

Article

Transcriptomic Analysis of the Carbon Fixation Pathway in Photosynthetic Organisms of *Pugionium cornutum* (L.) under Drought Stress

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Abstract: In recent years, the problem of crop yield reduction caused by drought has become increasingly serious in countries around the world. China, in particular, is facing a pressing issue of water resource scarcity that is limiting agricultural production and food security. To address this, studying the drought resistance of plants is crucial to understanding the limitations of cultivated plants in dealing with drought. It can also contribute to an improvement in plant drought resistance theory and provide a theoretical foundation for sustainable agricultural development. In this study, we used *Pugionium cornutum* (L.) Gaertn. as the experimental material and analyzed the transcriptome data of *P. cornutum* under drought stress using high-throughput Illumina sequencing technology. Under the simulated drought environment, we compared *P. cornutum* with the control and observed that the number of differentially expressed genes involved in the carbon fixation pathway in photosynthetic organisms was 14 and 30 under moderate and severe drought stress, respectively. Our findings revealed the presence of genes related to the C₄ cycle pathway in *P. cornutum*, which effectively explains its adaptation mechanism to arid desert environments. This adaptation mechanism alleviates the negative impact of drought on photosynthesis in seedlings.

Keywords: transcriptomics; C₃ plants; C₄ pathway; DEGs



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1. Introduction

In recent years, with the deterioration of the global environment, drought has become a major non-biological limiting factor for agricultural production worldwide [1–3], and it is also one of the most severe challenges facing the world's agricultural production [4,5]. With the further deterioration of the global environment, the scope and extent of drought tend to further increase [6]. At the same time, the world's water shortage has also sounded the alarm for world food production. Therefore, identifying how to obtain the maximum economic, ecological, and social benefits with efficient water consumption is a very important development strategy issue.

Photosynthesis is the primary process by which plants generate energy and accumulate substances. When photosynthesis is disrupted, it affects the overall growth, development, and physiological and biochemical mechanisms of the plant [7–11]. Drought stress significantly impacts photosynthesis, as it is highly sensitive to water scarcity. The decrease in light energy use efficiency is a major factor contributing to the loss of crop yield under water stress conditions [12,13]. The main effect of drought stress on plants is a reduction in their CO₂ assimilation capacity, which in turn restricts plant growth. [14].

In recent years, there has been a growing number of studies focusing on the molecular mechanisms underlying plant photosynthesis, specifically exploring the genes associated with metabolic adjustment [15–18]. These studies primarily investigate the enzymes involved in plant photosynthesis [19,20]. Throughout the process of long-term species evolution, certain plants have developed adaptive strategies by altering their metabolic pathways to suit their environment. For instance, in C_3 barley (*Hordeum vulgare*), the glume exhibits a higher content of PEPCase compared with the leaves. PEPCase is a crucial enzyme in the C_4 pathway, suggesting the potential existence of the C_4 pathway in C_3 plants [21]. Similarly, in the leaves of *Glycine max*, a C_3 crop, key enzymes in the C_4 pathway are present, forming a relatively complete C_4 pathway [22]. Consequently, it can be inferred that the C_4 pathway exists in C_3 plants.

P. cornutum (L.) Gaertn. is endemic to China. Growing on the sandy land of the Inner Mongolia Plateau, it has strong vitality and excellent drought resistance. This plant possesses rich nutritional value and can be utilized as food [23], feed, and medicine and also for health care, windbreak, and sand fixation [24,25]. Additionally, it demonstrates good stress resistance [26,27] and plays a crucial role in preventing desertification, protecting the ecological environment, and maintaining ecological balance [28]. However, *P. cornutum* is a wild plant that is distributed in deserts. Unfortunately, its natural resources are diminishing, and the species is currently endangered.

In this study, we investigated the transcriptome sequencing and biological carbon sequestration pathways in the leaves of *P. cornutum* under drought stress. The aim of this study is to understand the molecular mechanism underlying biological carbon sequestration under drought stress and to contribute to the development of high-quality germplasm for plant water-saving technologies. Additionally, the findings of this study can serve as a theoretical foundation for sustainable agricultural development.

2. Materials and Methods

2.1. Basic Condition of the Test Site

The experiment was conducted in the awning of Science and Technology Park of Inner Mongolia Agricultural University. The culture substrate was composed of sand and decomposed barnyard manure at a volume ratio of 4:1. The substrate contained total N ($2.20 \text{ g}\cdot\text{kg}^{-1}$), available P ($0.94 \text{ g}\cdot\text{kg}^{-1}$), and rapid available K ($1.13 \text{ g}\cdot\text{kg}^{-1}$), and the maximum water holding capacity (WHC) in the field was $13.74 \text{ g}\cdot 100 \text{ g}^{-1}$. The seedling bowl for planting was 12.5 cm high $\times$ 12.5 cm diameter. During the test, the temperature range was $20 \pm 5^\circ\text{C}$ and the humidity range was $60 \pm 5\%$.

2.2. Materials and Experimental Design

Seeds of *P. cornutum* were collected from the Mu Us Desert in Ordos, Inner Mongolia Autonomous Region of China. *P. cornutum* seeds were disinfected using a 2% hypochlorous acid solution for 10 min, rinsed with distilled water, and then placed in an incubator for germination. In April of the following year, the germinated seeds were sown in a seedling bowl containing 600 g of substrate. From the time of sowing until the start of the experiment, the soil water content in the pots was maintained at 70–80% of the water holding capacity (WHC) using a weighing method. Seedlings with identical morphology were selected at the 6-leaf stage. The degree of drought stress was determined based on the soil water content, which was divided into the following categories: control ($73.65 \pm 2.33\%$), moderate drought stress ($50.22 \pm 3.65\%$), and severe drought stress ($32.47 \pm 1.37\%$). The evening before the experiment, the pots were filled with water to reach 70–80% WHC after being weighed. Samples were taken at 7:00 every morning, with eight pots randomly selected as one treatment. The first day of sampling served as the control.

2.3. RNA Isolation and Library Preparation

A Quick RNA Isolation Kit (Huayueyang, Beijing, China) was utilized for the extraction of total RNA, adhering to the protocol provided by the manufacturer. Following extraction, the integrity of the RNA was assessed using an Agilent 2000 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Subsequent analyses were performed exclusively on samples exhibiting an RNA integrity number (RIN) of 7 or higher. For the creation of libraries, a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) was used, following the manufacturer's instructions. An Illumina sequencing platform (HiSeq™ 2000) was then utilized to sequence these libraries, generating paired-end reads of 125 bp/150 bp.

2.4. Analysis of RNA Sequencing and Differentially Expressed Genes (DEGs)

The sequencing of the libraries was conducted on an Illumina HiSeq X Ten platform, resulting in the generation of paired-end reads with a length of 150 bp. Raw reads for each sample from *P. cornutum* (L.) Gaertn. were obtained. To process the raw data (raw reads), the Trimmomatic software [29] (v0.40) was used. The reads that contained ploy-N and those with low quality were eliminated to obtain clean reads. After the removal of adaptor sequences and low-quality reads, the clean reads were assembled into clusters of expressed sequence tags (contigs) and further de novo assembled into transcripts with the application of Trinity [30] (version: 2.4) using the paired-end method. The longest transcript in terms of similarity and length was selected as the unigene for subsequent analyses.

2.5. Functional Annotation

Functional annotation of the unigenes was achieved by aligning the unigenes with the NCBI non-redundant (NR), Swiss-Prot, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) and Clusters of Orthologous Groups for eukaryotic complete genomes (KOG) databases using the Blastx method [31] with a threshold E-value of 10^{-5} . The proteins showing the highest similarities to the unigenes were utilized to assign functional annotations. Additionally, the unigenes were subjected to mapping in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [32] database to provide annotations on potential metabolic pathways. The Gene Ontology (GO) classification was performed by establishing a mapping relationship between the Swiss-Prot and GO term.

After annotation, FPKM [33] and the read count value of each unigene were calculated using bowtie2 [34] and eXpress [35]. Differentially expressed genes (DEGs) between different groups were identified using the DESeq [36] (2012) functions estimate Size Factors and nbinom Test. A threshold *p*-value < 0.05 and fold change > 2 or fold change < 0.5 was established to determine significantly differential expression. The expression pattern of genes in different groups and samples was showcased with the implementation of hierarchical cluster analysis on DEGs. Subsequently, DEGs underwent GO enrichment and KEGG pathway enrichment analysis separately using the hypergeometric distribution method.

2.6. Analysis of Quantitative Real-Time PCR

While RNA sequencing (RNA-seq) is a robust technique utilized to investigate the complete transcriptome, when examining billions of short reads obtained from RNA-seq, some errors may occur in the assembly of the transcriptome. Therefore, 8 single genes were randomly selected to verify the sequencing and transcription abundance results of the expressed products, and the Power SYBR® Premix Ex Taq™ II Kit (TaKaRa) was used for qRT-PCR analysis. The experimental parameters included heating the reaction mixture at a temperature of 95 °C for a duration of 10 min. Subsequently, a series of 40 cycles were performed, comprising a denaturation step at 95 °C for 15 s, followed by an annealing step at 60 °C for 5 min. Finally, the reaction was subjected to dissociation at 94 °C for a period of 90 s, then cooled to 45 °C for three minutes, followed by a final denaturation step at 94 °C for 10 s. R1_Unigene_BMK.22570 was used as a reference [25]. The $2^{-\Delta\Delta C_t}$ method was

used to calculate the relative expression level of the selected single gene for standardization analysis. Primers were selected using Premier 5 (Table 1).

Table 1. Name and primer sequences of the eight major genes.

GENE ID	Forward Primer	Reverse Primer	Product Length
Unigene4787	CACCTGAACCGGAAAAACCT	CTCTGATGGGATTCGCTGAG	108
CL8376.Contig2	CATAGCCCACCTTCCATTGA	CGGTTTAGCATCGCCTGGTT	136
Unigene4754	TGCGCATGGATCAAGTTAGG	TTCGGAGAAGCTGTGTGGTTC	92
CL6196.Contig1	AGAGCTTCCCGGTGAGTTCT	AGCTTGGCCATACGTTTCAGA	128
CL6196.Contig2	TCCGTCTCAGGGCTTCTTAC	CTTCGCCGCATAATCCAGAT	137
CL1132.Contig2	CTCAAGCCGCTGGAATCTTC	ATTGGGACGGTGGGTGTGTT	96
CL9467.Contig2	TTTCTCAGGCGTCTGCTGCT	TGCTATTGTTCTTGAGGACGG	81
CL981.Contig2	CCTACGGTGAAGCTGCAAAT	AGCTGGCACCTGCACTTTGA	95

3. Results

3.1. Illumina Sequencing and Reads Assembly

The cDNA of *P. cornutum* leaves under three treatments was mixed and sequenced. Table 2 shows that the transcriptome sequencing results are good, providing reliable original data for data assembly in subsequent analysis.

Table 2. RNA transcriptome sequencing statistics of *P. cornutum* (L.) Gaertn. leaves.

Reads	Clean Reads	Total Base Number/nt	Q20 Percentage	N Percentage	GC Percentage
72,423,700	64,820,356	5,833,832,040	97.89%	0.00%	46.15%

The obtained unigenes were annotated into NR, NT, Swiss-Prot, KEGG, COG, and GO databases, respectively. The number of genes annotated to each database and the number of all annotated genes were counted, and the results are shown in Table 3. The number of annotated genes was 54,851, among which the number of annotated genes in NT was the highest (52,570), and the number of annotated genes in COG was the lowest (17,832).

Table 3. Summary of annotation results for *P. cornutum* (L.) Gaertn. leaves transcriptomic unigenes.

Results Distribution	NR	NT	Swiss-Prot	KEGG	COG	GO	Total
Number of sequences	50,957	52,570	32,042	28,436	17,832	47,530	54,851

3.2. Analysis of Differentially Expressed Genes in Response to Drought Stress

According to Table 4, moderate drought stress resulted in 1275 DEGs compared with the expression profile of CK. Out of these, 463 genes were upregulated, with 377 being annotated in the public databases and 86 not annotated. Additionally, 812 genes were downregulated, with 709 being annotated and 102 not annotated. On the other hand, extremely severe drought stress led to 2780 differentially expressed genes. Among these, 1296 genes were upregulated, with 1181 being annotated and 115 not annotated. Furthermore, 1484 genes were downregulated, with 1368 being annotated and 116 not annotated. Under extremely severe drought stress, the quantity of differentially expressed genes was significantly greater, in contrast with the lower count observed during moderate drought stress, suggesting that the leaves of *P. cornutum* are less affected by genes under moderate drought stress.

Table 4. Number of differentially expressed genes in *P. cornutum* (L.) Gaertn. leaves under different drought stress (FDR ≤ 0.001 and $|\log_2 \text{Ratio}| \geq 1$).

Treatment	Differentially Expressed Genes	Upregulated Genes		Downregulated Genes	
		Know	Unknown	Know	Unknown
Moderate drought stress	1275	377	86	709	103
Severe drought stress	2780	1181	115	1368	116

3.3. Analysis of the Number and Type of Carbon Fixation Pathways in the Photosynthetic Organisms of *P. cornutum* (L.) Gaertn. Leaves under Drought Stress

Moderate drought stress affects the carbon sequestration of photosynthetic organisms in the leaves of *P. cornutum*. There were 14 DEGs involved in the carbon fixation in photosynthetic organisms (Table 5). Under severe drought stress, significant enrichment of differential genes for carbon fixation was observed in the photosynthetic organisms of leaves (Q-value ≤ 0.05), and the number of DEGs reduced to 30 (Table 5).

Table 5. Carbon fixation pathway in photosynthetic organisms of *P. cornutum* (L.) Gaertn. leaves under drought stress.

Treatment	Number of Gene	Ratio of All Differential Genes	p-Value	Q Value	Path ID
Moderate drought stress	14	1.89%	0.00231	0.050811	ko00710
Severe drought stress	30	1.88%	9.63×10^{-6}	1.96×10^{-4} *	ko00710

Note: * differentially expressed genes were significantly enriched (p -value ≤ 0.05).

In conclusion, moderate drought stress had little effect on photosynthetic carbon fixation in *P. cornutum*; under extremely severe drought stress, there was a higher impact on carbon fixation in photosynthetic organisms. This indicates that photobioenergy transformation is inhibited during photosynthesis in *P. cornutum* under extremely severe drought stress. The inhibition degree of carbon fixation in photosynthetic organisms in *P. cornutum* was different under different drought stress.

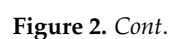
3.4. Analysis of Carbon Fixation in Photosynthesis under Drought Stress

3.4.1. Pathway of Photosynthetic Carbon Fixation in Leaves under Drought Stress

As shown in Figures 1 and 2, after analyzing the transcriptome photosynthetic carbon fixation pathway in photosynthetic organisms, it was found that the C_4 pathway exists. Studies on the differentially expressed genes related to the photosynthetic carbon sequestration pathway under extremely severe drought stress showed that the activities of AST [EC: 2.6.1.1] and ATP [EC: 4.1.1.49] in the cytoplasm were upregulated, indicating the existence of the C_4 pathway in seedlings.

Under moderate drought stress, nine enzyme sites related to carbon fixation in photosynthetic organisms were affected, of which 14 DEGs were downregulated. Under severe drought stress, 14 enzyme sites were affected, including two upregulated, two partially upregulated, and 10 downregulated. There were 30 genes involved, including five upregulated and 25 downregulated. These results show that under the aggravation of drought stress, photosynthesis has a certain self-regulation to ensure carbon fixation in photosynthetic organisms.

Under extremely severe drought stress, the AST [EC: 2.6.1.1] and ATP [EC: 4.1.1.49] activity increased in DEGs related to carbon fixation in photosynthetic organisms. The results showed that the C_4 photosynthetic pathway existed in *P. cornutum* leaves. However, the pathway of rhodiolic acid needs to be further studied.



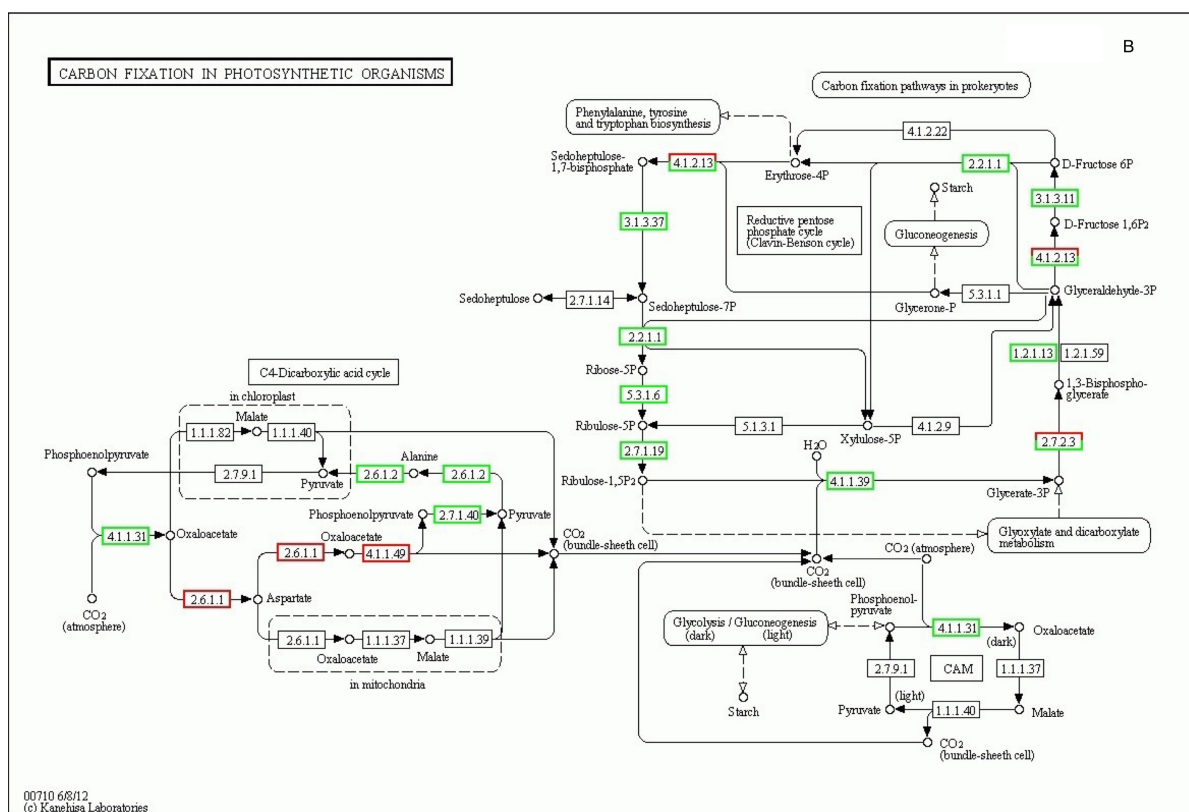


Figure 2. Pathway analysis of carbon fixation in photosynthetic organisms under different treatments. Notes: (A) represents moderate drought stress and (B) represents severe drought stress. Boxes with solid edges indicate that the differentially expressed gene was upregulated, and boxes with dotted edges indicate that the differentially expressed gene was downregulated.

3.4.2. Analysis of DEGs in Carbon Fixation under Drought Stress

In this paper, the analysis of DEGs under moderate drought stress was combined with a plant physiology analysis of carbon fixation pathways in photosynthetic organisms, according to the CO_2 assimilation step in the plant body. Thus, the results were divided into the following two steps: the C_4 pathway and C_3 pathway, where the C_3 pathway was divided into the carboxylation stage, reduction stage, and regeneration extreme.

In the C_4 photosynthetic pathway, PEPC [EC: 4.1.1.31], PK [EC: 2.7.1.40], and MDH [EC: 1.1.1.37] were inhibited. Under extremely severe drought stress, PEPC [EC: 4.1.1.31], PK [EC: 2.7.1.40], ALT [EC: 2.6.1.2], AST [EC: 2.6.1.1], and ATP [EC: 4.1.1.49] were affected.

As shown in Table 6, the differentially expressed gene CL9316.Contig1_CKA was inhibited under drought stress, but the expression multiple increased with increasing drought stress. For DEGs in MDH under moderate drought stress, the expression of CL4031.Contig1_CKA was inhibited. The expression of CL4031.Contig1_CKA was not affected under extremely severe drought stress. The differentially expressed genes CL7886.Contig1_CKA in ATP and CL10.Contig5_CKA in AST were not differentially expressed under moderate drought stress, but their expressions were upregulated under extremely severe drought stress. Overall, the regulation mechanism needs to be further studied.

Under drought stress, the carboxylation stage of the C_3 photosynthetic pathway in the leaves of *P. cornutum* is the stage of binding CO_2 . Only RuBP [EC: 4.1.1.39] at this enzyme site decreased under moderate and extremely severe drought stress. Under drought stress, three genes were affected, and their expression was inhibited (Table 7).

Table 6. Analysis of differentially expressed genes in the C₄ pathway under drought stress.

Treatment	Enzyme Site	Gene	Multiple of Difference Target	GO Biological Function	GO Biological Process
Moderate drought stress	Phosphoenolpyruvate carboxylase	CL2362.Contig2_CKA	−1.60	Phosphoenolpyruvate carboxylase, 2-alkene reductase activity; Mg ²⁺ binding	Metabolic process of oxaloacetate; carbon fixation; tricarboxylic acid cycle
		CL4235.Contig2_CKA	−1.37	Phosphoenolpyruvate carboxylase, 2-alkene reductase activity; Mg ²⁺ binding	
	Keto acid kinase	CL9316.Contig1_CKA	−2.73	Ion binding; pyruvate kinase activity	Glycolysis; phosphorylation
		CL3229.Contig3_CKA	−1.43	Mg ²⁺ , K ⁺ , ATP binding; pyruvate kinase activity	Maltose metabolism, starch biosynthesis process;
	Malate dehydrogenase	CL4031.Contig1_CKA	−3.68	L-malate dehydrogenase activity	glycolysis; phosphorylation
Severe drought stress	Phosphoenolpyruvate carboxylase	CL2362.Contig2_CKA	−2.71	Phosphoenolpyruvate carboxylase, 2-alkene reductase activity; Mg ²⁺ binding	Metabolic process of oxaloacetate; carbon fixation; tricarboxylic acid cycle
		CL4235.Contig2_CKA	−1.88	Phosphoenolpyruvate carboxylase, 2-alkene reductase activity; Mg ²⁺ binding	
	Keto acid kinase	CL9316.Contig1_CKA	−2.28	Ion binding; pyruvate kinase activity	Glycolysis; phosphorylation
		CL3229.Contig3_CKA	−2.80	Mg ²⁺ , K ⁺ , ATP binding; pyruvate kinase activity	Maltose metabolism, starch biosynthesis process; glycolysis; phosphorylation
	Alanine aminotransferase	CL6891.Contig1_CKA	−2.18	Alanine: 2-ketoglutarate aminotransferase, γ -glutamyl transferase, ACC synthase, glyoxylate alanine aminotransferase activity, pyridoxal phosphate binding	Hydrogen peroxide decomposition, glutathione metabolism, cysteine biosynthesis process
		Unigene27952_CKA	−2.02	Alanine: 2-ketoglutarate transaminase, γ -glutamyl transferase, alanine acetalate transaminase, glycine: 2-ketoglutarate transaminase activity; pyridoxal phosphate binding	Photorespiration; hydrogen peroxide decomposition, glutathione metabolism, cysteine biosynthesis
		CL6891.Contig2_CKA	−1.75		Hydrogen peroxide decomposition process; glutathione metabolism process; cysteine biosynthesis process
	Phosphoenolpyruvate carboxylase (ATP)	CL7886.Contig1_CKA	2.12	Kinase, phosphoenolpyruvate carboxylase (ATP) activity; DNA, ATP binding	Phosphorylation; sugar dysplasia
	Aspartate aminotransferase				
		CL10.Contig5_CKA	1.97	L-aspartic acid: 2-ketoglutarate aminotransferase activity; phenylalanine: 2-ketoglutarate aminotransferase activity; pyridoxal phosphate, copper ion combination	Ethylene biosynthesis, cell amino acid metabolism, leaf senescence; in response to cytokinin stimulation

Table 7. Analysis of differentially expressed genes in the carboxylation stage of the C₃ pathway under drought stress.

Treatment	Enzyme Site	Gene	Multiple of Difference Target	GO Biological Function	GO Biological Process
Moderate drought stress	Ribulose diphosphate carboxylase	CL1780.Contig1_CKA	−2.50	Cu ²⁺ binding; ribulose diphosphate carboxylase, monooxygenase activity	Complex biosynthesis of chloroplast diphosphate carboxylase; carbon fixation; photosynthesis
		CL1780.Contig2_CKA	−1.52	Cu ²⁺ binding; ribulose diphosphate carboxylase, monooxygenase activity	
		Unigene20141_CKA	−1.03	Hydrogen ion transport ATP synthase, proton transport ATPase activity and rotation mechanism; hydrogen export ATPase activity, phosphorylation mechanism; ribulose diphosphate carboxylase, monooxygenase activity; magnesium ion, ATP binding	Photorespiration; ATP hydrolysis coupled proton transport; production of mass and energy; synthesis coupled proton transport; reduced pentose phosphate cycle; transcription elongation, DNA-dependent;

Table 7. Cont.

Treatment	Enzyme Site	Gene	Multiple of Difference Target	GO Biological Function	GO Biological Process
Severe drought stress	Ribulose diphosphate carboxylase	CL1780.Contig1_CKA	−9.24	Cu ²⁺ binding; ribulose diphosphate carboxylase, monooxygenase activity	Complex biosynthesis of chloroplast diphosphate carboxylase; carbon fixation; photosynthesis;
		CL1780.Contig2_CKA	−5.10	Cu ²⁺ binding; ribulose diphosphate carboxylase, monooxygenase activity	Complex biosynthesis of chloroplast diphosphate carboxylase; carbon fixation; photosynthesis
		Unigene20141_CKA	−1.78	Hydrogen ion transport ATP synthase, proton transport ATPase activity and rotation mechanism; hydrogen export ATPase activity, phosphorylation mechanism; ribulose diphosphate carboxylase, monooxygenase activity; magnesium ion, ATP binding	Photorespiration; ATP hydrolysis coupled proton transport; production of mass and energy; synthesis coupled proton transport; reduced pentose phosphate cycle; transcription elongation, DNA-dependent;

Under drought stress, there are two enzyme sites involved in the reduction stage of the C₃ photosynthetic pathway, which are PGK [EC 2.7.2.3] and GAPD (NADP+) (phosphate change) [EC: 1.2.1.13] (Figure 2). The DEGs at enzyme site PGK [EC 2.7.2.3] were all downregulated under moderate drought stress, while some DEGs were upregulated under extremely severe drought stress (Figure 2B). Under severe drought stress, four additional DEGs exhibited significantly higher expression compared with moderate drought stress. Among these DEGs, the gene Unigene17559_CKA demonstrated an increasing pattern (Table 8).

Table 8. Analysis of differentially expressed genes in the reduction stage of the C₃ pathway under drought stress.

Treatment	Enzyme Site	Gene	Multiple of Difference Target	GO Biological Function	GO Biological Process
Moderate drought stress	Phosphoglycerate kinase	Unigene6226_CKA	−2.90	-	-
	Glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation)	CL4562.Contig3_CKA	−1.15	Protein, NAD, and NADP binding; glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation) activity	Proton transport regulation; protein dephosphorylation regulation; photosystem II assembly; photosystem I photosynthetic electron transport; reduced pentose phosphate cycle
Severe drought stress	Phosphoglycerate kinase	Unigene6226_CKA	−10.71	-	-
		Unigene17559_CKA	1.08	Phosphoglycerate kinase activity	Glycolysis; dephosphorylation photosynthesis, photoreaction; pentose phosphate shunting; hydrogen peroxide decomposition, salicylic acid biosynthesis, starch biosynthesis, isopentenyl pyrophosphate mevalonate biosynthesis, independent pathways
		Unigene23493_CKA	−8.41	-	Proton transport regulation; protein dephosphorylation regulation; photosystem II assembly; photosystem I photosynthetic electron transport; reduced pentose phosphate cycle
	Glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation)	CL4562.Contig3_CKA	−3.64	Protein, NAD, and NADP binding; glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation) activity	Proton transport control; pentose phosphate shunt; hydrogen peroxide decomposition process; photosystem II assembly; chlorophyll biosynthesis process; photosystem I photosynthetic electron transport; PSII-related photo-harvesting complex process II; reduced pentose phosphate cycle
		Unigene23466_CKA	−3.84	Combination of NAD and NADP; glyceraldehyde 3-phosphate dehydrogenase (phosphorylation) activity; glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation) activity	Proton transport regulation; protein dephosphorylation regulation; photosystem II assembly; photosystem I photosynthetic electron transport; reduced pentose phosphate cycle
		CL4562.Contig1_CKA	−2.51	Protein, NAD, and NADP binding; glyceraldehyde 3-phosphate dehydrogenase (NADP+) (phosphorylation) activity	photosystem II assembly; photosystem I photosynthetic electron transport; reduced pentose phosphate cycle

Note: “-” represents unknown.

During the regeneration stage of the C_3 photosynthetic pathway in the leaves of *P. cornutum*, three enzyme sites were affected under moderate drought stress including FBPA [EC:4.1.2.13], TK [EC:2.2.1.1], and RPK [EC:2.7.1.19], all of which were downregulated. Under extremely severe drought stress, six enzyme sites were affected. These included the three enzyme sites affected by moderate drought stress as well as FBP [EC:3.1.3.11], SBPase [EC:3.1.3.37], and Rpi [EC:5.3.1.6] (Figure 2A). Among them, the upregulated DEGs under extremely severe drought stress were CL8368.Contig1_CKA and Unigene19893_CKA of FBA [EC:4.1.2.13], and its main biological function is fructose diphosphate aldolase activity (Table 9).

Table 9. Analysis of differentially expressed genes in the regeneration of the C_3 pathway under drought stress.

Treatment	Enzyme Site	Gene	Differential Expression	GO Biological Function	GO Biological Process
Moderate drought stress	Fructose diphosphate aldolase	Unigene26991_CKA	−1.68	Fructose bisphosphate aldolase activity	Protein dephosphorylation regulation; chlorophyll biosynthesis process; photosystem II assembly;
		CL8368.Contig3_CKA	−2.11	Fructose bisphosphate aldolase activity; Cu ²⁺ binding	Pentose phosphate shunt; acetyl CoA biosynthesis process
	Transketolase	CL1429.Contig1_CKA	−1.20	Transketolase activity	Ca ²⁺ transport; glycolysis; cysteine biosynthesis process; Pentose phosphate shunt;
	Phosphoribulose kinase	Unigene26317_CKA	−1.19	Phosphoribulose kinase activity; protein, ATP binding	photosynthetic electron transport in photosystem I; protein dephosphorylation regulation; kinase cascade; photosystem II assembly;
Extremely severe drought stress	Fructose diphosphate aldolase	CL8368.Contig1_CKA	1.63	Fructose bisphosphate aldolase activity; Cu ²⁺ binding	Pentose phosphate shunt; acetyl CoA biosynthesis process
		Unigene19893_CKA	1.10	Fructose bisphosphate aldolase activity	Pentose phosphate shunt
		Unigene26991_CKA	−3.60	Fructose bisphosphate aldolase activity	Protein dephosphorylation regulation; chlorophyll biosynthesis process; photosystem II assembly
		Unigene26990_CKA	−2.38	Fructose bisphosphate aldolase activity	Hydrogen peroxide decomposition process; PSII-related photo-harvesting decomposition process complex II; protein dephosphorylation regulation; response to calcium ions; chloroplast migration; chlorophyll biosynthesis process; photosystem II assembly
		CL8368.Contig3_CKA	−1.69	Fructose bisphosphate aldolase activity; Cu ²⁺ binding	Pentose phosphate shunt; acetyl CoA biosynthesis process
	Transketolase	CL1429.Contig1_CKA	−3.18	Transketolase activity	Ca ²⁺ transport; glycolysis; cysteine biosynthesis process
	Phosphoribulose kinase	Unigene26317_CKA	−4.13	Phosphoribulose kinase activity; protein, ATP binding	Pentose phosphate shunt; photosystem I photosynthetic electron transport; protein dephosphorylation regulation; kinase cascade; photosystem II assembly
		Unigene20074_CKA	−1.77	2-alkene reductase activity; fructose 1,6 diphosphate phosphatase activity; Mg ²⁺ binding	Pentose phosphate shunt; carotenoid biosynthesis process; protein dephosphorylation regulation; photosystem II assembly; chloroplast migration; fructose 1,6 diphosphate metabolism process; chlorophyll biosynthesis process; photosynthetic electron transfer in photosystem I; reduced pentyl sugar phosphate cycle; Salicylic acid biosynthesis process; reduced pentose phosphate cycle; sucrose biosynthesis process;
	Fructose-1,6-bisphosphatase I/II/sedum heptulose-1,7-bisphosphatase	CL2268.Contig3_CKA	−8.50	Mg ²⁺ binding; fructose 1,6 diphosphate phosphatase, sedum heptulose diphosphatase activity	regulation of hydrogen peroxide metabolism process; electron transfer of photosynthetic photosystem I; protein dephosphorylation regulation; kinase cascade; photosystem II assembly;
	5-phosphate ribose isomerase	Unigene4656_CKA	−1.92	Phosphoribose isomerase activity	Cell death

4. Discussion

Photosynthesis, a crucial plant process, is significantly impacted by drought stress. Plants have different photosynthetic mechanisms, namely, the C₃, C₄, and CAM pathways. *P. cornutum*, an edible and medicinal plant, is a wild species found in deserts, and these plants are widely distributed in the arid and semi-arid regions of China. They have developed a variety of physiological and biochemical strategies to adapt to and tolerate conditions of drought stress. The molecular mechanism underlying drought stress tolerance in *P. cornutum* is currently not understood. In order to gain insight into this, we utilized Illumina DNA sequencing technology to identify genes that are involved in the response to drought stress. This discovery will serve as a starting point for future research aimed at understanding the molecular mechanism and eventually contributing to the development of sustainable agricultural practices for *P. cornutum* under drought conditions.

In this investigation, we used advanced Illumina sequencing technology to examine the transcriptome of *P. cornutum* seedlings experiencing drought-related pressures. The total number of sequenced bases was 5,833,832,040 nt, with a Q20 percentage of 97.89%. After filtration, the N percentage was 0, and the GC percentage was 46.15%. These results indicate that the transcriptome sequencing data are highly reliable and can provide valuable original data for subsequent data assembly. It is worth noting that our Illumina sequencing results for reads and unigenes differed from those of Wang et al. [25]. This discrepancy may be attributed to variations in the organs and treatments used in the same plant, which can lead to significant differences in the number of reads and unigenes.

Under moderate drought stress, 463 genes were upregulated, and 812 genes were downregulated. On the other hand, under extremely severe drought stress, 1296 genes were upregulated and 1484 genes were downregulated. These findings indicate that as the intensity of drought increases, the number of differentially expressed genes also increases. Interestingly, the number of genes affected by moderate drought stress was smaller compared with extremely severe drought stress. This suggests that *P. cornutum* is less affected by genes under moderate drought stress, which could explain its ability to withstand extremely severe drought. Similar results were observed in maize seedlings by Min H et al. [37]. Additionally, it has been demonstrated that plants are more vulnerable to drought stress and regulate the expression of a larger number of genes in response to stress [38].

Previous studies have shown that CO₂ assimilation occurs using the high-energy substances ATP and NADPH, which provide assimilation power to convert CO₂ into stable chemical energy, such as sugar and starch [39]. This study discovered the presence of a C₄ photosynthetic pathway in *P. cornutum*, which enables this species to effectively utilize CO₂ even under water stress.

Under moderate drought stress, the uptake of CO₂ by *P. cornutum* was inhibited, but it had little effect on the CO₂ assimilation stage, light energy acceptance, and conversion of light energy into chemical energy. However, under extremely severe drought stress, the photosynthetic pathway of *P. cornutum* was severely inhibited. In the C₄ photosynthetic pathway, some sites showed upregulation of DEGs, and the gene expression in some sites of the C₄ cycle was only upregulated during the CO₂ assimilation stage. This suggests that under extremely severe drought stress, although the photosynthetic acceptance and transmission ability of *P. cornutum* were inhibited, its ability to assimilate CO₂ using high-energy substances was enhanced to some extent. This can partially alleviate the inhibition of drought on photosynthesis and ensure the material demand of seedling under severe drought stress.

5. Conclusions

In conclusion, our findings uncover the genetic information and molecular functions of specific genes involved in carbon sequestration pathways in photosynthetic organisms under varying drought stress conditions. Notably, we identified genes associated with the C4 cycle pathway in leaves under drought stress. This research sheds new light on the link between drought and biological carbon sequestration pathways and has significant implications for understanding adaptation to arid desert environments and promoting sustainable agricultural development.

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