



# Analysis of the *Citrullus colocynthis* Transcriptome during Water Deficit Stress

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## Abstract

*Citrullus colocynthis* is a very drought tolerant species, closely related to watermelon (*C. lanatus* var. *lanatus*), an economically important cucurbit crop. Drought is a threat to plant growth and development, and the discovery of drought inducible genes with various functions is of great importance. We used high throughput mRNA Illumina sequencing technology and bioinformatic strategies to analyze the *C. colocynthis* leaf transcriptome under drought treatment. Leaf samples at four different time points (0, 24, 36, or 48 hours of withholding water) were used for RNA extraction and Illumina sequencing. qRT-PCR of several drought responsive genes was performed to confirm the accuracy of RNA sequencing. Leaf transcriptome analysis provided the first glimpse of the drought responsive transcriptome of this unique cucurbit species. A total of 5038 full-length cDNAs were detected, with 2545 genes showing significant changes during drought stress. Principle component analysis indicated that drought was the major contributing factor regulating transcriptome changes. Up regulation of many transcription factors, stress signaling factors, detoxification genes, and genes involved in phytohormone signaling and citrulline metabolism occurred under the water deficit conditions. The *C. colocynthis* transcriptome data highlight the activation of a large set of drought related genes in this species, thus providing a valuable resource for future functional analysis of candidate genes in defense of drought stress.

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. Raw sequence data is available for download at NCBI Sequence Read Archive under the accession numbers SAMN02769576, SAMN02769577, SAMN02769578 and SAMN02769579.

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## Introduction

Water is essential for plant growth in modern agriculture [1]. Drought delays the development of crops, and strongly affects morphology, as well as physiological processes like transpiration, photosynthesis, respiration and translocation of assimilates [2]. Drought avoidance can be achieved through morphological changes in plants, such as decreased stomatal conductance, reduced leaf area, and extensive root systems [3]. Drought tolerance is achieved by physiological and molecular mechanisms, including osmotic adjustment, antioxidant and scavenger compounds [4]. Both strategies involve the induction of specific genes and proteins, such as dehydrins (dehydration-induced proteins), key enzymes for osmolyte biosynthesis, and detoxification enzymes [5,6].

Plant species have developed diverse strategies to adapt and thrive in all kinds of climates and terrains to deal with extreme changes in the environment. These strategies are supported by rich and complex metabolic networks that enable the plant to synthesize a wide range of compounds. Plant responses to abiotic stresses involve interactions and crosstalk between many molecular pathways. High throughput screening techniques such as transcriptome sequencing have been used to study the adaptability of plants to drought [7]. This led to the discovery of many drought related genes. For example, PIP aquaporins were found to fine-

tune the environment in response to declining water availability [8]. However, few natural allelic variants have been cloned for drought related traits, so QTL, RNA sequencing, and other trait isolation methods are needed to improve methodology for exploring complex multivariate data [9,10].

The cucurbit family is a large family with several economically important species, such as watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and several *Cucurbita* species with edible fruits [11]. *Citrullus colocynthis* (L.) Schrad (2n = 2x = 22), the bitter apple, closely related to domesticated watermelon (*Citrullus lanatus* var. *lanatus*), is a very drought-tolerant perennial herbaceous species in the Cucurbitaceae family [12]. It can survive arid environments by maintaining its water content under severe stress conditions. *C. colocynthis* is an important medicinal plant and a source of valuable oil [12]. Its seeds were found in several early Egyptian, Libyan and Near Eastern sites from about 4000 BC. This species grows in sandy areas throughout northern Africa, southwestern Asia and the Mediterranean region [11,12]. The species has been used as a model to elucidate the function of genes implicated in the stress response ultimately leading to enhancement of stress tolerance in cucurbit crops through genetic manipulation. Si et al. [13] found dynamic gene expression changes in *C. colocynthis* root tissues

**Table 1.** Paired end read statistics before and after trimming of the *Citrullus colocynthis* transcriptome at 4 time points.

Time point	Day 1		Day 2		Day 3		Day 4	
	Before	After	Before	After	Before	After	Before	After
Reads	21,194,778	20,339,776	23,566,720	22,626,577	23,435,694	22,531,826	27,388,920	26,288,785
Average	21,379,596	20,537,526	23,774,222	22,846,047	23,626,206	22,738,550	27,737,584	26,649,390
% Trimmed	21,287,187	20,438,651	23,670,471	22,736,312	23,530,950	22,635,188	27,563,252	26,469,088
	96.15		96.11		96.19		96.03	

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using cDNA amplified fragment length polymorphism (cDNA-AFLP) technique.

Several research groups have used next generation sequencing technologies to study gene expression profiles in species of the cucurbit family. Guo et al [14] used 454 sequencing technology to study the comprehensive profile for watermelon fruit flesh tissues, Grassi et al [15] studied carotenoid pathway regulators in ripening watermelon fruit. The draft genome of watermelon (*C. lanatus*, 2n = 2x = 22, ~425 Mb) was analyzed by Guo et al [16] using three different watermelon subspecies. Comparative genomic analysis provided an evolutionary scenario for the origin of the 11 watermelon chromosomes derived from a 7-chromosome paleohexaploid eudicot ancestor. The genome sequence of cucumber (*Cucumis sativus*, 2n = 2x = 14) has been completed, and the genome of melon (*Cucumis melo*, 2n = 2x = 24) is being sequenced under the Spanish Genomics Initiative (MELONOMIC) [17,18]. Liu et al [19] used sequencing techniques to identify conserved and novel miRNA in watermelon, while Wincker [20] used comparative analysis of genomes between watermelon and sweet orange to detect the traits related to their domestication.

Here, high-throughput sequencing of the leaf transcriptome from *C. colocynthis* provides a glimpse at drought related genes in this uniquely drought tolerant cucurbit species. The lack of extensive genomic and functional genomic resources in *Citrullus* species has hampered research and breeding of the cultivated and economically important *C. lanatus*. This study should facilitate the identification of valuable multiple genes, needed for complex interactions of plant species with the environment.

**Materials and Methods**

**Plant Materials and RNA Extraction**

*C. colocynthis* seedlings were grown in Sunshine Mix #8 under a 16 h light/8 h dark photoperiod at 26°C day, 22°C night temperature. Seedlings with 2–3 true leaves (2–3 week old) in 50 ml containers were exposed to drought by withholding water for 0 (day 1, D1), 24 (day 2, D2), 36 (day 3, D3) or 48 hours (day 4, D4). True leaf samples were collected each day at noon, flash frozen in liquid nitrogen and stored at -80°C. RNA was subsequently extracted using the TRIzol method [21].

**Preparation of cDNA Library and Sequencing**

Illumina sequencing was performed at the HudsonAlpha Institute of Biotechnology (Huntsville, AL) following manufacturer’s instructions. RNA-Seq reads were first processed to remove rRNA sequence contamination. First strand cDNA was synthesized with reverse transcriptase and random primers using the small fragment RNAs as template. Second strand cDNA was then synthesized followed by phosphorylation by T4 DNA polymerase. The cDNA fragments were 3’ adenylated and ligated to the Illumina’s paired-end adapters. Enrichment of cDNA templates was conducted following fifteen cycles of PCR amplification. In total, over 20\*4Mp were sequenced for mapping assembly and differential expression analysis. Raw sequence data are available for download at NCBI Sequence Read Archive (SAMN02769576, SAMN02769577, SAMN02769578, and SAMN02769579).

**Assembly**

Raw sequencing data were filtered Z (0.05) using the CLC Genomics workbench (CLC Bio, Aarhus, Denmark). Paired-end sequences from the four samples were used to construct the *de novo* *C. colocynthis* transcriptome assembly with default parameters. Assembly reads were also assembled against the watermelon

**Table 2.** Summary details of sequences produced after assembly of *Citrullus colocynthis* reads.

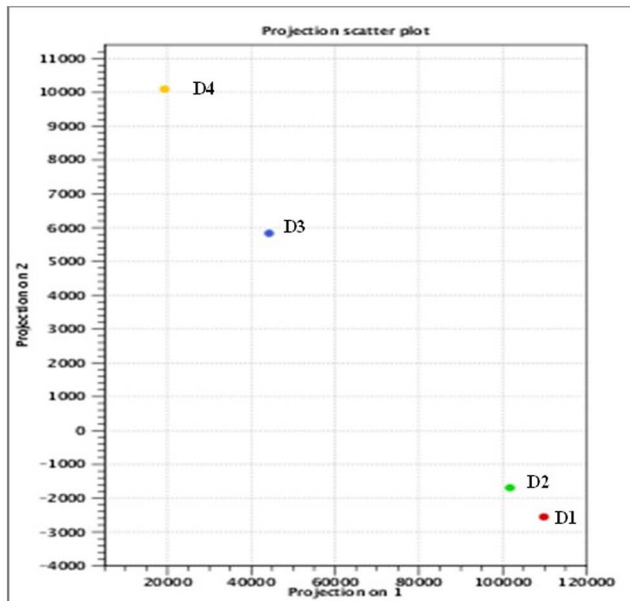
	Length/Number of reads
N50	1870 bp
Average length	1350 bp
Minimum length	201 bp
Maximum length	1956 bp
Total number of contigs	20581
Full length cDNAs	5038

doi:10.1371/journal.pone.0104657.t002

(*C. lanatus*) genome sequences, which were downloaded from the Cucurbit Database (<http://www.icugi.org/cgi-bin/ICuGI/index.cgi>). Reads were filtered and assembled using the CLC genomics workbench. The parameters used were as follows: 2 points of mismatch cost, 2 points of insertion cost, 2 points of deletion cost, 0.5 as length fraction, 0.95 as similarity fraction. After the *de novo* assembly and watermelon mapping assembly, we used Trinity to assemble all the contigs with the default parameters.

**Identification of Full Length cDNAs**

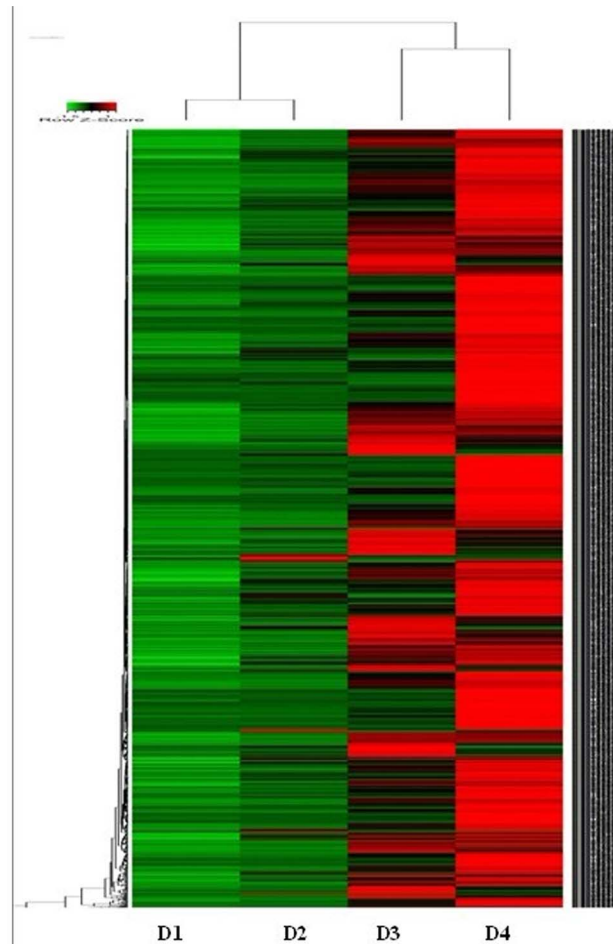
Two methods were used to identify full length cDNAs. First, BLASTX searching (E value:  $1e^{-10}$ ) was used to detect the matched cDNAs in SwissProt database; second, ESTScan 2.0 was used to identify the translated sequences. Sequences with either start codon (ATG) and stop codon (TAG/TGA/TAA), or sequences with start codon (ATG) and homologue to a known protein with  $\geq 80\%$  similarity, were chosen as full length cDNAs.



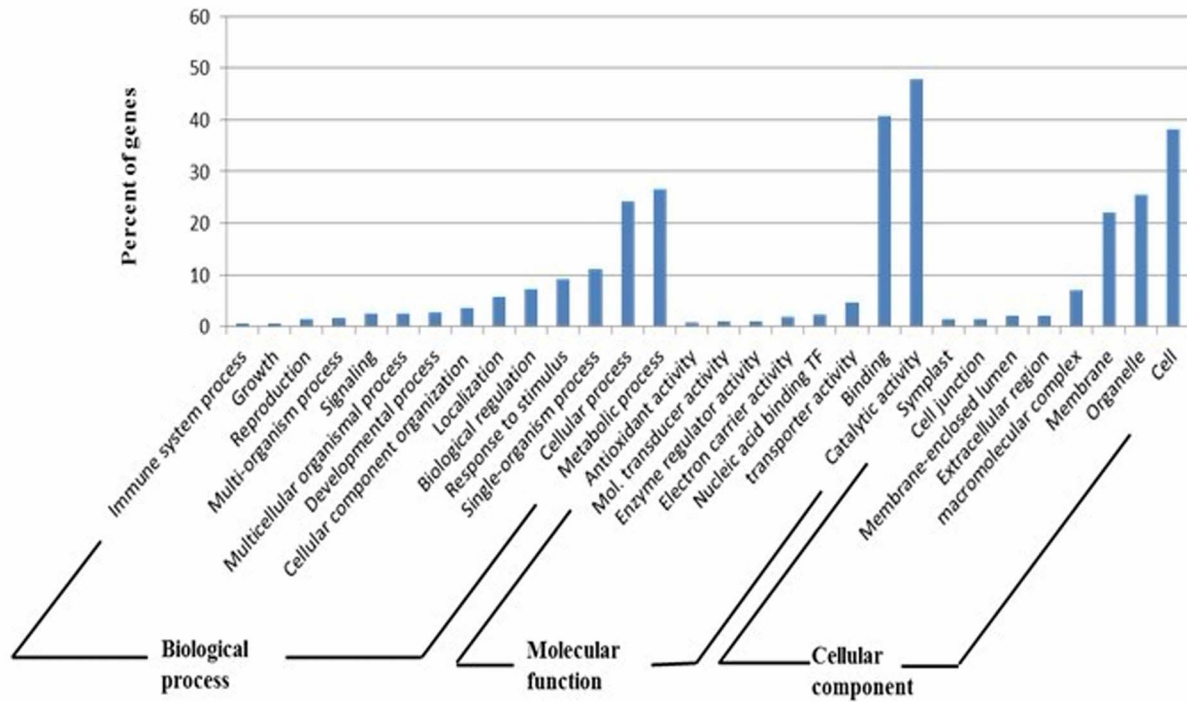
**Figure 1.** Principal component analysis of the leaf transcriptome of *Citrullus colocynthis* at 0 (D1), 24 (D2), 36 (D3) and 48 (D4) hours of withholding water. PCA analysis was conducted using the CLC workbench. The X-axis indicates principal component 1, the Y-axis principal component 2. Data from D1 and D2 are more closely related than data from D3 and D4. doi:10.1371/journal.pone.0104657.g001

**Expression Analysis Using Transcriptome Reference**

Pair-end sequencing reads of the four libraries were filtered using CLC Genomics workbench (0.05) before mapping to the references sequences from assembled cDNAs. First, read counts of each unigene were converted to reads per million (RPM).



**Figure 2.** Heat map of the leaf transcriptome of *Citrullus colocynthis* depicting changes in transcript patterns of 2545 gene transcript clusters under water deficit treatment. Heatmap was produced using R. Each red or green block represents normalized expression value of a gene detected at 0 (D1), 24 (D2), 36 (D3) and 48 (D4) hours of withholding water. Red indicates higher gene expression values across treatment, while green indicates lower expression values across treatment. doi:10.1371/journal.pone.0104657.g002



**Figure 3. Gene Ontology (GO) of significantly changed *Citrullus colocynthis* genes under drought stress.** Based on high-score BLASTX matches in the NR (non-redundant) plant protein database, *C. colocynthis* genes were classified into three main GO categories. The y-axis indicates the percentage of a specific category of genes in each main category. doi:10.1371/journal.pone.0104657.g003

Secondly, statistical analysis using Kal’s test [22] provided in CLC Genomics workbench ( $P < 0.05$  and fold change  $\geq 1.5$ ) was conducted. These transcripts were annotated against the reference sequences.

**Gene Ontology Analysis**

The functional annotation software BLAST2GO (<http://www.blast2go.com/b2ghome>) was used to conduct gene ontology (GO) analysis of *C. colocynthis* genes in this study. The databases used were SwissProt and NCBI. BLAST E-value was set at  $1.0e^{-3}$ . The major GO analysis was determined by BLAST, mapping, and annotation. Results are presented as a bar chart showing the percent of genes belonging to each group.

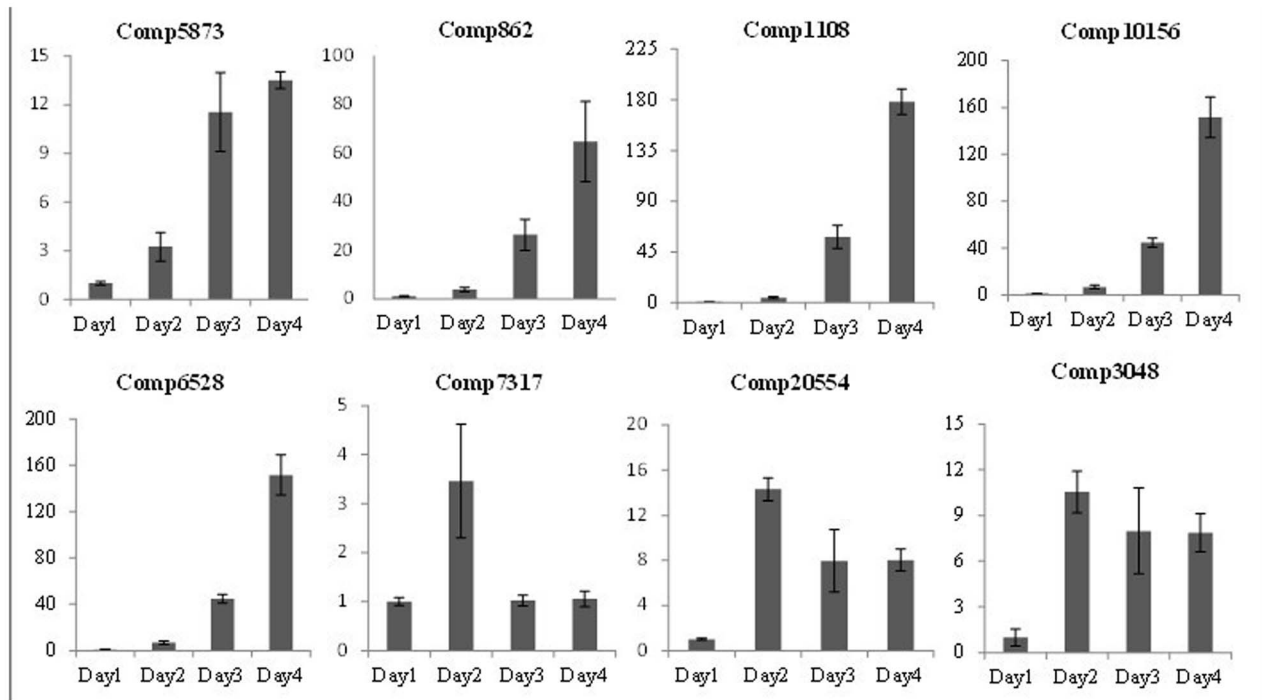
**qRT-PCR Analysis**

For cDNA synthesis, 500 ng of the total RNA for each sample (the same RNA was used for RNA-seq analysis) was used in reverse transcription with ProtoScript First Strand cDNA synthesis kit from New England BioLabs (Ipswich, MA). qRT-PCR was performed with SYBR-Green Supermix from Bio-Rad (Hercules, CA) in an Eppendorf Mastercycler ep realplex (Hauppauge, NY). Table S1 contains gene specific primer sequences. Each reaction contained 10  $\mu$ l of SYBR-Green supermix, 1  $\mu$ l of cDNA template, 1  $\mu$ l forward primer (4  $\mu$ m), 1  $\mu$ l reverse primer (4  $\mu$ m), and 7  $\mu$ l ddH<sub>2</sub>O. The qRT-PCR program consisted of one cycle at 95°C for 15 sec, followed 40 cycles of 15 sec at 95°C, 15 sec at 55°C, and 30 sec at 72°C. The relative expression data was compared with actin [13] from *C. colocynthis*. Quantification

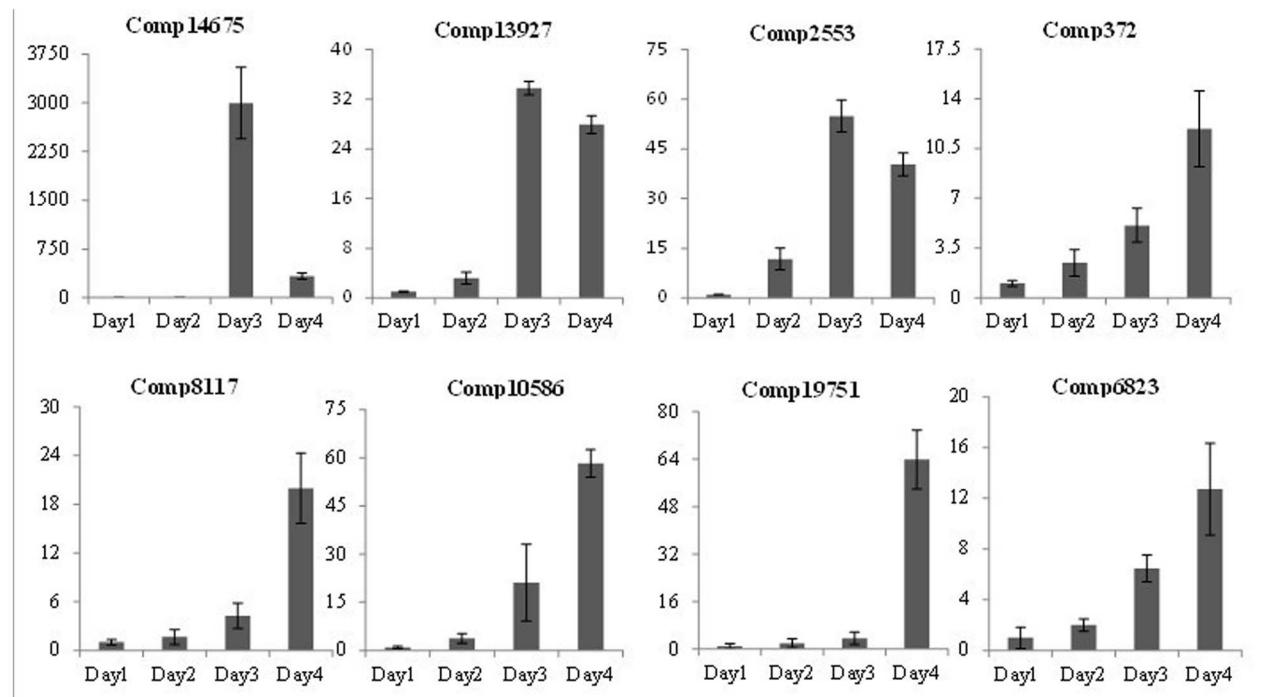
**Table 3.** Validation of the RNA-Seq expression profiles of selected *C. colocynthis* genes by qRT-PCR.

Gene ID	Hit ID	Annotations	Log2FC*-RNA seq	Log2 FC-qRT-PCR
Comp14675	Cla000300	Heat shock protein	7.60	8.37
Comp13927	Cla001351	Cold shock protein	3.41	4.8
Comp2553	Cla002576	MA3 domain-containing protein	2.15	5.33
Comp372	Cla002814	Bax inhibitor-1	1.90	3.56
Comp8117	Cla012143	Drought stress related gene	2.87	4.31
Comp10586	Cla013687	MYB	4.09	5.86
Comp19751	Cla022362	WRKY	6.27	6.00
Comp6823	Cla022169	MADS	2.07	3.67

Transcripts identified as D4 significantly changed genes were compared to transcripts at D1. The table shows the log2 fold change calculated from D4 expression vs D1 expression for RNAseq and qRT-PCR analysis. FC = fold change. Gene ID is gene named in *C. colocynthis*, and Hit ID is blastx of gene with watermelon ID number. doi:10.1371/journal.pone.0104657.t003



**Figure 4. qRT-PCR analysis of 8 selected *Citrullus colocynthis* genes under drought treatment.** The y-axis indicates relative expression compared with Day 1 expression of each gene. The x-axis shows days of water-withholding time points. doi:10.1371/journal.pone.0104657.g004



**Figure 5. qRT-PCR analysis of 8 additional *Citrullus colocynthis* genes under drought treatment.** The y-axis indicates relative expression compared with Day 1 expression of each gene. The x-axis shows days of water-withholding time points. doi:10.1371/journal.pone.0104657.g005



**Table 4.** List and number of several drought stress signaling pathway members detected in the *Citrullus colocynthis* transcriptome.

	NO.
<b>Ion Signaling</b>	
Calcium Binding Protein	10
<b>Protein Kinase Pathways For Osmotic Signaling</b>	
MAP kinase	16
<b>Osmotic Stress-Activated Phospholipid Signaling</b>	
PLC-like Phosphodiesterase	9
DAG	8
PIP2-like Aquaporin	5
<b>Detoxification Signaling</b>	
Heavy Metal Transport/Detoxification Protein	32
<b>Transcription Regulators</b>	
<b>Regulatory Proteins</b>	
MYB	131
MYC	43
NAC	42
Leucine-rich Repeat Proteins	150
<b>Functional Proteins</b>	
Heat Shock Protein 70	70
Heat Shock Protein 22	1
grpE Like Protein	4
RBOHD (respiratory burst oxidase)	2
VIP2 (VIRE2-INTERACTING PROTEIN2)	1
ABATransporter-like Protein	100
Synaptobrevin-related protein	5
Toc34-1 (Translocon outer envelope of chloroplast)	1
Beta-amylase	9
Puruvate Kinase	11
TIP1 (TIP GROWTH DEFECTIVE 1)	1
RD22	2

doi:10.1371/journal.pone.0104657.t004

of the relative transcript levels was performed using the comparative  $C_T$  method. The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to  $2^{-\Delta\Delta C_T}$ , where  $\Delta\Delta C_T = (C_{T, target\ gene} - C_{T, actin})_{treatment} - (C_{T, target} - C_{T, actin})_{control}$ . All experiments were replicated three times.

## Results and Discussion

### Transcriptome Assembly Results

The transcriptome of *C. colocynthis* leaves following 4 days of drought stress was assembled and assessed following paired-end (2\*50 bp) Illumina sequencing. The Illumina platform yielded an average of 24 million high-quality reads per sample (Table 1). All sample reads were used to construct a *de novo* assembly. A reference assembly was constructed using the completely sequenced watermelon genome. A total of 20,581 contigs were generated (Table 2). The contigs had an average length of 1350 bp and N50 of 1870 bp. BLASTX was used against SwissProt and ESTscan of translated protein sequences to detect full length cDNAs. A total of 5,038 full length cDNAs were detected in our sequencing assembly (Table 2).

Principle component analysis (PCA) was implemented in the CLC workbench. The results (Figure 1) illustrate differential gene expression patterns in *C. colocynthis* seedlings following four days of withholding water. Gene expression patterns on D1 and D2 were nearly similar, while results from D3 and D4 showed substantial differences as compared to D1 and D2, and gene expression patterns detected on D4 were very different from all other time points as a result of drought stress.

### Differentially Expressed Genes Involved in Response to Drought Stress in *C. colocynthis*

The paired-end reads were mapped to the reference genomes after filtration. Read counts of each unigene were converted to reads per million (RPM). The read number of each cDNA was divided by the total number of reads per day (1, 2, 3, or 4) from the data set, and multiplied by  $10^6$ . Statistical analysis was conducted using Kal's test ( $p < 0.05$  and fold change  $\geq 1.5$ ). Genes showing non-significant and significant changes in read counts are shown in Table S2. Each sample was compared to the day 1 sample reads for analysis of their significance level of gene expression. The read results indicated that 59 genes showed significant changes at D2, D3, and D4 as compared to D1; 13 genes showed significant differential expression at D2 and D3 as compared to D1; 30 genes

**Table 5.** Overview of *Citrullus colocynthis* genes involved in phytohormone signaling.

Function	Ethylene Pathway Major Members	Number
Ethylene-insensitive protein	EIN	5
Ethylene receptors	ETR	3
Constitutive triple response proteins	CTR	1
EIN3-like (EIL) transcription factors	EIL	1
Ethylene response factors	ERF	118
Function	Auxin Pathway Major Members	Number
Receptors/F-box proteins	TIR	1
Ubiquitin ligase component	SCF	5
Target proteins	Aux/IAA	30
Auxin response factors	ARF	30
Auxin transport protein	PIN	6
Function	JA Pathway Major Members	Number
Receptor/F-box proteins	COI1	1
Target proteins	JAZ	1
JAZ interacted proteins	NINJA	4
Activator transcription factors	R2R3-MYB transcription factor	3
Activator transcription factors	MYC2,3,4	9
Function	SA Pathway Major Members	Number
Regulatory proteins	NPR1	2
SA mainly induced genes	WRKY	46
SA mainly induced genes	TGA2,3,5,6	6
Function	ABA Pathway Major Members	Number
ABA receptors	PYR/PYL/RCAR	19
PYR/PYL/RCAR interacted proteins	PP2C	3
Serine/threonine-protein kinase	SnRK2	1
SnRK2 target	ABI5	1
Function	GA Pathway Major Members	Number
GA receptors	GID1A/B/C/-like	11
E3 ubiquitin ligase	SLY1/SNZ	2
DELLA proteins	GA1, RGA, RGL1, 2, 3	4

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showed significant differential expression early, at D2, as compared to D1; 897 genes showed changes at D3 and D4 as compared to D1; 341 genes were only differentially expressed at D3; 1191 genes were regulated late, at D4 only, under drought stress. In conclusion, the *C. colocynthis* gene expression patterns showed dramatic changes with 2545 genes showing significant changes, mostly occurring late under drought conditions (D3 and D4 of withholding water).

The heat map depicted in Figure 2 corresponded to the principal component analysis. D3 and D4 transcripts were clustered together, and D1 and D2 transcripts were clustered. Also significant changes were seen at D3 and D4 suggesting that the transcriptional response of many genes was up-regulated during drought. Strong effects were especially observed on D3 and D4.

### Gene Ontology (GO) Classification

To functionally categorize significantly changed genes in *C. colocynthis* under drought treatment, gene ontology analysis by BLAST2GO was performed. *C. colocynthis* unigenes were categorized in three main GO categorizes: biological process

(2672), molecular function (1368) and cellular component (1053). These GO terms were further divided into several sub-categories (Figure 3). In the biological process category, single organism process genes accounted for more than 20% of the biological process genes. In the molecular function category, more than 40% of genes were associated with a catalytic activity. In the cellular category, more than 35% of the genes were associated with the cellular component.

### Validation of Illumina Expression Patterns by qRT-PCR Analysis

To confirm the reliability of the Illumina sequencing read analysis, 8 candidate genes were selected and their expression was compared at D4 and D1 using qRT-PCR. The expression patterns resulting from qRT-PCR showed general agreement with those from the Illumina sequencing analysis (Table 3). Discrepancies with respect to ratio of fold changes between sequencing and qRT-PCR analysis can be attributed to the essentially different algorithm and sensitivity of the two techniques [23]. In the deep-sequencing method the absolute expression rather than relative expression as in qRT-PCR analysis is used. Transcrip-

**Table 6.** Photosynthesis-related and chlorophyll-related gene expression profiles.

Gene ID	Hit ID	Annotations	D1RPM	D2RPM	D3RPM	D4RPM
Comp808	Cla002545	Photosystem I psaA	1180	1066.9	513.6 ↓	418.8 ↓
Comp2604	Cla021635	Photosystem I subunit II	309	319	114 ↓	22.8 ↓
Comp9446	Cla002576	Photosystem I subunit III	470	428 ↓	97 ↓	39.1 ↓
Comp4444	Cla012670	Photosystem I subunit IV	274	219 ↓	70.4 ↓	8.3 ↓
Comp4514	Cla007871	Photosystem I subunit IV A	104.9	98.1 ↓	41.2 ↓	9.9 ↓
Comp2126	Cla004483	Photosystem I subunit V	292.9	281.8 ↓	95.6 ↓	23.5 ↓
Comp4727	Cla007940	Photosystem I subunit XI	257.2	229.5 ↓	61.4 ↓	9.6 ↓
Comp7482	Cla009814	Photosystem I subunit X	225.3	194.1 ↓	60.3 ↓	12.3 ↓
Comp5307	Cla011174	Photosystem I subunit X	199	176 ↓	44 ↓	4.6 ↓
Comp1999	Cla005420	Photosystem II polypeptide	225	194.1 ↓	60.3 ↓	12.3 ↓
Comp2011	Cla008554	Photosystem II 5 kDa protein	138.9	118.7 ↓	45.4 ↓	2.5 ↓
comp7744	Cla013942	Photosystem II Protein	495.8	515.8	331.4 ↓	116.7 ↓
Comp8614	Cla014815	Photosystem II reaction center W protein	310.2	265.5	117.3 ↓	28 ↓
Comp2016	Cla022723	Photosystem II core complex proteins psbY	78	76.3 ↓	24.7 ↓	7.7 ↓
Comp12757	Cla011748	Chlorophyll a–b binding protein 13	290.5	246.2 ↓	3.1 ↓	0.3 ↓
Comp6677	Cla013483	Chlorophyll a–b binding protein 3C	130.6	89.5 ↓	7.9 ↓	3.8 ↓
Comp1476	Cla013826	Chlorophyll a–b binding protein	671.4	583.9 ↓	42.9 ↓	8.8 ↓
Comp7589	Cla015680	Chlorophyll a–b binding protein 37	737.2	489.3 ↓	16.6 ↓	10.5 ↓
Comp2286	Cla017325	Chlorophyll a–b binding protein 3C	577.4	230.8 ↓	4.1 ↓	1 ↓
Comp544	Cla017983	Chlorophyll a–b binding protein 6	235.2	219.1 ↓	72.3 ↓	48.2 ↓
Comp3797	Cla018117	Chlorophyll a–b binding protein 6	632.9	502.3 ↓	154.8 ↓	38.3 ↓
Comp15099	Cla019595	Chlorophyll a–b binding protein 21	228.8	126.3 ↓	0.1 ↓	1
Comp1922	Cla001764	Chlorophyll a–b binding protein 8	609.1	470 ↓	241.6 ↓	58.6 ↓
Comp1584	Cla012368	Chlorophyll a–b binding protein 8	1082.2	884.8 ↓	111.1 ↓	281.1 ↓
Comp940	Cla009752	Chlorophyll a–b binding protein 21	652.6	514.8 ↓	43.3 ↓	3.6 ↓
Comp13569	Cla009753	Chlorophyll a–b binding protein 21	827.5	676.3 ↓	68.1 ↓	5.8 ↓
Comp4958	Cla022963	Chlorophyll a–b binding protein 7	538.2	404.7 ↓	110.1 ↓	18.8 ↓
Comp4043	Cla001790	Oxygen-evolving enhancer protein 1 of photosystem II	1088.6	795.9 ↓	334.6 ↓	82.8 ↓
Comp3077	Cla005429	Oxygen-evolving enhancer protein 2, chloroplastic	604.2	531.1 ↓	395.2 ↓	122.6 ↓
Comp5901	Cla019423	Oxygen-evolving enhancer protein 3	545.6	414.8 ↓	86.7 ↓	17 ↓

D1RPM–D4RPM are gene expression reads per millions for each day. ↓ indicates the down-regulation of each gene at each time point. doi:10.1371/journal.pone.0104657.t006

tional qRT-PCR analyses of 16 genes during the drought treatments are shown in Figures 4 and 5.

Gene Comp5873 is homeobox-leucine zipper protein, with significant upregulation during all days of drought in *C. colocynthis*. It is known that the expression of several homeobox-leucine zipper proteins is correlated to stress. Athb-12, a homeobox-leucine zipper domain protein from *Arabidopsis*, is functionally involved in salt tolerance in yeast [24]. Hahb-4, a homeobox-leucine zipper gene is potentially involved in water stress in sunflower [25]. Comp862 belongs to the glutathione S-transferase (GST) family, which contains heterogeneous, multi-functional dimeric proteins. This gene is highly up-regulated (60x) during drought in *C. colocynthis*. It is thought that GSTs are involved in cellular detoxification [26]. Comp1108 is a member of the NAC (NAM/ATAF1, 2/CUC2) gene family and NACs are known to be involved in numerous biological processes, including drought stress [27].

Comp10156 is a member of the GID1 (GIBBERELLIN INSENSITIVE DWARF1) family in *C. colocynthis* with high expression under drought conditions. GID1 is a soluble GA receptor in rice [28]. GA-GID1 complex interacts with DELLA proteins, which are negative regulators of GA signaling pathway. RD22, a known dehydration responsive gene in *Arabidopsis*, which is mediated by abscisic acid (ABA), may have physiological and molecular significance for processes underlying memory functions of plants in response to ABA and light pulses [29,30]. One RD22-like protein from soybean can alleviate salinity and osmotic stress [31]. RD22-like genes (Comp 6528) with significant up-regulation (>160x) under drought in *C. colocynthis* were confirmed by qRT-PCR, especially after days of withholding water.

NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE1) plays an important role in coordinating broader cellular processes in response to stress and bacterial pathogen infection [32]. The chemical β-aminobutyric acid, which is known to induce resistance



**Table 7. Expression profiles of some citrulline metabolic genes during drought stress.**

Gene ID	Hit ID	Annotations	D1RPM	D2RPM	D3RPM	D4RPM
Comp1556	Cla006970	Carbamoyl-phosphate synthetase	64.6	70.9 ↑	159 ↑	175.8 ↑
Comp1556	Cla005591	Carbamoyl-phosphate synthetase	196.1	162.5	846.5 ↑	1542 ↑
Comp3843	Cla014787	Carbamoyl-phosphate synthetase	15	20.5 ↑	49.8 ↑	63.3 ↑
Comp11009	Cla016474	Proline dehydrogenase	2.2	3.9 ↑	11.6 ↑	31.4 ↑
Comp01186	Cla023055	Arinosuccinase	5.6	6.2	3.7	5.3
Comp5521	Cla020781	Ornithine carbamoyltransferase	25.2	19.1 ↓	52.5 ↑	32.5
Comp6244	Cla015337	Acetylornithine aminotransferase	39	45.3 ↑	127 ↑	147.6 ↑
Comp6587	Cla008748	Glutamine amidotransferase	25.6	19.7	12.4	13.8
Comp15261	Cla017928	Glutamate 5-kinase	4.3	5.7	1.7	1.6
Comp3961	Cla019569	Ornithine-oxo-acid transaminase	19.3	30.5	28.1	17.1
Comp7707	Cla023055	Argininosuccinate lyase	5.6	6.2	3.7	5.3
Comp3468	Cla003592	Argininosuccinate lyase	22.9	26.8	35.4	25.6
Comp776_seq2	Cla002611	Arginosuccinate synthase	12.5	25.4	6	7.7
Comp776_seq1	Cla002609	Arginosuccinate synthase	18.2	18.9	2.4 ↓	2 ↓
Comp1556	Cla022915	Carbamoyl-phosphate synthetase	115.4	103.2	53.4 ↓	42 ↓

D1RPM-D4RPM indicate gene expression reads per millions for each day. ↑ and ↓ indicate the significant up-regulation and down-regulation of each gene at each time point.

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in plants, primed the expression of many genes, and NDR1/NHL10 was one of them [33]. Comp7317 gene, which is a member of NDR1/NHL10 showed significant changes at D2 only during the early stage of water deficit stress.

GRAS (for GA Insensitive, REPRESSOR of gal-3 (RGA), SCARECROW (SCR)) transcription factors, have a major function in plant development and environmental adaption. These TFs are particularly implicated in the modulation of plant tolerance to stressors as cold, drought, salinity by crosstalks via GA to ABA-dependent and ABA-independent pathways [34]. For example, SCL7 confers salt and drought tolerance in *Arabidopsis* [35]. SCL14 is involved in the detoxification of xenobiotics and possibly endogenous harmful metabolites [36]. Comp20554, which belongs to the GRAS transcription factor family, showed significant up-regulation especially on D2.

The expression profile of Comp3048, which codes for a methyltransferase, maintained high levels during drought stress, especially on D2. It was found that myo-inositol-O-methyltransferase (Imt1) responded to low temperature stress in transgenic *Arabidopsis* [37]. Trithorax-like H3K4 methyltransferase from barley is drought inducible [38]. The methylation of myo-inositol catalyzed by myo-inositol methyltransferase (IMT) occurs when plants are under abiotic stress. Over-expression of IMT resulted in improved tolerance to dehydration and salt stress treatment in *Arabidopsis* [39].

Heat-shock proteins (HSPs) are environmentally induced proteins that enable plants to cope with heat and other environmental stresses. For example, *Trichoderma harzianum* Hsp70 transgenic *Arabidopsis* is abiotic stress tolerant [40]. Similarly HSP22 was found to be highly upregulated in *C. colocynthis* roots during drought conditions [13]. Overexpression of GmHsp90S can decrease damage of abiotic stresses in *Arabidopsis* [41]. Comp14675 belongs to the HSP family, and showed up-regulation at later stages of D3 and D4 (Figure 5).

Plant cold shock proteins (CSP) are very conserved among various plant genera [42]. The first CSP identified was WCSP1 from winter wheat, which did accumulate in response to low-

temperature stress [43]. Similarly in *C. colocynthis* CSP (Comp13927) was up-regulated during later stages of water deficit.

Comp2553, one MA3 domain-containing protein gene, showed marked changes on D3 and D4 under drought conditions (Figure 5). Loss-of-function of ECIP1 (one MA3 domain-containing protein) resulted in enhanced ethylene response but altered salt response [44] in *Arabidopsis*.

Overexpression of plant BL-1 in *Arabidopsis* resulted in the attenuation of cell death induced by biotic stresses (pathogens) and abiotic stresses such as heat, cold, drought, salt and chemical-induced oxidative stresses [45]. BL-1 might function to control the level of the “pro-survival and pro-death” signals under multiple stress conditions in plants [46]. For example, cucumber BAX inhibitor-1 is a conserved cell death suppressor induced by cold stress and a negative regulator of programmed cell death (PCD) [47]. Comp372 encodes one BL-1 gene, which was up-regulated by drought at later stages (D3 and D4).

Comp8117, which is a homologue of a *Populus* EST (CU233481.1), is a drought stress related gene, up-regulated at D4. Comp10586, which encodes a member of the MYB transcription factor family, is up-regulated during the late stage of drought stress. Some MYB members have been shown to regulate plant responses to biotic and abiotic stress conditions [48]. For example, AtMYB96 acts through the ABA signaling pathway to induce pathogen resistance by promoting salicylic acid biosynthesis, and thus regulating stomata movement, drought tolerance and disease resistance in *Arabidopsis* [49,50]. MYB88 might function directly or indirectly, as positive regulator of stress-responsive genes [51]. TaMYB30-B from wheat did improve drought stress tolerance in transgenic *Arabidopsis* [52]. Another family of transcription factors, the WRKY family, named for the WRKY domain of about 60 amino acids, contains a highly conserved WRKYGQK heptapeptide at its N-terminus and a zinc-finger-like motif at its C-terminus [53]. WRKY transcription factors are involved in multiple aspects of plant growth, development and stress [54]. Several TaWRKY in wheat with roles in the abiotic stress response acted in an ABA-dependent

manner [55]. Here, gene Comp19751 encodes a WRKY gene, which showed remarkable up-regulation at D4.

MADS-box family members function in reproductive development and stress [56]. For example, OsMADS25 and OsMADS27 transcripts accumulate in response to osmotic stress [57]. Comp6823, which encodes for a MADS-box gene in *C. colocynthis*, is up-regulated markedly at the last stage of drought (D4; Figure 5).

### Analysis of the Drought Stress Signaling Transcriptome in *C. colocynthis*

Drought stress signal transduction consists of several pathways including ionic and osmotic homeostasis signaling, detoxification response and growth regulation pathways [58]. Genes detected in *C. colocynthis* leaves during water deficit are listed in Table 4.

In the ion signaling pathway, calcium binding proteins are well known for their involvement in both biotic and abiotic stress response pathways [59]. The calcium ion ( $\text{Ca}^{2+}$ ) as a secondary messenger in plants is sensed by calmodulins (CaMs)/CaM-like protein (CMLs), the caldineurin B-like proteins (CBLs) and  $\text{Ca}^{2+}$ -dependent protein kinases (CDPKs). CaM binds to CaM-binding proteins (CBPs), which function in different pathways under biotic and abiotic stresses [60]. A total of 10  $\text{Ca}^{2+}$  binding proteins were detected in the *C. colocynthis* transcriptome.

Protein phosphorylation is a central theme in the cell's response to stress. The MAP kinase cascade in transcript levels consist of a number of protein kinases, such as two-component histidine kinase, MAPKKK, MAPKK, MAPK etc. [61]. Here we detected 16 MAP kinases in the *C. colocynthis* transcriptome.

Membrane phospholipids can activate several types of phospholipases that cleave certain phospholipids to generate lipid messengers (eg. PA, DAG, IP<sub>3</sub>), which further regulate stress tolerance through modulation of stress-responsive gene expression [62]. Several members in this pathway, such as phospholipase C (PLC), diacylglycerol (DAG) and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>)-like aquaporin were detected (Table 4).

Detoxification signaling can ameliorate the damage in plants under stresses [63], as noted in many other plant species. A total of 32 detoxification proteins were detected in the *C. colocynthis* transcriptome.

Molecular mechanisms regulating gene expression in response to drought stress have been studied by analyzing the functional transcription factors in ABA-dependent and ABA-independent pathways [6]. Several regulatory proteins in ABA-dependent or -independent pathway were detected, among which NAC, MYB/C and leucine-rich repeat proteins (LRR). These regulatory proteins can further modulate many responsive transcription factors. Several functional proteins such as heat shock protein (HSP) 70, HSP22, grpE like protein, respiratory burst oxidase D (RBOHD), VIRE2-Interacting protein2 (VIP2), ABA transporter-like protein, synaptobrevin-related protein, translocon outer envelope of chloroplast (Toc34-1), beta-amylase, TIP1 (TIP GROWTH DEFECTIVE 1), and RD22 were detected.

### Analysis of Phytohormone Signaling Mediators in *C. colocynthis*

Phytohormones play important roles in regulating plant responses under biotic and abiotic stress. Elaborate phytohormone signaling networks mediate the adaptability of plants to different environmental conditions [64]. Many phytohormones such as ABA, salicylic acid (SA), jasmonic acid (JA), auxin, ethylene and gibberellic acid (GA) are being studied for their role in abiotic stress responses [33,65,66]. It is known that downstream signaling

proteins for auxin, GA, JA, and ABA are subjected to ubiquitin-dependent degradation [67]. Putative phytohormone signaling genes detected in *C. colocynthis* during the drought response are listed in Table 5.

Ethylene signaling pathway components were ordered into a hypothetical linear pathway based on both genetic (epistasis) analysis and biochemical interactions [68]. Almost all of the ethylene signaling homologous members (118 ethylene response factors or ERFs) were detected in the *C. colocynthis* transcriptome. APETALA2/ethylene responsive factor (AP2/ERF) transcription factors are well-known for mediating stress responses and development in plants [69].

The auxin response factor (ARF) family contains transcription factors that bind to auxin-responsive elements (AREs) in the promoters of primary auxin-responsive genes. Aux/IAAs are early auxin-response proteins that bind ARFs, therefore inhibiting ARE-mediated gene transcription. Aux/IAAs are involved in ubiquitin-mediated degradation, which is catalyzed by SCF E3 ubiquitin ligase. TIR1 can stimulate Aux/IAA proteolysis by binding auxin to this protein [70]. All of the major auxin signaling related transcription factors found in the *C. colocynthis* transcriptome are shown in Table 5. Similar to the auxin pathway, a novel family of transcriptional regulators, the jasmonate ZIM-domain (JAZ) proteins play a target part as Aux/IAA. In addition, COI1 plays a similar role as TIR1, while MYC and R2R3-MYB transcription factors work as ARFs [71]. Several JA signaling pathway related genes exist in the *C. colocynthis* transcriptome.

In the SA pathway, NPR1 (non-specific disease resistance 1) is a key regulator in SA-dependent defense signaling [72]. Similarly WRKY and TGA play major roles as transcriptional regulators in the SA pathway. We detected 2 NPR1 related proteins, 46 WRKYs and 6 TGAs in *C. colocynthis*, which might function in the *C. colocynthis* SA pathway.

Drought triggers the production of the phytohormone ABA, which in turn causes stomatal closure and induces expression of stress-related genes. The soluble PYR/PYL/RCAR receptors function at the apex of a negative regulatory pathway to directly regulate PP2C phosphatases, which in turn directly regulate SnRK2 kinases [73]. Several of the core transcription factors in the ABA pathway are listed in Table 5.

We also detected 11 GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), 4 DELLA growth inhibitors (DELLAs) and 2 F-box proteins (SLY1) and SNEEZY (SNZ), which play important roles in GA signaling pathways [74,75].

### Cellular Metabolism under Drought Stress in *C. colocynthis*

Global gene expression analyses have shown substantial down-regulation of many photosynthetic genes under drought not only in *Arabidopsis* [76], but also several other species such as indica rice [77,78]. Similarly, many photosystem I and II, chlorophyll a, b binding protein, and oxygen evolving enhancer protein genes [79], showed down-regulation during water deficit stress in *C. colocynthis* (Table 6).

Citrulline, a non-protein amino acid intermediate in the arginine biosynthetic pathway, has been found to accumulate in leaves of drought tolerant watermelon under water deficit conditions [80,81,82]. Thus several factors related to the response of this model drought tolerant species to stress have been identified (Table 7, Figure S1). Citrulline metabolic genes (carbamoyl-phosphate synthetase, acetyl glutamate synthase, acetylornithine aminotransferase, aminoglutamate decarboxylase, acetylornithine deacetylase, and glutamate dehydrogenase) were found to be significantly up-regulated during drought.

One of the major research goals is to understand the molecular mechanisms underlying drought tolerance in plants. It is clear that

drought triggers a wide variety of responses in *C. colocynthis*. Down regulation of many photosynthetic genes was observed especially at the later stages of drought. Up regulation of many transcription factors, stress signaling factors, detoxification genes, and genes involved in phytohormone signaling occurred throughout the water deficit experiment. Systematic approaches using genomic analyses should lead to the discovery of additional stress factors and provide us with a better understanding of the stress tolerance mechanism of this drought tolerant plant species.

### Supporting Information

**Figure S1 Citrulline metabolism pathway in *C. colocynthis*.**  
(TIF)

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**Table S1 qRT-PCR primer sequence information.**  
(DOCX)

**Table S2 Non-significant and significantly changed *C. colocynthis* genes expressed as read counts during drought.**  
(XLSX)

### Author Contributions

Conceived and designed the experiments: ZW FD. Performed the experiments: ZW FD. Analyzed the data: ZW FD HH LG. Contributed reagents/materials/analysis tools: FD LG SM. Contributed to the writing of the manuscript: ZW FD.

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