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Original Article

Transcriptome Profiling and Activity Pathway Identification of Iranian Medicinal Plant Safflower (*Carthamus tinctorius* L.)

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Abstract

Introduction: Safflower (*Carthamus tinctorius* L.) is a medicinal and crop plant rich in phyto-compounds such as unsaturated fatty acids (UFAs) and flavonoids with a known pharmacological activity. Therefore, defining its activity pathways and functional genes involved in the main biological process during plant growth and development is of high importance. The objective of this study was to define the transcriptome profile and identification of genes, activity pathways, and important proteins/enzymes of safflower flower bract at the flowering stage.

Materials and Methods: RNA was extracted from flower bracts for RNA-Seq assay, and the *De novo* assembly method was used to reconstruct the safflower transcriptome. Protein identification was run against the UniProt database. Gene ontology (GO) analysis was done for identified unigenes.

Results: 125,544 contigs were generated and 100,652 CDS coding for 12,941 proteins were identified. 8,113 proteins were selected for further downstream analyses. Functional annotation could identify 298 records for molecular function, 1,574 for biological process, 257 for cellular components, and 99 for KEGG pathways. Important pathways were metabolic pathways (991 genes), biosynthesis of secondary metabolites (496 genes), biosynthesis of cofactors (131 genes), endocytosis (93 genes), and glycerophospholipid metabolism (62 genes), respectively. In "biosynthesis of secondary metabolite" pathways, three activity sub-pathways related to biosynthesis/metabolism of vitamins (B_2, B_6, and D) were detected and the associated genes were identified. Five KEGG pathways related to fatty acids (FA) were identified including FA metabolism, FA degradation, FA biosynthesis, FA elongation and biosynthesis of UFAs. In this research, we didn't identify any active pathway related to flavonoid biosynthesis in flower bract.

Conclusions: Using *De novo* assembly, several GO terms and KEGG pathway were enriched for detected uingenes in safflower transcriptome in flower bract at flowering stage.

Keywords: Fatty Acids, Flowers, Gene Ontology, Metabolic Networks and Pathways, RNA-Seq

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Introduction

Safflower (*Carthamus tinctorius* L.) is a multipurpose economic plant, which is rich in UFAs and is consumed in Iran and other countries. Its seed is rich in oil (30 to 50%), the plant has high resistance to environmental stresses, and commonly is cultivated for edible oil, biofuel and medicinal compounds.[1] Safflower seed oil and flavonoids have great economic value that make it a suitable plant source to alleviate some of important diseases such as cardiovascular and cerebrovascular diseases, as well as to protect cardiomyocyte and brain cell functions.[2] Despite of existence of abovementioned medicinal compounds, the pharmacological potential of safflower has not been fully studied.

The RNA sequencing (RNA-seq) is a high-throughput expression profiling method preferably used for discovering important genes in plants and animals.[3] The fluctuations in transcriptome under different conditions regulate all aspects of plant growth and development. After RNA sequencing the short reads are assembled using a suitable assembler, which produces good-quality assemblies. The Trinity assembler is better than other *de novo* assemblers for reconstructing full-length transcripts.[4,5] The *de novo* method is used for RNA-seq analysis of organisms with no reference genome.[6] After *de novo* assembly and quality assessments, a functional annotation (gene ontology and KEGG pathway) is performed and regulatory proteins including transcription factors are determined. Gene ontology (GO) is used to describe the properties of genes and their products within an organism using a dynamic-updated controlled vocabulary. GO comprises three main categories including MF (molecular function), CC (cellular component), and BP (biological process).[7] Using Kyoto encyclopedia of genes and genomes (KEGG), metabolic pathways and gene signaling networks are determined.[8]

In this research, we generated and annotated the transcriptome

profile of Iranian safflower at flowering stage for the first time to uncover the genes and respective activity pathways especially involving in oil and metabolic production and also in its natural drought tolerance. Data generated in present work can be utilized in gene transfer and genome editing approaches in safflower for improving its value-added medicinal components. Nevertheless, there are limited molecular studies on safflower. Only a few studies on genetic diversity and gene expression in this plant have been reported.[9-11] In NCBI database, the genome and transcriptome profile of this plant is recorded but does not have a high-throughput data for genes and proteins. Therefore, in order to mine and annotate transcriptome profile of this plant, it is necessary to sequence the RNAs, assemble the reads (*de novo* assembly) and create reference transcriptomes. The present study aimed to construct a transcriptome map for safflower and unraveling its protein profile and activity pathways using *de novo* transcriptome assembly and functional annotations.

Materials and Methods

Sample Preparation

Seeds of spiny safflower (cultivar Arak 2811) were planted in pots at greenhouse of Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany. At flowering stage, margins of flower bracts were cut and collected (4 replicates), frozen in liquid nitrogen, powdered and used for RNA extraction.

Total RNA Extraction and Transcriptome Sequencing

Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN), and Qubit™ 4 Fluorometer was used for evaluating RNA quality. RNA samples with high quality (OD260/280 ratio > 1.8, 28S/18S ratio > 2 and RIN > 8) were selected for RNA-seq. For library preparation, Illumina Stranded mRNA Prep Ligation protocol was used. Libraries were sequenced using Illumina Novaseq 6000 (paired end with 100bp from each direction of the fragments).

Preprocessing, Quality Control and Read Trimming

Raw reads were checked using the Trimmomatic software (Version 0.39)[12] to remove the adaptor and low-quality nucleotides/sequences. Next, quality control was conducted using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to examine the characteristics of the libraries and to verify the trimming efficiency. Finally, the high-quality reads were used for downstream analyses. *De novo* assembly of the clean reads was conducted using Trinity (Release 2021-03-04)[4] with default parameters (k-mer size = 32, min_contig_length = 200). The resulted constructed contigs were clustered using CAP3 package by identity cutoff 95% and min_contig_length 200 nt. The candidate coding sequence (CDS) regions within all transcript sequences, were defined using the TransDecoder tool (http://transdecoder.github.io).

Functional Annotation of Safflower Assembled Unigenes

For annotation and functional analysis, the protein sequences of predicted ORFs were extracted and submitted to the Trinotate software (http://trinotate.github.io/). Next, the assembled unigenes were subjected to BLASTx against UniProt (Swiss-Prot and TrEMBL) protein database with E-value cutoff of 1e-5. Metabolic pathways were defined using KEGG (http://www.genome.jp/kegg/).[13,14] GO functional classification for all assembled unigenes was conducted by gProfiler (https://biit.cs.ut.ee/gprofiler/gost). A protein-protein interaction (PPI) network was created using STRING database (https://string-db.org/) for biosynthesis of secondary metabolites pathway. The Plant Transcription Factor Database (http://planttfdb.gao-lab.org/) was used to determine the transcription factors.

Results

RNA-seq data was deposited in SRA database (under accession numbers SRX15039676 to SRX15039683) consisting 282,600,000 raw reads. After initial quality control by FASTQC and trimming, the absolute majority of short reads showed phred score > 36, GC content of ~47% and sequence length of 109 bp (Figure 1). In *de novo* assembly analysis using Trinity, 125,543 contigs were generated with GC content of 40.4%. 59,652 CDS were identified using Transdecoder tool. After BLASTp search against Uniprot database, 8,113, 17 and 42 proteins were detected based on Arabidopsis, lettuce and sunflower unigenes, respectively.

The annotated transcriptome of safflower was subjected to BLASTx against Arabidopsis protein database. Based on this mining, the hits were sorted by protein classes which resulted to identification of major protein classes at flowering stage (Table 1). As seen in the table, transcription factors, transferases and kinases are most frequent among safflower protein classes. This result confirms the vital role of regulatory machinery (including TFs and kinases) in safflower at flowering stage.

Transcription factor (TF) analysis among Arabidopsis-based unigenes was investigated using plantTFDB. 57 TF families were identified with different frequencies. The most abundant TF families were bHLH, MYB, ERF, bZIP, NAC, WRKY, and C2H2 (Figure 2).

Functional annotation of Arabidopsis-based unigenes showed that 298 records for molecular function (MF), 1,574 for biological process (BP) and 257 for cellular component (CC) (Figure 3) were detected. Additionally, 99 KEGG pathways were detected, 20 of which are shown in Figure 4. Top four records for MF were related to binding, catalytic activity, transporter activity and double-stranded DNA binding; top BP enriched terms were cellular process, anatomical

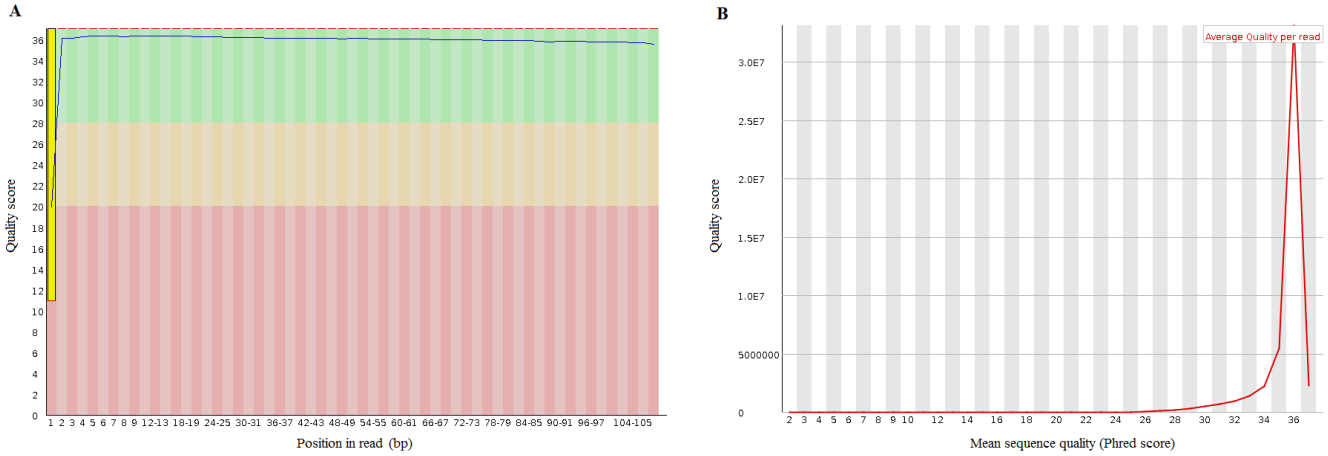

Figure 1. FASTQC Quality Control Report on RNA-seq data. **A)** Quality scores across all bases; **B)** Quality score distribution over all sequences.

Table 1. Main Protein Classes Found in Annotated Safflower Transcriptome at Flowering Stage

Protein class	Frequency	Protein class	Frequency
Transcription factor	813	Helicase	86
Transferase	695	Lipase	81
Kinase	630	Synthetase	73
Transporter	332	Oxygenase	69
Pentatricopeptide	322	Nuclease	65
Synthase	214	U-box	37
Phosphatase	203	Methylase	34
Ligase	186	Hydroxylase	28
ATPase	186	Phosphorylase	26
Actin	182	Decarboxylase	23
Hydrolase	173	Glucanase	21
F-box	167	HSP	16
Dehydrogenase	130	Acetylase	14
Protease	119	MADS-box	13
Polymerase	95	Amylase	13
Isomerase	88	Peroxidase	10

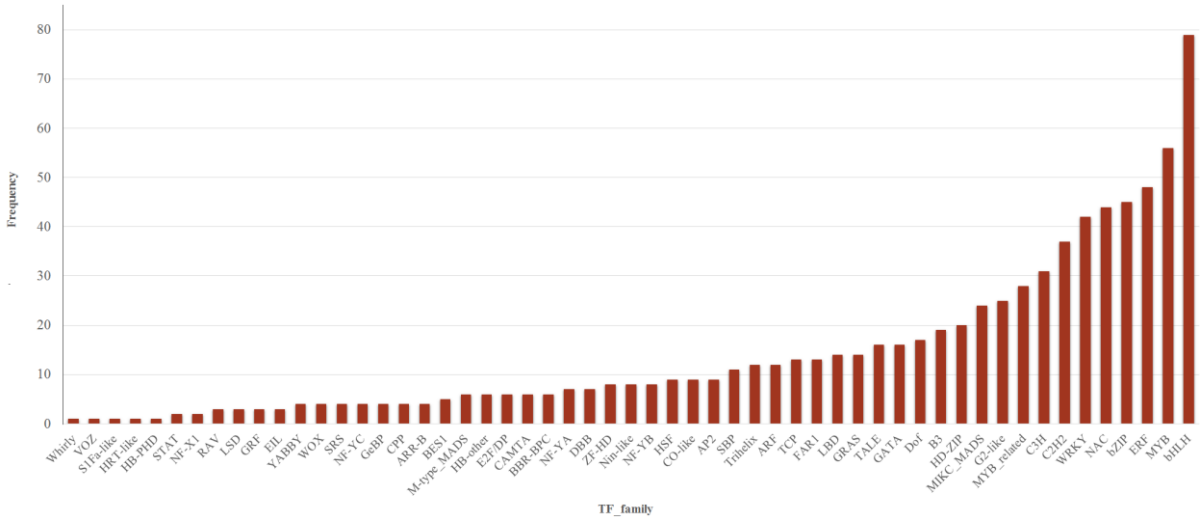

Figure 2. Abundance of Transcription Factor (TF) Families among Unigenes Detected in Transcriptome of Safflower at Flowering Stage.

anatomical structure development, metabolic process and developmental process; intracellular anatomical structure, organelle, cytoplasm and cellular anatomical entity were top CC enriched terms (Table 2).

Among KEGG pathways, six pathways (inositol phosphate metabolism, glycerophospholipid metabolism, endocytosis, biosynthesis of cofactors, biosynthesis of secondary metabolites and metabolic pathways) were important detected pathways. 49, 62, 93, 131, 496, and 991 genes were classified in these pathways, respectively. The pathway of secondary metabolites

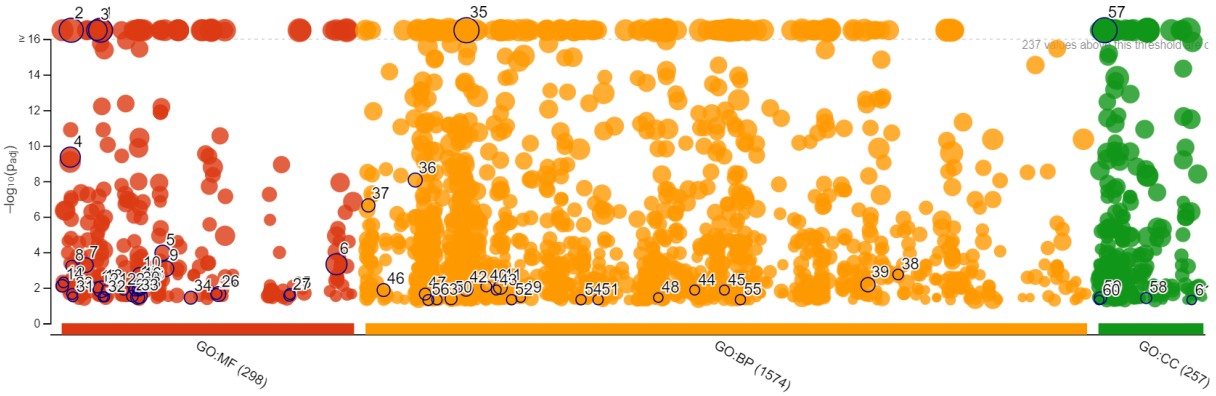

Figure 3. Results of GO Annotation of Safflower Unigenes against *Arabidopsis thaliana* Protein Database.

Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$
Metabolic pathways	KEGG:01100	1.081×10^{-85}	
Biosynthesis of secondary metabolites	KEGG:01110	1.069×10^{-29}	
Biosynthesis of cofactors	KEGG:01240	5.291×10^{-23}	
Endocytosis	KEGG:04144	8.973×10^{-19}	
Glycerophospholipid metabolism	KEGG:00564	2.091×10^{-14}	
Inositol phosphate metabolism	KEGG:00562	3.555×10^{-12}	
Valine, leucine and isoleucine degradation	KEGG:00280	3.771×10^{-12}	
Carbon metabolism	KEGG:01200	6.514×10^{-11}	
Nucleocytoplasmic transport	KEGG:03013	6.694×10^{-11}	
beta-Alanine metabolism	KEGG:00410	6.243×10^{-10}	
Various types of N-glycan biosynthesis	KEGG:00513	3.013×10^{-9}	
Amino sugar and nucleotide sugar metabolism	KEGG:00520	3.245×10^{-9}	
Ubiquitin mediated proteolysis	KEGG:04120	2.043×10^{-8}	
Phosphatidylinositol signaling system	KEGG:04070	2.403×10^{-8}	
mRNA surveillance pathway	KEGG:03015	2.403×10^{-8}	
Biosynthesis of nucleotide sugars	KEGG:01250	2.403×10^{-8}	
RNA degradation	KEGG:03018	5.341×10^{-8}	
Protein processing in endoplasmic reticulum	KEGG:04141	6.127×10^{-8}	
Glycerolipid metabolism	KEGG:00561	6.654×10^{-8}	
Fatty acid degradation	KEGG:00071	8.198×10^{-8}	

Figure 4. 20 top KEGG Pathways Detected in Safflower Transcriptome at Flowering Stage.

Table 2. Top GO Terms Enriched for Safflower Unigenes against *Arabidopsis thaliana* Protein Database

GO class	Term ID	Term Name
GO:MF	GO:0005488	Binding
GO:MF	GO:0003824	Catalytic activity
GO:MF	GO:0005215	Transporter activity
GO:MF	GO:0003690	Double-stranded DNA binding
GO:BP	GO:0009987	Cellular process
GO:BP	GO:0048856	Anatomical structure development
GO:BP	GO:0008152	Metabolic process
GO:BP	GO:0032502	Developmental process
GO:CC	GO:0005622	Intracellular anatomical structure
GO:CC	GO:0043226	Organelle
GO:CC	GO:0005737	Cytoplasm
GO:CC	GO:0110165	Cellular anatomical entity

was created using STRING database, indicating a complex protein-protein interaction network of this pathway in safflower (Figure 5). Additionally, five KEGG pathways were identified which are related to fatty acids (FA), including FA metabolism (35 genes), FA degradation (30 genes), FA biosynthesis (20 genes), FA elongation (16 genes), and

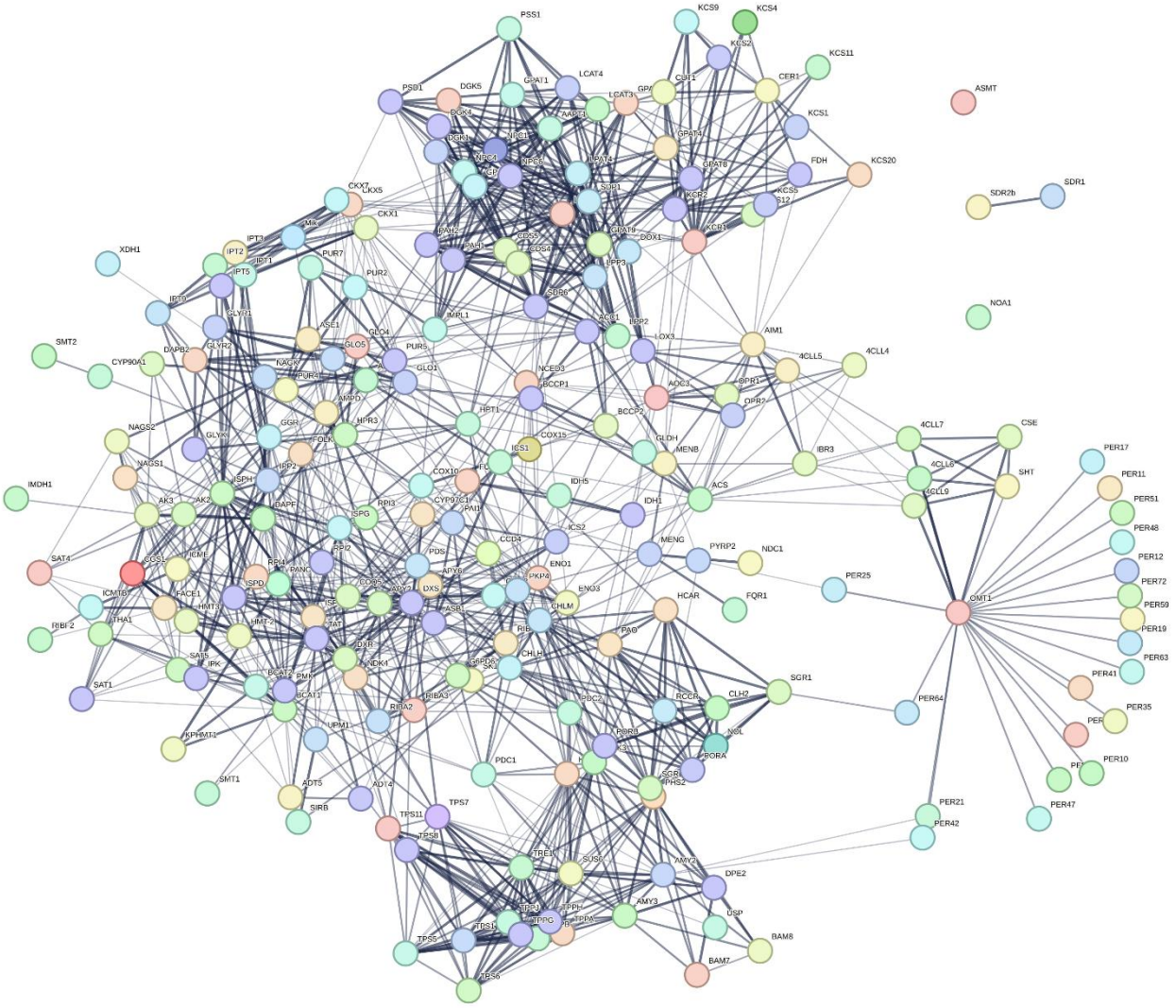

Figure 5. Protein-protein Interaction Network of Major Genes Enriched for Biosynthesis of Secondary Metabolites in Safflower.

biosynthesis of UFAs (12 genes). One another enriched KEGG pathway with intermediate significance is riboflavin biosynthesis (15 genes).

Discussion

Phyto-compounds and flavonoids are important ingredients of safflower with a wide spectrum of pharmacological activities.[15,16] Due to the lack of a comprehensive genome annotation reference for the analysis of safflower transcriptome, it was necessary to use *de novo* assembly method. Therefore, we analyzed the functional activity of the constructed contigs, including gene ontology (GO) classification, identification of functional KEGG pathways and transcription factors. The results showed that most genes of safflower at flowering stage are involved in metabolic (GO:0008152) and cellular biological (GO:0009987) processes as well as in developmental processes (GO:0032502). Metabolic processes include various components, such as organic substance metabolic process and primary metabolic process. The above activities are the main functions of plants, which were enriched for a large part of safflower genes (>5000).

An important group of secondary metabolites are vitamins such as vitamins B_2, B_6, and D, whose biosynthesis or metabolism pathways were classified under the KEGG term of "biosynthesis of secondary metabolites". For vitamin B_6 biosynthesis pathway, a gene with hydrolase activity (*DXS*) was detected among our transcripts which is localized to chloroplasts. Furthermore, for its metabolism 4 genes were detected (*PSAT2*, *PLR1*, *PK*, and *TS1*). In vitamin D biosynthesis pathway, a gene with chaperone activity (*COX15*) was detected. Another enriched KEGG pathway was riboflavin (vitamin B_2) biosynthesis. In its biosynthesis pathway 5 genes (bifunctional riboflavin kinase/FMN phosphatase, 6,7-dimethyl-8-ribityllumazine synthase, bifunctional riboflavin biosynthesis protein RIBA 1, riboflavin biosynthesis protein PYRR and monofunctional riboflavin biosynthesis protein RIBA 2) were detected. Previous studies indicated that riboflavin application can confer pathogen resistance in

plants.[17,18]

Safflower is one of the most drought tolerant oil seed crops which produces reasonable seed yield in semi-arid areas.[19] In this study, there were two specific KEGG pathways related to drought tolerance including glycerophospholipid metabolism (KEGG:00564) and inositol phosphate metabolism (KEGG:00562). Myo-inositol-1-phosphate synthase (MIPS) is a key enzyme in myo-inositol biosynthesis pathway. The MIPS gene improves tolerance to abiotic stresses in different plant species.[20] The biosynthetic pathway of glycerophospholipid metabolism plays an important role in drought tolerance via scavenging reactive oxygen species (ROS) and reconstructing cell membranes.[21]

UFAs are associated with a reduced risk of developing certain cancers such as the colon, breast and prostate cancers.[22] As UFAs are now an important healthy nutritional source, and both public and industrial interest is towards their presence in foods,[22] we were eager to define the putative pathways in our transcriptome profile. We identified a KEGG pathway for UFAs, namely biosynthesis of UFAs (KEGG:01040). Twenty-five genes were annotated for the UFA pathway in KEGG database (Supplementary file 1), 12 of which were identified in our transcriptome profile (including *ACX1*,*2*,*3*,*4*, *FAD2*, *PKT3*, *PAS2*, *FTM1*, *KCR1*,*2*, *CER10*, and *ACTE2*).

The predominant protein classes in safflower (at flowering stage) were TFs, transferases and kinases which play crucial roles in plant growth and development. The most abundant transcription factors in this plant were bHLH, MYB, ERF, bZIP, NAC, WRKY, and C2H2 which has various rules in plant growth, development and defense. Majority of MYB/bHLH complexes have been previously described, and the parallel evolution of them is associated with the developmental and metabolic plasticity in plants.[23] One of the most important transcription factors identified in safflower was ERF, which plays a crucial role in growth and stress response processes in the plant.[19,24]

Conclusion

Using *de novo* assembly, several GO terms and KEGG pathway were enriched for detected uingenes in safflower transcriptome in flower bract at flowering stage. The findings of this research can extend our knowledge about molecular genetics and activity pathways of safflower at flowering stage.

Authors' Contributions

SKM conducted the experiment and formal analysis, and prepared the original draft of manuscript. AA did conceptualization, supervised the project, contributed in formal analysis, and reviewed the manuscript. ZN co-supervised the project, shared resources and reviewed the manuscript. RH conducted formal analysis, shared resources, and reviewed the manuscript.

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Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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