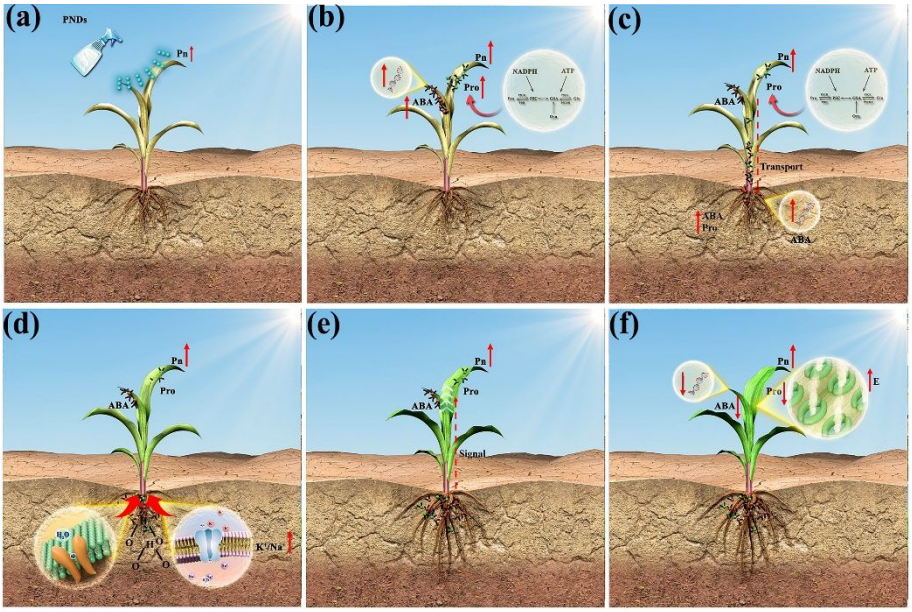

Figure 4. The dynamic process of PNDs enhanced maize drought tolerance. (a), improving Pn; (b), synthesizing signal molecules; (c), signal transport from shoots to roots; (d), increasing water uptake; (e), transmitting weakened drought signal from the roots back to the leaves; (f), opening the stomata, increasing the transpiration rate, and returning to normal growth.

3.5 Molecular mechanism of enhancing maize drought tolerance by PNDs

As discussed above, PNDs could enhance maize drought tolerance through scavenging ROS, improving photosynthesis, and inducing ABA and Pro. In order to explore the molecular mechanism of these processes, transcriptomic and metabolomic profiles in maize were investigated after foliar spraying PNDs. In general, 6811 genes (3115 up-regulation, 3696 down-regulation) were discovered to be changed significantly after PNDs (5 mg·L^{-1}) exposure

(Figure S16). The differentially expressed genes (DEGs) in maize treated with foliar-sprayed PNDs were already enriched in signal response pathway (Figure S17 and S18).

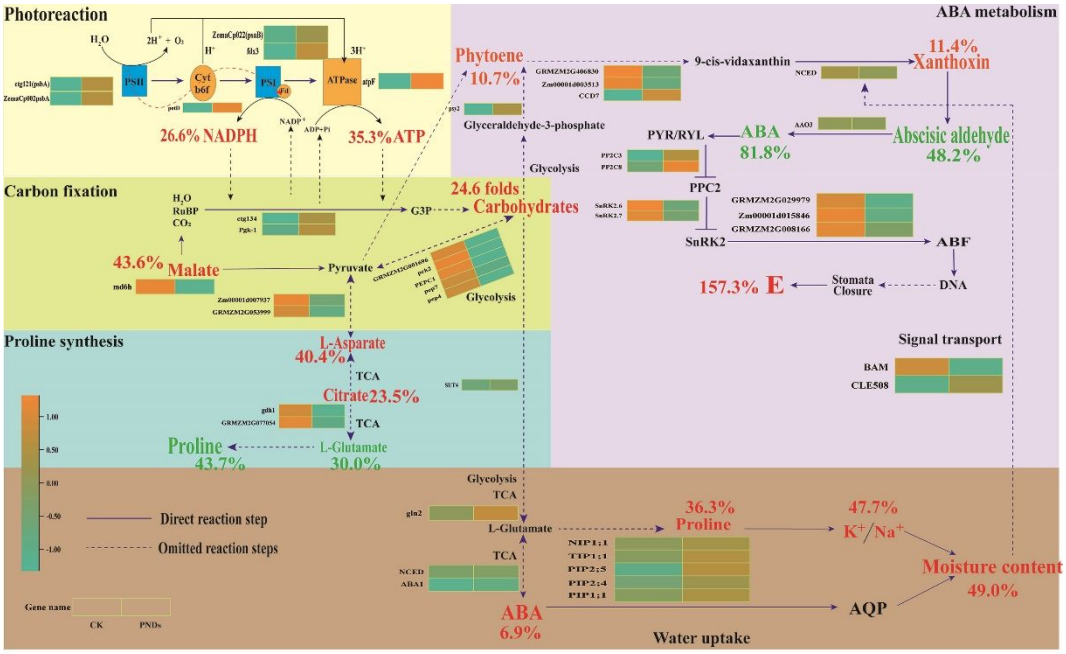

Figure 5. Molecular mechanism of enhancing maize drought tolerance after foliar spraying PNDs. Significant increase ($p < 0.05$) and significant decrease ($p < 0.05$) are highlighted with red and green texts, respectively. Orange represents no statistical difference.

As well known, photosynthesis is essential for plant growth. Previous studies demonstrated photosynthesis would be limited under abiotic stresses.[2,3] Therefore, increasing photosynthesis of plants can enhance their ability to resist adverse stresses. As shown in Figure 5, over-expression of *ctg121 and ZemaCp002* (21.4~55.1 folds) contributed to the D1 protein synthesis, which could maintain the stability of PSII.[5] Expression of *petD*, *ZemaCp022*, *fdx3*, *atpF* located on Cyt b6f, PS Ⅰ and ATPase were up-regulated by 2.2~33.4%. Thus, PNDs exposure could enhance the photo reaction activity under drought stress, causing more energy release (NADPH and ATP, 26.6% and 35.3%). The photo reaction and dark reaction are closely connected. The NADPH and ATP produced in the photo reaction can be used as energy to participate in the Calvin cycle (carbon fixation).[41] Key genes for CO_2 fixation, such as *ctg134* and *Pgk-1*, were up-regulated significantly (76.9 and 18.3 folds). Besides, the content of carbohydrates increased by 24.6 folds. Notably, foliar sprayed PNDs also up-regulated (2.3 folds) the transport gene

(*SUT4*), which improved transportation of carbohydrate from shoots to roots, and further promoted the roots growth (Figure 5 and S19).[30] Carbohydrate could also provide precursor substances for synthesizing signal substances through glycolysis and tricarboxylic acid cycle (TCA) in the roots.[42]

Signals including Pro and ABA are essential for plant in response to abiotic stresses.[10] Pro can be as an osmotic pressure regulator in roots to maintain cell stability on abiotic stress, [10] which increased ratio of K^+/Na^+ (47.7%) for low osmotic pressure (Figure 3c and 5). The precursor (L-glutamate) synthetic gene (*gln2*) of Pro increased by 2.7 folds, suggesting that Pro was accumulated in the roots. A recent study revealed that over-expression of *NCED, ABA1* and *AAO3* could increase ABA content in the plant.[43] ABA accumulation in roots could upregulate AQP gene expression in maize and *Arabidopsis*, respectively.[44] In our case, the major AQP genes (*PIP1;1, PIP2;4, PIP2;5, TIP1;1* and *NIP1;1*) related to water uptake were over-expressed by 2.3~7.6% (Figure 3g and 5). These results showed that foliar-applicated PNDs increased water uptake (89.3%) of maize roots through accumulating Pro (36.3%) and ABA (6.9%). Thus, water uptake alleviated the drought stress and helped crops normal growth.

Root-to-shoot signaling is important for plant to adapt water-deficient conditions.[45] The drought signal generated in the roots can regulate the synthesis of ABA in shoots.[45] However, no mobile signaling molecules have yet been identified that can trigger ABA change in maize shoots. Fuminori *et al.* proved the small peptide CLAVATA3/EMBRYO-SURROUNDING REGIONRELATED 25 (CLE25) in *Arabidopsis* can transmit the drought stress signal from the roots to the shoots through the vascular tissue, thereby regulating the biosynthesis of ABA and the closure of stomata in the shoots.[45] By comparing the homologous gene families between *Arabidopsis* and maize, the polypeptides produced by these families that function as signal molecules were investigated.[46] Herein, we screened out the only significant change in the homologous gene CEL508. Particularly, the expression of the receptor gene BARELY ANY MERISTEM (BAM) also changed significantly (sole receptor).[47] On this basis, we speculated

that the signal molecular pathway that can transport ABA signals from roots to shoots for long distances was CEL508-BAM in maize, which might be worth further investigation in a separate study.

Genes related ABA-synthesis in shoots, including *GRMZM2G406830*, *Zm00001d003513*, *NCED, AAO3*, were down-regulated by 63.2~87.5% (Figure 5 and S19). Some proteins like phytoene and xanthoxin (Figure 5) involved in synthesis increased by 10.7% and 11.4% respectively. ABA synthetic intermediates (abscisic aldehyde) also decreased by 48.2%. CLE peptides can induce expression of *NCED* gene that encodes a key enzyme for ABA synthesis under water-deficient stress in shoots.[47] In Figure S19, the overexpression of CLE508 gene (25.5 folds) may positively respond to drought stress of maize. However, the degree of drought stress on maize was reduced and the gene expression of BAM (the receptor that binds to CLE) was decreased (by 68.1%) since the water uptake increased. Therefore, the amount of CEL transported to the shoots was decreased, which down-regulated the expression of NCED, and thereby ABA synthesis was reduced. ABA can be involved in the opening of stomata by regulating DNA, and increased opening of stomata renders a greater transpiration rate in plants.[47] In the downstream pathway of stomata opening mediated by ABA, expression of *PP2C3* and *PP2C8* were up-regulated by 2.16~3.12 folds. Furthermore, expression of *SnRK2.6*, *SnRK2.7*, *GRMZM2G029979*, *Zm00001d015846*, *GRMZM2G008166* related to decomposition of ABA were improved by 50.3~82.3% (Figure 5 and S19). The pathway of ABA metabolism after foliar-spraying PNDs could support the above findings on the decreasing content of ABA in shoots under drought stress. In addition, the content of Pro in the shoots also decreased (43.7%). L-glutamate as a substrate for Pro synthesis decreased by 30.0% because glutamate synthetase gene (*GRMZM2G077054*) down-regulated by 72.6%. In general, foliar application of PNDs would enhance the ability of plants to resist abiotic stress by regulating signal synthesis and transduction. However, it is unknown whether Pro synthesis in the shoots is also regulated by signals from the roots, which is similar to the long-distance regulation of ABA signals.

3.6 Correlation network analysis

Towards understanding the metabolic scenario of the drought tolerance mechanism, we performed gene and metabolites interaction network analyses (targeting genes with |log2FC| ≥ 1 and metabolites with VIP ≥1 in response to drought stresses).[43] The correlation network consisted of 54 nodes (genes and metabolites) and 116 edges (Figure S20 and Figure 6). These nodes were assigned to genes, metabolites and others factors (E, K^+/Na^+, and moisture). When the distribution of nodes was modularized, all nodes were classified as five major modules (five metabolic pathways). The most highly connected pathway could potentially act as the keystone pathway that could be considered as major influencer in the formation of signal path response to drought.[44] Therefore, the carbon fixation and ABA metabolism had high connectivity (Pi=0.67), illustrating that ABA metabolism was closely related to carbohydrates. Carbohydrates can participate the reactions of other pathways through glycolysis and TCA, and provide substrates for the synthesis of signal substances.[48] Notably, 11 nodes were defined as highly connected (bigger nodes), representing the intermediate correlation factors.[49] For example, citrate was a key substance in TCA, which was involved in the syntheses of different substances through participating the metabolism of other pathways.[50] Surprisingly, most correlations observed between genes and metabolites were positive. Such phenomenon suggests specific interactions in different metabolic pathways. [51] Some signal molecules can positively induce the expression of resistance genes, thereby increasing the ability of plants to resist stress.[52] Therefore, foliar application of PNDs could increase maize drought tolerance by regulating the signal molecular pathways, which illustrated PNDs had great potential in responding to global climate change, and can be a paradigm for further development of engineered nanomaterials for agriculture.

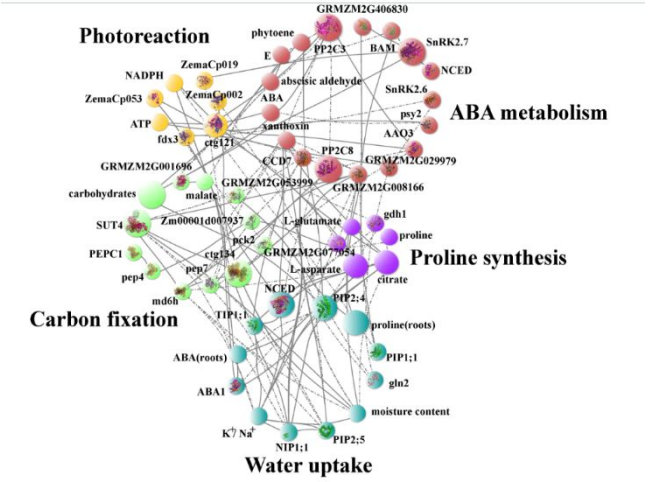

Figure 6. Correlation network showing genes and metabolites correlated to foliar sprayed PNDs under drought. The solid lines represent significant positive correlations ($p \leq 0.05$). The dashed lines represent significant negative correlations ($p \leq 0.05$).

4. CONCLUSIONS

The synthesized PNDs (size, 2.8±0.4 nm, N doping and negative charge, –15.04 mV) could enhance maize drought tolerance. Foliar-applied PNDs could promote the photosynthesis and growth of maize under drought stress. This is particularly evidenced by the significant increase of FW and DW in shoots and roots upon exposure to PNDs (5 mg·L^{-1}). Also, through the detailed investigations we found that PNDs could improve photosynthesis through scavenging ROS. Moreover, foliar-applied PNDs could promote water uptake by increasing ABA and Pro levels in roots, resulting in up-regulated AQP and high osmotic pressure. These results demonstrated PNDs can enhance maize drought tolerance through multiple pathways including modulating photosynthesis and plant signal molecules. Our identifications of the molecular pathways, regulation patterns of the related genes, and correlations between the genes and metabolites, inform upon the key influencers in the resistance mechanism on the molecular level. Therefore, PNDs have great potential in responding to global climate change, and can be a paradigm for further development of engineered nanomaterials for agriculture.

CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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