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Transcriptome analysis of safflower (*Carthamus tinctorius* L.) reveals the roles of osmotic adjustment and regulatory mechanisms in response to drought stress

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Abstract

Although safflower (*Carthamus tinctorius* L.) is a momentous medicinal and industrial crop with intrinsic stress tolerance, molecular mechanism response to drought stress at the transcriptome level has been rarely scrutinized. Here, comprehensive transcriptome analysis was executed in the seedling stage to decipher responsive genes to drought stress. 138 M (90.9%) out of 152 M clean reads were mapped to the reference genome, generating 74,491 contigs. 1,096 differentially expressed genes (DEGs) were identified under drought stress, containing 454 genes up-regulated and 642 genes down-regulated. Transferases ($n=58$), kinases ($n=51$), and transcription factors ($n=47$) were key genetic players under drought stress. Thirty-nine transcription factors (belonging to the HD-ZIP, ERF, CO-like, bZIP, TALE, NAC, C2H2, FAR1, GRAS, MYB-related, and Trihelix families) were upregulated, and 59 transcription factors (belonging to the bHLH, GATA, HD-ZIP, WRKY, G2-like, DBB and LBD families) were downregulated. The results illustrated that drought stress dramatically diminished photosynthesis rate and chlorophyll content. The functional enrichment analysis indicated that in addition to canonical pathways of response to drought stress (such as metabolic pathways and secondary metabolite biosynthesis), revealed reconnaissance of more specific pathways, including peroxisome, autophagy, photosynthesis, glycerolipid metabolism, beta-alanine biosynthesis, and protein processing. In addition to distinguishing the classical drought stress-responsive genes in safflower, several novel genes were recognized in drought stress transcriptome analysis that have not been reported in safflower and have been less studied in other plants. These candidate genes could be promising in future breeding programs for the extension of tolerant cultivars and as molecular markers.

Keywords Abiotic stress, Comparative transcriptomics, Differentially expressed genes (DEGs), Drought tolerance, Real-time qRT-PCR, RNA-seq, Transcription factors

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Introduction

Agriculture of the 21st century is expected to have diverse arrays of answers to the daunting task of feeding future generations of humans and animals. Environmental factors, especially abiotic stresses such as drought, act against achieving the goal of a 70% increase in crop yield by 2050. With the increasing human population in recent years and demand for more food, expenditure of agricultural products has increased worldwide. Drought stress threatens crop yield, food security, and causes economic losses [1] and it is a main limiting factor in crop cultivation in Iran with 80% of the land locating in the arid and semi-arid regions [2]. Thus, identifying genetic factors that help plants withstand drought stress and understanding the stress-responsive mechanisms would be paramount in breeding programs to develop tolerant cultivars. At the molecular level, many stress-related proteins, defense responses, detoxifying agents, carbohydrate metabolism, production of ROS, and reduction in chlorophyll content and photosynthesis rate perform key roles in this process [3]. Safflower (Asteraceae: *Carthamus tinctorius* L.) is an annual herbaceous oilseed crop suitable for arid and semi-arid regions, with an extensive root system and global production of more than 800,000 tons and a cultivated area of more than one million hectares [4]. It is considered a relatively new crop for its valuable oil content with high amounts of oleic and linolenic acids. Drought stress decreases oil content, fatty acid composition, yield [5], chlorophyll content [6, 7], and photosynthetic activity [8]. Although safflower is a drought-tolerant crop, the most tolerant among oilseeds [9], its molecular adaptation mechanisms at the transcriptome level have been rarely studied due to its genome complexity and low cultivated area, compared to other oilseed plants are almost unknown. In an integrated transcriptomics and metabolomics of drought-tolerant vs. drought-intolerant safflower varieties under drought stress, the key candidate responsive genes to drought were identified [10]. It was reported that *A. thaliana* lines overexpressing a safflower MYB gene (*CtMYB63*) had pronounced drought tolerance [11]. In our earlier work, *CAT2*, *ZAT10*, and *CAMTA* were introduced as candidates for drought response [12]. Safflower is a naturally stress-resilient oilseed and medicinal crop. Compared to other drought-tolerant plants such as sorghum and millet, safflower utilizes hormonal regulation and ABA signaling under drought stress conditions to ROS scavenging and activate the antioxidant defense system, while millet (a C4 plant), due to early maturation, asynchronization in tillering and early flowering [13], and sorghum (a C4 plant) with physiological parameters such as waxy leaves and less production of ROS [14], minimize water loss under drought stress. Given that the safflower genome has only recently been sequenced and not much has been studied about the response of this inherently tolerant plant to abiotic stresses, including drought stress, studying the differential response of the safflower transcriptome to drought stress can greatly help identify genes and stress response pathways and pave the way for future targeted studies. In this study, we investigate differential transcriptome profile of safflower (cv. ARAK 2811) under drought stress to identify involved genes and biological pathways in response to drought stress via an RNA-seq analysis at the seedling stage, and report here.

Materials and methods

Plant growth and treatment

Safflower seeds (cv. Arak 2811) were surface-sterilized with sodium hypochlorite (1%) for 5 min and washed several times with dH_2O. Seeds were planted in 800 g dark pots in the National Plant Protection Research Institute greenhouse at 25 ± 2 °C and a dark/light regime (16h/8h). The pots were regularly watered every three days. At the 5–6 leaf stage, seedlings were stressed via water withholding for 10 days to have a soil with 40% field capacity (F.C), when twisting, wilting, and leaf rolling symptoms were seen in safflower leaves (occurrence of drought stress), fresh leaves were collected and snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Total RNA was extracted from green leaves 38 d post-planting (5–6 leaf stage) from both control (CT) and drought-treated (DT) seedlings with three biological replicates. The SPAD device was utilized to measure leaf chlorophyll content in three replicates. The Li-Cor 6400 portable infrared gas analyzer (IRGA) was used to assess the photosynthesis rate of safflower seedlings in CT and DT treatments. Soil field capacity (FC) was measured based on the moisture content using the following formula [15] Supplementary Table S1. The Arak 2811 is a commercial variety that is cultivated as a second crop in summer after wheat and barley in most agricultural lands in Iran due to its favorable agronomic and economic traits, such as higher thousand-grain weight, capitulum number per sub-branches, short growth period, drought tolerance, and lower water requirement compared to corn.

RNA extraction and cDNA synthesis

Total RNA extraction was performed with three biological replicates using the GenUP Total RNA Kit, following the manufacturer's protocols. RNA quality was assessed using 1% agarose gel, and the quantity was obtained by a NanoDrop spectrophotometer. RNA integrity number (RIN) was determined via microcapillary electrophoresis. To select high-quality RNA for sequencing, the following criteria were used: 28 S/18S ratio ≥ 2, OD 260/280 ≥ 1.8, and RIN > 8. cDNA synthesis was performed using the AddScript kit according to the manufacturer's protocol.

Sequencing and mapping short reads to the reference genome

RNA sequencing was executed with two out of three biological replicates (based on RNA quality tests) using the Illumina Novaseq 6000 platform equipped by flow cell SP XP (with 100× two cycles, paired-end), and short reads were saved in FASTQ format. Short reads were trimmed to remove adapter sequences and reads with inappropriate quality. FASTQC v0.11.9 was used to check the quality of trimmed reads [16]. The GC content, reads length, N content, and adaptor content were reported. The safflower's reference genome (FASTA) and respective annotations (GFF3) were downloaded from the safflower genome database. The Trimmomatic v.0.39 was used to filter out low-quality reads (SLIDINGWINDOW:4:20) and retaining the reads with a minimum length of 35 bases (MINLEN:35). The genome index was built using HISAT2. All clean reads were aligned against the indexed genome using HISAT2 with default parameters (--score-min L,0,-0.2 --mp 6,2). StringTie v.2.2.1 was used with default parameters (-c 1 -m 200 -M 50) to obtain the transcriptome counts of each annotated gene of safflower [17]. Additional explanations about methods/devices and software/websites used in the study are provided in Supplementary Table S1.

Differentially Expressed Genes (DEGs) identification and co-expression analysis

The genes with read counts < 10 were omitted to filter out low-expressed genes, and then counts of each sample were converted to count per million (CPM) values [18, 19]. To identify and determine the distance between the expression profile of genes in both control (C) and drought stress (S) seedlings, principal component analysis (PCA) was executed and presented as a biplot. To remove any batch effect, CPM data were normalized using the NormalizeBetweenArrays() function in edgeR package in R [20]. DEGs were identified using the glmFit function in R with edgeR and limma packages [21] in which a generalized linear model (GLM) was fitted with treatment as a factor. The dispersion was estimated using estimateGLMCommonDisp(), estimateGLMTrendedDisp() and estimateGLMTagwiseDisp() functions. To claim the significance of differentially expressed genes (DEGs), the |fold change| ≥ 2 and adjusted $P \leq 0.05$ (calculated based on BH method for multiple-testing correction) were considered. Co-expression analysis in response to drought stress was conducted using DiffCorr package in R [22]. Coefficients of variation (CV) of the genes in each module were calculated, and the gene modules having more than 50% of genes with CV < 0.5 were considered as stable. A heatmap was generated for genes in each module using the gplots package in R [23].

Table 1 Characteristics of mapped reads on the safflower reference genome

Samples	Raw reads	Total mapped reads	Mapped reads (%)
CT1	42,109,745	37,576,272	89.23
CT2	40,623,835	37,394,573	92.05
ST1	37,220,181	34,001,669	91.35
ST2	32,540,561	29,614,352	91.01
Total	152,494,322	138,586,866	90.91

CT1, CT2: two biological replicates of normal (untreated) samples

ST1, ST2: two biological replicates of drought stressed (treated) samples

Functional annotation of DEGs in response to drought stress

The statistical analysis of gene ontology with adjusted $P < 0.05$, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed using the g: profiler online tool to define drought stress-affected pathways and gene enrichment analyses [24].

Protein-protein interaction network

To understand the interactions of DEGs, homologous genes/proteins with Uniport IDs were searched in STRING (https://string-db.org/) using *A. thaliana* as a model plant to construct a PPI network, and data were transferred to Cytoscape (v3.10.1) for visualization. The *CytoHubba* plugin (http://apps.cytoscape.org/apps/cytohubba) gene network was used to plot the network based on the degree criterion [25].

Quantitative real-time PCR analysis

To validate the reliability of RNA-seq results, six genes, including three upregulated and three downregulated DEGs, were chosen to be scrutinized by real-time qRT-PCR with three replicates using gene-specific primer pairs (Supplementary Table S2) and the CFX-96 Real-time PCR device. The *EF1* gene was used as a housekeeping gene [26].The REST software was utilized to calculate the relative expression of the selected genes [27].

Results

Statistical analysis of short reads and transcript mapping

Comparative transcriptome profiling of Arak 2811 under drought stress vs. control condition resulted in 152 M short reads with lengths of 35–150 bp across all samples using the Illumina Novaseq 6000 platform (Supplementary Table S3). The average read length after trimming was 150 bp, and average GC content ranged between 46 and 48%. Library sizes/sequencing depths were 37.6, 37.4, 34.0, and 29.6 M reads, respectively, for CT1, CT2, ST1, and ST2 samples (Table 1).

Quality evaluation confirmed that reads were of high reliability after trimming (Figure S1). The total number of clean reads that were mapped to the reference genome

was 138 M for all samples. Mapping efficiency ranged from 89.2% to 92.1%, with an average of 90.9% (Table 1).

Identification of differentially expressed genes involved in drought regulation

DEGs were recognized in different treatments on unique pairs of reads. After counting the assembled reads, 74,491 contigs were identified when checked against the safflower genome. Of these, 45,325 were mapped to annotated genes of safflower. Before DEG identification, the genes with more than 2 zero counts in four samples were filtered. As a result, 20,918 genes remain for DEG identification. Data normalization was conducted with the CPM algorithm, and PCA demonstrated no batch effect (Figure S2). Expression analysis of *C. tinctorius* cv. Arak 2811 genes under continuous drought stress revealed 1,096 significant DEGs between treated vs. untreated samples (Fig. 1A). The heatmap graph demonstrates the expression profile of genes in normal and stress states. The change in the expression pattern heatmap is related to the effect of drought stress, and a different expression pattern between the normal and stressed samples is

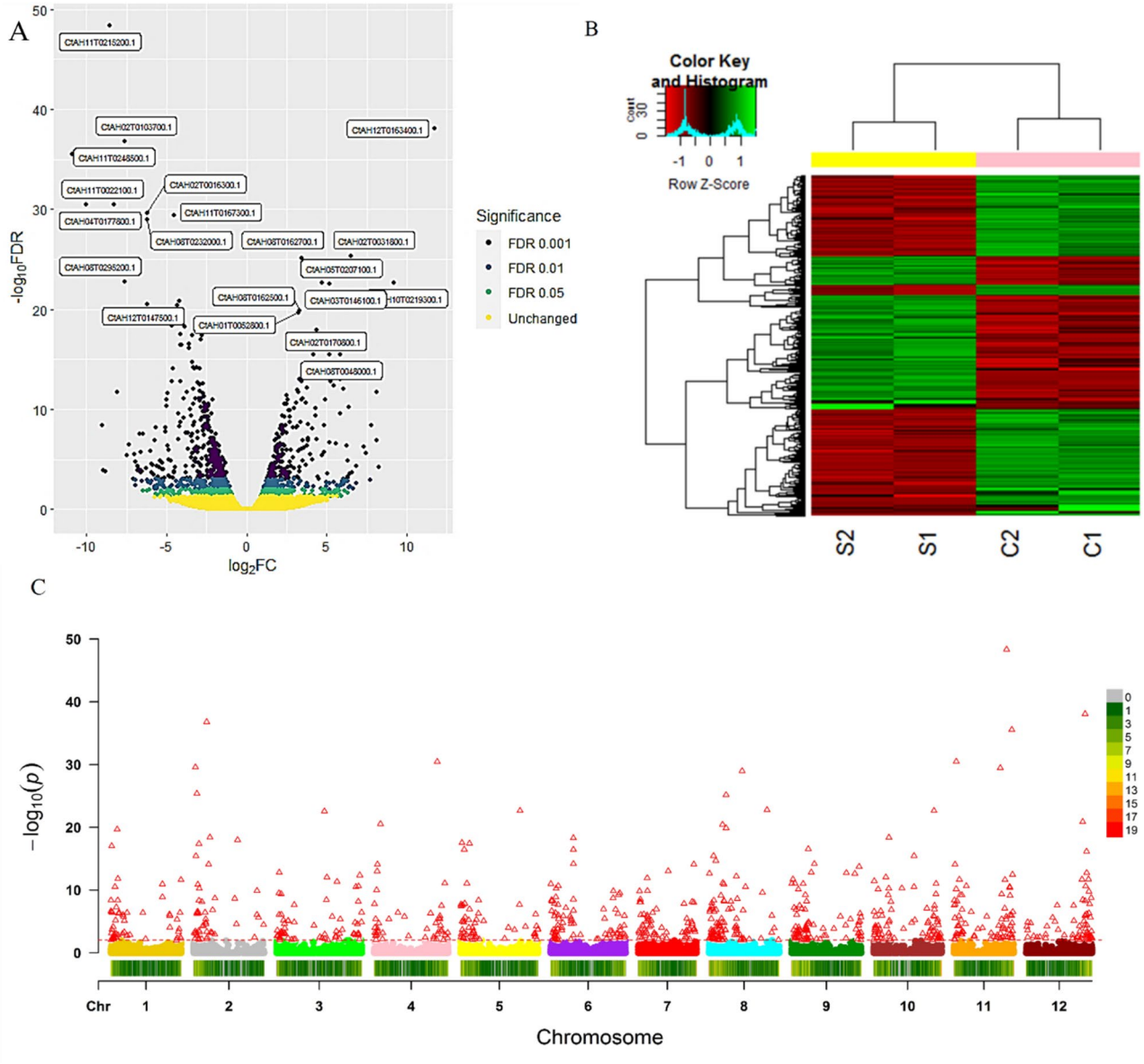

Fig. 1 **A** Volcano plot indicating transcripts with increased expression (on the right) and decreased expression (on the left) in safflower under continuous drought stress. The top DEGs were marked with rectangles. X axis: log_2 FC, and Y axis: -log_{10} FDR. **B** Heatmap of DEGs under drought stress. Upregulated genes (green color) and Downregulated genes (red color) under drought stress of safflower (repetitions of drought stress: S1 & S2 and normal repetitions: C1 & C2). **C** Chromosomal position of safflower DEGs under drought stress. The red triangles are the significantly differentially expressed genes (DEGs). X axis: chromosome number, and Y axis: -log_{10} (p)

observed (Fig. 1B). The 20 top genes comprising 10 up- and 10 down-regulated genes are brought in Table 2.

The *RAB18*, *RAB19*, and *XERO1* belonging to the dehydrin family genes are involved in biological pathways of response to water deprivation and response to abscisic acid. The *ATHB7* gene is a HD-ZIP transcription factor and involved in response to water, response to water deprivation, and response to abscisic acid. The *BAG6* protein is implicated in apoptosis, senescence, and response to biotic and abiotic stress [28]. The mechanism of action of the *CBSDUF5* gene is not entirely understood. Studies have shown that some *DUF* genes play a role in the response to drought stress [29]. *XTH* genes are participated in the xyloglucan metabolic pathway, which is essential for cell wall regeneration and development during drought stress [30]. The *HSP*s are a group of proteins that are activated when cells are exposed to environmental stresses, and their expression increases in the cell. They are key genes in the response to abiotic stress in plants [31].

Positioning the DEGs on safflower chromosomes

The chromosomal position plot of the identified DEGs illustrates the minimum number of genes close to the centromeres, and most DEGs are distributed on chromosome arms (Fig. 1C). The highest number of DEGs was found on chromosomes 8 (126 genes; 11.4%) and 10 (113 genes; 10.3%). The lowest number of DEGs was on chromosomes 2 (68 genes; 6.20%) and 4 (60 genes; 5.4%).

Co-expression network of DEGs under drought stress

The safflower orthologs of up- and downregulated genes were 436 and 604 in *A. thaliana*, respectively. Co-expression analysis exhibited six gene modules, and only results of modules 1 (upregulated) and 2 (downregulated) demonstrated to be stable in the replicates of each condition (Fig. 2). The expression profiles of genes in each module were highly correlated ($r^2 \geq 90\%$). The number of genes in each module is depicted in Supplementary Table S4.

Functional enrichment analysis

GO enrichment

GO (gene ontology) enrichment analysis with non-redundant (Nr) annotation and adjusted p-value < 0.05 was conducted. Upregulated genes were classified into three main categories, including biological processes (BP: 380 terms), molecular function (MF: 110 terms), and cellular component (CC: 54 terms) (Figures S3 A & S4 A). In the category of BP, the largest number of genes belong to the cellular process (186 genes), response to chemicals (185 genes), metabolic process (173 genes), response to stimulus (134 genes), and stress response (93 genes). For MF, the largest number of genes belongs to molecular function (233 genes), binding (187 genes), catalytic activity (114 genes), protein binding (113 genes), ion binding (109 genes), and organic cyclic compound binding (107 genes). In the category of CC, cellular component (234 genes), cellular anatomical entity (231 genes), intracellular anatomical structure (201 genes), intracellular

Table 2 Identified 10 top down- and upregulated genes under continuous drought stress and their descriptions in safflower seedlings

Gene id	Expression type	Protein id	Gene symbol	Description
CtAH08G0162700.1	up	P30185	*RAB18*	Dehydrin Rab18
CtAH08G0162500.1	up	P30185	*RAB19*	Dehydrin Rab18
CtAH01G0052800.1	up	P25863	*XERO1*	Dehydrin Xero 1
CtAH02G0031800.1	up	P46897	*ATHB7*	Homeobox-leucine zipper protein ATHB-7
CtAH05G0207100.1	up	P46516	*HSP21*	17.9 kDa class II heat shock protein
CtAH03G0146100.1	up	O82345	*BAG6*	BAG family molecular chaperone regulator 6
CtAH02G0170800.1	up	Q9C9W9	*ADO3*	Adagio protein 3
CtAH08G0048000.1	up	P13853	*HS17C*	17.6 kDa class I heat shock protein 3
CtAH10G0219300.1	up	Q9LTD8	*CBSDUF5*	DUF21 domain-containing protein At5g52790
CtAH12G0163400.1	up	P05478	-	HSP16
CtAH02T01G03700.1	down	A0A067XRK9	*XTH6*	Xyloglucan endotransglucosylase protein 6
CtAH11G0248500.1	down	Q9M015	-	Uncharacterized protein At5g01610
CtAH11G0022100.1	down	Q8LF99	*XTH6*	Probable xyloglucan endotransglucosylase/hydrolase protein 6
CtAH08G0295200.1	down	Q8LB81	-	GDSL esterase/lipase
CtAH12G0147500.1	down	Q38910	*XTH23*	Probable xyloglucan endotransglucosylase/hydrolase protein 23
CtAH02G0016300.1	down	P18025	*TUBB1*	Tubulin beta-1 chain
CtAH11G0167300.1	down	O64964	*TIP1-1*	Aquaporin TIP1-1
CtAH08G0232000.1	down	G5DAC6	*XTH1*	Xyloglucan endotransglucosylase protein 1
CtAH11T0215200.1	down			
CtAH04T0177800.1	down			

up upregulated, *down* downregulated

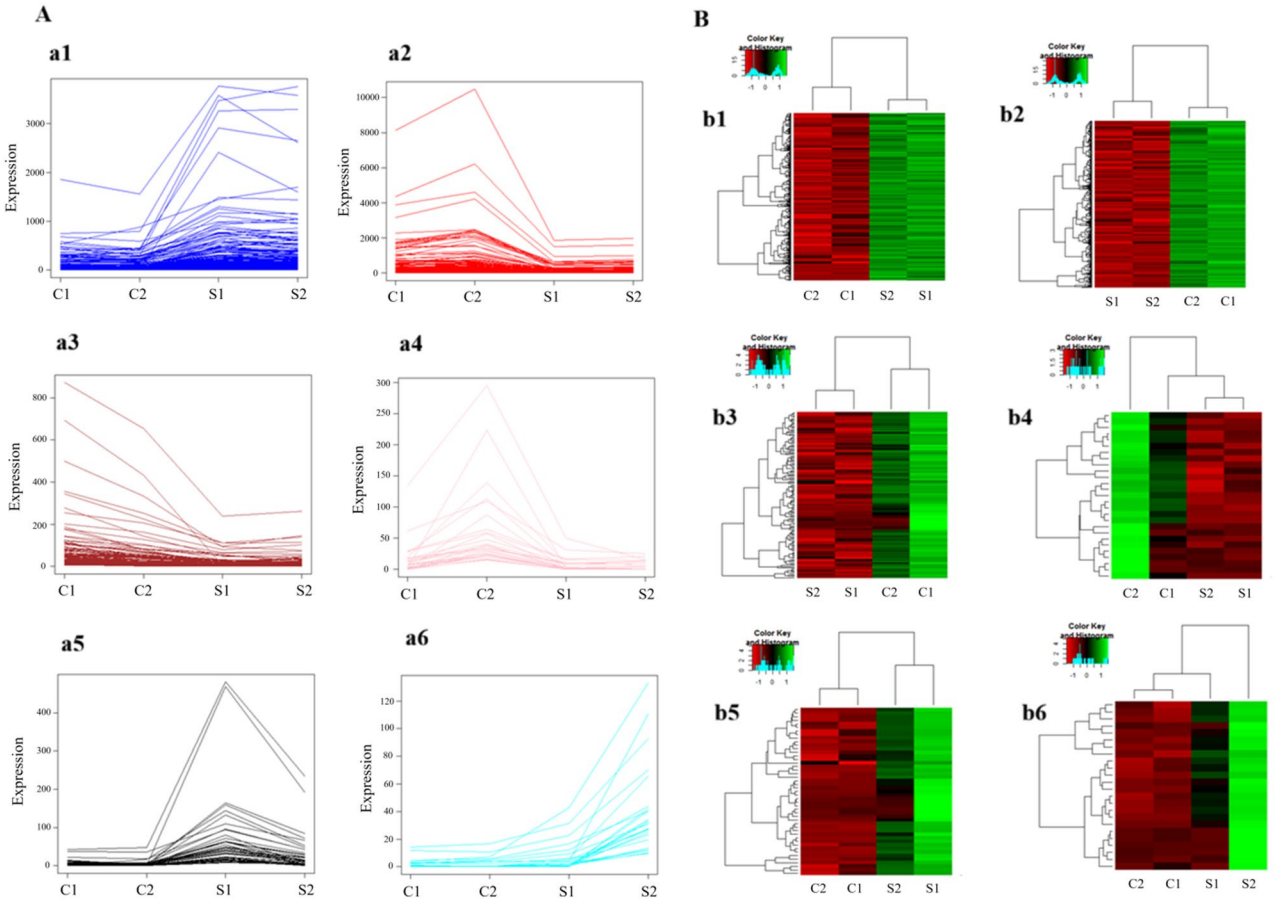

Fig. 2 **A** Co-expression profiles of DEGs in six modules (a1-a6). X axis: samples, and Y axis: expression values. **B** The respective heat maps of genes in six modules (b1-b6). Modules 1 and 2 are the most stable

organelle (182 genes), organelles (182 genes), and cytoplasm (150 genes) were enriched in the ontology analysis.

Similarly, GO enrichment analysis for downregulated genes resulted in the identification of three main GO categories, including BP (194 terms), MF (58 terms) and CC (89 terms) (Figures S3 B & S4 B). In the category of BP, the most important terms were biological processes (282 genes), cellular processes (236 genes), metabolic processes (198 genes), cellular metabolic processes (173 genes), and response to stimuli (107 genes). In the category of MF, the most important terms included molecular function (277 genes), binding (205 genes), catalytic activity (158 genes), protein binding (124 genes), ion binding (106 genes), organic cyclic compound binding (105 genes), and transport activity (68 genes). For CC, the most important terms were cellular component (291 genes), the anatomical entity of the cell (287 genes), the structure of the intracellular anatomy (219 genes), organelles (202 genes), and intracellular membrane-bounded organelle (197 genes).

KEGG pathway determination of DEGs under drought stress

For upregulated genes, eleven associated KEGG pathways to drought stress (adjusted p-value < 0.05) were discovered, namely metabolic pathways (KEGG:01100, 41%), biosynthesis of secondary metabolites (KEGG:01110, 24%), protein processing in the endoplasmic reticulum (KEGG:04141, 6%), nucleotide metabolism (KEGG:01232, 5%), fatty acid degradation (KEGG:00071, 4%) and fatty acid metabolism (KEGG:01212, 4%), peroxisome (KEGG:04146, 4%), tyrosine metabolism (KEGG:00350, 3%), α-linolenic acid metabolism (KEGG:00592, 3%), autophagy (KEGG:04136, 3%) and β-alanine metabolism (KEGG:00410, 3%) (Fig. 3; Supplementary Table S5). The metabolic pathway and biosynthesis of secondary metabolites consist of important genes including The *LACS7* (long-chain Acyl-CoA synthetase 7), *ALDH12A1* (Delta-1-pyrroline-5-carboxylate dehydrogenase 12A1) that implicated ROS metabolic process, proline metabolic and catabolic process, and stress response, the expression these genes were induced in metabolic pathway. trehalose-phosphate synthase 6 (*TPS6*), 10 (*TPS10*), and 11 (*TPS11*), the *CYP707A2* (abscisic acid 8'-hydroxylase 2) is involved in the abscisic

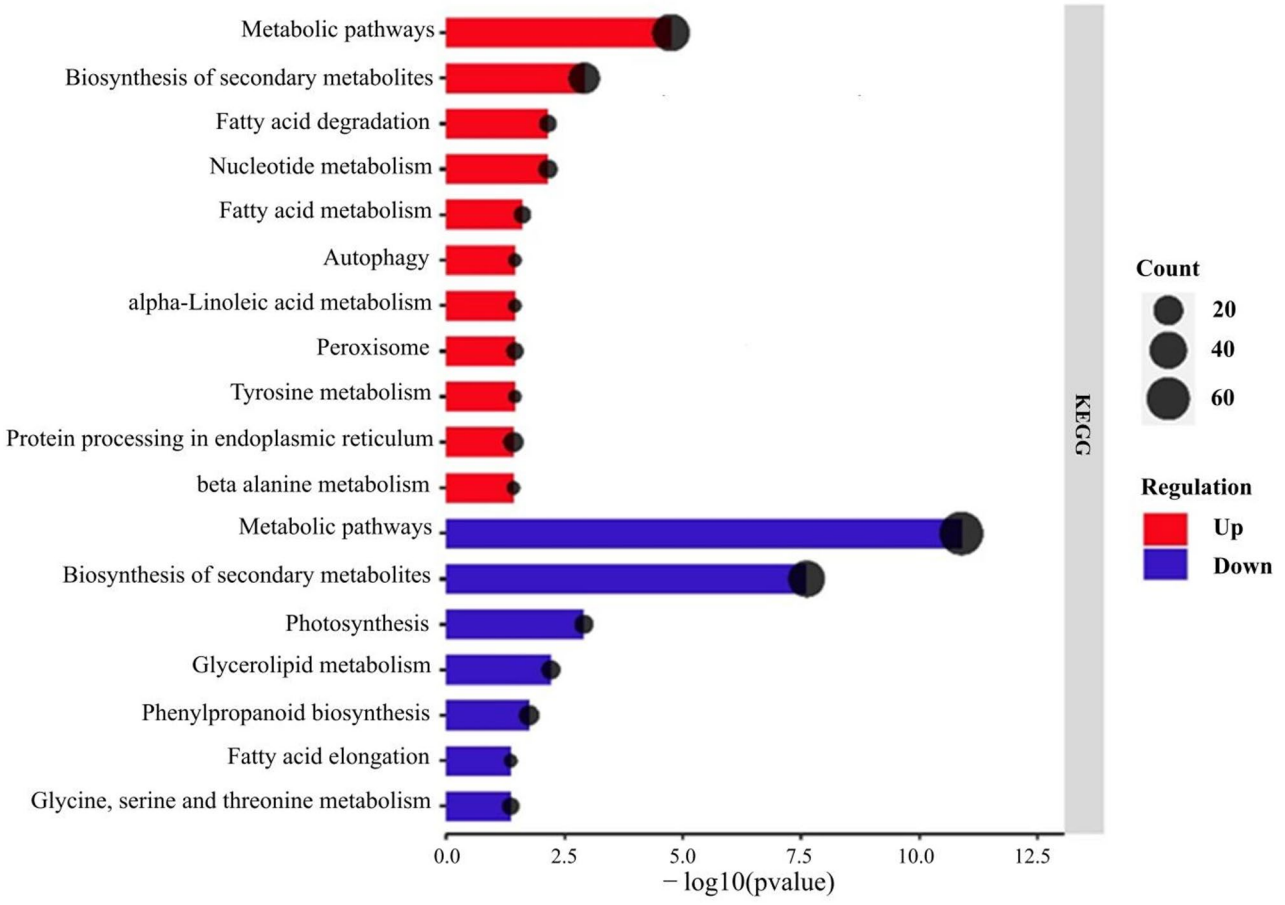

Fig. 3 Results of analysis of KEGG pathways for up- and down-regulated genes based on -$\log_{10}$*p-value* (X axis). Black dots in different sizes indicate the number of genes in each pathway

acid metabolic and catabolic process. Beta amylase 1 (*BAM1*) is one of the key enzymes of the starch hydrolysis pathway. The expression of above genes was increased in the pathway of secondary metabolites (upregulated). The *ACOX2*/*ACX2* and *ACOX4*/*ACX4* genes, which encodes acyl-coenzyme A oxidase-2&4 enzymes, was found in several pathways, including β-alanine metabolism, α-linoleic acid metabolism, biosynthesis of secondary metabolites, fatty acid breakdown, fatty acid metabolism, metabolic pathways, and peroxisome functions. *HSP*s (heat shock proteins) are involved in endoplasmic reticulum protein processing in response to drought stress. In the metabolic pathway *APX3* and *GSTU17* genes that play serious role in ROS scavenging and detoxification, were upregulated.

Seven KEGG pathways were found for downregulated genes with adjusted p-value < 0.05 including metabolic pathways (KEGG:01100, 50.4%), biosynthesis of secondary metabolites (KEGG:01110, 30.89%), phenylpropanoid biosynthesis (KEGG:00940, 4.88%), photosynthesis (KEGG:00195, 4.07%), glycerolipid metabolism (KEGG:00561, 4.07%), glycine, serine and threonine metabolism (KEGG:00260, 3.25%) and fatty acid elongation (KEGG:00062, 2.44%) (Fig. 3; Supplementary Table S6). GPAT (glycerol 3-phosphate acyltransferase) is involved in metabolic, biochemical, and physiological pathways, response to stress, oil content, and seed growth (118). In our research, *GPAT6* (*CtAH10g0151200.1*) and *GPAT8* (*CtAH03g0148300.1*) were downregulated in the pathways of glycerolipid metabolism and biosynthesis of secondary metabolites.

Recognition of protein classes in DEGs including TFs

BLASTp exhibited 603 downregulated genes (with 21.8%-99.7% identity to Uniprot proteins) and 436 upregulated genes (with 24.7%-100% identity to Uniprot proteins). Orthologs found in *A. thaliana* for only 665 DEGs, including 294 (67.43%) upregulated and 371 (61.5%) downregulated genes. In total, 24 protein classes were recognized for the safflower seedlings transcriptome in response to drought stress. Proteins with the highest abundance were associated with transcription factors (TFs, $n = 47$), protein kinases (PKs, $n = 51$), and transferases ($n = 58$) (Fig. 4A; Table 3).

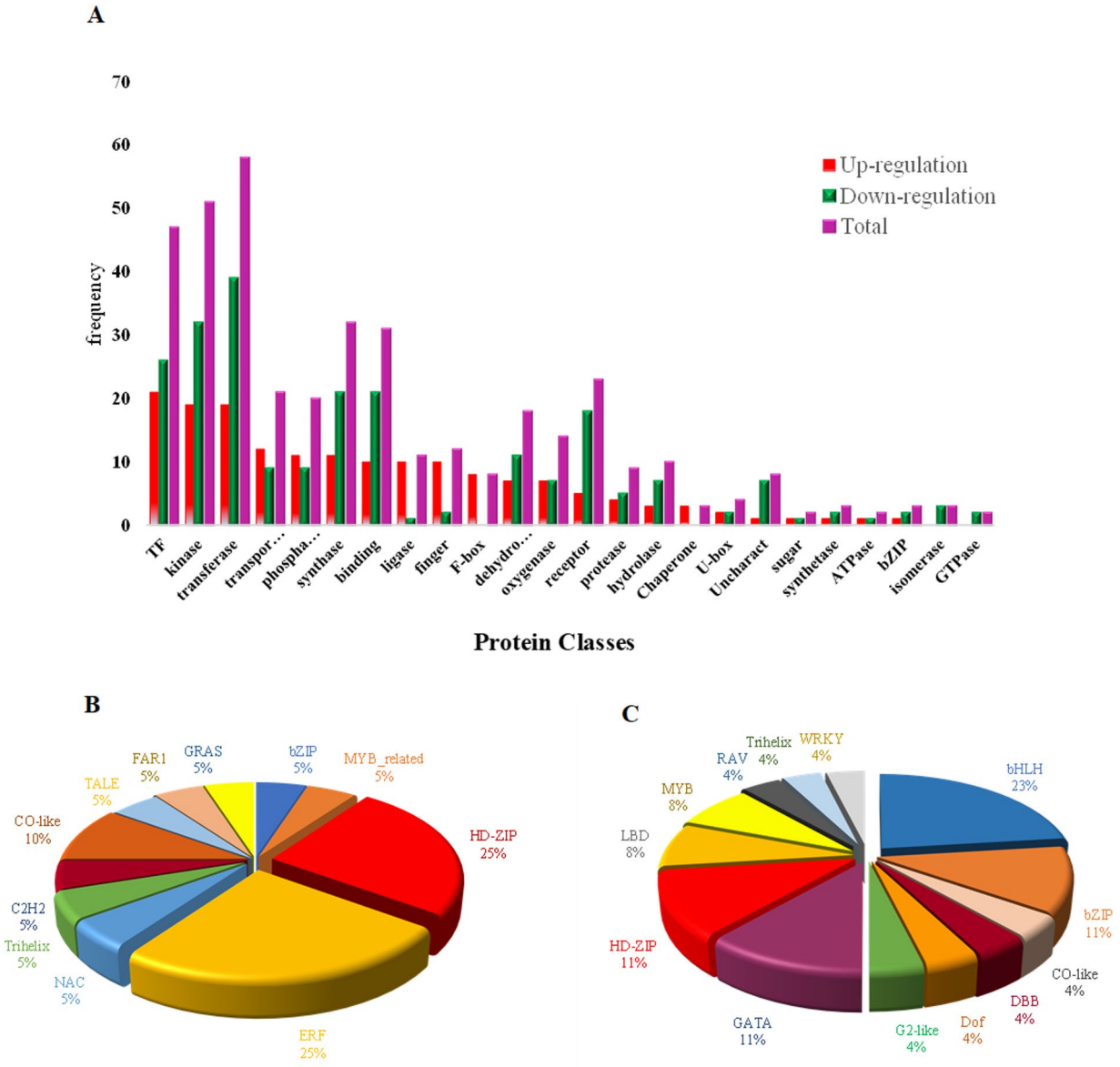

Fig. 4 **A** Abundance of different functional protein classes within DEGs in safflower seedlings under drought stress based on homology search against *A. thaliana* proteins. X axis: protein classes, and Y axis: frequency of protein classes. **B** &**C** The pie charts demonstrating the frequency of up-regulated and down-regulated transcription factors in safflower seedlings under drought stress

Thirty-nine upregulated TFs (in the order of gene frequency belonging to *HD-ZIP*, *ERF*, *CO-like*, *bZIP*, *TALE*, *NAC*, *C2H2*, *FAR1*, *GRAS*, *MYB-related*, and *Trihelix* families) (Fig. 4B), and 59 TFs with decreased expression (in the order of gene frequency belonging to *bHLH*, *GATA*, *HD-ZIP*, *bZIP*, *MYB*, *LBD*, *WRKY*, *G2- like*, *DBB*, and *CO-like* families) were recognized among DEGs (Fig. 4C). The protein kinases were achieved in this study correspond to 61 safflower gene IDs in a homology search with *A. thaliana* (Supplementary Table S7). Twenty-six (40.9%) kinase proteins belong to the serine/threonine kinase (STK) subgroup of plants' receptor protein kinases (RPKs) and one was found to be related to histidine kinase (HK2). The STK and AHK2 implicate the phosphorylation of serine/threonine and histidine residues. The HKs are important in signal transduction in response to abiotic and biotic stresses [32]. The calcium-dependent protein kinase 7 (*CDPK7*), CBL-interacting protein kinases consisting of *CIPK6*, *CIPK14 (CIPKE)*, and *CIPK25* genes (6.5%), and two *SPHK2*s (sphingosine kinase) were the other protein kinases obtained in the transcriptomic analysis of drought-stressed safflower. Seven RLKs (receptor-like protein kinases; 11.4%) were also found. Safflower guanylate kinase

Table 3 Abundance of different functional protein classes (24 top classes shown here) within DEGs based on homology search against *A. thaliana* proteins

Protein class	Upregulated	Downregulated	Sum	Protein class	Upregulated	Downregulated	Sum
TF	21	26	47	receptor	5	18	23
kinase	19	32	51	protease	4	5	9
transferase	19	39	58	hydrolase	3	7	10
transporter	12	9	21	Chaperone	3	0	3
phosphatase	11	9	20	U-box	2	2	4
synthase	11	21	32	Uncharacterized	1	7	8
binding	10	21	31	sugar	1	1	2
ligase	10	1	11	synthetase	1	2	3
Zinc finger	10	2	12	ATPase	1	1	2
F-box	8	0	8	bZIP	1	2	3
dehydrogenase	7	11	18	isomerase	0	3	3
oxygenase	7	7	14	GTPase	0	2	2

genes [*GMK2/GK-2* and *CK3* (*CtAH09T0105400.1*, *CtAH12T0178900.2;* KEGG:01232)] were upregulated under drought stress.

The transferase protein class with abundance 58 was achieved in a homology search with *A. thaliana* (Supplementary Table S8). Seven (22.5%) transferase proteins belong to methyltransferase, aminotransferase (16.1%), and glutathione *S*-transferase (6.4%) subgroups. DNA methylation, with the help of methyltransferase enzymes, is important in regulating plant gene expression under abiotic and biotic stresses [33].

Identification of hub genes

The photosynthesis pathway was inquired to decipher hub genes involved in safflower's response to drought stress. In this pathway (Supplementary Figure S5), Q8LCQ4 (*LHCA6*: light-harvesting complex A) and Q9SUI5 (*PSAK*: Photosystem I reaction center subunit *psaK*) had the highest rank and were considered as the hub genes. The result obtained from the Licor device confirmed that the photosynthesis rate in normal samples was measured at 33 µmol/s, and reduced to 24.5 µmol/s under drought treatment (25.8% reduction). The average chlorophyll content in normal leaves was 43.4, and was decreased to 32.4 in drought-treated leaves (25.3% reduction) (Supplementary Figure S6).

Real-time PCR analysis for validation of RNASeq results

To validate the expression pattern of DEGs identified by RNASeq analysis, real-time qRT-PCR was carried out using the top genes, including three up- and three down-regulated genes (Fig. 5). The relative expressions of all up- (3.2 to 6.3-fold changes) and down-regulated (-1.8 to -5.9 -5.9-fold change) candidate genes were significant ($p < 0.01$) and revealed a high correlation with the RNA-Seq outcomes.

Discussion

Drought stress, which negatively impacts the growth and development of safflower and other plants, is the most devastating abiotic stress in the era of climate change. It has been the leading factor of famine in the past and continues to appear as a serious threat to global food security in the future [34]. Safflower, because of its resilience, can be developed as a vital alternative oilseed option for Iran and similar ecosystems [35]. We conducted comparative transcriptome profiling of leaves collected from the Arak 2811 genotype of *Carthamus tinctorius* L. under continuous drought stress at the seedling stage to understand plant molecular mechanisms in response to drought stress.

Proteins involved in drought stress adaptation

Among the identified DEGs similar to [36], *dehydrin* (*DHN*) genes (*RAB18*, *RAB19*, and *XERO1*) were highly upregulated. *DHN*s are prevalent response factors to environmental stresses, such as drought and cold [37]. *RAB18* transcripts demonstrated increased abundance in response to drought. Previously, it was illustrated that *AtRAB18* expression was enhanced during drought stress in *A. thaliana* [38].

Most genes of the *aldehyde dehydrogenase* (*ALDH*) family are involved in responses to drought and oxidative stress in plants [39, 40]. Significant increases in the expression of *ALDH12A1* in transgenic Arabidopsis [41] and *Oryza sativa* [42] have been reported in response to drought stress. In *Cleistogenes songorica*, *CsALDH12A1* transcripts increased 6-fold under drought stress. Overexpression of *CsALDH12A1* in transgenic varieties of *A. thaliana* facilitates increased tolerance to drought stress [41].

The *ACOX2*/*ACX2* gene is potentially involved in the production of ABA under drought stress conditions [43]. The *LACS7* genes play an important role in plant defense mechanisms, especially drought stress-producing

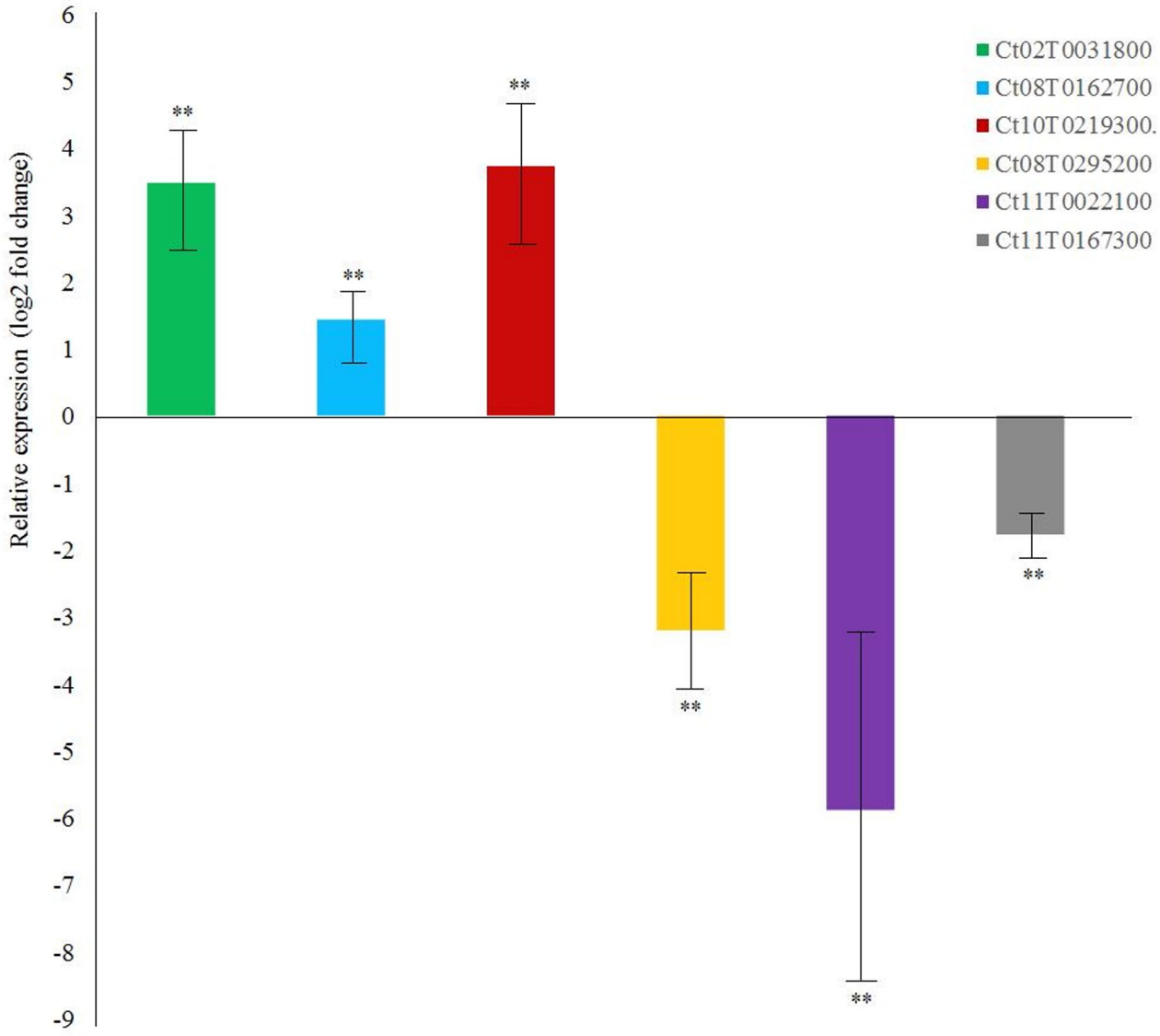

Fig. 5 Real-time qRT-PCR for three up- and three down-regulated DEGs. All genes showed significant relative expression at 1% level under drought stress in safflower seedlings. The Y-axis shows relative expression (log_2 fold change)

cuticular wax. Upregulation of *LACS* genes in sunflowers enhanced drought tolerance and reduced the destructive effects of stress [44].

Osmotic regulation under drought stress

Genes potentially involved in the β-alanine biosynthesis were upregulated in response to drought stress in safflower seedlings, similar to what was observed in *A. thaliana* [45]. Transcriptomic profiling analysis in *Sorghum bicolor* revealed the importance of β-alanine biosynthesis in drought tolerance [46]. Studies indicates that *BAM1* upregulation in stomatal guard cells was associated with starch degradation and modulates stomatal activity [47], and potentially is involved in the biosynthesis of sugars and proline in response to water stress [48]. In addition, the *BAM1* mitigates the negative effects of carbon accumulation during drought stress and is crucial for osmotic regulation within the cell [49]. Trehalose metabolism and *TPS6* as its key gene [50] are positively associated with abiotic stress tolerance, including drought, as reported in *A. thaliana* [51], wheat, cotton [52], and *Brassica napus* [53].

Reactive Oxygen Species (ROS) scavenging genes in response to drought stress

Detoxifying enzymes such as APX (ascorbate peroxidase) and GSTU17 (glutathione *S*-transferase U17) play decisive roles in ROS scavenging, detoxification, and protecting vital macromolecules in drought stress [54–57]. In our study, *APX* and *GSTU17* demonstrated significant

increases in expression. The *AtGSTU17* gene was identified in signal transduction pathways in response to drought and salinity stresses [58]. The genes of *OsGGCT-1* and *OsGGCT-2* (γ-glutamylcyclotransferase) in rice were upregulated in response to drought and salt stress, suggesting their crucial roles in response to drought stress in plants. The Gamma-glutamyl cyclotransferase probably plays a role in glutathione homeostasis and reduces the adverse effects of oxidative stress in plants exposed to drought stress [59].

Genes involved in ABA metabolism and signaling in response to drought stress

The *CYP707A2* gene (ABA 8'-hydroxylase 2) more probably involves in ABA hydroxylation under drought stress in plants. In addition, other genes such as ABF, PP2C, PYR/PYL, and SnRK2 are important in ABA signaling in drought stress. In this study, the expression of genes *CYP707A2*, *P2C25*, *P2C75*, *P2C73*, *P2C12*, and P2C16 were upregulated. In contrast, the *P2C47*, *P2C12*, and *P2C65* were downregulated. Similar to [60], *CYP707A2* overexpression of *CtCYP71A1* ameliorated drought tolerance and lignin accumulation in transgenic safflower [61]. The results showed that ABA signaling in safflower is essential for drought stress response, which is consistent with previous findings in other plants [62, 63].

Our study revealed that three tyrosine aminotransferases were upregulated in response to drought stress in safflower. The tyrosine aminotransferase overexpression in callus tissue of *Malus domestica* was associated with the enhanced drought-tolerance and osmotic stress [64]. Studies displayed that the expression of Arabidopsis ornithine aminotransferase (*AtOAT*) was associated with the increased proline content and peroxidase activity, and is also associated with drought tolerance in wheat [65].

Regulatory proteins related to responses to drought stress

MYB, NAC, WRKY, and bZIP transcription factors are reported to be the primary regulators of stress responses [66]. Overexpression of the HD-ZIP (*OsTF1*) gene in transgenic varieties of rice induced lignin biosynthesis, drought-responsive genes, and stomatal closure [67]. Members of the MYB family contribute in response to drought stress via ABA signaling and leading to improvements in drought tolerance [68]. The *HB1* gene of *Helianthus annuus* and its Arabidopsis homolog, *AtHB13*, are induced in response to drought stress [69]. Under normal conditions, *WRKY60*, *WRKY18*, and *WRKY40* directly bind to promoters, leading to the negative regulation of ABA-responsive genes (ABI4, ABI5, ABF4). Drought stress and abscisic acid induced the expression of these TFs [70]. The *ZmNAC1* overexpression in *A. thaliana* induced drought tolerance and increased chlorophyll content in transgenic plants. *ZmNAC1* acts as a positive regulator in signal transduction in abiotic stress [71]. Overexpression of *NAC1* in rice induced stomatal closure in the early stages of drought stress, which is an important factor in reducing water loss and improving drought tolerance [72]. The *ATHB-7* gene, a member of the HD-ZIP TFs family, was upregulated, which facilitates plant adaptation to drought stress via the regulation of the ABA signaling pathway [73, 74]. In the case of the basic helix-loop-helix (*CtbHLH*) TF in safflower, three expression patterns, namely upregulated (*CtbHLH12*, *CtbHLH20*, and *CtbHLH26*), downregulated (*CtbHLH24*), and bell-shaped with distinct peak timings (*CtbHLH70 and CtbHLH99*), were reported [75]. Similarly, in a comprehensive analysis of the safflower, WRKY transcription factor family, a *CtWRKY55* was detected to rapidly become upregulated by eightfold during drought (for 3 h) and rehydration (48 h of rewatering) treatments. Additionally, it was inferred that other *CtWRKY* genes, including *CtWRKY9*, *-15*, *-17*, *-37*, *-54*, and *– 72*, had a probable role in response to drought stress [76].

Protein kinases play a remarkable role in plant signaling and response to drought stress. One of the important and effective mechanisms in the signaling pathways in response to drought stress is phosphorylation and dephosphorylation of proteins regulated by kinases and phosphatases [77]. The role of *CDPK* in these pathways is well established, and directly/indirectly regulates TFs involved in response to drought stress [78]. Aadditionally, *CDPKs* have been demonstrated to have a role in drought tolerance and signaling, as reports are accumulating on their increased expression in varieties of plants like *Cajanus cajan* [79]. A protein kinase in our study with increased abundance was Sphingosine kinase 2 (*SPHK2*; F2Y4A3: KEGG:01100). *SPHK* phosphorylates sphingolipids to form sphingosine-1-phosphate (S1P), causing the closure of stomata in response to drought stress in plants [80, 81]. Investigations showed that histidine kinase proteins are involved in drought stress in cotton, and gene *GhHK8* is a negative regulator in response to drought stress in cotton [32]. The *ZmHK9* from maize plays an important role in tolerance to drought stress and stomatal development in Arabidopsis via an ABA-dependent signaling pathway [3]. Overexpression of the *AHK1* gene caused drought tolerance in Arabidopsis transgenic plants under water stress [82].

Drought stress effect on photosynthesis pathway

The gene expressions related to photosynthesis were significantly downregulated during drought stress in the current study. The *LHCA6* encodes chlorophyll a/b binding protein 6 in photosystem I. *LHCA6* is essential for chloroplast function, and its downregulation leads to a reduced photosynthesis rate in plants [83]. Here, safflower *LHCA2* and *LHCA6* were downregulated,

similar to what reported in [84]. The expression of pepper's *PSAK* was downregulated in response to drought stress. This is associated to significant limits in light harvesting, energy transfer, and photosynthetic electron transfer during stress [85]. The expression of the *PSAK* gene in *Prunus persica* L. was significantly downregulated during drought stress [86]. In maize, drought stress led to the downregulation of the ferredoxin and *PSAK* genes in drought-sensitive genotypes [87]. Downregulation of identified genes in safflower seedlings was consistent with findings from measuring the photosynthesis rate using Licor and SPAD devices (Figure S6). Reduced photosynthesis rate and average stomatal conductance during drought stress were evident (data not shown). The Average chlorophyll content significantly declined in safflower leaves 10 days after continuous drought stress.

The *XTH 6,1,23* genes are involved in the xyloglucan metabolic pathway and are less reported as drought-responsive genes; It seems they are crucial for the reconstruction and expansion of cell walls during drought stress [88]. These genes are associated with the reconstruction of stomata cell walls in water-deficient conditions to barricade water and protect plants during drought stress [89]. *XTH*s connect and disconnect xyloglucans to cellulose microfibrils [90] Their downregulation during drought stress remarkably minimizes cell wall structural changes. Additionally, water transport through the membrane diminishes due to the downregulation of aquaporin *TIP1*. The *XTH6* expression was downregulated in leaves of Arabidopsis in response to drought stress [91]. Overexpression of the *TaXTH12.5a* gene in *A. thaliana* led to the regulation and promotion of drought tolerance [88].

TIP1-1 (tonoplast intrinsic protein) is located in the tonoplast and implicated in water transport across cellular membranes. In barley, under drought stress, the *HvTIP* gene was downregulated significantly, and re-watering reversed the gene expression. The *TIP1-1* gene is essential for responding to drought stress and facilitating plant adaptation in appropriate conditions [92]. Overexpression of the *TIP* gene in transgenic tomatoes caused increased tolerance to drought stress [93]. *GPAT6* and *GPAT8* play essential roles in the cuticle synthesis of the flower and leaf, respectively [94, 95]. *GPAT* genes in response to drought stress were downregulated in *Phaseolus vulgaris* [96].

In general, responsive genes to drought stress in the investigation of the transcriptome profile of cultivated safflower under stress conditions are divided into two categories: classical genes, such as dehydrin, aldehyde dehydrogenase, *BAM1*, *TPS 6,10,11*, and novel genes such as *FLA* (fasciclin-like arabinogalactan protein), *PUB4* (U-box domain-containing), *KTI* (Kunitz trypsin inhibitor 5), *PYL6* (abscisic acid receptor PYL6) which have not been recognized in safflower transcriptome studies under drought stress and seem to be suitable candidate genes for further studies to reveal their roles in promoting/reducing drought stress tolerance. *PYLs* can play an essential role in ABA signaling in response to drought stress. The investigation of barley [97] and potatoes [98] demonstrated the *PYL* genes were potentially involved in response to abiotic stress. Transcriptome profiling provides an effective tool for identifying important drought-tolerant/resistant genes and physiological and transcriptional processes in safflower and with the help of this tool, efficient and effective strategies can be provided for improving safflower cultivars through breeding and genetic engineering methods.

Conclusion

In conclusion, comparative transcriptome profiling of the safflower under continuous seedling-stage stress unveiled key molecular mechanisms underpinning resilience to drought stress. Upregulated DEGs encompassed dehydrins (*RAB18/19*, *XERO1*), *ALDH12A1*, *HSPs* (*HSP23.6/22.0/17.6 C*), *ACOX2*, *LACS7*, β-alanine biosynthesis genes, *BAM1*, *TPS6*, *ROS* scavengers (*APX*, *GSTU17*), ABA pathway components (*CYP707A2*, *PP2Cs*), methyltransferases, tyrosine aminotransferases, and regulatory TFs/kinases (MYB, NAC, WRKY, bZIP, HD-ZIP, CtbHLH, CtWRKY, CDPK, CIPK, SPHK2, histidine kinases, RLKs), collectively bolstering osmotic adjustment, ROS detoxification, ABA signaling, and stomatal modulation. Conversely, downregulation of photosynthesis (*LHCA2/6*, *PSAK*), cell wall remodeling (*XTHs*), aquaporins (*TIP1*), and cuticular lipid (*GPAT6/8*) genes aligned with observed physiological declines in chlorophyll content and photosynthetic rates. This study identifies both canonical drought-responsive genes and novel candidates (e.g., *FLA*, *PUB4*, *KTI5*, *PYL6*) in safflower, previously unrecognized in its drought transcriptomes, offering prime targets for functional validation and breeding. Leveraging these insights, alongside genetic engineering of resilient loci like *CtCYP71A1* or *CtWRKY55*, promises to enhance safflower's durability/tolerance as an alternative oilseed crop for arid ecosystems, fortifying global food security against escalating drought threats.

Abbreviations

APX	Ascorbate peroxidase
BAM	Beta amylase
BP	Biological process
CC	Cellular component
CPM	Count per million
CT	Control treatment
DEG	Differentially expressed gene
DT	Drought treatment
FC	Field capacity
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

MF	Molecular function
PAR	Photosynthetically active radiation
PK	Protein kinase
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
ROS	Reactive oxygen species
TF	Transcription factor

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
F.S. conducted formal analyses, conducted the experiments, and wrote the original draft. A.A. conceptualized, supervised the research, conducted some analyses, and investigated the results. N.F. co-supervised the research, contributed to analyses, and investigated the results. R.H. supported software facility, contributed to the analyses, and investigated the results. All authors read and approved the final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files or available from the corresponding author upon reasonable request. The RNAseq metadata are available in the NCBI repository under the accession number ID PRJNA1230082.

Declarations

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Not applicable.

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