

Article

Exogenous DCPTA Increases the Tolerance of Maize Seedlings to PEG-Simulated Drought by Regulating Nitrogen Metabolism-Related Enzymes

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Abstract: 2-(3,4-Dichlorophenoxy) triethylamine (DCPTA) regulates plant development; however, the molecular basis of this regulation is poorly understood. In this study, RNA sequencing (RNA-seq) analysis and physiological indexes of maize seedlings (three-leaf stage) treated with 15% polyethylene glycol (PEG) with/without DCPTA were investigated to explore the possible mechanism of exogenous DCPTA-improved drought tolerance. In the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG, totals of 19, 38 and 20 differentially expressed genes (DEGs) were classified as being involved in metabolic processes, respectively; totals of 5, 11, and 6 DEGs were enriched in the nitrogen (N) metabolic pathway, respectively. The genes encoding nicotinamide adenine dinucleotide-nitrate reductase (NADH-NR), *ferredoxin*-nitrite reductase (Fd-NiR), reduced ferredoxin- glutamate synthase (Fd-GOGAT), and chloroplastic *glutamine synthetase* (GS 2) were common in response to PEG-simulated drought stress with/without DCPTA treatment. Moreover, DCPTA maintained stable gene relative expression levels and protein abundances of NADH-NR, Fd-NiR, GS2, and Fd-GOGAT. Moreover, exogenous DCPTA partially mitigated PEG-simulated drought-induced reductions in the enzymatic activities of NR, nitrite reductase (NiR), glutamine synthase (GS), glutamine oxoglutarate aminotransferase (GOGAT), and transaminase, as well as in the contents of nitrate (NO₃⁻), nitrite (NO₂⁻) and soluble proteins and increases in the contents of ammonium (NH₄⁺) and free amino acids. Together, our results indicate that exogenous DCPTA improved plant growth and drought tolerance by regulating N-mechanism enzymatic activities involved in transcription and enzymatic protein synthesis.

Keywords: DCPTA; maize seedlings; drought tolerance; RNA-seq; N metabolism; enzymatic activities

1. Introduction

Worldwide food security has been threatened by the continuously increasing population and ongoing climatic change [1]. Because of drought, approximately 15% of the potential maize (*Zea mays* L.) yield is lost annually [2]. Thus, increasing the drought tolerance of maize is urgently needed to guarantee global food security [3].

Plant growth and productivity are largely determined by nitrogen (N) metabolism [4]. As an essential macronutrient, N is a major component of a variety of cellular metabolites, including photosynthetic pigments [5]. Moreover, most plant drought-responsive physiological processes involve N metabolism, such as the protection of subcellular structures and stabilization of internal metabolic processes [6]. Although several forms of N are available for maize, nitrate (NO₃⁻) is the predominant

N source [7]. NO_3^- is converted to nitrite (NO_2^-) and further reduced to ammonium (NH_4^+). The two successive reactions require reductants generated by respiration or photosynthesis and are catalysed by nitrate reductase (NR) and nitrite reductase (NiR), respectively [8]. The majority of NH_4^+ is subsequently used for glutamine formation by the glutamine synthase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle in which reduced ferredoxin (Fd) or NADH is used as a reductant [9]. Consistent with the variety of metabolic roles and different specificities towards the reductants, NiR, GS, and GOGAT are present in maize in several isoforms; specifically, there are two types of NiR isoforms (Fd-NiR and NADH-NiR), two types of GOGAT isoforms (Fd-GOGAT and NADH-GOGAT), and two types of GS isoforms (cytosolic and chloroplastic GS-GS1 and GS2) [10,11]. In maize, the GS1 isoforms are encoded by five genes, and the GS2 isoforms are encoded by a single gene [12]. Glutamate is the N donor used for transamination reactions, which are catalysed mainly by alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plants [13,14].

Numerous studies have shown that drought strongly influences N metabolism, such as by disrupting NO_3^- uptake and/or long-distance transport, and influences N assimilation-related enzyme activity [15–17]. These phenomena could partly explain the plant growth inhibition under drought stress.

Various attempts have been made to increase maize drought tolerance. Plant growth regulators have been widely applied to prevent or minimize the negative influence induced by stress on plants [18]. 2-(3,4-Dichlorophenoxy)triethylamine (DCPTA) can accelerate plant growth [19,20], increase root development [21], increase chloroplast volume, increase leaf photosynthetic enzyme activity [22], promote CO_2 fixation [23], increase photosynthetic pigment biosynthesis [24], and stimulate carotenoid biosynthesis [25]. However, the molecular basis of the DCPTA effect on plants and its integrated effects on N metabolism under stress conditions remain unclear.

Our previous study demonstrated that DCPTA applications could increase the drought tolerance of maize by improving the photosynthetic capability [26], modulating the antioxidant system [27], and ameliorating the soil drought effects on N metabolism in maize during the pre-female inflorescence emergence stage [28]. To further investigate the possible mechanism of the exogenous DCPTA-promoted drought tolerance of maize seedlings, this work was designed to address the following questions: (1) How do transcripts change in maize seedlings in response to drought stress and/or exogenous DCPTA? (2) What metabolic pathway does DCPTA regulate under drought stress?

2. Materials and Methods

2.1. Material and Growth Conditions

The experiment was carried out in a controlled growth chamber at Northeast Agricultural University, Harbin, China. Maize (*Zea mays* L.) seeds of inbred line Chang 7–2 were kindly donated by the Henan Academy of Agricultural Sciences, China. DCPTA was obtained from China Zhengzhou Zhengshi Chemical Company, Ltd.

After germination for 72 h at 28 °C in the dark, the seeds were placed uniformly in plastic containers (inner length, 50 cm; width, 30 cm; and height, 18 cm; totals of 50 plants/container) that contained 10 L of 1/2-strength modified Hoagland's nutrient solution (H-NS), containing 6.0 mM KNO_3 , 4.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.0 mM KH_2PO_4 , 0.05 mM KCl , 1.0 mM MgSO_4 , 0.02 mM Fe-EDTA , 25 μM H_3BO_3 , 0.2 μM MnSO_4 , 0.2 μM ZnSO_4 , 0.05 μM $(\text{NH}_4)_2\text{MoO}_4$, and 0.05 μM CuSO_4 . The pH of the nutrient solution was adjusted to 6.0 by the addition of 0.1 mol/L NaOH or HCl and was continuously aerated. The ambient conditions were as follows: the temperature was 25/18 °C (day/night); the relative humidity was 60%–70%; and light intensity and duration were 350 $\mu\text{mol}/\text{m}^2/\text{s}$ and 16 h per day, respectively.

2.2. Experimental Design and Sampling

Maize seedlings at the three-leaf stage were exposed to the following treatments: (1) control = 1/2-strength H-NS, (2) PEG = 1/2-strength H-NS containing 15% (*w/v*) PEG-6000, (3) DCPTA = 1/2-strength H-NS containing 15 mg/L DCPTA, and (4) PEG + DCPTA = 1/2-strength H-NS containing 15% (*w/v*) PEG-6000 and 15 mg/L DCPTA. The experiment was repeated five times, and container positions were randomised.

After 24 h of PEG and/or DCPTA treatment, the 3rd leaf (numbered basipetally) from 3 plants of each treatment was sampled randomly from 10:30 to 11:00 a.m., frozen in liquid N for 15 min and then placed at -80°C used for RNA sequencing (RNA-seq) analysis. The 3rd leaf was sampled from 6 plants of each container, divided into 2 equal parts along the direction of venation. One of the half leaves was used for quantitative real-time polymerase chain reaction (qRT-PCR), and the other half leaves was used for Western blot analysis. Subsequently, the whole 3rd leaf was sampled and stored in the same manner used for determination of physiological indexes. After 7 days of PEG and/or DCPTA treatment, 10 plants were harvested from each container for the determination of the relative growth rate (RGR).

2.3. Relative Growth Rate (RGR)

The harvested seedlings were divided into roots and leaves, oven dried at 105°C for 30 min, and then heated at 80°C for 24 h. The RGR was determined as follows: $\text{RGR} = [\ln(\text{final dry weight}) - \ln(\text{initial dry weight})]/(\text{duration of treatment days})$ [29]. The mean values of 10 plants were considered one replication.

2.4. Ribonucleic Acid (RNA) Extraction and Sequencing

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the samples. An Illumina HiSeq 2000 instrument with a 100 bp paired-end protocol was used for RNA-seq at GENEWIZ Biotechnology Co., Ltd. (Suzhou, China). The control, DCPTA, PEG and DCPTA + PEG treatments generated 34,345,494; 30,834,696; 34,282,646; and 40,784,960 raw reads, respectively, and 28,503,138; 26,180,040; 28,759,526; and 34,278,670 clean reads, respectively, after the adaptor sequence and low-quality reads were filtered and removed. The clean reads of each sample were then mapped to the maize genome (<http://www.plantgdb.org>) using Burrows-Wheeler Alignment (BWA-0.7.4). The clean read statistics of the RNA-seq and mapping parameters are shown in Table 1.

Table 1. Statistics of clean reads in RNA-seq and mapping parameters.

Sample	Length	Total Reads	Total Mapped	Uniquely Mapped	Mapped (%)	Unique Mapped (%)	Seq Depth
Control	142.98	28,503,138	23,512,659	21,635,820	82.49%	75.91%	2.57
DCPTA	141.32	26,180,040	21,689,777	19,780,924	82.85%	75.56%	2.30
PEG	140.78	28,759,526	23,719,008	21,795,481	82.47%	75.79%	2.57
PEG + DCPTA	141.45	34,278,670	28,164,459	25,444,256	82.16%	74.23%	2.03

The nonparametric algorithm (*NOIseq-real*) package (<http://bioinfo.cipf.es/noiseq/>) was used to statistically identify the differentially expressed genes (DEGs) according to the provided instructions. The DEGs revealed from 5 library pair comparisons including DCPTA vs. the control, PEG vs. the control, PEG + DCPTA vs. the control, PEG + DCPTA vs. PEG, and PEG + DCPTA vs. DCPTA were identified. A Venn diagram tool (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) was used to determine the up-/downregulated transcripts. Blast2go (<http://www.blast2go.org/>) and GO-TermFinder (0.86) based on the BLASTx results were used for Gene Ontology (GO) enrichment analysis of the DEGs. The genes were assigned to metabolic pathways by the KEGG Automatic Annotation Server (KAAS; <http://www.genome.jp/tools/kaas/>; <http://www.genome.jp/kegg/>) databases, as described by Wei et al. [30].

2.5. qRT-PCR

The qRT-PCR experiments were conducted on the 4 common DEGs in the library comparisons of PEG vs. the control and PEG + DCPTA vs. PEG related to the N metabolism selected based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses: ZmNADH-NR (id: XM_008680224.1), ZmFd-NiR (id: XM_008648080.1), ZmFd-GOGAT (id: NM_001112223.1), and ZmGS2 (id: NM_001111973.1). The total RNA from the collected samples was extracted using a TransZol™ Up Plus RNA Kit (TransGen Biotech, Beijing, China). After measuring the purity and concentration of RNA using an ultraviolet spectrophotometer, the total RNA was reverse-transcribed using a TransScript® One-Step gDNA Removal Kit and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The gene-specific primers used for qRT-PCR are shown in Table 2. The synthesis of cDNA and real-time PCR was performed as described by Liu et al. [31]. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative transcript levels.

Table 2. Gene-specific primers used for qRT-PCR.

Gene	Gene ids	Forward Sequence	Reverse Sequence
ZmNADH-NR	XM_008680224.1	5'-CTACCATTCAAGGACAACCG-3'	5'-GCGTCGTTATACCGAGTTT-3'
ZmFd-NiR	XM_008648080.1	5'-CAAGTGGCTCGGCTCT-3'	5'-GCGTCTGCTCGCTGTC-3'
ZmFd-GOGAT	NM_001112223.1	5'-ACGCAGCACATTGATCTGGATAC-3'	5'-TGTCTCCATACCTCTTGGAATCA-3'
ZmGS2	NM_001111973.1	5'-CAGGAAGGGGCAGAACATA-3'	5'-CTGAGGGCCAGGGTACG-3'
ZmActin	NC_008332.1	5'-ACATGCGCCTAAGGAGAAATAG-3'	5'-ACCTCCATGCTCACTGGTACTT-3'

2.6. Western Blot Analysis

After washing the leaves with phosphate-buffered solution (PBS, pH 7.3), proteins were extracted from the samples with lysis buffer (100 mmol/L Tris-HCl, pH 7.0, 40 mmol/L β -mercaptoethanol, 10 mmol/L $MgCl_2$, 100 mmol/L KCl, 15% glycerol). Following centrifugation (13,000 \times g for 25 min, 4 °C), the protein content was detected via a BCA protein assay (Thermo, Massachusetts, USA). 10 μ L of each about 500 ng protein was boiled in loading buffer (100 mmol/L Tris-HCl, pH 6.8, 4% (*w/v*) SDS, 10% (*v/v*) β -mercaptoethanol, 0.2% (*w/v*) bromophenol blue, 20% (*v/v*) glycerol), subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with nonfat dry milk (5% M/V) in Tris-buffered saline containing Tween-20 (TBST) for 1 h. The blots were then incubated for 24 h at 4 °C with anti-NADH reductase, anti-Fd-NiR, anti-GS2, and anti-Fd-GOGAT (1:500, Abcam, Shanghai, China) as primary antibodies. The blots were subsequently washed four times for 5 min each in TBST at room temperature and then incubated for 60 min in peroxidase-labelled goat anti-rabbit antibody IgG-HRP at a dilution ratio of 1:10,000 (Abcam, Shanghai, China) in TBST. The membranes were visualized by enhanced chemiluminescence (ECL kit, Thermo, Wilmington, DE, USA) and detected using a bioimaging system (MicroChemi 4.2 Chemilumineszenz-System, Tel Aviv, Israel). The film was scanned, and the optical density value of the target strip was analysed using a gel image processing system (Gel-Pro Analyzer software, Media Cybernetics, Inc., Rockville, MD, USA).

2.7. Enzymic Activities

The activities of NR, NiR, GS, and GOGAT were determined as described by Barro et al. [32], Ida and Morita [33], O'neal and Joy [34], and Groat and Vance [35], respectively. The ALT and AST activities were determined according to the methods of Jia et al. [36]. Enzymic activity was expressed as micromoles per gram per minute.

2.8. The Contents of Foliar NO_3^- , NO_2^- , NH_4^+ , Proteins, and Amino Acids

The foliar NO_3^- content was determined via the salicylic acid method described by Cataldo et al. [37]. The contents of NO_2^- , NH_4^+ and proteins were determined as described by Barro et al. [32], Bräutigam et al. [38] and Bradford [39], respectively.

Amino acid determination was performed according to a description by Du et al. [40]. The samples were homogenized with 2% (*w/v*) sulfosalicylic amino acid. The pH of the homogenate was adjusted to 2.0 (± 0.05) with 0.02 mol/L HCl, after which the samples were centrifuged ($10,000\times g$ for 10 min, 4 °C), and the supernatants were used for analysis. The amino acid content was determined using an amino acid analyser (H835-50, Hitachi, Tokyo, Japan).

2.9. Statistical Analysis

The data were analysed via SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), and the results are presented as the means (\pm SDs) of five replications. Origin 9.0 was used to create the figures. The NOISeq method was used to assess the DEGs in the library pair comparisons using the following criteria as defaults: fold-change ≥ 2.0 and diverge probability ≥ 0.8 .

3. Results

3.1. RGR

After seven days of exposure to PEG-simulated drought, the plants exhibited growth inhibition, which was partially alleviated by exogenous DCPTA, which also exerted a positive influence on the root growth of maize seedlings under non-stress conditions (Figure 1, Table 3). Compared with that in the control treatment, the RGR of the shoots and roots in the PEG treatment decreased by 35.26% and 28.72%, respectively, and that in the PEG + DCPTA treatment decreased by 21.60% and 16.81%, respectively; moreover, the root RGR in the PEG + DCPTA treatment increased by 7.50%.

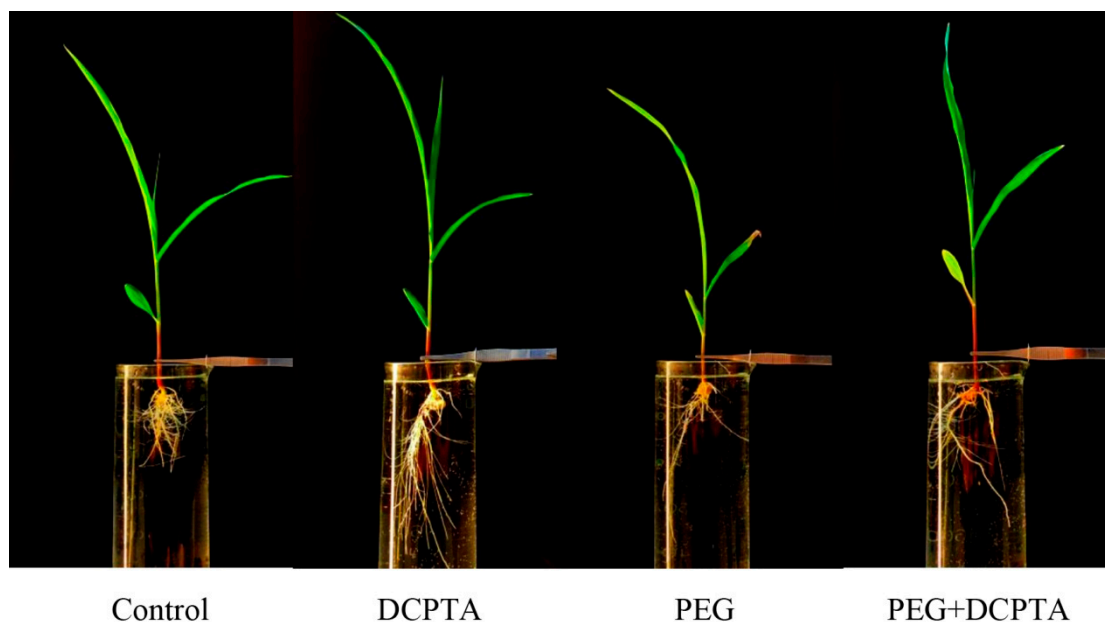


Figure 1. Phenotypes of the maize seedlings after seven days of treatment with polyethylene glycol (PEG) and/or 2-(3,4-Dichlorophenoxy)triethylamine (DCPTA).

Table 3. Effects of polyethylene glycol (PEG) and/or 2-(3,4-Dichlorophenoxy)triethylamine (DCPTA) treatment on the relative growth rate (RGR) of the shoots and roots of maize seedlings after 7 days of PEG and/or DCPTA treatment.

Treatment	Shoots	Roots
Control	3.98 ± 0.20 a	3.95 ± 0.17 b
DCPTA	4.03 ± 0.17 a	4.24 ± 0.13 a
PEG	2.58 ± 0.26 c	2.81 ± 0.17 d
PEG + DCPTA	3.12 ± 0.14 b	3.28 ± 0.14 c

The values represent the means ±SEs (n = 50). The values with the same letters in the columns are not significantly different at p < 0.05, and the differences were determined by the least significant difference (LSD) test.

3.2. Transcriptome Analysis

To identify DEGs putatively affected by DCPTA and/or PEG treatment, an RNA-seq analysis was performed. Compared to those in the control treatment, 149 and 336 genes in DCPTA treatment were up- and downregulated, respectively; 220 and 240 genes in the PEG treatment were up- and downregulated, respectively; and 174 and 286 genes in PEG + DCPTA treatment were up- and downregulated, respectively. Compared with those in the PEG treatment, 265 and 209 genes in PEG + DCPTA treatment were up- and downregulated, respectively, and compared with those in the DCPTA treatment, 254 and 265 genes in PEG + DCPTA treatment were up- and downregulated, respectively (Figure 2A). As a potential response to environmental changes, 22 and 64 genes were found to be commonly up- and downregulated in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. the control, respectively (Figure 2B). There were 15 and 33 genes commonly up- and downregulated, respectively, in both the DCPTA treatment and the PEG + DCPTA treatment and 18 and 14 genes commonly up- and downregulated, respectively, in both the PEG treatment and the PEG + DCPTA treatment (Figure 2C).

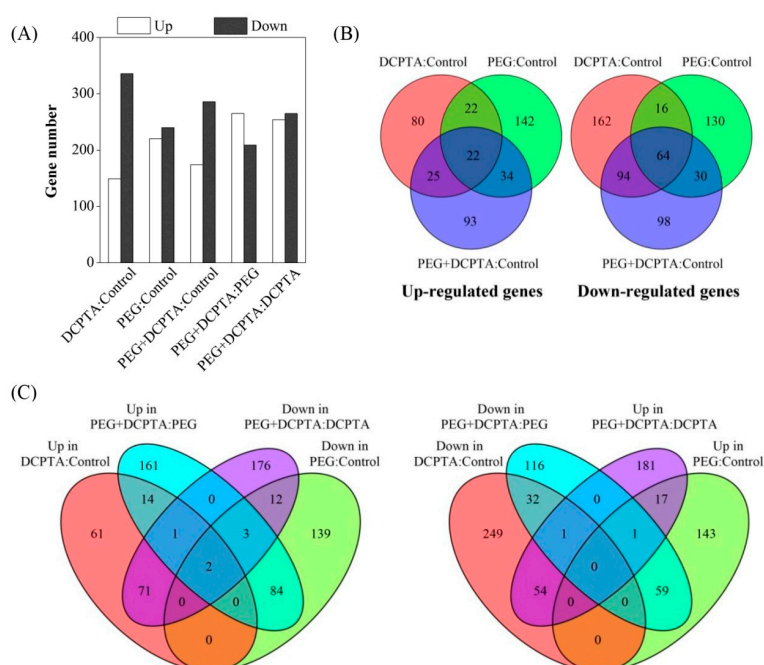


Figure 2. Analysis of the RNA-seq data. (A) Number of DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, PEG + DCPTA vs. the control, and PEG + DCPTA vs. PEG. (B) Numbers of common DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG. (C) Comparison of the number of DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG according to a Venn diagram.

To classify the functions of the DEGs and identify the major metabolic pathway affected by DCPTA and/or PEG treatment, GO analysis and KEGG analysis were performed. GO analysis showed that all DEGs could be categorized into 19 functional groups. There were 7, 4 and 8 functional groups in the following three main categories, respectively: biological process, cellular component and molecular function (Figure 3). Moreover, in the biological process category, the DEGs were enriched mainly in the metabolic process (GO:0008152), as there were 19, 38 and 15 DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control and PEG + DCPTA vs. PEG, respectively.

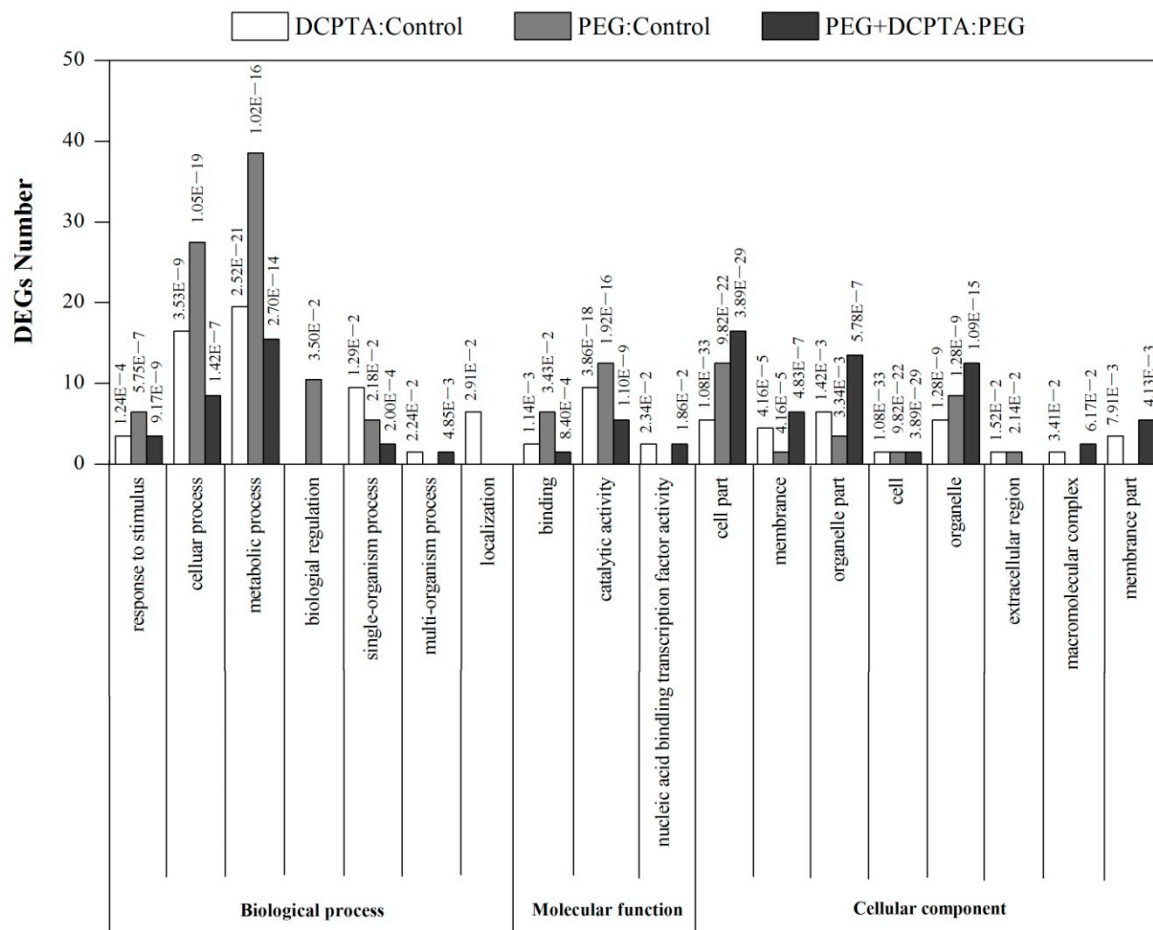


Figure 3. GO analysis of DEGs (fold change > 2) in response to PEG-simulated drought and/or exogenous DCPTA. The values on the bars are *p* values of enriched DEGs population vs. the background population of all genes in the corresponding GO term.

KEGG analyses revealed that the DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG were enriched in 16, 22, and 15 pathways, respectively (Tables S1–S3). Each library pair comparison had DEGs enriched in the N metabolism pathway (KO00910): there were 5, 11, and 6 DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG, respectively. The common DEGs in the library pair comparisons of PEG vs. the control and PEG + DCPTA vs. PEG included ZmNADH-NR (id: XM_008680224.1), ZmFd-NiR (id: XM_008648080.1), ZmFd-GOGAT (id: NM_001112223.1), and ZmGS2 (id: NM_001111973.1), and these DEGs were selected as candidate genes for qRT-PCR.

3.3. Relative Expression Levels of Genes Encoding N Metabolism-Related Enzymes

Based on results of RNA-seq analysis, NR, NiR, GOGAT, and GS were selected as candidate enzymes for the subsequent study. The enzymatic transcriptional results showed the same variation

trend with respect to PEG and/or DCPTA treatment. Compared to those in the control treatment, the relative expression levels of NADH-NR, Fd-NiR, Fd-GOGAT, and GS2 in the PEG treatment were downregulated by 66.78%, 34.95%, 54.45%, and 37.37%, respectively, and those in the PEG + DCPTA treatment were downregulated by 19.02%, 15.96, 21.53%, and 18.38%, respectively (Figure 4). Moreover, the relative expression levels of NADH-NR and Fd-NiR were upregulated by 26.38% and 17.58% in DCPTA treatment compared to those in the control treatment.

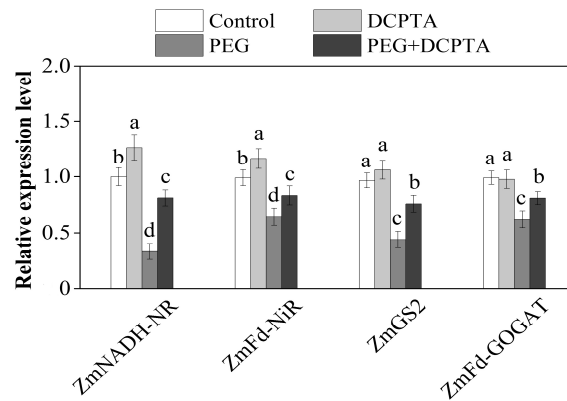


Figure 4. Effects of PEG and/or DCPTA treatment on the relative expression levels of ZmNADH-NR, ZmFd-NiR, ZmFd-GOGAT and ZmGS2 in the leaves of maize seedlings. The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

3.4. Protein Abundance of Genes Encoding N Metabolism-Related Enzymes

Compared to those in the control treatment, the protein abundances of NADH-NR, Fd-NiR, Fd-GOGAT, and GS2 in the PEG treatment were downregulated by 64.93%, 41.76%, 52.89%, and 50.30%, respectively, and those in the PEG + DCPTA treatment were downregulated by 24.85%, 15.29%, 22.36%, and 33.92%, respectively (Figure 5, Table S4). The protein abundances of NADH-NR and Fd-NiR were upregulated by 19.44% and 20.59%, respectively; however, exogenous DCPTA had no significant effect on the protein abundances of Fd-GOGAT and GS2 under non-stress conditions.

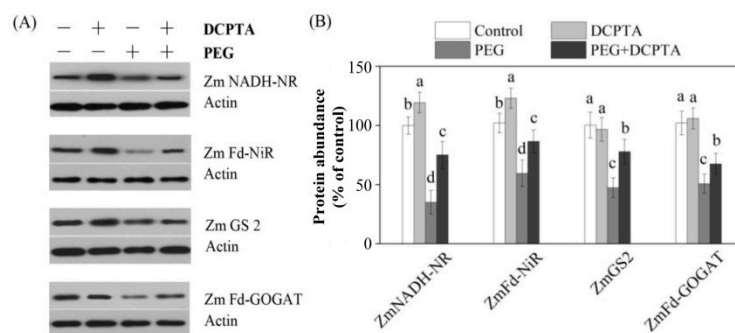


Figure 5. (A) Western blotting of ZmNADH-NR, ZmFd-NiR, Zm-GS2, and ZmFd-GOGAT protein abundances. (B) The protein abundances of ZmNADH-NR, ZmFd-NiR, Zm-GS2, and ZmFd-GOGAT were detected by Western blot analysis (% of control). The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

3.5. Activities of N Metabolism-Related Enzymes

Exogenous DCPTA reduced the effects of PEG-simulated drought stress on the activities of foliar NR, NiR, GS, and GOGAT. Compared to those in the control treatment, the NR, NiR, GS, and GOGAT activities in the PEG treatment decreased by 37.11%, 28.25%, 39.20%, and 39.68%, respectively, and those in the PEG + DCPTA treatment decreased by 15.91%, 11.13%, 21.54%, and 21.54%, respectively. Moreover, the NR, NiR and GS activities increased by 8.09%, 7.90%, and 10.11% in the DCPTA treatment, respectively (Figure 6).

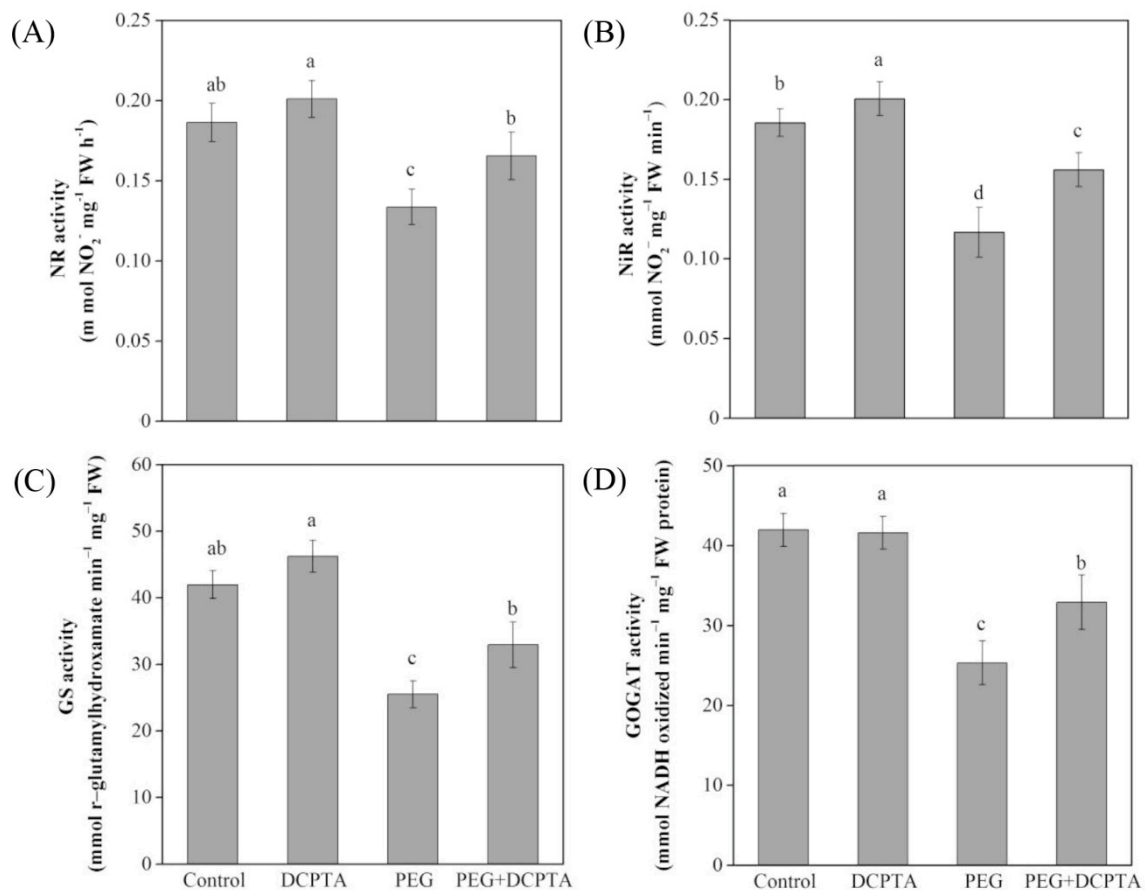


Figure 6. Effects of PEG and/or DCPTA treatment on the NR (A), NiR (B), GS (C), and GOGAT (D) activities in the leaves of maize seedlings. The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

3.6. Contents of Foliar NO_3^- , NO_2^- and NH_4^+

Exogenous DCPTA partially reversed the decline in the contents of foliar NO_3^- and NO_2^- and suppressed the rise in foliar NH_4^+ content induced by PEG-simulated drought stress (Figure 7). Compared with those in the control treatment, the contents of foliar NO_3^- and NO_2^- in the PEG treatment decreased by 43.81% and 48.54%, respectively, and those in the PEG + DCPTA treatment decreased by 20.31% and 25.52%, respectively. The foliar NH_4^+ content increased by 81.09% in the PEG treatment and by 36.10% in the PEG + DCPTA treatment. Likewise, exogenous DCPTA alone also resulted in marked increases in the contents of foliar NO_3^- and NO_2^- . Compared to those in the control treatment, the contents of foliar NO_3^- and NO_2^- in the DCPTA treatment increased by 13.66% and 5.91%, respectively.

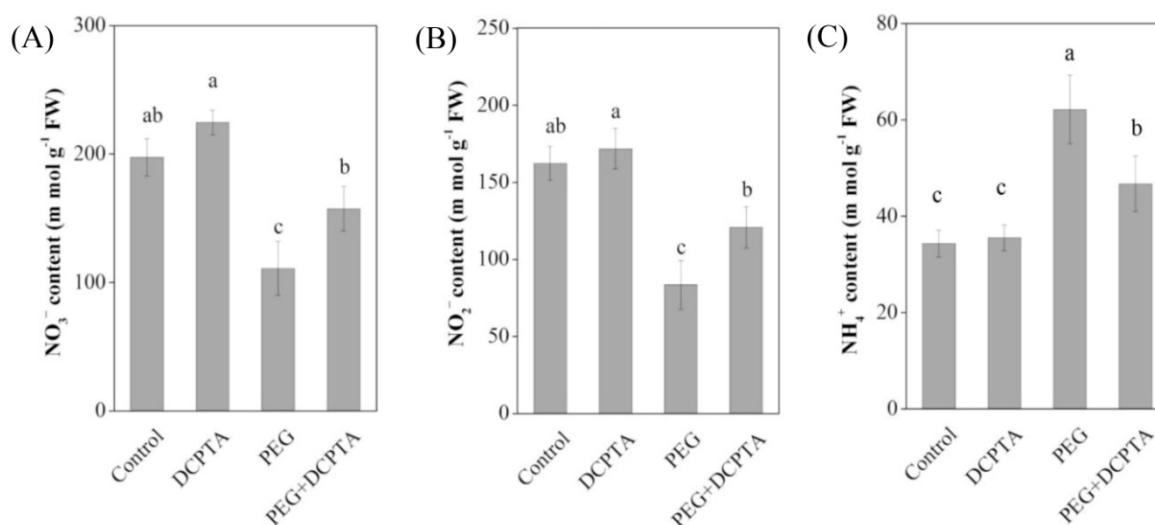


Figure 7. Effects of PEG and/or DCPTA treatment on the contents of NO_3^- (A), NO_2^- (B) and NH_4^+ (C). The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

3.7. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities

To further explore the effects of DCPTA and/or PEG treatment on transamination, activities of AST and ALT were investigated. Exogenous DCPTA decreased the activities of foliar AST and ALT induced by PEG-simulated drought stress. Compared to those in the control treatment, the AST and ALT activities in the PEG treatment decreased by 45.26% and 50.43%, and those in the PEG + DCPTA treatment decreased by 24.43% and 23.96%, respectively (Figure 8). Exogenous DCPTA alone significantly enhanced the foliar AST activity.

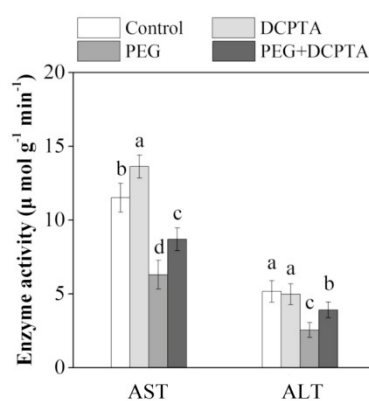


Figure 8. Effects of PEG and/or DCPTA treatment on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the leaves of maize seedlings. The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

3.8. Contents of Soluble Protein and Free Amino Acids

The contents of soluble protein and amino acids were also investigated in this study. The soluble protein content significantly decreased in the PEG treatment and remained relatively stable in the PEG + DCPTA treatment (Figure 9). Compared with that in the control treatment, the soluble protein

content in the PEG treatment decreased by 43.48%, that in the PEG + DCPTA treatment decreased by 16.39%, and that in the DCPTA treatment increased by 9.20%.

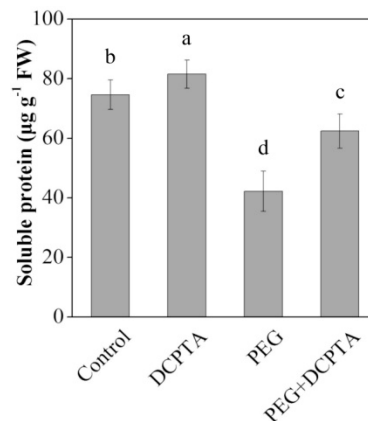


Figure 9. Effects of PEG and/or DCPTA treatment on the soluble protein content in the leaves of maize seedlings. The data represent the means of independent measurements of five biological replicates, and standard deviations are indicated by the vertical error bars. The values with the same letters on the bars are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

Exogenous DCPTA mitigated the decreases in the contents of glutamate, aspartate, alanine, cysteine, and methionine and the increases in the contents of total amino acids, serine, glycine, valine, isoleucine, leucine, tyrosine, lysine, histidine, and arginine induced by PEG-simulated drought stress; moreover, exogenous DCPTA further increased the proline content (Table 4). Significant increases in glutamate, aspartate, and alanine contents were observed in the DCPTA treatment compared with the control treatment. Exogenous DCPTA had no effect on the threonine or phenylalanine content with or without PEG-simulated drought stress.

Table 4. Effects of PEG-simulated drought and/or exogenous DCPTA on amino acids [% dry weight (DW)] in the leaves of maize seedlings.

Amino Acid (mg g ⁻¹ DW)	Treatment			
	Control	DCPTA	PEG	PEG + DCPTA
Alanine	14.34 ± 0.95 b	15.44 ± 0.45 a	7.85 ± 1.05 d	12.03 ± 0.94 c
Arginine	11.52 ± 0.53 b	11.74 ± 0.83 b	13.58 ± 0.74 a	11.93 ± 1.03 b
Aspartate	18.48 ± 1.51 b	19.85 ± 0.45 a	10.63 ± 1.01 d	13.33 ± 1.05 c
Cysteine	2.64 ± 0.21 a	2.52 ± 0.27 a	1.63 ± 0.20 c	2.10 ± 0.11 b
Glutamate	29.82 ± 0.69 b	32.10 ± 1.02 a	20.71 ± 0.95 d	26.18 ± 1.23 c
Glycine	11.67 ± 0.89 b	11.39 ± 1.09 b	18.85 ± 0.64 a	12.24 ± 0.58 b
Histidine	4.98 ± 1.03 b	5.34 ± 1.02 b	8.38 ± 1.05 a	5.37 ± 1.05 b
Isoleucine	8.19 ± 0.89 c	8.59 ± 0.89 c	10.95 ± 0.90 a	9.79 ± 0.47 b
Leucine	13.94 ± 0.92 c	14.73 ± 0.93 c	18.41 ± 0.91 a	16.20 ± 1.20 b
Lysine	7.33 ± 0.79 c	8.02 ± 0.51 c	16.84 ± 0.60 a	12.01 ± 0.98 b
Methionine	3.82 ± 0.66 b	3.69 ± 0.47 b	8.01 ± 0.27 a	4.49 ± 0.30 b
Phenylalanine	11.79 ± 0.89 b	12.61 ± 0.88 b	15.10 ± 0.88 a	14.11 ± 0.88 a
Proline	10.35 ± 0.90 c	11.06 ± 1.01 c	20.06 ± 0.94 b	24.31 ± 1.17 a
Serine	10.95 ± 0.90 c	11.57 ± 1.00 bc	14.22 ± 0.47 a	12.50 ± 1.18 b
Threonine	7.17 ± 0.99 c	7.96 ± 1.05 bc	10.03 ± 1.10 a	9.20 ± 1.05 ab
Tyrosine	4.67 ± 0.54 b	5.21 ± 0.35 b	10.08 ± 0.94 a	4.27 ± 1.05 b
Valine	10.70 ± 0.88 c	10.63 ± 0.65 c	16.65 ± 2.23 a	14.27 ± 0.72 b
Total	182.37 ± 5.12 d	192.46 ± 4.26 c	221.96 ± 5.24 a	204.33 ± 5.25 b

The values represent the means ±SEs ($n = 5$). The values with the same letters in the columns are not significantly different at $p < 0.05$, and the differences were determined by the least significant difference (LSD) test.

4. Discussion

In our previously reported results, DCPTA was proven to increase the drought tolerance of maize seedlings under hydroponic conditions [26,27]. However, the underlying molecular mechanisms remain poorly understood. In this study, transcriptome sequences of maize seedlings treated with PEG and/or DCPTA were obtained by RNA-seq technology and then mapped to the maize genome. Compared with the edgeR, DESeq, SAMseq and baySeq approaches, NOISeq is a nonparametric approach that can be used to identify DEGs; it maintains a stable false positive rate and is suited for different data of various sizes [41–43]. In this study, DEGs of each library pair comparison were statistically identified by the NOISeq method after sequencing to obtain accurate and reliable data for subsequent analyses (Figure 2).

GO analysis was performed to classify the functions of the DEGs. The results showed that the main term associated with the DEGs in the biological process category (GO:0008152) was metabolic process, which was true for 19, 38, and 20 DEGs in the library pair comparisons of DCPTA vs. the control, PEG vs. the control, and PEG + DCPTA vs. PEG, respectively (Figure 3). This indicated that the effect of PEG and/or DCPTA on seedlings may be related to metabolic processes. On the basis of the KEGG analysis, the results showed that the common enriched pathway was the N metabolism pathway (ko00910). The common DEGs in the library pair comparisons of PEG vs. the control and PEG + DCPTA vs. PEG included ZmNADH-NR, ZmFd-NiR, ZmFd-GOGAT, and ZmGS2 (Tables S1–S3). These results indicated that exogenous DCPTA promoted the drought tolerance of maize seedlings possibly via regulation of N metabolism-related enzymes.

N assimilation is crucial for plant stress tolerance [44]. In higher plants, NR uses NADH as a reductant to catalyse the conversion of NO_3^- to NO_2^- in the cytoplasm [45]. Similar to that which occurs in tobacco, PEG-simulated drought stress inhibited NR activity and gene relative expression levels in maize leaves (Figure 4, Figure 6) [46]. Significant reductions were also noted in the enzymatic protein relative expression level of NADH-NR in response to PEG, which may be attributed to excessive levels of reactive oxygen species (ROS) induced by stress (Figure 5) [47]. Moreover, the negative effects of PEG-simulated drought stress on foliar NADH-NR activity was ameliorated to some extent by exogenous DCPTA, and the increased NR activity matched the increase in the corresponding gene relative expression levels and protein abundances of NADH-NR. These data show that exogenous DCPTA affected NR-involved mRNA levels and enzymatic protein synthesis rather than simply causing enzymatic activity increases, and the de novo synthesis of enzymatic proteins may be key to the increased NR activity under PEG-simulated drought conditions. Our previous study showed that exogenous DCPTA significantly decreased the accumulation of ROS by increasing their scavenging via elevation of antioxidant enzyme activities and improving the redox state of ascorbate and glutathione [27]. The increased NR activity may partly be attributed to exogenous DCPTA alleviating drought-induced oxidative damage by modulating the antioxidant system in maize seedlings. Moreover, the reduction in foliar NO_2^- content was also significantly reversed by exogenous DCPTA (Figure 7). This indeed shows that exogenous DCPTA could help maintain the conversion of NO_3^- to NO_2^- in maize seedlings under PEG-simulated drought stress via increased NR activity. After it is taken up, NO_3^- is stored in the vacuole, reduced in the cytoplasm, or transported to the leaves. Similar to the findings of previous reports, PEG-simulated drought significantly reduced the foliar NO_3^- content (Figure 7) [48,49]. However, exogenous DCPTA prevented the decrease in foliar NO_3^- content to a certain extent. This phenomenon may be attributed to the promotion of NO_3^- intake due to the strong root system (Table 3, Figure 1) and flux of NO_3^- from the roots to the leaves induced by exogenous DCPTA under PEG-simulated drought stress. The transcription, translation, and activity of NR, a typical substrate-inducible enzyme, are primarily regulated by the NO_3^- concentration in higher plants [50]. Another major reason for the increased NR activity induced by exogenous DCPTA may be the increased foliar NO_3^- content. The Fd-NiR gene relative expression level and protein relative expression level in DCPTA-treated seedlings were notably greater, and the activity remained stable. This is beneficial for promoting the conversion of NO_2^- to NH_4^+ in maize seedlings under

PEG-simulated drought stress. In addition, the gene relative expression levels and protein abundances of NADH-NR and the NR activity slightly increased in seedlings treated with DCPTA compared with the control, which implies that NO_3^- assimilation was promoted, and this may favour plant growth under non-stress conditions.

Previous studies have shown that drought stress can increase the NH_4^+ content in bamboo [51] and tomato [46]. NH_4^+ is produced by the NO_3^- assimilation and oxidation of glycine during the photorespiration process under stress conditions, and excessive accumulation of NH_4^+ is detrimental to plants, as it can trigger protein extrusion and disturb cytosolic pH [7,52]. GS is responsible for glutamine synthesis in the NH_4^+ assimilation process as the entry point of inorganic N into the organic form, and GOGAT is responsible for catalysing the transfer of the amide group of glutamine to 2-oxoglutarate, generating glutamate simultaneously [46]. In leaves, chloroplastic glutamine synthetase (GS 2) and Fd-GOGAT are the predominant forms, and the combination of GS2 and Fd-GOGAT is responsible for the assimilation of NH_4^+ derived from photorespiration in leaves [53]. In this study, although NO_3^- assimilation was inhibited, the foliar NH_4^+ content significantly increased under PEG-simulated drought stress, which may be due to the activated photorespiration process and inhibited GS/GOGAT cycle (Figure 7) [9]. The increased glycine content may be due to the glycine generation by proteolysis being more than the glycine degradation by oxidation in the photorespiration process under PEG-simulated drought stress; moreover, exogenous DCPTA diminished the effect of drought (Table 4). In the DCPTA-treated seedlings, the foliar GS and GOGAT activities increased (Figure 6), which may have been due to upregulated GS 2 and Fd-GOGAT gene expression levels and higher GS 2 and Fd-GOGAT protein abundances (Figure 4, Figure 5), which prevented the accumulation of NH_4^+ originating from photorespiration by efficient re-assimilation of NH_4^+ , alleviating NH_4^+ toxicity under PEG-simulated drought conditions and maintaining a steady glutamate content.

Glutamate serves as a donor of the amino group for the biosynthesis of major N-containing compounds [54]. It has been reported that AST and ALT play an important role in the process of amino acid synthesis [13,55]. High activities of AST and ALT were found in the PEG + DCPTA treatment, which may be associated with the high activities of GS/GOGAT, which generated more glutamate for the substrate of transamination reactions (Figure 6, Figure 8). Stable AST and ALT activities were detected in the PEG + DCPTA treatment, accelerating the synthesis of aspartate and alanine; this was reflected by the suppressed PEG-simulated drought stress-induced decrease in alanine and aspartate contents (Table 4). The energy and C skeletons required for the transamination process are generated by photosynthesis, so another reason for the stable alanine and aspartate contents under PEG-simulated drought stress may be related to the stable photosynthesis induced by exogenous DCPTA, which was proven in our previous study [26,28]. Glutamate is also the primary amino acid for the synthesis of many other amino acids. Previous studies have shown that the accumulation of glutamate is a common physiological response to drought [56]. In this study, the effective regulation of GS/GOGAT by exogenous DCPTA might lead to increased amounts of glutamate for proline synthesis, contributing to osmotic adjustment and alleviating the negative effects induced by PEG-simulated drought stress by scavenging ROS [57]. As one of the limiting substrates of glutathione biosynthesis, cysteine plays an important role in protecting plants from the damaging effects of ROS [58]. In this study, the increased cysteine content induced by exogenous DCPTA might promote glutathione biosynthesis, enhancing oxidation resistance under PEG-simulated drought stress.

Decreased soluble protein contents have also been observed in drought-stressed plant species such as barley [59], cotton [60], and maize [61]. Zhang et al. [52] reported that the protein degradation of tomato seedlings exposed to saline-alkaline stress could be inhibited by exogenous spermidine by relieving disturbances in N metabolism. In this study, the increased contents of total amino acids, serine, glycine, valine, isoleucine, leucine, tyrosine, lysine, histidine, methionine, and arginine and the decreased contents of soluble protein in seedlings exposed to PEG-simulated drought stress may have been due to the promotion of protein degradation and the suppression of protein biosynthesis by inadequate supplies of reducing potential (in the form of NADPH and ATP) due

to reduced photosynthesis (Table 4, Figure 9) [62,63]. Exogenous DCPTA weakened these effects induced by PEG-simulated drought stress, which may be attributed to the alleviation of stress-induced photosynthetic inhibition and oxidative damage, as demonstrated in our previous studies [26,27].

5. Conclusions

The effects of DCPTA on seedlings is related to metabolic processes, and N metabolism is the major metabolic pathway. The DEGs enriched in this pathway included ZmNADH-NR, ZmFd-NiR, ZmFd-GOGAT, and ZmGS2 in response to PEG and/or DCPTA. Maize seedlings treated with exogenous DCPTA maintained a high N assimilation capability via regulation of the activity of enzymes related to N assimilation (NR, NiR, GOGAT and GS) at the transcriptional and translational levels under PEG-simulated drought stress. Moreover, exogenous DCPTA suppressed the negative effects of PEG-simulated drought on AST and ALT activities to maintain transamination, stabilized the soluble proteins, and maintained a relatively high RGR of the plants. Moreover, exogenous DCPTA had a positive influence on N assimilation under non-stressed conditions.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/9/11/676/s1>, Table S1: KEGG enrichment analysis of DEGs in the library pair comparison of DCPTA vs. the control. Table S2: KEGG enrichment analysis of DEGs in the library pair comparison of PEG vs. the control. Table S3: KEGG enrichment analysis of DEGs in the library pair comparison of PEG + DCPTA vs. PEG. Table S4: The data of ZmNADH-NR, ZmFd-NiR, Zm-GS2 and ZmFd-GOGAT protein abundances detected by Western blot analysis (% of control).

Author Contributions: T.X. performed the entire experiment, analysed the results, and drafted the manuscript. S.W. and W.G. designed the entire experiment and were in charge of manuscript revisions. C.L. and J.L. interpreted the results and prepared the manuscript. All authors read and approved the final manuscript.

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