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Research article

Endogenous NO-mediated transcripts involved in photosynthesis and carbohydrate metabolism in alfalfa (*Medicago sativa* L.) seedlings under drought stress

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is an important perennial legume and used as a forage crop worldwide, and has extensive resistance to various abiotic stresses. Nitric oxide (NO) plays a critical role in response to external and internal cues to regulate plant growth and development. However, endogenous NO-mediated molecular mechanisms of drought tolerance in alfalfa is poorly understood. To get a deeper insight into the regulate pathway of NO, RNA-Seq was used to profile transcriptome changes of alfalfa seedlings, which were treated with NO scavenger under normal and drought conditions. A total of 1,025 and 3,461 differently-expressed genes ($FDR < 0.0001$; fold change ≥ 2) were observed while NO absence under normal and drought conditions, respectively. Based on GO enrich and KEGG pathway analysis, we found NO absence induced photosynthesis, carbon fixation in photosynthetic organisms and primary metabolism were significantly up-enriched. Most oxidoreductase, dehydrogenase, reductase and transferase genes were down-regulated in the above processes. Moreover, NO absence restrained chlorophyll biosynthesis and decreased different sugar content. Therefore, this work provides insights into the mechanism that NO-mediated enhanced photosynthesis and carbohydrate metabolism in alfalfa under drought stress.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a widely adapted perennial forage crop and plays a key role in livestock. Drought is one of the most important natural factors that influences on plant growth, and causes considerable crop losses worldwide. To survive, plant has evolved a complex network supporting perception and transmission of stress signal. The one is local intracellular signaling network in the cells perceiving the stress signal, the other is systemic long-distance extracellular communication pathway (Foyer et al., 2016; Mittler, 2006; Nakashima et al., 2009).

As a gaseous diatomic radical, nitric oxide (NO) is an major endogenous signaling biomolecule associated with signal transduction in plants (Sami et al., 2018). Extensive research findings revealed that exogenous application of NO donor exerts various plant functions, such as seed germination, root growth, stomatal movement, photosynthesis, and cell transportation (Montilla-Bascón et al., 2017; Silveira et al., 2016; Yu et al., 2017). Recently, exogenous NO-mediated transcriptional modulation response to abiotic stress has been reported (Chen et al., 2017; Li et al., 2018; Singh et al., 2017). These studies have identified thousands of NO-induced different expressed genes, which have involved in multiple regulatory pathways (Hussain et al., 2016). However, most of these results were obtained by exogenous application of NO donor such as sodium nitroprusside and S-nitrosocysteine (Hussain et al., 2016; Singh and Bhatla, 2016). Moreover, it is worth mentioning that in combination with NO donor may lead to the formation of artifacts not connected with the biological function of NO. Arasimowicz-Jelonek et al. (2011) noted that the form of NO emitted from the donor structure shown inconsistent metabolomic changes, indicating while making inferences based on the use of these NO-modulating compounds needs to be cautious.

Interestingly, several oxidative and reductive pathways of NO biosynthesis have been discovered in plants (Arasimowicz and Floryszak-Wieczorek, 2007; Besson-Bard et al., 2008; Sami et al., 2018). Under environmental stress, plant cells synthesize NO and significantly increased concentrations were observed (Sun et al., 2014). In the past few years, the biochemistry and biological process of NO have been extensively studied, emerging evidence demonstrates that NO interplays with signal transduction of abscisic acid (Lozano-Juste and Leon, 2010),

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ethylene (Liu et al., 2017; Wang et al., 2009), auxins (Lanteri et al., 2006), brassinosteroids (Li et al., 2017). At molecular level, NO activates many NO-responsive transcripts (Parani et al., 2004). Furthermore, increasing evidences corroborate that NO can mediate post-translational changes through mechanisms such as S-nitrosylation or tyrosine nitration of proteins (Begara-Morales et al., 2014). The advantages of 2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (c-PTIO) are specific for NO and cell permeable (Vitecek et al., 2007), and is widely used as a NO scavenger in plant experimental systems to validate the involvement of NO in pathways triggered by different external/internal stimuli (D'Alessandro et al., 2013). Xiong et al. (2012) have demonstrated that drought treatment increased endogenous NO content, and c-PTIO treatment remarkably reduced NO content in well-watered and drought-treated plant by fluorometric approach. Li et al. (2012) also found alfalfa seedlings exposed to c-PTIO under Cd stress increases of lipid peroxidation, and a total of antioxidant enzymes activities as well as corresponding transcripts were modulated differentially.

Understanding the underlying mechanisms of NO regulates alfalfa's response to drought is important for developing drought-tolerant germplasm through genetic modification or molecular breeding. To date, there are limited studies investigating endogenous NO role in gene expression level. To gain insights into endogenous NO-mediated drought stress tolerance, the transcriptional responses of alfalfa to drought stress (polyethylene glycol-6000, PEG) and the absence of NO under drought stress (PEG + cPTIO) were investigated by RNA-Seq analysis. We aimed to further investigate the following questions: (I) provide insight into the alfalfa seedlings transcriptome response to drought stress and the change absence of endogenous nitric oxide. (II) uncover the genes and pathways that are associated with endogenous NO-induced stress tolerance in alfalfa.

2. Materials and methods

2.1. Sample preparation

Seeds of alfalfa (*Medicago sativa* cv. Sanditi) were sterilized with NaClO (5%) for 15 min, immersed in dH_2O for 24 h at 25 °C, and then germinated in Petri dishes (two-filter paper) with 50 seeds per dish. Firstly, in order to investigate the effect of different drought stresses on seed germination, PEG-6000 solutions (dH_2O, 5%, 10%, 15%, 20% and 25%) were added into the above dishes; after 7 days, seedling growth characteristics (Fig. S1) including root length, shoot and root fresh weight (FW) were measured. Secondly, with the aim of revealing the effect of endogenous nitric oxide (En-NO) mediated-drought stress on seed germination, treatments including dH_2O, 200 μM cPTIO (NO scavenger), 10% PEG as well as 10% PEG + 200 μM cPTIO were performed; seeds germination rate and whole seedling FW were measured (Fig. S2) 7 days later; 30 seedlings each treatment were randomly collected and rapidly frozen in liquid nitrogen for RNA-seq analysis. The remaining seedlings were used for physiological analysis.

2.2. RNA extraction, cDNA library construction, and sequencing

The total RNA of 12 samples was extracted using an RNA reagent (Invitrogen) according to the manufacturer's protocol, then quality and quantity were assessed using NanoPhotometer spectrophotometer (IMPLEN, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). RNA integrities number of all samples were greater than 7.0. Next, poly(A) mRNA was enriched and then used to prepare paired-end cDNA library following manufacturer's recommendations (Illumina, San Diego, USA). After cDNA libraries passing the check, Illumina Hiseq X Ten platform was performed and 150 bp paired-end reads were generated. The entire process was commissioned and completed by the Beijing Novogene Co. Ltd experimental department.

2.3. Raw data processing, sequence assembly and functional annotation

Raw reads from each library were filtered separately by removing adaptor sequences, reads containing 'N' larger than 10%, and low-quality reads ($Q_{phred} \leq 20$ bases more than 50%) with Cutadapt (https://doi.org/10.14806/ej.17.1.200). Clean data was used for all the further analyses. *De novo* assembly of the transcriptome through Trinity software as the reference genome sequence, with the min_kmer_cov set to 2 by default (Grabherr et al., 2011). For annotations, unigenes were searched against the NCBI non-redundant protein (NR) (Deng et al., 2006), non-redundant nucleotide (Nt) (Pruitt et al., 2007), Protein family (Pfam) (Finn et al., 2014), Clusters of Orthologous Groups of proteins (KOG) (Tatusov et al., 2000), Swiss-Prot (Apweiler et al., 2004), Gene Ontology (GO) (Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2004) using BLASTx.

2.4. Differential expression genes identify and functional analysis

Differential expression genes (DEGs) between two treatments were detected through the DESeq (Anders and Huber, 2010). False discovery rate (FDR) was controlled through the adjusted *p*-values (Benjamini et al., 2001). The standard of DEGs screening was FDR < 0.0001 and fold change ≥2. GO analysis of the DEGs were performed using the GOseq R packages (q-value $<$ 0.05) to describe gene function (Young et al., 2010). KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathway, and pathway with q-value < 0.05 deem to be significantly enriched (Mao et al., 2005).

2.5. qRT-qPCR validation

Nine significant DEGs were chosen for validation using qRT-PCR. *PDF2* was amplified as internal standard (Kakar et al., 2008), and PrimerQuest Tool was used to design the specific primer sequence (Table S1). Relative gene expression amount was normalized by comparing with control expression and calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schnmittgen, 2001). qRT-PCR analysis was conducted using abm®Eva Green qPCR Master Mix-No Dye (Takara, Dalian, China). PCR amplification was performed in a 96-well platform according the following program: one cycle at 95 °C for 10 min, followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min. Melting curve analysis was implemented after incubation at 60 °C for 34 s.

2.6. Determination of chlorophyll and carbohydrate metabolites content

Chlorophyll content was determined as described by Coste et al. (2010). The amounts of non-phosphorylated soluble sugars (sucrose, fructose and glucose) were extracted and determined as described in Jones et al. (1977). The starch content was measured according to the method described by Gibon et al. (2004).

2.7. Statistical analysis

Data on growth and physiology parameters were expressed as mean values ± SD from at least three independent experiments. Statistical analysis was performed via ANOVA and Duncan multiple tests. A value of $P < 0.05$ was considered as the basis for statistical differences with the aid of SPSS software.

3. Results

3.1. Illumina sequencing analysis

To identify En-NO-mediated molecular mechanism under drought stress in alfalfa, comparison of gene transcription for seeds exposed to PEG, cPTIO PEG + cPTIO and dH_2O were performed. As a result, a total

Table 1
Summary of the RNA-Seq data analysis.

Sample	Raw Reads	Clean Reads	Clean Bases	Q20 (%)	Q30 (%)	GC Content (%)	Total mapped
CK-1	71661552	69281712	10.39G	96.67	91.69	42.03	47744062(68.91%)
CK-2	50020752	48263526	7.24G	96.69	91.77	41.75	33237572(68.87%)
CK-3	59453348	57402392	8.61G	96.77	91.96	41.76	39309154(68.48%)
cPTIO-1	43788772	41222450	6.18G	96.39	91.16	41.77	27904410(68.35%)
cPTIO-2	57564822	55193040	8.28G	96.84	92.11	41.87	37834008(68.55%)
cPTIO-3	64684534	62217194	9.33G	96.76	91.91	42.03	42959692(69.05%)
PEG-1	57406932	55142796	8.27G	96.7	91.77	41.92	38042564(68.99%)
PEG-2	61708120	59510056	8.93G	96.95	92.32	41.86	41295848(69.39%)
PEG-3	44385528	42860552	6.43G	96.24	90.49	41.56	29252724(68.25%)
PEG + cPTIO-1	66164154	63529774	9.53G	96.46	91.29	41.86	34247392(64.79%)
PEG + cPTIO-2	58219566	56007858	8.4G	96.85	92.13	41.7	30499094(65.45%)
PEG + cPTIO-3	52999534	50894624	7.63G	96.89	92.27	42.06	34572086(67.93%)
Total	688057614	661525974	99.22G				

of 661,525,974 clean reads were ultimately obtained from 688,057,614 raw reads after filtration, constituting 99.22G bases, with each library constituting more than 6G bases. An average of 68.08% of clean reads were individually mapped to the alfalfa assembled transcriptome (Table 1). The clean reads were assembled into 464,141 transcripts and 286,481 unigenes. The transcript and unigenes length interval analyses show that transcripts length less than 300 bp taking the largest proportion of the five length distribution categories, with a mean length of 695bp. Most unigenes have lengths between 500 and 1000bp, and the mean of unigenes length was 952bp (Fig. S3).

The percentage of genes that could be mapped into public databases is shown in Fig. S4A. NR database annotation analysis indicated that 173,018 unigenes were mapped into five identified plant species (86.2%) and other (13.8%) species (Fig. S4B). GO, KOG and KEGG database annotation analyses found that 120,442 unigenes were classified into 56 functional group (Fig. S5), 39698 unigenes were grouped into 26 categories (Fig. S6), and 60738 unigenes were mapped onto 19 pathways (Fig. S7), respectively.

3.2. *Identification of different expression genes (DEGs)*

To estimate the genes expression difference in the absence of En-NO under normal and drought conditions, the changes in gene expression were determined by comparing PEG versus CK, c-PTIO versus CK and PEG + c-PTIO versus PEG. We detected both unique and overlapping sets of DEGs, a total of 6,430 DEGs were identified, and 30 genes were common between cPTIO vs CK and cPTIO + PEG vs PEG (Fig. 1A). Table S2 through Table S4 list detail of these DEGs. A total of 2,240 DEGs were found between PEG and CK, including 1,333 up-regulated (UR) and 907 down-regulated genes (DR) (Table S4). Then, 3,461 DEGs were observed between PEG + c-PTIO and PEG treatment with 479 UR genes and 2,982 DR genes (Table S5). Finally, 1,025 DEGs, which including 536 UR and 489 DR genes, were identified between c-PTIO and CK (Table S6). We used hierarchical clustering of all the DEGs to observe the gene expression patterns, and the expression levels of each gene were calculated using the FPKM method (Fig. 1B).

3.3. *Validation of DEGs expression by qRT-PCR analysis*

qRT-PCR was used to confirm the expression profiles of nine unigenes identified from RNA-sequencing analysis, with *PDF2* serve as the reference gene. Two unigenes were up-regulated and seven unigenes were down-regulated in PEG + cPTIO as compared to PEG. The results of qRT-PCR analysis were coincided with those from the Illumina sequencing data (Table S5), which confirmed the transcriptome analysis was reliable.

3.4. *GO and KEGG enrichment analysis of DEGs received different treatment*

To study the function of DEGs, GO function analysis and KEGG pathway enrichment were adopted for these identified genes. We performed GO assignments in the three main categories: biological process, cellular component and molecular function. We found “organonitrogen compound metabolic process” terms were shared in PEG + cPTIO vs PEG and cPTIO vs CK. Two cellular component term “apoplast”, “external encapsulating structure” and one molecular function term “xyloglucosyl transferase activity” were common in PEG vs CK and cPTIO vs CK. Significantly enriched KEGG pathway “carbon fixation in photosynthetic organisms” were shared in PEG + cPTIO vs PEG and cPTIO vs CK.

3.4.1. *Differentially expressed genes (DEGs) in response to drought stress*

GO functional analysis shows that UR DEGs were significantly involved in 12 functional terms. Among these, “oxidation-reduction process”, “inositol biosynthetic process” and “lipid biosynthetic process” is in the category of biological process. Nine terms including “oxidoreductase activity”, “carbon-oxygen lyase activity, acting on phosphates”, “iron ion binding”, “terpene synthase activity”, “catalytic activity”, “heme binding”, “tetrapyrrole binding”, “inositol-3-phosphate synthase activity” and “oxidoreductase activity” are in the category of molecular function. For DR DEGs, mainly enriched in cellular component domain containing “apoplast”, “transcription factor complex”, “external encapsulating structure” and molecular function domain including “xyloglucosyl transferase activity”, “transcription factor activity” terms (Table S6). This result indicated that under drought stress cell membrane protected from damage.

Through a KEGG pathway annotation, DEGs between PEG vs CK involved in 12 pathways. PEG treatment affected secondary metabolites biosynthesis, such as isoflavonoid, flavonoid, phenylpropanoid, monoterpenoid, sesquiterpenoid and triterpenoid. This result suggests that activation of secondary compounds biosynthesis is an integral part of plant adaption to drought conditions. Photosynthetic related pathway including circadian rhythm and photosynthesis-antenna proteins were affected. Furthermore, “plant hormone signal transduction”, “cyanoamino acid metabolism”, “plant-pathogen interaction”, “pentose phosphate”, “glutathione metabolism” were also enriched ($q < 0.05$; Table S7). Pentose phosphate pathway provides nicotinamide adenine dinucleotide phosphate (NADPH) for fatty acids and steroids biosynthesis.

3.4.2. *Transcriptional profiling regulated by En-NO in response to drought stress*

GO functional enrichment analysis of the DR DEGs in PEG + cPTIO vs PEG found that 19 and 26 major groups related to the category of biological process and molecular function, respectively. In the biological process category, DEGs mainly enriched in “cellular amino acid

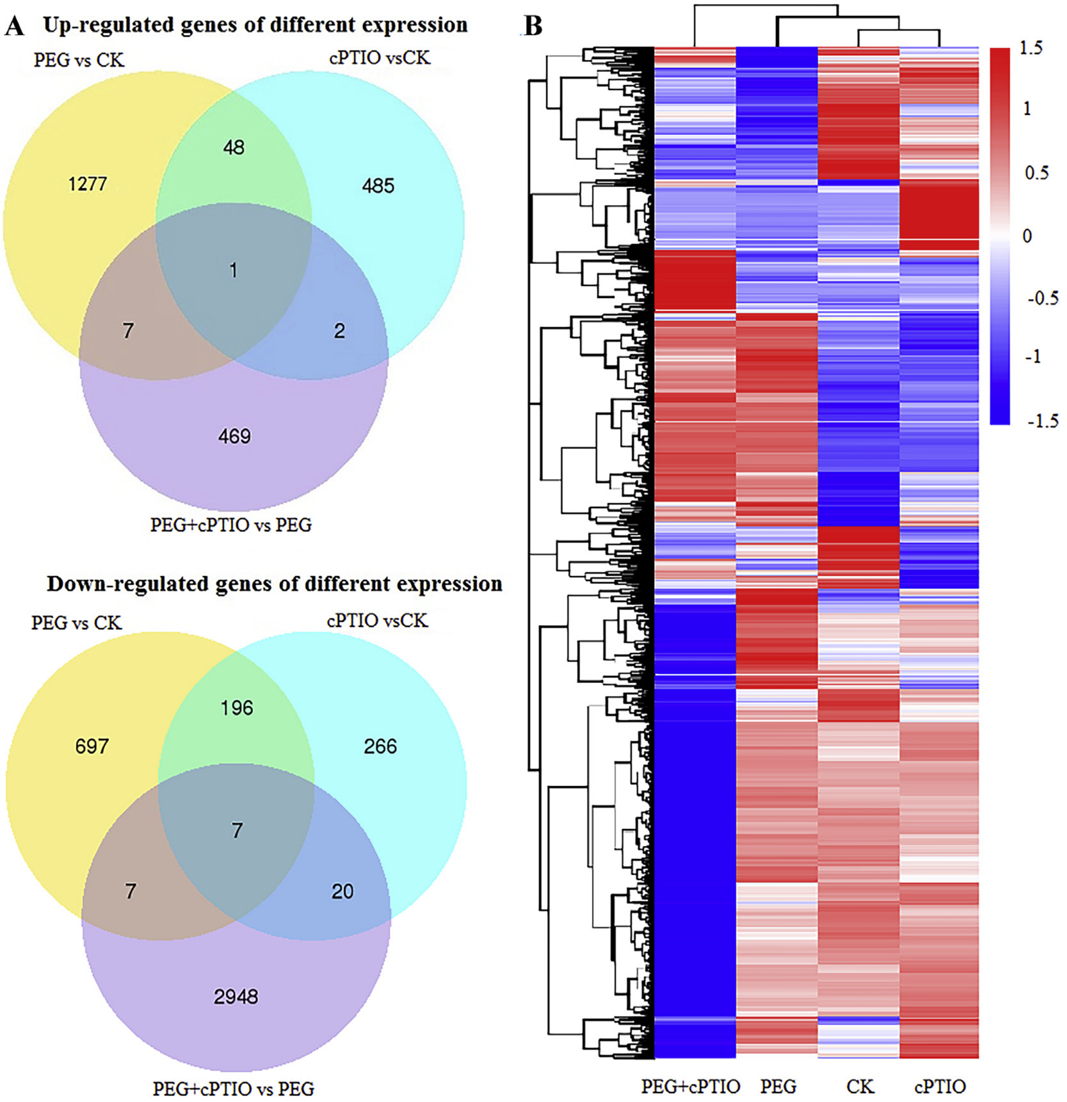

Fig. 1. Differentially expressed genes (DEGs) analysis. (A) Venn diagram of numbers of the DEGs in different samples. (B) Heatmap and cluster analysis of DEGs among different samples using complete linkage. The gene expression level increases with blue to red. CK, samples treated with distill water; cPTIO, samples treated with 200 μM cPTIO; PEG, samples treated with 10% PEG-6000; PEG + cPTIO, samples treated with 10% PEG-6000 and 200 μM cPTIO.

metabolic", "glucose metabolic process", "glycosyl compound metabolic" and "cellular protein localization". In the cluster of molecular function, DEGs mostly related to "catalytic activity", "nucleoside binding", "transferase activity", "hydrolase activity". In addition, 'membrane coat' and 'coated membrane' in the category of cellular component were also affected when NO is absent under drought stress (Table S6). No UR DEGs were significantly enriched.

Significantly enriched KEGG pathway including "alanine, aspartate and glutamate metabolism", "arginine biosynthesis", "propanoate metabolism", "pyruvate metabolism" and "glyoxylate and dicarboxylate metabolism". Above metabolic pathways are re-synthesized using carbohydrates. In addition, a large set of DEGs involved in Aminoacyl-tRNA biosynthesis, proteasome. Meanwhile, "circadian rhythm-plant", "carbon fixation in photosynthetic organisms" and "citrate cycle" pathways were also enriched, and the genes involved in these pathways were down-regulated, suggesting NO absence inhabited energy translated and photosynthesis (Table S7).

3.4.3. Transcriptional profiling regulated by En-NO under normal conditions

The GO enrichment analysis of DEGs obtained from c-PTIO vs CK revealed that DR GO classifications were far more than UR GO classifications. Within the biological process category, the UR DEGs mapped to "peptide biosynthetic and metabolic", "amide biosynthetic and metabolic", "ribonucleoprotein complex biogenesis" and "translation", this result indicated that c-PTIO treated induced UR genes related to protein production. On the contrary, DR genes most commonly belonged to photosynthesis, including light harvesting and reaction, response to light intensity and stimulus. In addition, "reactive oxygen species metabolic process" and "amino acid metabolic process" were also detected. Within the cellular component domain, genes were UR associated with "structural constituent of ribosome", "ribonucleoprotein complex" and "non-membrane-bounded organelle", genes were DR related to photosynthetic organs, such as chloroplast and chloroplast stroma, plastid and plastid stroma, thylakoid and thylakoid membrane, cell wall, photosynthetic membrane. Within the molecular function category, for genes that were UR and DR were involved in 'structural constituent of ribosome' and 'xyloglucosyl transferase activity', respectively. (Table S6). These results suggested that En-NO plays a positive role in alfalfa photomorphogenesis.

Four pathways with a KEGG analysis were significantly enriched ($q < 0.05$), including 'ribosome', 'photosynthesis-antenna proteins', 'photosynthesis', and 'carbon fixation in photosynthetic organisms' (Table S7). The result indicated that in the normal environment, NO

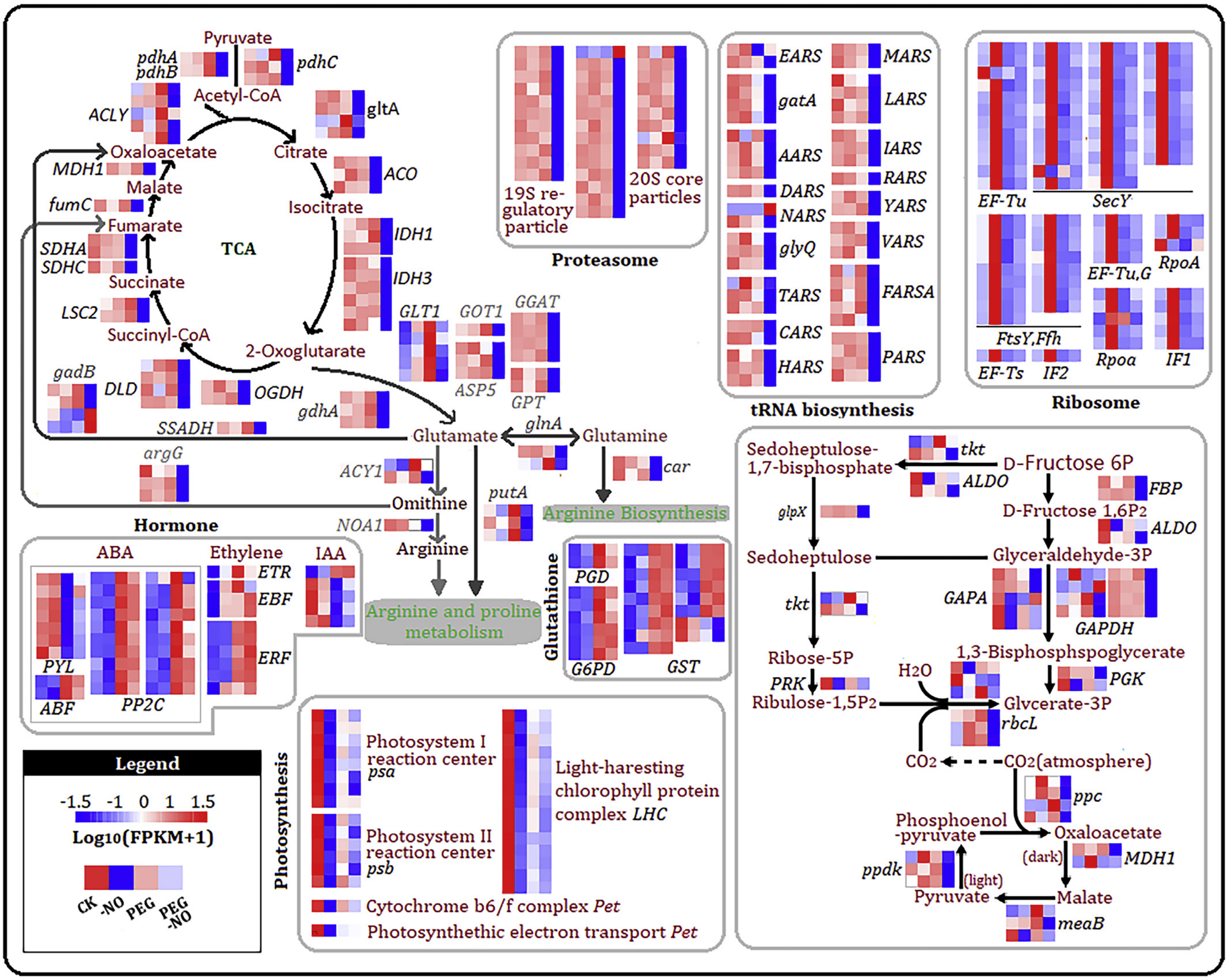

Fig. 2. Endogenous NO-mediated biological process in alfalfa responded to drought stress through transcriptional regulation. The $\log_{10}$ (FPKM + 1) color scale ranges from −1.5 to 1.5, with blue indicating a decrease and red an increase gene expression (see color set scale on bottom left corner). Expression values of four treatments are presented as FPKM normalized $\log_{10}$ transformed counts.

mainly regulated the structural constituent of ribosome and photosynthesis.

In order to overview the genome-wide transcriptomic changes between control and treatment samples, we draw a heatmap of DEGs assigned to NO-mediated metabolic pathways and biological processes under normal and drought conditions (Fig. 2). The map showed that genes were associated with ribosome, tRNA biosynthesis, proteasome, photosynthesis, and enzymes involved in TCA cycle and carbon fixation in photosynthetic were all down-regulated in NO absence under drought stress. While genes involved in hormone signal transduction and glutathione metabolism were mostly up-regulated.

3.5. Putative photoreaction-related and carbon fixation-related DEGs regulated by En-NO

Endogenous NO affected the multi-step process in the light phase of photosynthesis (Fig. 3). Under normal conditions, the absence of En-NO induced 32 DR DEGs, which include light-harvesting chlorophyll (LHC) protein, photosystem II oxygen-evolving enhancer protein and photosystem I subunit. Under drought conditions, the absence of En-NO induced 22 DR DEGs, which contain LHC, photosystem II reaction center, ferredoxin and F-type ATPase subunit (Table S8). In the dark phase of photosynthesis, genes encoding enzymes involved in carbon-fixation pathway were all DR under drought conditions with the absence of En-NO (Table 2).

3.6. Starch and sucrose metabolism related DEGs were mediated by En-NO

The carbohydrate metabolism is reflected by the expression level of related enzyme genes. Twenty beta-glucosidase genes and one beta-amylase gene were UR in PEG compared to that in CK; however, the two enzymes genes were DR in cPTIO treatment. Under drought condition, the absence of En-NO induced most of enzyme genes DR, except for one beta-glucosidase and glucose-1-phosphate adenylyltransferase gene for each, and two 1,4-alpha-glucan branching enzyme genes were UR (Table 3).

3.7. Effect of En-NO on phenotypic change, chlorophyll and carbohydrate content

The exposure to drought stress treatment influenced the morphological traits in alfalfa seedlings, and cPTIO application significantly inhibited its growth (Fig. 4A). The root length was significantly increased under PEG treatment; thus it is possible that through increase

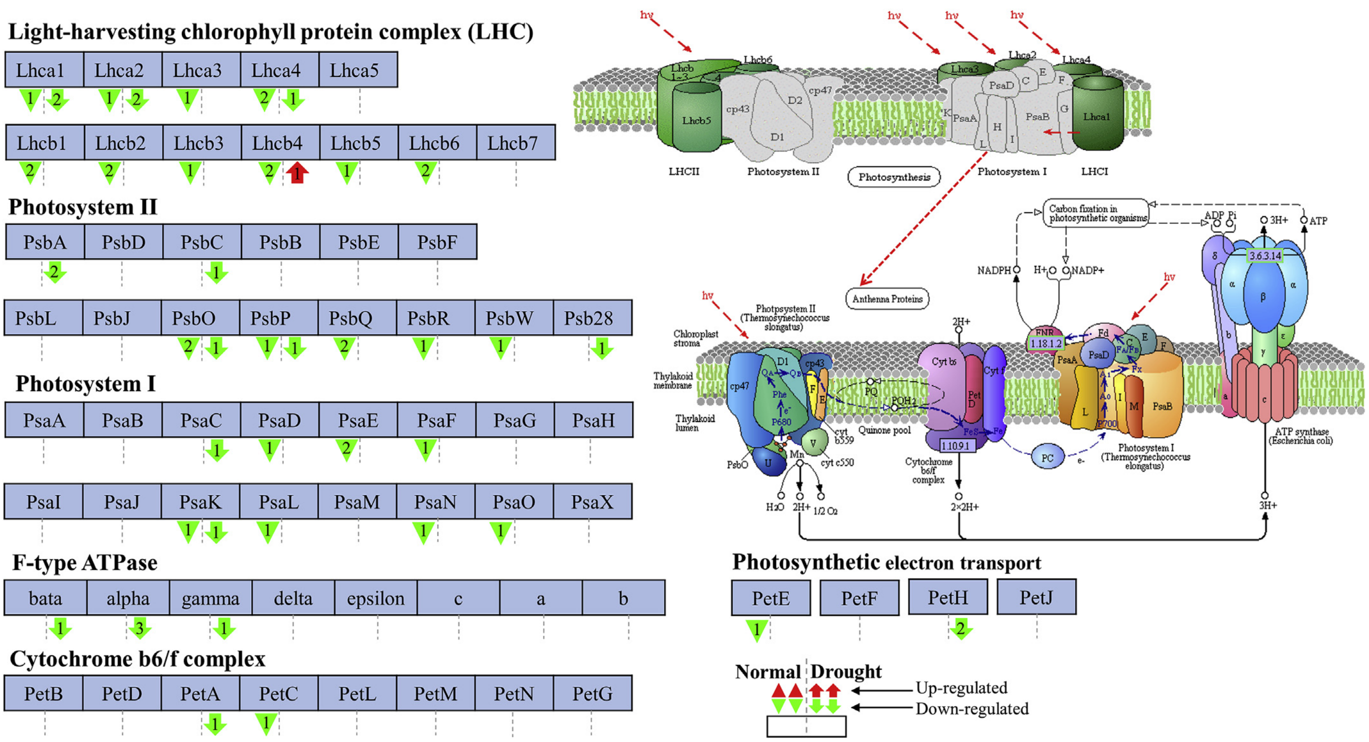

Fig. 3. Schematic representation of genes associated with photosynthesis and antenna protein pathways in alfalfa with the absence of NO under normal and drought conditions. The numbers of transcripts differently expressed are reported for each gene category.

Table 2
Key enzyme DEGs involved in carbon-fixation in photosynthetic.

Enzyme	Enzyme No.	No. of unigenes (DR/UR)		
		PEG vs CK	cPTIO vs CK	PEG + cPTIO vs PEG
Transketolase	EC: 2.2.11	2 UR	1 DR	2 DR
Phosphoenolpyruvate carboxykinase	EC: 4.1.1.49	2 UR	—	2 DR
Phosphoenolpyruvate carboxylase	EC: 4.1.1.31	—	—	4 DR
Glyceraldehyde 3-phosphate dehydrogenase (NADP+)	EC: 1.2.1.13	—	3 DR	2 DR
Glyceraldehyde 3-phosphate dehydrogenase	EC: 1.2.1.12	—	1 UR	7 DR
Alanine transaminase	EC: 2.6.1.2	—	1 DR	6 DR
Malate dehydrogenase (decarboxylating)	EC: 1.1.1.39	—	—	4 DR
Malate dehydrogenase	EC: 1.1.1.40	3 UR	1 DR	6 DR
Ribulose-bisphosphate carboxylase	EC: 4.1.1.39	2 DR	2 DR	3 DR

root length and surface area, plant improves water absorption capacity to adapt stress environment. However, cPTIO remarkably attenuated this response (Fig. 4B). Together, cPTIO exhibited negative effect on chlorophyll biosynthesis. Chlorophyll content decreased by 16.68% and 13.40% under cPTIO and PEG + cPTIO treatment, respectively (Fig. 4B). Subsequently, carbohydrate content analysis show that sucrose, fructose and glucose content significantly increased in shoot and accompanied by a decrease in root under PEG treatment compared to control. Furthermore, the application of cPTIO weaken the above three sugars level under both normal and PEG condition. For starch level,

Table 3
Key enzyme DEGs involved in starch and sucrose metabolism.

Enzyme	Enzyme No.	No. of unigenes (DR/UR)		
		PEG vs CK	cPTIO vs CK	PEG + cPTIO vs PEG
Beta-glucosidase	EC: 3.2.1.21	20 UR	2 DR	2 DR 1UR
Beta-amylase	EC: 3.2.1.2	2 DR 1UR	2 DR	4 DR
Alpha-amylase	EC: 3.2.1.1	—	—	2 DR
Trehalose 6-phosphate synthase	EC: 2.4.1.15	—	—	4 DR
Sucrose synthase	EC: 2.4.1.13	—	—	4 DR
Glucose-1-phosphate adenylyltransferase	EC: 2.7.7.27	—	—	3 DR 1 UR
1,4-alpha-glucan branching enzyme	EC: 2.4.1.18	—	—	4 DR 2 UR
Glycogen phosphorylase	EC: 2.4.1.1	—	—	4 DR
Phosphoglucomutase	EC: 5.4.2.2	—	—	4 DR
Glucose-6-phosphate isomerase	EC: 5.3.1.9	—	—	4 DR

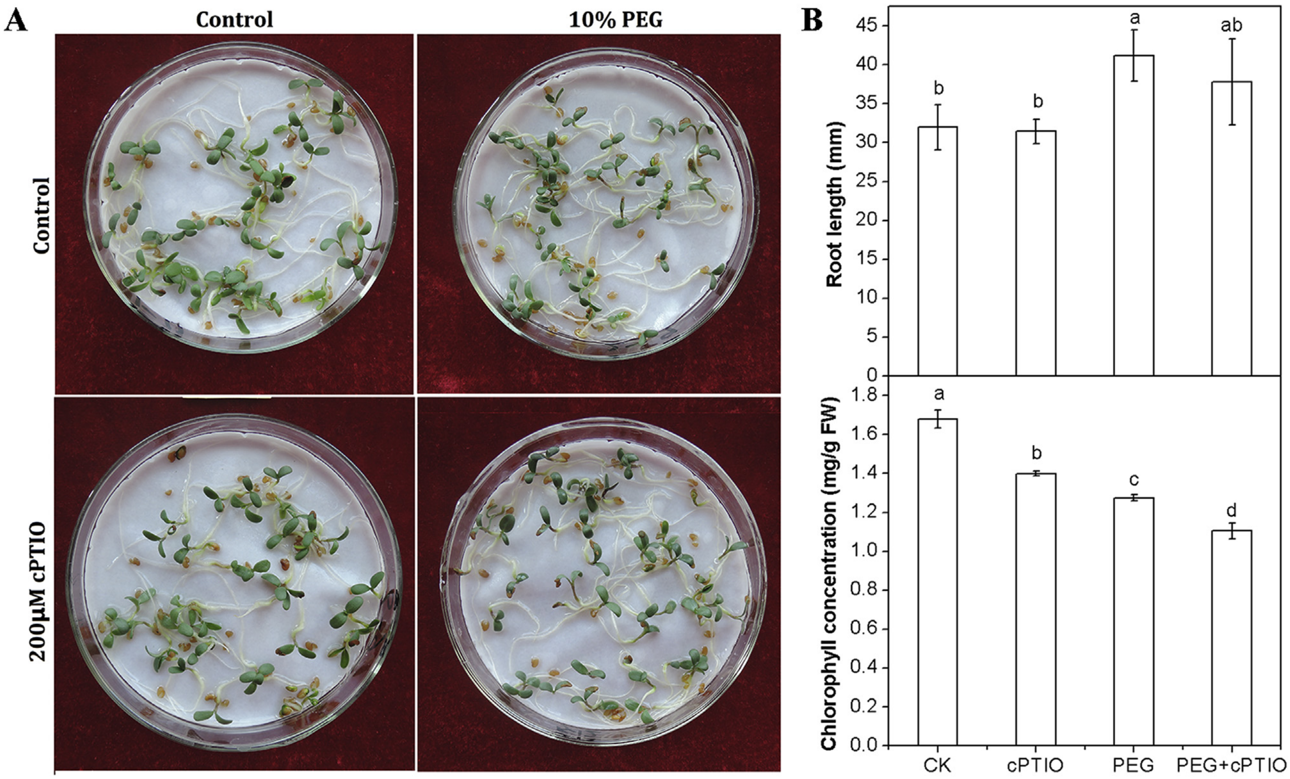

Fig. 4. Phenotypic changes (A) and root length and chlorophyll content (B) in alfalfa seedlings with or without endogenous NO under normal and drought conditions. Data are shown as mean ± SD of three individual experiments. Within each set of experiments, bars denoted by the different letters are significantly different at $P < 0.05$ according to Duncan's multiple test.

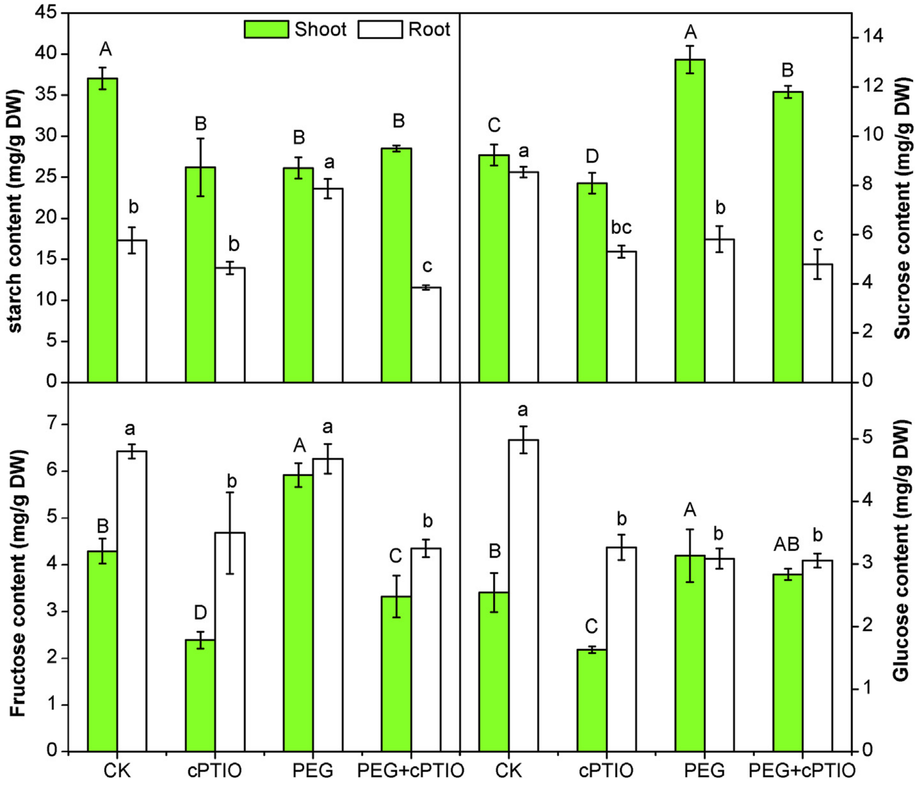

Fig. 5. Starch and different sugars content in alfalfa seedling with or without endogenous NO under normal and drought conditions. Data are shown as mean ± SD of three individual experiments. Different uppercase letters indicate significant differences between shoots, different lowercase letters represent significances between roots.

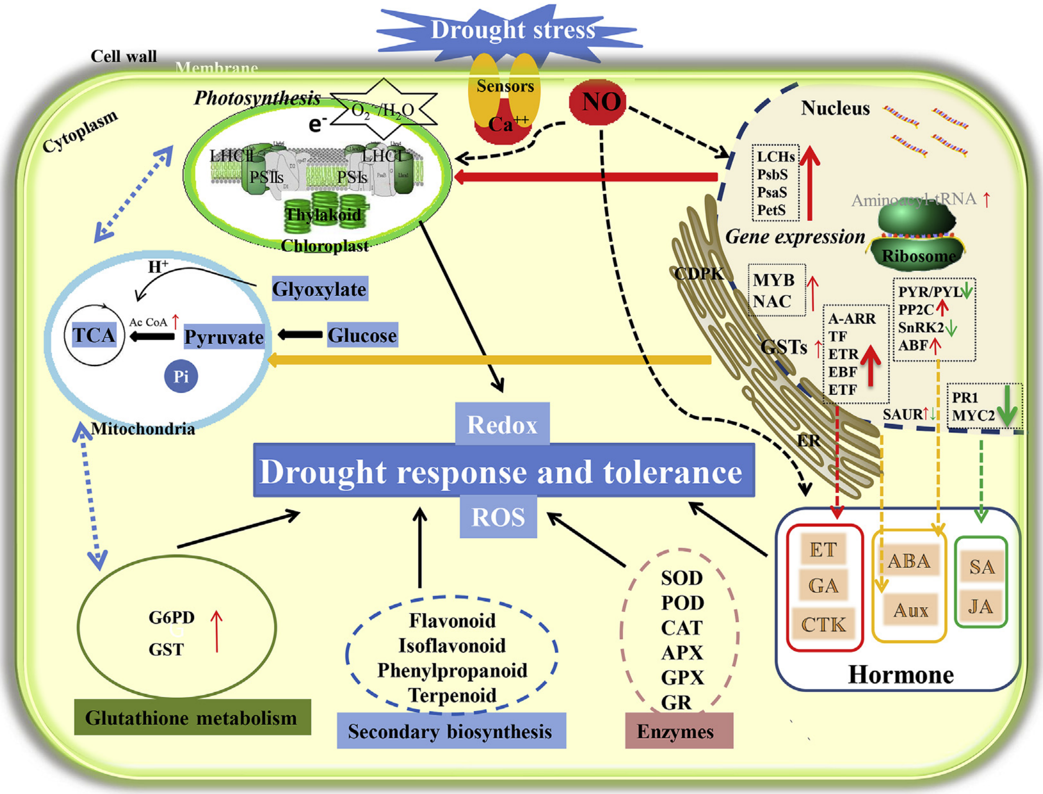

Fig. 6. Overview of the proposed model for nitric oxide-mediated drought stress response in alfalfa. Green lines indicated down-regulated, Red lines indicated up-regulated. The thickness of the arrows indicates the number of genes.

were strongly decreased in shoot and increased in root under PEG treatment, cPTIO-applied decreased starch content in root under normal and PEG condition (Fig. 5).

4. Discussion

Medicago sativa is a worldwide cultivated forage legume exhibit excellent ecological adaptability, but their germination and development still threatened by extremely drought stress. Accumulating evidence illustrated that NO serve as a signal molecule in *vivo* plant, where it has proved to promote plant development and response to abiotic stress (Khan et al., 2014). NO is also required for root organogenesis, including lateral root (Correa-Aragunde et al., 2004) and adventitious rooting formation (Pagnussat et al., 2002; Li et al., 2012). Up to date, most of studies focused on NO function in enhancing the plant tolerance based on supplication exogenous NO donner, little information is available for the role of En-NO stems from plant itself. Understanding the molecular mechanisms of En-NO in response to abiotic stress is important for drought-tolerance crop breeding program. In the present study, we analyzed the transcript of alfalfa whole seedlings in response to drought and NO scavenger treatment by using the Illumina Hiseq X Ten system and identified a total of 286,481 unigenes, which was more than previously reported for alfalfa whole seedlings transcriptome analysis (An et al., 2016). Using a more stringent criteria of both FDR ≤ 0.0001 and FC $\geq$ 2, our study detected 2,240 stress response DEGs under PEG treatment, thus indicating that these genes responded to drought stress in alfalfa. We further find 1,025 and 3,461 DEGs with the presence of cPTIO under normal and drought conditions, the number of genes UR by cPTIO are similar in cPTIO vs CK and PEG + cPTIO vs PEG, whereas the number of DR genes between PEG + cPTIO and PEG increased 6 times more than that between cPTIO and CK (Fig. 1A). These results demonstrate that a diverse and complex mechanism of En-NO modulated gene expression in alfalfa seedlings under drought stress.

When the alfalfa seedlings exposed to drought stress, 1,333 UR DEGs between PEG and CK were enriched in molecular function and biological process. GO functional enrichment found these genes involved in oxidation-reduction process and oxidoreductase activity, and they are positive to the reactive oxygen species (ROS) homeostasis. An et al. (2016) found alfalfa respond to saline-alkaline stress by modulating genes encoded antioxidant activity and lipid biosynthesis. In our study, genes related to lipid and inositol biosynthetic was UR under PEG stress, they are main components of cell membrane and cell wall. Oxidative compounds are known to have a damaging effect on membranes, organelles and macromolecules. Plants typically produce a diverse group of antioxidants as a protective mechanism against various stresses (Oh et al., 2009). KEGG pathway analysis shows that much DEGs involved in secondary metabolites biosynthesis, including isoflavonoid, flavonoid, phenylpropanoid and terpenoid, glutathione and cyanoamino acid metabolism relative genes were up-regulated in alfalfa exposed to drought. Furthermore, plant hormone signal transduction pathways were also activated, and play an important role in plant growth regulation (Sanz et al., 2015). Recently, it has been proposed that NO might negatively regulate cytokinin signaling by blunting phosphorelay activity through S-nitrosylation (Feng et al., 2013). Researchers have shown that ABA signaling participate in regulating plant drought stress response (Seo et al., 2009), abscisic acid receptor PYR/PYL family (PYL) and ABA responsive element binding factor (ABF) were differently expressed. Resent study shows that plant hormone affect photosynthesis in response to different abiotic stress conditions (Peleg and Blumwald, 2011). In our study, most of photosynthesis-antenna proteins genes (LHCs) were highly DR under PEG stress (Fig. 3), and chlorophyll content was decreased. Our findings agree with previous studies that alfalfa defend abiotic stress through plant hormone interactions, ROS homeostasis and lipid metabolism (An et al., 2016; Aranjuelo et al., 2011; Lei et al., 2018).

When supplementation with NO scavenger, the total fresh biomass of alfalfa under cPTIO and PEG + cPTIO treatment was declined when compared with the CK and PEG, respectively, indicating a positive impact of En-NO on alfalfa growth and development. This result is consistent with previous studies, NO role in alleviation cadmium and salt stress induced oxidative damage (Li et al., 2012; Wang et al., 2012). The mechanism of En-NO alleviation drought-induced oxidative damage was revealed at transcriptomic modulation level. Under normal conditions, NO mainly regulated ribosome pathway that engaged in protein synthesis thus are indispensable for metabolism, cell division, and growth (Wang et al., 2013). In our experiment, the genes of chloroplast and plastid cellular component, as well as photosynthesis process were down-regulated when En-NO was absent (Fig. 6). Previous studies also demonstrated that NO modulates ultrastructure of chloroplasts and photosynthetic rate (Chen et al., 2010; Si et al., 2017). When intercellular NO concentration was eliminated under drought stress (PEG + cPTIO), key enzymes, such as dehydrogenase, decarboxylase and transferase, involved in carboxylic acid metabolic, amino acid and glucose metabolic process were significantly down-regulated. Li et al. (2018) found these oxidation-reduction process-related enzymes were extremely up-regulated in *Pleurotus eryngii* exposed to exogenous NO, suggesting NO may regulated energy metabolism process to defense stress. Previous studies have shown that some plants species can resist to drought stress by releasing organic acids, such as citrate, oxalate, succinate and malate (Fougère et al., 1991; Ullah et al., 2017). In the present study, TCA cycle and pyruvate metabolism were mainly enriched based on KEGG analysis. We found most of genes encoded enzymes in TCA cycle showed a lower expression level under PEG and NO scavenger treatment. This agrees with the earlier observation (Gupta et al., 2012) that the levels of TCA intermediates citrate, succinate, fumarate, malate and oxaloacetate were decreased under hypoxia along with lower NO content in plant. Reduction of alternative oxide (*AOX*) to its more active form is known to be mediated by oxidation of specific TCA cycle substrates (Vanlerberghe et al., 1995). NO mediated an increase expression of *AOX1a* gene and pyruvate content in wheat leaves experiencing drought (Wang et al., 2016). Our results showed that 4 genes encoding mitochondrial alternative oxidase were significantly down-regulated in presence of cPTIO under drought stress (Cluster-9801.121480; Cluster-9801.147573; Cluster-9801.121481; Cluster-9801.133623). Additionally, pyruvate kinase and phosphoenolpyruvate carboxylase related genes also down-regulated, indicating that NO mediated drought-induced alternative pathway in alfalfa seedlings.

In conclusion, we demonstrated that alfalfa respond to drought stress by increasing secondary metabolites biosynthesis and regulating hormone transduction. When NO scavenger was added, ribosome structure, chloroplast, plastid and cell wall structure were affected, and organic acids metabolism and carbon fixation were restrained. Furthermore, En-NO was related to the link between photosynthesis and plant carbohydrate metabolism. Based on the above observations, a model for NO-mediated drought stress response in alfalfa was proposed (Fig. 6). To the best of our knowledge, this study provides the first evidence of the potent roles of En-NO at transcriptional level, which in turn, enhanced our understanding of the molecular mechanism of En-NO in alfalfa tolerance.

Availability of supporting data

All the raw data are available in the NCBI Sequence Read Archive (http://www.ncbi.nlm.gov/sra) database (Accession Number 416829).

Contributions

Conceived and designed the experiment: XHW. Performed the experiments: YZ and WJM. Analysis the data: YZ and XZJ. Wrote the paper: YZ. All authors read and approved the final manuscript.

Conflicts of interest

All the authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.023.

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