3 Biotech (2020) 10:381
https://doi.org/10.1007/s13205-020-02374-3

ORIGINAL ARTICLE

The influence of soil drought stress on the leaf transcriptome of faba bean (*Vicia faba* L.) in the Qinghai–Tibet Plateau

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Received: 12 March 2020 / Accepted: 31 July 2020 / Published online: 7 August 2020

Abstract

Water deficit has a significant impact on growth, development and yield of fava bean (*Vicia fava* L.) in arid and semi-arid climates. The aim of this study was to identify differentially expressed genes in the Qinghai 13 genotype under soil drought through leaf transcriptome analysis. A total of 256.95 M clean reads were obtained and assembled into 176334 unigenes, with an average length of 766 bp. A total of 9126 (4439 upregulated and 4687 downregulated) differentially expressed genes (DEGs) were identified in faba bean leaves under soil drought. In total, 324 putative transcription factors were identified and classified as belonging to different transcription factor families. According to GO and KEGG analysis, the soil drought stress-inducible DEGs encoded proteins mainly involved in regulating photosynthesis, osmotic adjustment, detoxification, autophagy and other functions. In addition, a large portion of DEGs appeared to be novel because they could not be annotated in any functional databases, therefore, suggesting a specific response to soil drought in faba bean. Finally, RNA-seq analysis was validated by quantitative reverse-transcription PCR analysis. This work provides comprehensive and valuable information for understanding the molecular mechanisms which faba bean uses to respond to soil drought.

Keywords Faba bean · Transcriptome · Soil drought · Differentially expressed genes · Function

Introduction

Faba bean (*Vicia faba* L.) is the most widely cultivated cool-season legume reported by the FAOSTAT (http://www.fao.org/faostat/en/). It is consumed worldwide as a plant protein source for humans and animals due to its high protein content (Abdelmula et al. 1999; Amede et al. 1999; Maalouf et al. 2019). In addition, faba bean is a globally important nitrogen-fixing legume (Sosulski and McCurdy 1987; Doyle and Luckow 2003; Cazzato et al. 2012; Webb et al. 2016). Thus, faba bean cultivation is widespread in the temperate and subtropical regions of the world (Torres et al. 2006).

The Qinghai province, which is located in the northwest area of the Qinghai-Tibet Plateau has an average altitude above 3000 m and is one of the main faba bean producing areas in China. Faba bean is grown in Qinghai because this area has intense sunlight, large diurnal temperature variation, and low pest pressure compared to other areas in China (Li et al. 2018). To adjust to the agricultural policy of local government in recent years, the major producing areas of faba bean have been extended from irrigated agricultural areas to rain-fed lands (dry areas or semi-arid areas), but the planting area of faba bean in rain-fed land still only accounts for 20% of the total area in Qinghai (Li et al. 2018). Water deficit is one of the most severe abiotic stresses that significantly impacts plant growth, development and yield (Chaves et al. 2003; Farooq et al. 2009; Hossain et al. 2016; Hong et al. 2020). Faba bean is known to have a low tolerance to water deficit compared with other grain legumes (McDonald and Paulsen. 1997; Amede et al. 2003; Khan et al. 2010), which limits its cultivation in rain-fed areas. Farmers have been forced to select faba beans with better drought tolerance when growing in rain-fed lands.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-020-02374-3) contains supplementary material, which is available to authorized users.

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In recent years, physiological and molecular mechanisms underlying plant drought resistance have been studied in several leguminous crops, including *Glycine max* (Kron et al. 2008; Du et al. 2009), *Medicago lupulina* (Küchenmeister et al. 2013), *Cicer arietinum* (Varshney et al. 2014), *Vigna radiata* (Sengupta et al. 2011) and *Cajanus cajan* (Varshney et al. 2013). Though the morphological changes and physiological responses to drought stress have been studied in faba bean (Khan et al. 2007; Abid et al. 2016; Ammar et al. 2015, 2017), the molecular mechanisms underlying drought tolerance still need to be investigated. This is especially important in Qinghai, which often experiences drought. Some genetic and genomic studies in faba bean have been carried out (Abid et al. 2015; Webb et al. 2016; Yang et al. 2019), though the genome of faba bean (approximated 13 Gb) is unknown so far. Identifying candidate genes is fundamental to unraveling the molecular mechanism of plant drought stress survival. There are several studies which have attempted transcriptomic profiling of faba bean recently (Ammar et al. 2015; Ray et al. 2015; Siddiqui et al. 2015; O'Sullivan et al. 2016; Braich et al. 2017; Cooper et al. 2017; Alghamdi et al. 2018). RNA-Seq, has frequently been applied for identifying stress-inducible transcripts and determining the complex networks that underly plant stress biology (Kolev et al. 2010; Mizuno et al. 2010; Siegel et al. 2010; Zhai et al. 2013). In the previous study, researchers attempted to identify and evaluate the drought-responsive genes of faba bean genotypes by RNA-Seq with a treatment of 15% polyethylene glycol (PEG) 6000 (Khan et al. 2019). PEG is widely used to simulate drought stress artificially (Skriver and Mundy. 1990), but the actual genes expression profile of plants responding to soil drought has been shown to significantly differ compared with PEG or mannitol treatment (Bray 2004; Forner-Giner et al. 2011). Little is known about the actual molecular responses of faba bean under soil drought stress.

In our previous work, we found Qinghai 13 was able to cope with water deficit better than other local varieties (Zhang et al. 2015). For this work, Qinghai 13 was cultivated in soil and then subjected to water deficit to simulate a natural drought. Although the leaves and roots have distinct developmental trajectories, plants also evolved highly concerted biological processes to combat drought conditions by fine-tuning energy production in leaves and nutrients in roots (Khan et al. 2012; Zhu 2016). It has been reported lots of DEGs showed tissue-specific expression and leaves are more sensitive to drought stress than roots when analyzing the drought resistance transcriptome in vetch (*Vicia sativa* L) (Min et al. 2020). In previous study, drought is linked to changes in leaves of Qinghai 13 including shrinkage in the size of leaves, chlorophyll content (Chl), superoxide dismutase activity (SOD), leaf relative water content (RWC) and increasement in soluble sugar content (SSC)(Zhang et al. 2015), which revealed obvious morphological and physiological changes in leaves of faba bean under drought stress. Therefore, the leaves are chosen for transcriptome analysis in faba bean under soil drought in this study. We aimed to investigate the drought stress transcriptome profile of faba bean under these more natural drought conditions, which could provide a comprehensive reference for the drought tolerance mechanism in faba bean.

Materials and methods

Plant materials, growth conditions and stress treatments

Qinghai 13, cultivated by Qinghai Academy of Agricultural and Forestry Science was used in this work because of the well adaptation to drought tolerance in the previous study (Zhang et al. 2015). The seeds were surface-sterilized in 5% (v/v) sodium hypochlorite solution for 10 min followed by three sterile distilled water washes and soaked for 7 days. After that, seedlings were sowed in pots containing a mixture of soil and vermiculite (2: 1, w/w) in an controlled growth chambers (temperature of 25/18 °C (day/night), the relative humidity of 70 ± 5%, and a 16 h/8 h (day/night) photoperiod (250 μmol/m^2/s light intensity)) (Li et al. 2019). Seedlings were equally irrigated with half-strength Hoagland solution for 10 days, then the pots were divided into two batches (control and water stress). After that, the control plants were irrigated regularly and the water-stressed plants were subjected to water deficit for 7 days after a sufficient irrigation. Leaves were collected and instantly frozen in liquid nitrogen, and stored at − 80 °C.

RNA isolation and sequencing

Total RNA was extracted from 100 mg leaf using the total RNA extraction kit (TIANGEN, China) according to the manufacturer's instructions. Total RNA of each sample was quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher, USA) and 1% agarose gel. 1 μg total RNA with RIN value above 7 was used for following library preparation. Next-generation sequencing library preparations were constructed according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®).

The poly(A) mRNA isolation was performed using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, USA) or Ribo-Zero™ rRNA removal Kit (Illumina, USA). First-strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA was then treated with End

Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends.

Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen, USA), and fragments of ~ 360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen, USA), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq X ten instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). The sequences were processed and analyzed by GENEWIZ (China).

Data analysis

Quality Control: To remove technical sequences, including adapters, polymerase chain reaction (PCR) primers, or fragments thereof and quality of bases lower than 20, pass filter data of fasta format were processed by Cutadapt (version 1.9.1) to be high-quality clean data.

Assembly: First, assembled by Trinity, which represents a novel method for the efficient and robust de novo reconstruction of transcriptomes from RNA-Seq data. Trinity combines three independent software modules: Inchworm, Chrysalis, and Butterfly were applied sequentially to process large volumes of RNA-seq reads. Second, remove the duplicated contigs by cd-hit, then get the unigene sequence file.

Expression analysis: With the unigene sequence file as a reference gene file, RSEMestimated gene and isoform expression levels from the pair-end clean data (Li and Dewey 2011).

Differential expression analysis: Differential expression analysis used the DESeq 2 Bioconductor package, a model based on the negative binomial distribution, and calculated by the fragments per kilobase of transcript per million mapped transcript (FPKM) method for each sample. After adjusted by Benjamini and Hochberg's approach for controlling the false discovery rate, differentially expressed genes (DEGs) were determined by setting the thresholds for false discovery rate (FDR) < 0.05 and the $|\log_2 \text{ration}| \geq 1$ by performing pairwise comparisons for the treatment and control samples.

GO and KEGG enrichment analysis: GO-TermFinder was used to identify Gene Ontology (GO) terms that annotate a list of enriched genes with a p value that less than 0.05. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (http://en.wikipedia.org/wiki/KEGG).

Annotation: Use blast software to annotate the unigene sequence. All the database includes NCBI nonredundant protein (Nr), clusters of orthologous groups (COG), Swissprot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO).

Quantitative qRT-PCR analysis

RNA-Seq results were verified by qRT-PCR with 12 randomly selected DEGs. RNA exacted from the stored sample at -80 °C which mentioned in the plant material above. The experiment was conducted using 2 × SYBR Premix Ex Taq™ II (TaKaRa) on a LightCycle®480II (Roche) Real-time Detection System. The primers were designed with Primer 5. The *ELF1A* gene of in faba bean was used as an internal control (Gutierrez et al. 2011). Three independent biological replicates were used in this assay and the relative mRNA expression level was calculated as $2^{-\Delta\Delta Ct}$.

Results

Analysis of transcriptome sequencing data

The leaves of Qinghai 13 that were drought-stressed for 7 days were selected because of significant differences observed in soil water content (SWC), relative water content (RWC) and physiological indices compared with that in the control leaves (Zhang et al. 2015). Six cDNA libraries were prepared from mRNA, that was extracted from the leaves of the control and drought-stressed plants of Qinghai 13. The libraries were termed as C-1, C-2, C-3 (three replications of the control group) and D-1, D-2, D-3 (three replications of the stress group). These libraries were sequenced by Illumina deep-sequencing, and a filtering process was performed on the raw sequencing reads.

The transcriptome sequencing and assembly are shown in Table 1. The cDNA libraries from C-1, C-2 and C-3 (control samples) produced a total of 42.5 M, 39.26 M and 40.48 M raw reads, with 42.45 M, 39.22 M and 40.43 M total clean reads. Clean reads had a Q20 of 97.69%, 97.85% and 97.35%, respectively (Table 1). The cDNA libraries from D-1, D-2 and D-3 (drought samples) produced total raw reads of 39.86 M, 47.77 M and 47.35 M, with total clean reads of 39.82 M, 47.73 M and 47.30 M. Clean reads had a Q20 of 97.76%, 97.46% and 97.33%, respectively (Table 1). The Q20 clean reads were consistently greater than 97%, indicating high-quality sequencing (Table 1).

Table 1 Summary of transcriptome sequencing

Sample no.	Total raw reads (M)	Total clean reads (M)	Bases	Q20 (%)	Q30 (%)	GC (%)
C-1	42504992 (42.5 M)	42450700 (42.45 M)	6298313539	97.69	93.38	43.36
C-2	39266402 (39.26 M)	39225018 (39.22 M)	5822251138	97.85	93.72	43.30
C-3	40480440 (40.48 M)	40438798 (40.43 M)	6002963474	97.35	92.64	43.26
D-1	39865584 (39.86 M)	39825266 (39.82 M)	5912995882	97.76	93.47	43.28
D-2	47778828 (47.77 M)	47730534 (47.73 M)	7091246545	97.46	92.89	43.17
D-3	47358562 (47.35 M)	47308692 (47.30 M)	7025370995	97.33	92.56	43.21

C-1, C-2, C-3: three replications of the control group leaves; D-1, D-2, D-3: three replications of the stress group leaves; Total raw reads: the number of reads before filtering; Total clean reads: the number of reads after filtering; Bases: total number of bases after filtration; Q: Q scores are used to measure base calling accuracy; GC: GC content

Table 2 Assembly statistics of unigenes

Type	Sequences	Bases	Min	Max	Average	N50	(A + T) %	(C + G) %
All_Contig	14042026	664050651	25	18087	47.29	47	58.91	41.09
All_Unigene	176334	135066464	201	20142	765.97	1239	61.52	38.48

Table 3 Annotation statistic of unigenes in different database

Database categories	Number	Percentage
All assembled unigenes	176334	100%
Nr	85057	48.23%
KOG	37597	21.32%
Swissprot	51948	29.46%
KEGG	18047	10.23%
GO	85206	48.32%
All annotated unigenes	88593	50.24%

All libraries were assembled with high stringency and the results are summarized in Table 2. 14042026 contigs consisting of 664050651 bases were assembled into 176334 unigenes with an average length of 766 bp and N50 length of 1239 bp (Table 2). The distribution of unigene length is shown in Table S1 and Fig.S1. A total of 97302 (55.18%) unigenes ranged from 200 to 500 bp in length. A total of 38346 (21.75%) unigenes ranged from 500 to 1000 bp in length and a total of 31369 (23.05%) unigenes were longer than 1500 bp. The proportions of A + T and G + C were 61.52% and 38.48%, respectively (Table S1). The transcriptome data of the six faba bean libraries were deposited in the NCBI-SRA database with the following accessions: SRX7873340, SRX7873341, SRX7873342, SRX7873343, SRX7873344 and SRX7873345.

Annotation statistic of unigenes in different databases

A sequence similarity search for all assembled unigenes was executed against the Nr, COG, Swissprot, KEGG and GO databases (Table 3 and Fig.S2). Of the 176334 unigenes, 85057 (48.23%) shares homology with members of the Nr database and 85206 unigenes had hits in the GO database (48.32%). Additionally, 37597 (21.32%) unigenes were annotated in COG, 51948 (29.46%) in SwissProt and 18047 (10.23%) in KEGG (Table 3 and Fig.S2).

Characterization of all unigenes

The distribution of the top annotated unigenes against the Nr database is shown in Fig.S3. Approximately 35540 (41.78%) unigene sequences were similar to the model legume *Medicago truncatula*, 19696 unigenes (23.15%) were similar to *Cicer arietinum*, 4599 unigenes (5.4%) were similar to *Cajanus cajan* and 4013 unigenes (4.71%) were similar to *Glycine max*. An additional 8.03% the sequences showed similarities to other organisms, including *Pisum sativum*, *Trifolium subterraneum*, *Glycine soga*, *Mus musculus*, *Vigna radiata var. radiata* and *Phaseolus vulgaris* (Fig. S3). Overall, nearly 70% of the assembled unigenes had high similarity with the leguminous plants.

To predict and classify the putative functions of these unigenes, all unigenes were compared against the COG database. A total of 37579 COG -annotated putative proteins were classified into 25 families (Fig. S4). The largest category among these families was the "general function prediction only" (5098 unigenes) followed by "signal transduction mechanisms" (4953 unigenes) and "posttranslational modification, protein turnover, chaperones" (4252 unigenes). In addition, there were a large number of unigenes belonging to "carbohydrate transport and metabolism" (2385 unigenes), "translation, ribosomal structure and biogenesis" (2271), "transcription" (2153), "intracellular trafficking, secretion, and vesicular transport" (1976), "RNA processing and modification" (1833), "lipid transport and metabolism" (1829),

"energy production and conversion" (1795) and "amino acid transport and metabolism" (1795). There were also 2521 unigenes belonging to the category of "function unknown".

For GO terms, a total of 85206 (48.32%) unigenes were assigned to GO ontologies based on their sequence similarity with genes that have known functions. These hits were categorized into 57 functional groups in the three main categories of molecular function, biological process and cellular components (Fig. 1, Table S2). In the molecular function category, the most represented GO terms were related to binding (15776) and catalytic activity (15433). In addition, the most frequent terms in the molecular function category were transporter activity (1576), structural molecule activity (712), and electron carrier activity (499). In the cellular component category, cell part (4250), membrane part (3144) and organelle (3052) were the most highly represented categories. Additionally, macromolecular complex (2306), organelle part (1938) and membrane (1836) were also highly represented in the cellular component category. In the biological process category, the most represented terms were associated with the metabolic process (11359) and cellular process (9029). In addition, single-organism process (4706),

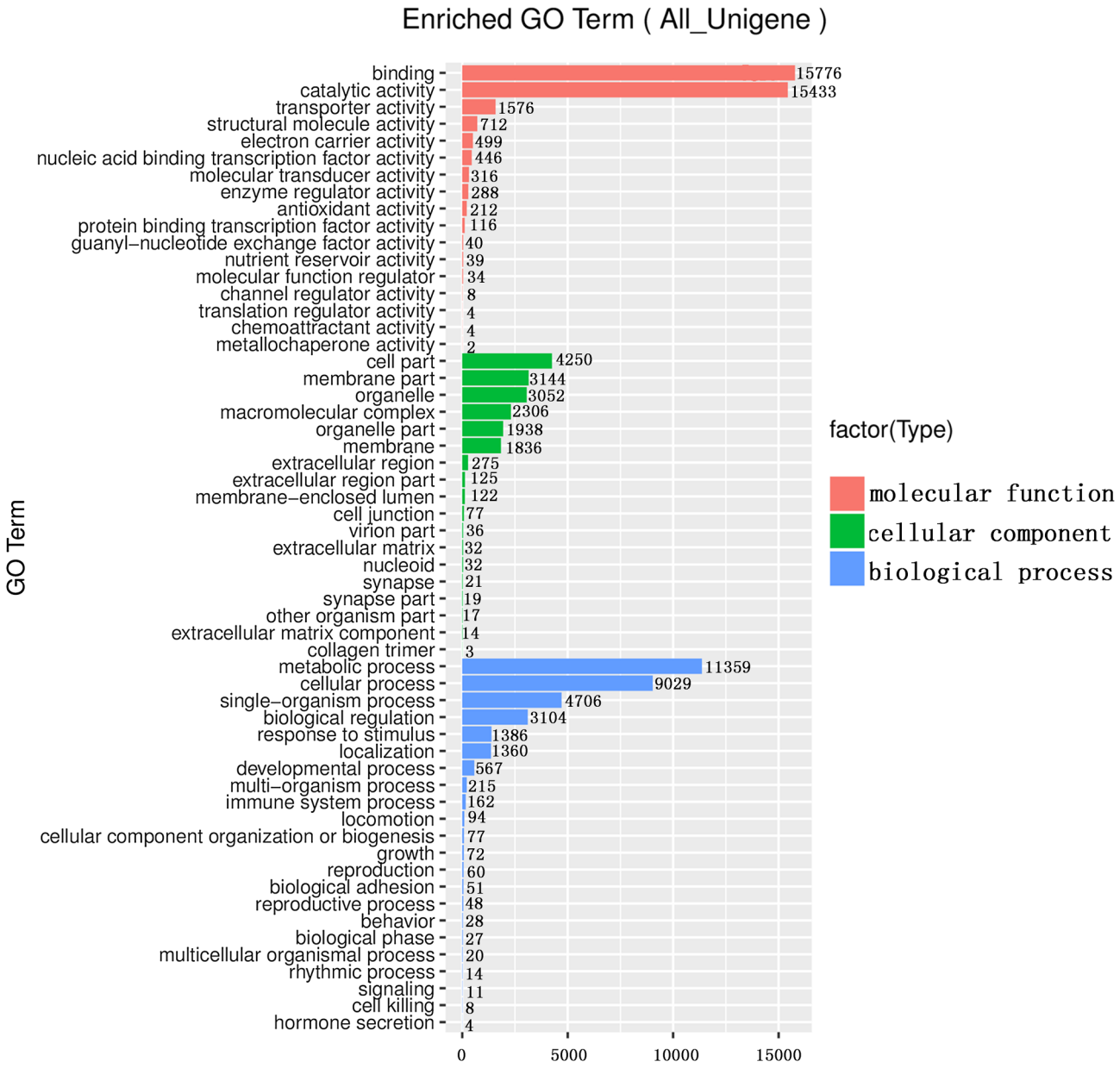

Fig. 1 Histogram of GO terms assigned to all assembled unigenes. X axis represents corresponding number of unigenes in certain category. Y axis represents unigenes are categorized into three main groups: molecular function, cellular components and biological process

biological regulation (3140), response to a stimulus (1386) and localization (1360) were the most frequent groups in the biological process category (Fig. 1, Table S2).

Next, the metabolic pathways that annotated unigenes participated in were investigated by comparing to the KEGG database. A total of 18047 annotated unigenes were mapped to 148 KEGG reference pathways. These can be classified into five different functional groups including metabolism, genetic information processes, environmental information processing, organismal system and cellular processes (Fig. 2). In the metabolism functional groups, unigenes were predominantly involved in "global and overview maps" (13592), "carbohydrate metabolism" (4190), "amino acid metabolism" (2179), "lipid metabolism" (2088), "energy metabolism" (1473) and "nucleotide matabolism" (1063). Among the genetic information processing functional groups, the majority of the unigenes were involved in "translation" (3673), "folding, sorting, and degradation" (2195), "transcription" (1098) and "replication and repair (912)". In the environmental information processing functional groups, "signal transduction" (932) and "membrane transport" (255) were most frequent. In the organismal systems functional groups, unigenes only involved in "environmental adaptation" (682) were found (Fig. 2). Meanwhile, in cellular processes functional groups, unigenes involved in only "transport and catabolism" (1694) were found (Fig. 2). Detailed information about functional pathways found by comparing against the KEGG database is shown in Table S3.

Identification of differentially expressed genes

The expression values of the unigenes were analyzed via the FPKM method (Table S4). Genes which were differentially expressed between the control group and the stress group were identified in pairwise comparisons with the following criteria: $\log_2$FoldChange ≥ 1 or ≤ -1, FDR ≤ 0.05. As shown in Fig.S5, a total of 4439 upregulated genes and 4687 downregulated genes were identified when comparing the drought stress group against the control group. The overall differential expression pattern was also visualized with a volcano plot (Fig. S6). The results above revealed a specific expression profile in response to soil drought stress in faba

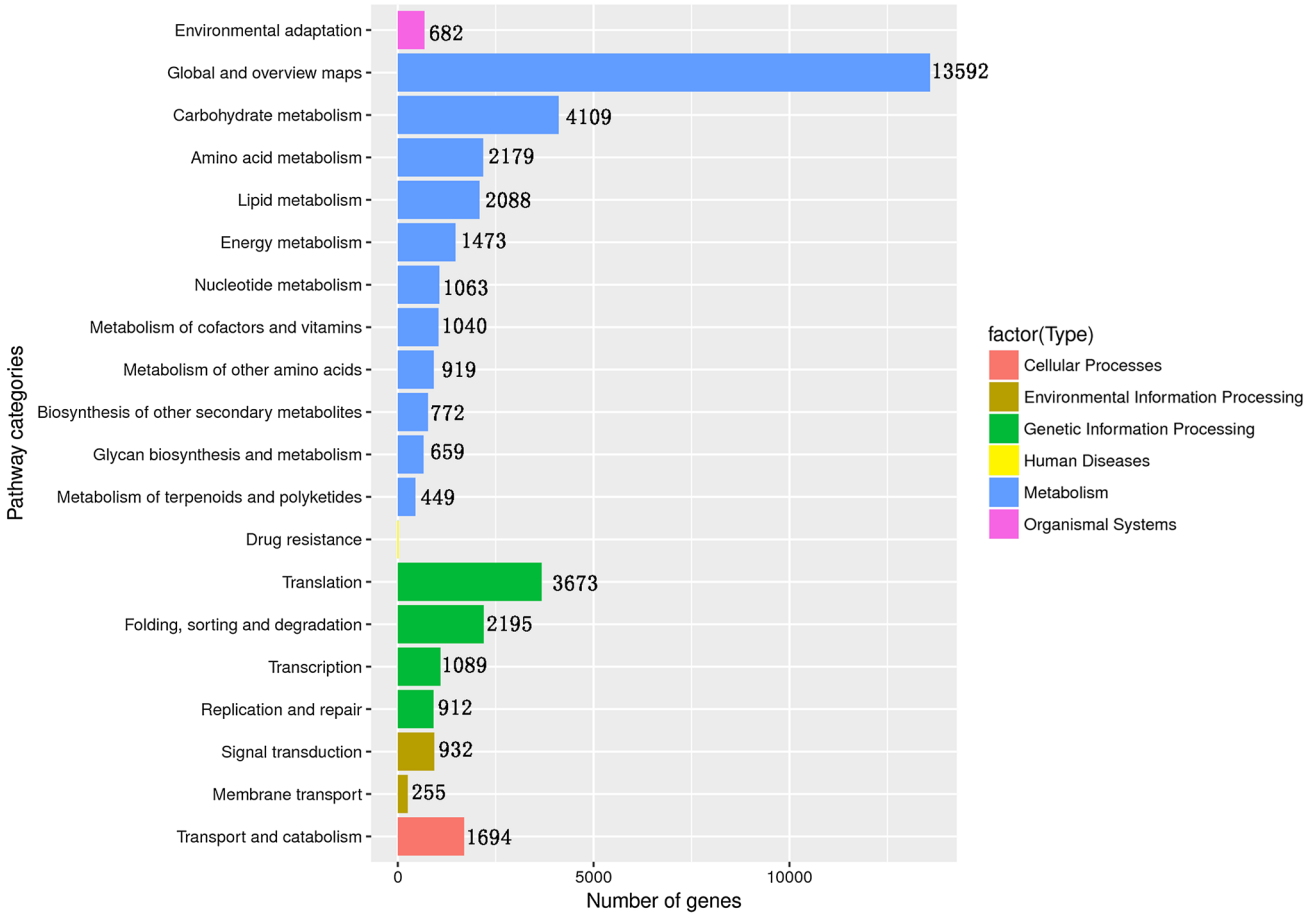

Fig. 2 Functional distribution of KEGG terms assigned to all assemble unigenes. X axis represents the number of unigenes in a certain category. Y axis represents the KEGG functional category

bean leaf. The genes with the strongest drought induction included a ribulose bisphosphate carboxylase small chain member, SLOW GREEN1 (chloroplastic), delta-1-pyrroline-5-carboxylate synthase, nuclear poly(A) polymerase and protein translocase subunit SECA2 (Table S4). Genes which were most repressed by drought included glutaredoxin, phosphatase 2C and cyclic nucleotide-binding and lysine-specific demethylase JMJ30 (Table S4).

In addition to the above genes, a total of 324 putative transcription factors (TFs) were found to be significantly regulated during drought stress. As shown in Fig. 3, the top 11 transcription factor families included 54 NAC, 36 bHLH, 26 MYB, 23 C3H, 18 GRAS, 17 WRKY, 9 FAR1, 6 HSF, 9 AP-BREBP, 4 Trihelix and 4 GRF. As is known, these transcription factor families play important roles during abiotic stress including salt, drought, cold, heat and so on (Udvardi et al. 2007). Finally, a hierarchical clustering heat map of DEGs was constructed to show control and soil drought transcriptomes (Fig. 4).

A total of 12 DEGs were randomly selected to conduct qRT-PCR for validation of the RNA-Seq data. Detail description and the primers of the 12 selected DEGs are listed in Table 4. Six upregulated DEGs, including D-TRINITY_DN33510 (ribulose-1,5-bisphosphate carboxylase small subunit), D-TRINITY_DN24736 (chitinase family protein), D-TRINITY_DN43139 (Rab GTPase activator), D-TRINITY_DN68230 (NAC transcription factor) and 2 genes of unknown function (D-TRINITY_DN47859 and D-TRINITY_DN47873), were assayed with qRT-PCR. Six downregulated DEGs, including C-TRINITY_DN38456 (myb transcription factor), C-TRINITY_DN32684 (transmembrane protein), C-TRINITY_DN37999 (ethylene response factor), D-TRINITY_DN46507 (auxilin-related protein) and 2 genes of unknown function (C-TRINITY_DN32373 and C-TRINITY_DN24815), were also screened for qRT-PCR. The expression levels of selected DEGs that were measured by qRT-PCR were quantified with $\log_2$foldchange value and all the DEGs showed an approximately similar expression compared with the transcriptome analysis (Table 4). These qRT-PCR results demonstrated the high quality and reliability of the RNA-seq data.

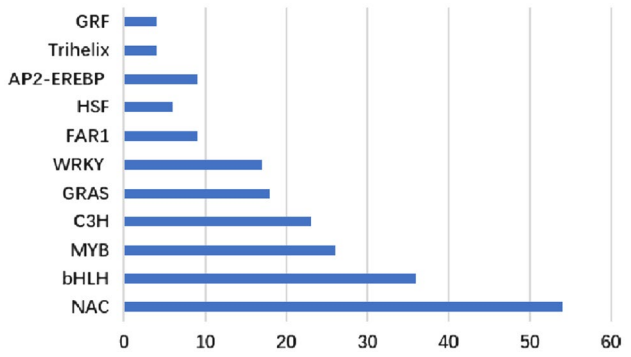

Fig. 3 Top 11 families of differentially expressed transcription factors response to soil drought stress in faba bean leaves. X axis represents the corresponding number of differentially expressed unigenes in certain transcription factor family. Y axis represents the transcription factor family classification

Functional annotation of DEGs

There were a significant amount of DEGs in response to soil drought. To classify the functions of the DEGs, we compared all the DEGs to the GO database to search for significantly enriched GO terms. This analysis revealed enrichment in major biological processes, molecular functions and cellular components (Fig. 5). In the molecular function category, the most significant GO terms were catalytic activity, binding and transport activity, electron carrier activity, nucleic binding transcription factor activity, structural molecule activity, enzyme regulator activity, antioxidant activity, molecular transducer activity, nutrient reservoir activity and protein binding transcription factor activity. The most enriched terms in the cellular component category were cell part, membrane part, organelle, membrane, macromolecular complex, organelle part, extracellular region, membrane-enclosed lumen and extracellular region part. In the biological process category, DEGs were enriched in metabolic process, cellular process, single-organism process, biological regulation, response to stimulus, localization, multi-organism process, development process, cellular component organization biogenesis, cell killing, immune system process and growth.

KEGG pathway enrichment of DEGs

As shown in Table 5, DEGs were mainly enriched in three major KEGG pathways, including "metabolism", "Environmental Information Processing" and "Genetic Information Processing". The largest number of unigene-containing pathways in the "metabolism" category were assigned to the metabolic pathway (ko01100; 486, 46.15%) and biosynthesis of secondary metabolites (ko01110; 281, 26.69%). The largest number of unigene-containing pathways in the "Genetic Information Processing" were assigned to DNA replication (ko03030; 20, 1.9%). There were also two pathways belonging to the "Environmental Information Processing" category, including MAPK signaling pathway-plant (ko04016; 38, 3.61%) and plant hormone signal transduction (ko04075; 74, 7.03%).

In addition to the pathways mentioned above, there were some pathways assigned to pentose and glucuronate interconversions (ko00040; 20, 1.9%), flavonoid biosynthesis (ko00941; 11, 1.04%), linoleic acid metabolism (ko00591; 16, 1.52%), photosynthesis-antenna proteins (ko00197; 16,

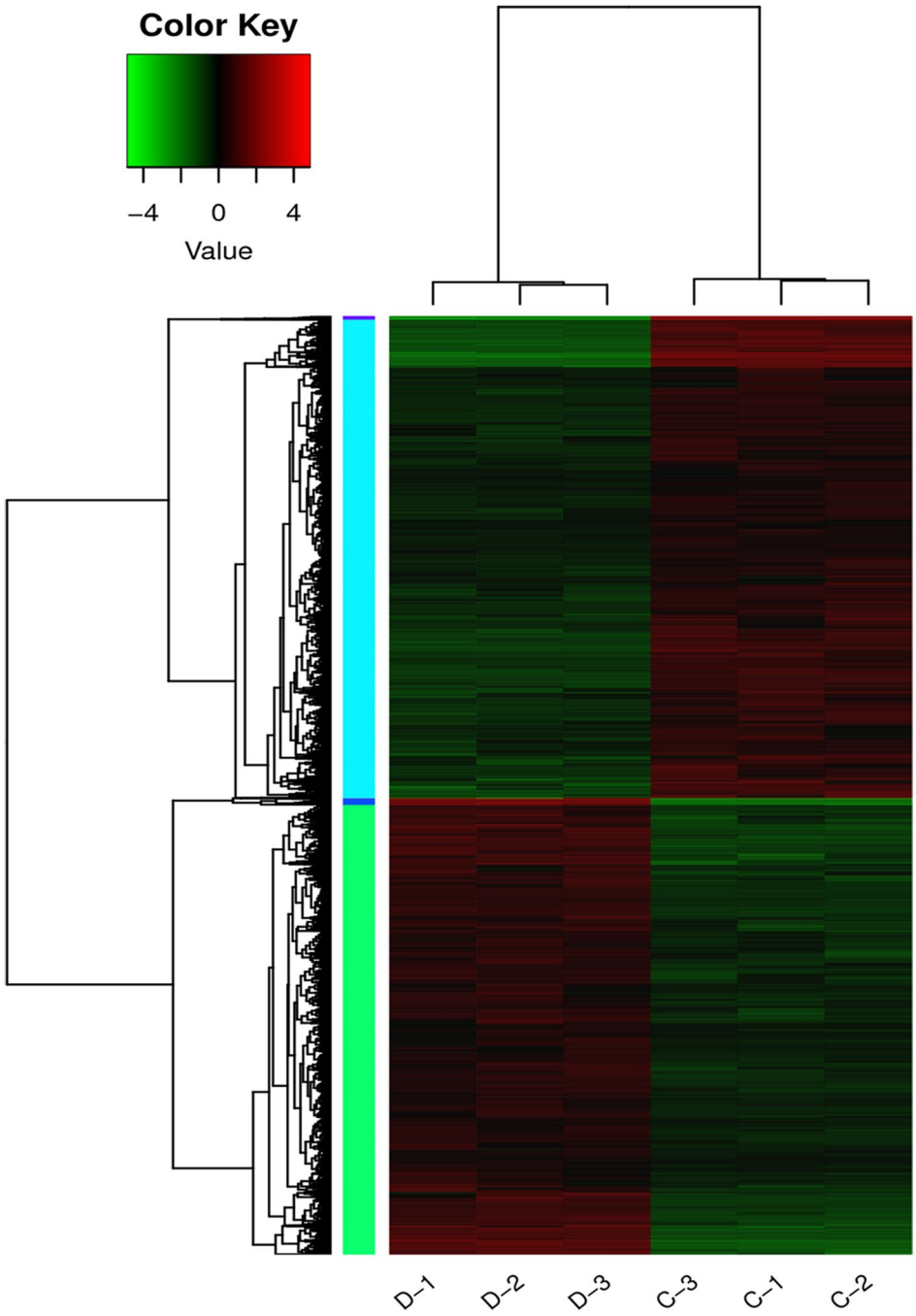

Fig. 4 A hierarchical clustering heat map showing DEGs identified in the transcriptomes of control samples (three replications:C-1, C-2, C-3) and soil drought-stressed samples (three replications:D-1, D-2, D-3). Relative gene expression fold-changes (log_2FPKM) are color coded (red upregulated and green downregulated, the deeper color representing greater difference in relative gene expression)

1.52%), cutin, suberine and wax biosynthesis (ko00073; 9, 0.85%), ubiquinone and other terpenoid-quinone biosynthesis (ko00130; 17, 1.61%), isoquinoline alkaloid biosynthesis (ko00950; 12, 1.14%), tropane, piperidine and pyridine alkaloid biosynthesis (ko00960; 13, 1.23%), tyrosine metabolism (ko00350; 18, 1.71%), phenylpropanoid biosynthesis (ko00940; 47, 4.46%), monoterpenoid biosynthesis (ko00902; 6, 0.57%), zeatin biosynthesis (ko00908; 6, 0.57%) and sesquiterpenoid and triterpenoid biosynthesis (ko00909; 4, 0.38%). As a result, a total of 1053 DEGs were identified as being associated with 18 most strongly represented KEGG pathways through mapping DGEs to the KEGG database (Fig. 6). These results are visualized in Fig. 6 and full information is available in Table S5.

Table 4 Detail of the primers of 12 randomly selected DEGs for qRT-PCR

Gene no.	Gene ID	Description	Primers sequence (5′–3′)	Production (bp)	log_2Foldchange (RNA-Seq)	log_2Foldchange (qRT-PCR)
1	D-TRINITY_DN33510_c1_g1_i1	ribulose-1,5-bisphosphate carboxylase small subunit	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	177	7.68	6.5
2	D-TRINITY_DN47859_c3_g5_i1	Unknown	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	169	6.39	5.4
3	D-TRINITY_DN24736_c1_g1_i1	chitinase family protein	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	167	6.1	5.9
4	D-TRINITY_DN47873_c3_g7_i6	Unknown	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	158	4.9	3.7
5	D-TRINITY_DN43139_c2_g1_i13	Rab GTPase activator	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	146	3.2	2.7
6	D-TRINITY_DN68230_c0_g1_i1	NAC transcription factor	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	166	3.03	1.8
7	D-TRINITY_DN32373_c1_g2_i1	Unknown	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	150	−6.15	−4.6
8	D-TRINITY_DN38456_c0_g1_i3	myb transcription factor	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	148	−5.34	−5.4
9	D-TRINITY_DN32684_c0_g1_i1	hitinase family protein	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	175	−5	−4.6
10	D-TRINITY_DN37999_c1_g1_i6	ethylene response factor	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	165	−3	−1.9
11	D-TRINITY_DN46507_c0_g1_i2	auxilin-related protein	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	151	−3.9	−4.3
12	D-TRINITY_DN24815_c0_g1_i1	Unknown	Forward:CTTTGAGTTGAAGCATGAAGCA Reverse:ATTGTTGGCCTTCTTGGTAACCG	148	−2.9	−2

Discussion and conclusion

Water scarcity greatly influences the yield of faba bean (Vicia faba L.) in the Qinghai province, but the mechanisms behind faba bean drought tolerance are not well-understood. Therefore, the main objective of this study was to determine how the drought-tolerant faba bean genotype Qinghai 13 alters its transcriptome in response to water deficit.

The leaves of Qinghai 13 were sequenced with an Illumina HiSeq X ten instrument, which resulted in 257.22 M raw reads and 256.95 M clean reads from six cDNA libraries. This is greater than the depth obtained by some previous

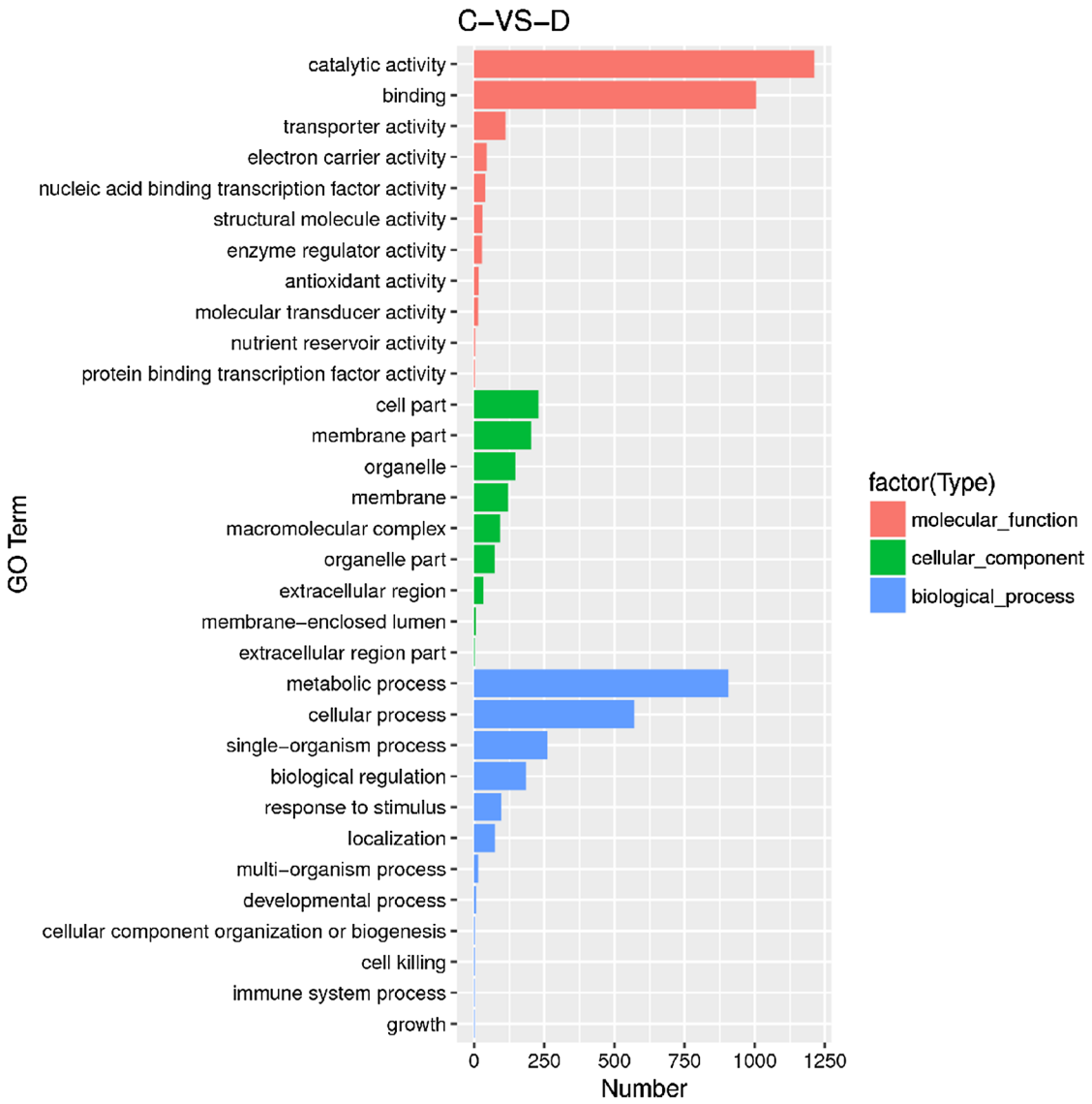

Fig. 5 GO enrichment distribution of differentially expressed genes. X axis represents the corresponding number of DEGs. Y axis indicates DEGs are categorized into three main groups: molecular function, cellular components and biological process

studies 65.8 M (Arun-Chinnappa and McCurdy. 2015), 33.023 M (Ocaña et al. 2015), 304680 (Kaur et al. 2012), but is smaller than others (606.35 M, Khan et al. 2019). However, the high Q20 percentage (97%) indicated that the sequencing was extremely high quality. In this study, we obtained a total of 176, 334 unigenes, which was larger than 164679 reported by Khan (Khan et al. 2019). Among the assemble unigenes, 88593 (50.24%) were annotated, while 87751 (49.76%) unigenes were not. The annotated unigenes showed high similarity with the model legumes *Medicago truncatula* (41.78%) and *Cicer arietinum* (23.15%), which is consistent with the result reported by Khan (Khan et al. 2019). GO terms of the annotated unigenes mainly involved categories associated with molecular function and biological process, which may indicate a common role in plant growth and development, and stress tolerance mechanisms.

DEGs were identified through transcriptome comparison between the control and drought-stressed leaf samples. A total of 9126 unigenes with significant differential expression were identified with the following criteria: $\log_2$FoldChange ≥ 1 or ≤ -1, FDR ≤ 0.05. The number of the DEGs in this work is far smaller than the number found in leaves of different developmental stages under PEG6000 stress, which shows that a different spectrum of drought-responsive genes in faba bean is present when using different water-deficit-stress experimental systems. Similar work in wheat has also found that its proteome exhibited obvious differences under soil drought compared to PEG stress (Cui

Table 5 KEGG pathway annotation and number of DEGs

Pathway category	Pathway ID	DEGs with pathway annotation (1053)	All genes with pathway annotation (18047)	Qvalue
Pentose and glucuronate interconversions	ko00040	20 (1.90%)	158 (0.88%)	4.30E − 03
Flavonoid biosynthesis	ko00941	11 (1.04%)	64 (0.35%)	4.01E − 03
Linoleic acid metabolism	ko00591	16 (1.52%)	103 (0.57%)	1.44E − 03
Photosynthesis—antenna proteins	ko00196	16 (1.52%)	100 (0.55%)	1.31E − 03
MAPK signaling pathway-plant	ko04016	38 (3.61%)	306 (1.70%)	1.55E − 04
Plant hormone signal transduction	ko04075	74 (7.03%)	707 (3.92%)	2.19E − 05
Cutin, suberine and wax biosynthesis	ko00073	9 (0.85%)	48 (0.27%)	4.30E − 03
Ubiquinone and other terpenoid-quinone biosynthesis	ko00130	17 (1.61%)	146 (0.81%)	1.58E − 02
Isoquinoline alkaloid biosynthesis	ko00950	12 (1.14%)	65 (0.36%)	1.44E − 03
Tropane, piperidine and pyridine alkaloid biosynthesis	ko00960	13 (1.23%)	89 (0.49%)	5.95E − 03
Tyrosine metabolism	ko00350	18 (1.71%)	149 (0.83%)	9.15E − 03
Biosynthesis of secondary metabolites	ko01110	281 (26.69%)	3670 (20.23%)	1.62E − 05
Phenylpropanoid biosynthesis	ko00940	47 (4.46%)	451 (2.5%)	1.05E − 03
Metabolic pathways	ko01100	486 (46.15%)	7274 (40.31%)	1.02E − 03
Monoterpenoid biosynthesis	ko00902	6 (0.57%)	31 (0.17%)	1.36E − 02
Zeatin biosynthesis	ko00908	6 (0.57%)	25 (0.14%)	4.50E − 03
Sesquiterpenoid and triterpenoid biosynthesis	ko00909	4 (0.38%)	21 (0.12%)	4.36E − 02
DNA replication	ko03030	20 (1.90%)	166 (0.92%)	6.12E − 03

et al. 2019). We concluded that the transcriptome response to drought in faba bean may be different due to a variety of factors, including stress time, osmotic pressure and others.

Meanwhile, GO enrichment analysis of DEGs showed enrichment in "catalytic activity" and "binding" in the molecular function category, as well as "cellular process" and "metabolic process" in the biological process category. This indicated a common response mechanism in both PEG stress and soil drought, which has also been reported in peanut (*Arachis hypogaea* L.) and *Glycine max* (Brasileiro et al. 2015; Tripathi et al. 2016; Yang et al. 2017).

There were many pathways (133) enriched under PEG in a previous study (Khan et al. 2019), but only 18 of them overlapped with our results. This lack of overlap could have several possible explanations. For example, carbon fixation in photosynthetic organisms and glycolysis/gluconeogenesis were significantly increased by PEG6000 stress in faba bean and other crops (Valluru and Van den Ende 2008; Yang et al. 2017; Fan et al. 2018; Wu et al. 2018). Additionally, arginine and proline metabolism were also significantly increased under PEG6000 stress in faba bean and other species (Armengaud et al. 2004; Wu et al. 2018; Cui et al. 2019). Genes involved in ribosome biogenesis have also been shown to be differentially expressed in PEG6000 stressed plants (Cui et al. 2019; Khan et al. 2019), but were unchanged in faba bean under soil drought stress. In a previous study, genes involved in the citrate cycle, glyoxylate and dicarboxylate metabolism and pyruvate metabolism were differentially expressed under PEG6000 stress, but they remained largely unchanged under soil drought in Jute (Yang et al. 2017).

Although drought and PEG stress elicit some similar response (such as phytohormonal balance and leaf water content), the osmotic stress caused by PEG causes the two to diverge for several reasons, including the different length of possible treatment (Cui et al. 2019). Undoubtedly, there are several common pathways that are shared by PEG stress and soil drought, because majority of the responsive pathways in soil drought can be annotated from the PEG stress. For example, phenylpropanoid biosynthesis, biosynthesis of secondary metabolites, photosynthesis-antenna proteins, MAPK signaling pathway-plant, plant hormone signal transduction, cutin, suberin and wax biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, phenylpropanoid biosynthesis are all regulated in both stresses. Considering these results, PEG stress may be redundant compared to simply testing soil drought directly. Soil drought could undoubtedly be better understood by the addition of experiment which uses multiple water-deficit-stress experimental systems, or studies which combine transcriptome, proteome and metabolome data (Hamanishi et al. 2015; Kosová et al. 2016; Shen et al. 2016; Savoi et al. 2017). Such work would enable a better understanding of the physiological or biochemical processes associated with drought stress and provide insight into the possible molecular mechanisms behind these responses in faba bean.

In this work, the drought-induced genes were mainly classified into two major categories: regulatory proteins

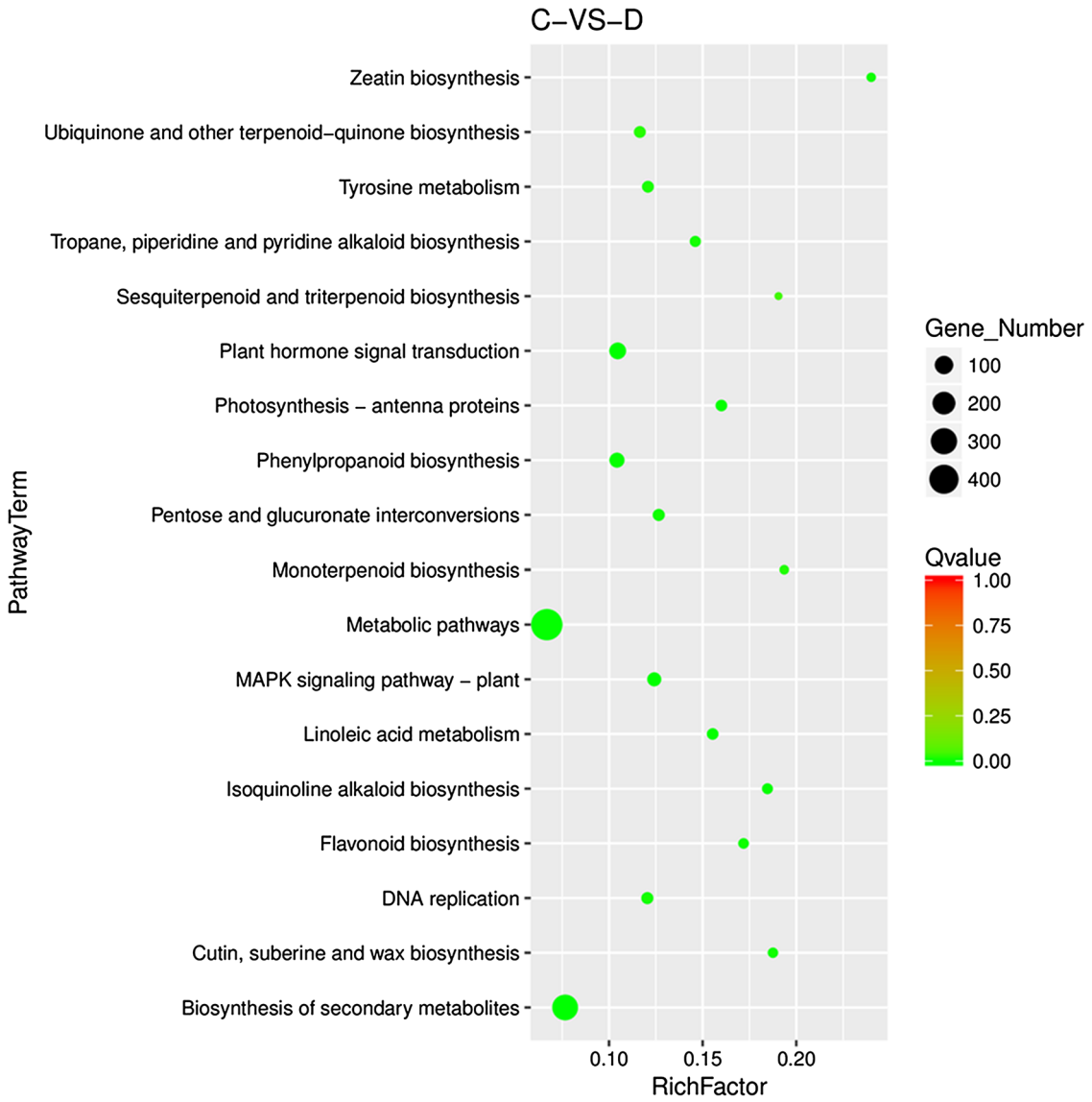

Fig. 6 Scatter diagram of enriched KEGG pathways for DEGs in soil drought-stressed samples (D) vs. control samples (C). The 18 most strongly representative pathways are displayed. X axis represents RichFactor, a result of the ratio of DEG number to all annotated genes in certain pathway. Y axis represents KEGG term. The colour of the dots represents the range of the -$\log_{10}$ (QValue). The area of black dot means the number of DEGs in certain pathway

and functional proteins. Regulatory proteins mainly included transcription factors, protein kinases, protein phosphatases and regulators in signal transduction. Differentially expressed transcription factors, including NAC, bHLH, MYB, WRKY, AP2-EREBP were identified under soil drought and also found to some extent in PEG stress (Khan et al. 2019). This overlap indicated a common function of the transcription factor families of NAC, bHLH, WRKY, MYB, AP2-EREBP during water deficit (Reddy et al. 2008; de Zélicourt et al. 2012; Bhatnagar-Mathur et al. 2014;Sosa-Valencia et al. 2017). Among the transcription factors, the expression of putative *MYB59* was found to be substantially reduced, which fits with its known role in calcium signal regulation in *Arabidopsis thaliana* during stress (Fasani et al. 2019). Members of NAC family are involved in abiotic stress response and symbiotic nodule senescence (de Zélicourt et al. 2012), and also represent the family with the most differentially expressed members in faba bean under soil drought. Transcription factors of the bHLH family were also differentially expressed in faba bean under drought stress. It has been reported that the bHLH member AtMYC2 can function as a transcriptional activator in ABA-inducible gene expression under drought stress in plants (Abe et al. 2003). DEGs also included members of AP2/EREBP family, which

is known to function in mediating cuticular permeability, sensitivity to abscisic acid (ABA), and drought resistance by regulating wax biosynthesis (Zhang et al. 2019).

DEGs encoding receptor-like kinases and LRR receptor-like kinases were found in our differential testing. DEGS encoding protein kinases including calcium-dependent protein kinases, serine/threonine protein kinases, light-sensor protein kinases, cysteine-rich receptor-kinase-like protein and mitogen-activated protein kinases were also differently expressed in faba bean leaves under soil drought. The protein kinase is known to function as sensor response genes for initiating phosphorylation cascades (Singh et al. 2015). Besides, DEGs involved in the regulation of signal transduction such as plant hormone regulation, signaling molecule regulation, redox reaction, and carbohydrate and sugar metabolism were differentially expressed in this work.

Additionally, functional genes encoding proteins involved in various functional processes were differently regulated under soil drought in faba bean. Several DEGs identified in this study belong to the aquaporin family, whose members facilitate water uptake across cell membranes in maintaining cellular water homeostasis (Javot and Maurel 2002). That would be accountable for the low water uptake and consequent reduction in relative water content (RWC) of leaves in Qinghai 13 faba bean under soil drought (Zhang et al. 2015). In addition, DEGs encoding soluble sugars synthetases and sugar transporter were also differentially expressed. The soluble sugar is also an osmolyte and signaling molecule expressed under drought stress. Furthermore, DEGs encoding functional proteins also included enzymatic compounds triggered by reactive oxygen species (ROS), ABC-transporter proteins, chloride channel, Na^+/K^+ transporter, late embryogenesis abundant protein (LEA) and other drought-induced proteins.

In this study, the most drought-induced gene encodes a ribulose-1,5-bisphosphate carboxylase small subunit. It is reported that severe drought limits the quantum efficiency of PS II during photosynthesis by reducing the activity of ribulose-1,5-bisphosphate carboxylase (Rubisco) (Carmo-Silva et al. 2012). Additionally, a group of genes encoding chloroplastic were identified, which fits with its known role as a major player in photosynthesis (Carmo-Silva et al. 2012). As a result, chlorophyll content showed a visible reduction in faba bean leaves under soil drought (Zhang et al. 2015). All of the above data indicated that drought-induced differential expression results in major changes to photosynthetic machinery.

In this study, we also identified genes encoding Δ1-pyrroline-5-carboxylate synthetase (P5CS1), which is a major component of proline biosynthesis, and proline dehydrogenase 1 (PDH1), which is related to proline catabolism. Proline is thought to contribute to osmotic adjustment and the stabilization of subcellular structures under stress conditions (Ashraf and Foolad 2007). The *Arabidopsis p5cs1* and *pdh1* are deficient in stress-induced proline synthesis, which lead to a reduction in proline catabolism (Sharma et al. 2011). This implies that P5CS1 and PDH1 play important roles in osmotic adjustment during water stress through regulating proline biosynthesis and catabolism (Khan et al. 2019). A homolog of the *BRCA1* gene 1(*NBR1*), was the most down-regulated DEG in faba bean during soil water deficit and the *Arabidopsis nbr1* mutant was previously shown to have reduced drought tolerance (Zhou et al. 2013). In faba bean, *NBR1* also play an important role in adapting to drought stress through mediating autophagy.

The production of reactive oxygen species (ROS) is an early consequence of plant defense response to water stress and acts as a secondary messenger to trigger subsequent adaptive responses (Miller et al. 2010). A large number of genes encoding detoxification enzymes such as glutathione S transferase (GST), ascorbate peroxidase (APX), mono dehydroascorbate reductase (MDAR), glutathione peroxidase (GPX) and glutathione reductase (GR) were differentially expressed to execute cell protection in faba bean under soil drought. ROS signaling under drought is linked to abscisic acid (ABA) and Ca^{2+} changes (Kaur and Asthir 2017). It is well known that abscisic acid (ABA) is a very important signaling molecule during drought stress. In this study, a group of 2C-type protein phosphatases, which affect the ABA pathway, were differentially expressed. In addition, 9-cis-epoxycarotenoid dioxygenases (NCED1 and NCED3), key enzymes in ABA biosynthesis, were also differentially expressed. Overexpression of several genes involved in ABA biosynthesis has been shown to result in improved drought tolerance in petunia plants (Iuchi et al. 2000).

Lastly, many non-annotated DEGs were identified from the assembled unigenes (Table S4). Although the function of these genes is still unknown, many of them were differentially expressed during drought stress. Elucidating the function of these drought-regulated unannotated genes may prove useful for improving drought tolerance in the future.

Acknowledgements This work was supported by The Project of Qinghai Science & Technology Department (2018-ZJ-940Q), The Open Project of State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University (2017-ZZ-12, 2016-ZZ-04), The China Agriculture Research System (CARS-09), Open Fund of Qinghai Province Key Laboratory of Physical Geography and Environmental Process (2018-QZH-K06) and National Natural Science Foundation of China (31960339).

Author contributions YL developed the experimental design; XW and YF prepared the samples for RNA-seq, conducted physiological experiments and RT-qPCR; YL and LL supervised all experiments; XW and LL wrote the article.

Availability of data and material All the sequencing data have been deposited in the NCBI-SRA database with the following

accessions: SRX7873340, SRX7873341, SRX7873342, SRX7873343, SRX7873344 and SRX7873345.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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