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Research article

Transcriptome analysis reveals novel insights into the continuous cropping induced response in *Codonopsis tangshen,* a medicinal herb

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ABSTRACT

Codonopsis tangshen Oliv. (*C. tangshen* Oliv.), a famous medicinal herb in China, is seriously affected by continuous cropping (C-cro). The physiological and biochemical results indicated that C-cro significantly affected the malonaldehyde (MDA) and chlorophyll content, as well as activities of catalase (CAT) and superoxide dismutase (SOD) when compared with the non-continuous cropping (NC-cro) group. Transcriptome profiling found 762 differentially expressed genes, including 430 up-regulated and 332 down-regulated genes by C-cro. In addition, pathway enrichment analysis revealed that genes related to 'Tyrosine degradation I', 'Glycogen synthesis' and 'Phenylalanine and tyrosine catabolism' were up-regulated, and genes associated with 'Signal transduction', 'Immune system', etc. were down-regulated by C-cro. The expression of target genes was further validated by Q-PCR. In this study, we demonstrated the effects of C-cro on *C. tangshen* at the transcriptome level, and found possible C-cro responsive candidate genes. These findings could be further beneficial for improving the continuous cropping tolerance.

1. Introduction

Codonopsis tangshen Oliv., a perennial herbaceous species, is one of the most important medicinal herbs used in traditional Chinese medicine (Lin et al., 2013). The plant mainly cultivated in Sichuan, Guizhou, Hunan, Hubei, and Shaanxi provinces of China. Chuan–Danshen, a traditional medicine made from *C. tangshen* dried root, is commonly used for improving appetite, replenishing qi (vital energy) deficiency, promoting gastrointestinal function, curing gastric ulcer, regulating blood sugar, lowering blood pressure, anti hypoxia, enhancing body immunity, and so on (Wang et al., 2007; He et al., 2014). Moreover, Chuan–Danshen has high medicinal value and low price and sometimes used as a substitute for ginseng (Tsai and Lin, 2008).

In addition to its medicinal use, *C. tangshen* is an important raw material for the production of modern cosmetics and health products, and consequently the demand for *C. tangshen* is increasing. However, the wild *C. tangshen* is limited, and furthermore various cultivation measures such as field management, growth years and harvest time, etc. have a remarkable effect on the yield and quality of Chuan–Danshen (Gao et al., 2016). In addition, from our long-term experience, we observed that continuous cropping is a significant limiting factor for the growth and development *C. tangshen*. Generally, plants grown in the continuously cropped land showed smaller leaves, chlorosis and senescence, shorter and thinner stems and lower lodging resistance, and becomes rigid than plants grown in the non-continuously cropped land. Furthermore, the root disease induced by pathogenic fungi was more serious, which all results a significant reduction in the yield and quality of Chuan–Danshen (unpublished data). The overall results indicate that continuous cropping seriously affected the cultivation and production of *C. tangshen* in China.

Abbreviations: DEGs, differentially expressed genes; Q-PCR, quantitative reverse transcription PCR; DE, differentially expressed; C-cro, continuous cropping; NC-cro, non-continuous cropping; FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced; KEGG, Kyoto encyclopedia of genes and genomes; GO, Gene Ontology; MDA, malonaldehyde; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; RSEM, RNA-Seq by Expectation Maximization

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Continuous cropping is a system in which certain crops are "replanted" in the fields that had previously cultivated the same or similar plant species (Xiong et al., 2015), and is a widely used practice in Chinese agricultural production. Consequently, continuous cropping obstacles, also known as replanted disease are usually observed in agricultural crops (Chen et al., 2011; Zhao et al., 2018). Several studies have reported the negative effects of continuous cropping on various plant species, especially for cash crops and medicinal plants. A study by Wang et al. (2015b) showed that continuous cropping reduced growth and stress resistance, postponed development and reduced yield and quality in eggplant (*Solanum melongena* L.). A recent study also indicated that long-term monoculture severely inhibited the growth of coffee (*Coffea arabica* L.), and resulted in yield loss and serious soil-borne disease (Zhao et al., 2018). Continuous cropping markedly reduced plant height, branch numbers, leave chlorophyll and dry-matter content, root vigor, and activities of ribulose diphosphate carboxylase and sucrose phosphate synthase in potato (*Solanum tuberosum* L.) (Liu et al., 2017). A study on *Rehmannia glutinosa* L., a medicinal plant, showed that continuously cropped plants exhibited lower growth rate, smaller leave size, declined root activity and activity of root ATPase (Yin et al., 2009). In the same species, Gu et al. (2013) found lower chlorophyll content, photosynthetic performance and root activity under continuous cropping condition.

Similarly, continuous cropping decreased growth, yield, essential oil content, chlorophyll content, photosynthetic rate, and activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), but increased electrolyte leakage (EL), malondialdehyde (MDA), proline and soluble sugar content in *Angelica sinensis* (Oliv.) Diels (Zhang et al., 2010). Likewise, Panax notoginseng (*Panax notoginseng* (Burk.)) plants grown under continuously cropped field showed reduced tuber quality and yield, and high seedling death rate (Tan et al., 2017). Consecutive monoculture also caused a serious disease and results in the decrease in biomass and quality of shoot parts, and lower harvest rate in *Pogostemon cablin* (Blanco) Benth (Zhang et al., 2018b). However, the impact of continuous cropping on the growth and development of *C. tangshen* plant is not well documented.

A number of studies have shown the effect of continuous cropping on the physiological and biochemical characteristics of various crop species, but studies on the molecular response of plants to continuous cropping are scant. For instance, Yang et al. (2011) compared the expression of miRNAs between the first year and second year planted *R. glutinosa* plants, and found 32 differentially expressed miRNAs. In addition, Wu et al. (2015) characterized the transcriptional response of ginseng (*Panax ginseng* C. A. Mey) to autotoxin (benzoic acid), which is a component of root exudates in the continuously cropped soils, and found important transcriptional factors. More recently, Zhang et al. (2018b) reported the leaf proteome change in response to continuous cropping in *P. cablin*, and showed that continuous cropping altered the expression of proteins related to energy, carbohydrate, and amino acid metabolism. Based on these limited reports, here we hypothesized that continuous cropping might directly affect the transcriptome profiles of medicinal plants, and therefore induce physiological and phenotypical variations.

RNA sequencing (RNA- Seq), a next-generation high throughput technique with greater depth and coverage, is an economical and quick method to obtain bulk information for species with (Zhang et al., 2018a) or without (Li et al., 2017) available genome data. The objectives of this study were to: (a) investigate the genome-wide transcriptome profiles of *C. tangshen* under two cropping systems; (b) identify candidate genes and pathways related to continuous cropping response in *C. tangshen*, and (c) understand the molecular mechanisms associated with continuous cropping response.

2. Materials and methods

2.1. Plant materials and sample collection

On March 2017, the fresh roots of *C. tangshen* were planted in a standardized planting base in Xinqiao village, Banqiao town, Enshi city, Hubei province, which is a famous place for *C. tangshen* cultivation. For continuous cropping (C-cro) treatment, the roots of *C. tangshen* were planted in the plot where *C. tangshen* was continuously grown for 3 years. For non-continuous cropping (NC-cro) treatment, the roots were planted in the plot where the previous crop was corn (*Zea mays*). The two plots were adjacent to each other, and all the other management measures were the same for both treatments. On June 16, 2017, young leaf samples were collected from five independent plants and mixed as one biological repetition. There were three biological replicates for each treatment. Samples were immediately frozen in liquid nitrogen and stored at −80 °C refrigerator until use. For physiological, biochemical and sequence analyses we followed the same cultivation and sample collection procedure.

2.2. Measurement of physiological and biochemical traits analysis

The chlorophyll content, malonaldehyde (MDA), soluble protein content, and the activities of antioxidant enzymes such as SOD and CAT were measured according to the previous method (Li et al., 2012). The experiment was performed with three biological replicates, and one-way ANOVA was used to analyze differences between the two treatment groups.

2.3. RNA extraction, library construction, and sequencing

Total RNA was extracted from the leaf samples using TRizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). DNA contaminant was removed using RNase free DNase I (Qiagen, Hilden, Germany), and the quality of RNA was monitored on 1% agarose gels, while the concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). The RNA integrity was assessed using the RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). About 1.5 μg high-quality RNA was used for each library construction. RNA-Seq libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer's instructions. Briefly, mRNA was enriched from total RNA using poly-T oligo-attached magnetic beads and then fragmented into short fragments by fragmentation buffer. Subsequently, the first strand cDNA was synthesized from the fragmented mRNA using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-), and the second strand cDNA was then synthesized and purified, followed by 3′ ends adenylation and sequencing adapters ligation. The cDNA fragments with suitable size (250–300 bp) were purified with AMPure XP system (Beckman Coulter, Beverly, USA), and amplified to produce the library. After purification of PCR products and assessment of library quality, the libraries were then sequenced on an Illumina Hiseq platform 2500 after cluster generation and paired-end reads were generated. To obtain clean reads, low quality reads and reads containing adapters or ploy-N were removed from raw reads. Thereafter, the Q20, Q30, and GC-content of the clean reads were calculated and high-quality clean reads were used for the downstream analyses.

2.4. De novo transcriptome assembly and functional annotation

De novo transcriptome assembly was performed using Trinity (Grabherr et al., 2011). The min_kmer_cov was set to 2 by default, and all the other parameters were set at default levels. For gene functional annotation, we used databases or softwares such as GenBank non-redundant protein sequences (Nr, http://www.ncbi.nlm.nih.gov), NCBI

nucleotide sequences (NCBI blast), Pfam (http://pfam.sanger.ac.uk/), KOG (diamond v0.8.22), Swiss-Prot (http://www.ebi.ac.uk/uniprot/), GO (Blast2GO v2.5), and KEGG (http://www.genome.jp/kegg/).

2.5. Gene expression level and the identification of DEGs

The read count value of each gene was quantified using RSEM (RNA-Seq by Expectation Maximization) (Li and Dewey, 2017), and then transformed into FPKM (Fragments Per Kilobase of exon per Million mapped fragments) for estimating the level of gene expression. Genes were considered as expressed genes at a cut off value of FPKM > 0.3 in at least one sample. Subsequently, differential expression analysis was performed with the DESeq2 Package (Anders and Huber, 2010; Love et al., 2014). The values of log2 Fold Change were calculated using the read count after DESeq normalization, and P values were corrected using Benjamini and Hochberg's approach to control the false discovery rate. Thus, genes with an adjusted P-value < 0.05 and |log2 Fold Change| > 1 were designated as differentially expressed genes (DEGs). Furthermore, the hierarchical clustering of DEGs was performed based on the FPKM expression values using gplots in the R program environment.

2.6. GO and pathway enrichment analysis

GO terms describe gene functions and usually occur in different quantities due to changing conditions. GO enrichment analysis was performed for all DEGs to identify the overrepresented GO terms by the GOseq R package according to Wallenius non-central hypergeometric distribution (Young et al., 2010). In addition, hypergeometric test/Fisher's exact test was employed to analyze the statistical enrichment of DEGs in functional pathways using the collective databases including BioCyc, Gene Ontology, KEGG PATHWAY, PANTHER, Gene Ontology Slim, and Reactome. The P values were corrected by using the Benjamini and Hochberg's approach.

2.7. Validation of RNA-Seq by Q-PCR

The expression level of target DEGs were determined by quantitative reverse transcription-PCR (Q-PCR) using ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA) and SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). The first-strand cDNA was synthesized from 1.5 μg total RNA. To verify the presence of the specific amplified product, melting curve analysis was performed at the end of each PCR reaction. The Q-PCR primers of the ten DEGs were listed in Table S1. *UBC* gene (encoding for the ubiquitin-conjugating enzyme) was used as a reference gene according to previous reports (Borges et al., 2014; Ye et al., 2018). All Q-PCR reactions were performed according to our previous method (Li et al., 2017). The $2^{-\Delta\Delta}$CT method was used to determine the relative expression of specific genes (Livaka and Schmittgen, 2001).

3. Results

3.1. Effect of continuous cropping on physiological and biochemical traits

Compared with the non-continuous cropping (NC-cro), continuous cropping (C-cro) significantly decreased the Chl a, Chl b and total Chl content of *C. tangshen* leaves ($p < 0.05$) (Table 1). In addition, plants grown under C-cro condition exhibited significantly higher MDA content and CAT activity, but markedly lower SOD activity than plants grown under NC-cro (Table 2). Continuous cropping also remarkably increased the total soluble protein content of *C. tangshen* leaves compared with the NC-cro group (Table 2).

Table 2
Antioxidant enzyme activities of *C. tangshen* leaves under two different cropping systems.

Treatment	CAT	SOD
… … ….(Unit min^{-1} mg^{-1} protein) … … …		
Non-continuous cropping	138.8 ± 28.74 b[z]	627.4 ± 77.52 a
Continuous cropping	232.0 ± 10.76 a	325.6 ± 48.26 b

[y] Means within a column followed by the different letters were significantly different at the 0.05 probability level based on Fisher's least significant difference (LSD).

3.2. Illumina sequencing and de novo assembly

For transcriptome profiling, six cDNA libraries were constructed from the leaves of *C. tangshen* cultivated in both C-cro and NC-cro plot and sequenced using the Illumina HiSeq platform 2500. The RNA-Seq raw and clean reads of the six libraries were presented in Table 3. All the clean reads were obtained after removing low-quality reads and adaptor sequences. Thus, we obtained 61,427,706, 59,773,454 and 56,403,580 raw reads, and 60,856,924, 58,963,520 and 55,699,552 clean reads from the three C-cro libraries (C-cro1, C-cro2, and C-cro3), respectively (Table 3). Similarly, 62,257,564, 60,771,514, and 65,912,858 raw reads, and 61,477,624, 60,049,566 and 64,993,228 clean reads were obtained from the three NC-cro libraries (NC-cro1, NC-cro2, and NC-cro3), respectively (Table 3). In summary, a total of 178 million and 189 million raw reads, and 176 million and 187 million clean reads were obtained from C-cro and NC-cro treatments, respectively. The average GC contents of C-cro and NC-cro were 45.05% and 44.94%, respectively. In addition, the Q20 values were 97.6% and 97.4, and the Q30 values were 93.23% and 92.8% for C-cro and NC-cro group, respectively, indicating the good quality of the transcriptome sequencing (Table 3). The raw sequencing reads of each library were deposited in the Sequence Read Archive (SRA) database at the NCBI. The SRA accession numbers were PRJNA525545, PRJNA525553, PRJNA525569, PRJNA525589, PRJNA525598, and PRJNA525612 for C-cro (C-cro1, C-cro2, and C-cro3) and NC-cro (NC-cro1, NC-cro2, and NC-cro3) groups, respectively. Due to the lack of *C. tangshen* reference genome sequence, *de novo* assembly was employed and a total of 240,522 transcripts were generated (Table 4). The maximum and the mean lengths were 13,738 bp and 1041 bp, respectively. While the N50 and N90 values were 1763 bp and 421bp, respectively. Accordingly, a total of 196,814 unigenes were assembled, with an average length of 1208 bp and the maximum length of 13,738 bp. At the same time, the N50 and N90 values of these unigenes were 1857 bp and 536 bp, respectively. All the unigenes are listed in Table S2.

Table 1
Content of chlorophyll, MDA and soluble protein in *C. tangshen* (*Codonopsis tangshen* Oliv) leaves under two different cropping systems.

Treatment	Chl_a	Chl_b	$Chl_{(a+b)}$	MDA	Soluble protein
	… … … … … … … … … … … … … … (mg g-1 FW) … … … … … … … … … … … ….				
Non-continuous cropping	2.23 ± 0.12 a[z]	0.70 ± 0.07 a	2.93 ± 0.18 a	27.9 ± 1.74 b	8.2 ± 0.88 b
Continuous cropping	1.93 ± 0.09 b	0.49 ± 0.03 b	2.42 ± 0.12 b	37.2 ± 2.31 a	11.5 ± 1.12 a

[y] Means within a column followed by the different letters were significantly different at the 0.05 probability level based on Fisher's least significant difference (LSD).

Table 3
Overview of the sequencing data.

Sample name	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC (%)
C_cro1	61,427,706	60,856,924	98.33	94.84	45.22
C_cro2	59,773,454	58,963,520	97.39	92.78	45.18
C_cro3	56,403,580	55,699,552	97.07	92.07	44.74
Subtotal/average	177,604,740	175,519,996	97.60	93.23	45.05
NC_cro1	62,257,564	61,477,624	97.30	92.54	44.74
NC_cro2	60,771,514	60,049,566	97.31	92.59	44.97
NC_cro3	65,912,858	64,993,228	97.60	93.26	45.12
Subtotal/average	188,941,936	186,520,418	97.40	92.80	44.94

Leaves of *C. tangshen* grown in continuous cropping plot (C_cro) and non-continuous cropping plot (NC_cro) were sampled respectively for RNA sequencing.

3.3. Functional annotation of C. tangshen transcriptome

The GO assignments were performed to classify the functions of the unigenes, and 81,584 (41.45%) unigenes were classified into the three GO categories including “biological process (BP)”, “molecular function (MF)”, and “cellular components (CC)” (Fig. 1). In the biological process category, ‘cellular process’, ‘metabolic process’ and ‘single-organism process’ were predominantly enriched. In the ‘metabolic process’, 60 unigenes were related to “reactive oxygen species”, while 10 unigenes were related to “hormone biosynthetic process” (Table S3). The cellular component category mainly comprised unigenes related to ‘cell’, ‘cell part’ and ‘organelle’. In the molecular function category, ‘binding’, ‘catalytic activity’, ‘nucleic acid binding transcription factor activity’ were highly represented (Fig. 1, Table S3).

Furthermore, all the unigenes were subjected to KOG functional classifications. Accordingly, out of the 196, 814 unigenes, 29,199 (14.83%) unigenes were assigned to 26 KOG functional classes (Fig. 2). The ‘Posttranslational modification, protein turnover, chaperones’ were the largest groups followed by ‘General function prediction only’, ‘Translation, ribosomal structure and biogenesis’, ‘RNA processing and modification’ ‘Function unknown’ ‘Signal transduction mechanisms’ and ‘Intracellular trafficking, secretion and vesicular transport’.

The KEGG database is powerful for systematic analysis of gene functions and metabolic pathways. Through blast analysis against KAAS (KEGG Automatic Annotation Server), the unigenes were annotated to 5 major KEGG pathways including ‘cellular processes’ (A), ‘environmental information processing’ (B), ‘genetic information processing’ (C), ‘metabolism’ (D) and ‘organismal systems’ (E). Category D was the dominant category, among which ‘Carbohydrate metabolism’ (3758 unigenes) and ‘Overview’ (2660 unigenes) were the highly represented subcategories. In category C, the most represented subcategory was ‘Translation’ (3521 unigenes) (Fig. S1). Category A comprised one subcategory ‘Transport and catabolism’, with 4 KEGG pathways, and ‘Peroxisome’ (ko04146) (645 unigenes) was the dominant pathway (Fig. S1, Table S4). Category B contained two subcategories including ‘Signal transduction’ and ‘Membrane transport’. In the ‘Signal transduction’ subcategory, ‘Plant hormone signal transduction’ had the highest unigenes representation (842 unigenes) (Table S4).

3.4. Differential expression analysis of assembled unigenes

Venn diagram analysis was performed to demonstrate both the commonly and specifically expressed genes of the two treatment groups. There were more specific unigenes in the NC-cro group (24,516) than in the C-cro group (20,403), but the commonly expressed genes (96, 312) accounted for the largest portion in the Venn diagram. The differentially expressed genes (DEGs) were screened using DESeq2 software at the threshold value of padj < 0.05 and $|\log2FoldChange| > 1$. Based on these criteria, 762 (430 up-regulated and 332 down-regulated) unigenes were differentially expressed in response to C-cro compared to the NC-cro. The up-regulated and down-regulated unigenes were listed in Table S5 and Table S6, respectively. In addition, the expression and annotation of each DEG were shown in Table S7.

Furthermore, the DEGs from three replicates of the two treatment groups were clustered based on the hierarchical clustering method. As shown in Fig. 3, all the DEGs were classified into two groups, with opposite expression profile between C-cro and NC-cro groups. The majority of the DEGs showed relatively high expression level in the C-cro treatment, but displayed low expression in the NC-cro treatment and vice versa. In addition, most of these DEGs had similar expression level among the different replicates of the two treatment groups.

3.5. GO and pathway enrichment analysis of DEGs

GO enrichment analysis was performed to reveal the significantly enriched GO terms. Thus, 15 GO terms were significantly enriched among the DEGs, In the cellular component category, “coated pit”, “trans-Golgi network transport vesicle membrane”, “clathrin coat of trans-Golgi network vesicle”, “membrane region”, “clathrin-coated vesicle membrane”, “RNA-directed RNA polymerase complex”, and “Golgi-associated vesicle membrane” were the highly enriched GO terms. Within the molecular function, “RNA-directed RNA polymerase activity” and “oleate hydratase activity” were significant enriched (Table S8). In addition, some DEGs were annotated to the GO terms related to “oxidoreductase activity”, but were not significant (Supplementary Table S8).

Furthermore, we determined the number of DEGs in each pathway, and the P-Values were corrected to further investigate the significantly enriched pathways. Thus, nine pathways including ‘Tyrosine degradation I’, ‘Phenylalanine and tyrosine catabolism’, ‘Tyrosine metabolism’, ‘Glycogen synthesis’, ‘Metabolic pathways’, and so on were significantly enriched in the C-cro versus NC-cro (Fig. S2; Table S9). The up-regulated DEGs were mainly related to ‘Tyrosine degradation I’, ‘Glycogen synthesis’ and ‘Phenylalanine and tyrosine catabolism’ pathways (Fig. S3; Table S10), while the down-regulated DEGs were mainly associated with ‘Signal transduction’, ‘Immune system’, ‘Signaling by GPCR’, etc (Fig. S4; Table S11). In addition, some DEGs were annotated to “Plant hormone signal transduction” and “Transcription regulation by bZIP transcription factor”, and “Basal transcription factors”, pathways, but were not statistically significant (Table S9).

3.6. DEGs related to transcription factors

In the present study, transcription factors (TFs), which were classified into 8 TF families including bHLH, bZIP, MYB, AP2-EREBP, MADS, Trihelix, WRKY, and Jumonji (Table S12) were identified from 20 DEGs (Table S12, Additional file 17). From the five *bHLH* genes, two were down-regulated, while the three were up-regulated in C-cro group compared to the NC-cro group. There were four DEGs coding *bZIP* family, of which one was significantly down-regulated and the other three were remarkably up-regulated in C-cro group compared with the

Table 4
Summary of the RNA-Seq data from the Trinity *de novo* assembly in *C. tangshen* leaves.

Assembly statistic	Total Length (bp)	Sequence No.	Max Length (bp)	Ave Length (bp)	N50	N90
Transcript	250,399,159	240,522	13,738	1041	1763	421
Unigene	237,831,423	196,814	13,738	1208	1857	536

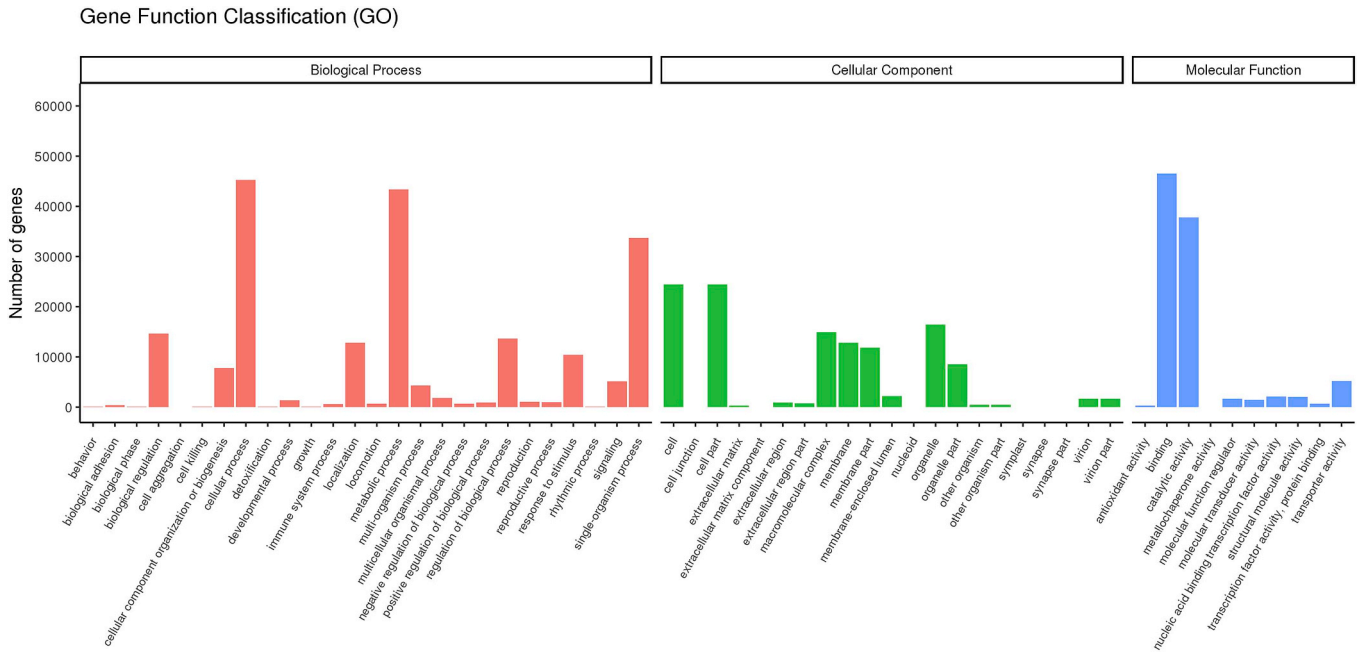

Fig. 1. Histogram of gene ontology classification. The results were summarized in three main categories: biological process, cellular component and molecular function. The y-axis indicated the number of genes in a category. The x-axis indicated the subcategories.

NC-cro group. All the 8 members of *MYB, MADS, Trihelix*, and *Jumonji* family were significantly down-regulated, whereas all the 3 members of the *AP2-EREBP* and *WRKY* family were remarkably up-regulated in C-cro compared to the NC-cro group.

3.7. Validation of the DEGs by Q-PCR analysis

Ten unigenes (five up-regulated and five down-regulated) related to photosynthesis or stress responses were used to confirm their expression profiles of the RNA-Seq results by Q-PCR. The results indicated that the expression level of the five up-regulated unigenes (*PR1*, *MYC2*, *DnaJ*, *PERK8*, and *RAP2-4*) was significantly higher in the C-cro group than in the NC-cro group. However, as shown in Fig. 4, C-cro greatly inhibited the expression of the five down-regulated unigenes (*apoprotein*, *psbA*, *psbW*, *BPS1,* and *SR34A*) compared with the NC-cro. Consequently, the Q-PCR results were consistent with the RNA-Seq results, indicating that our RNA-Seq results were reliable and valid.

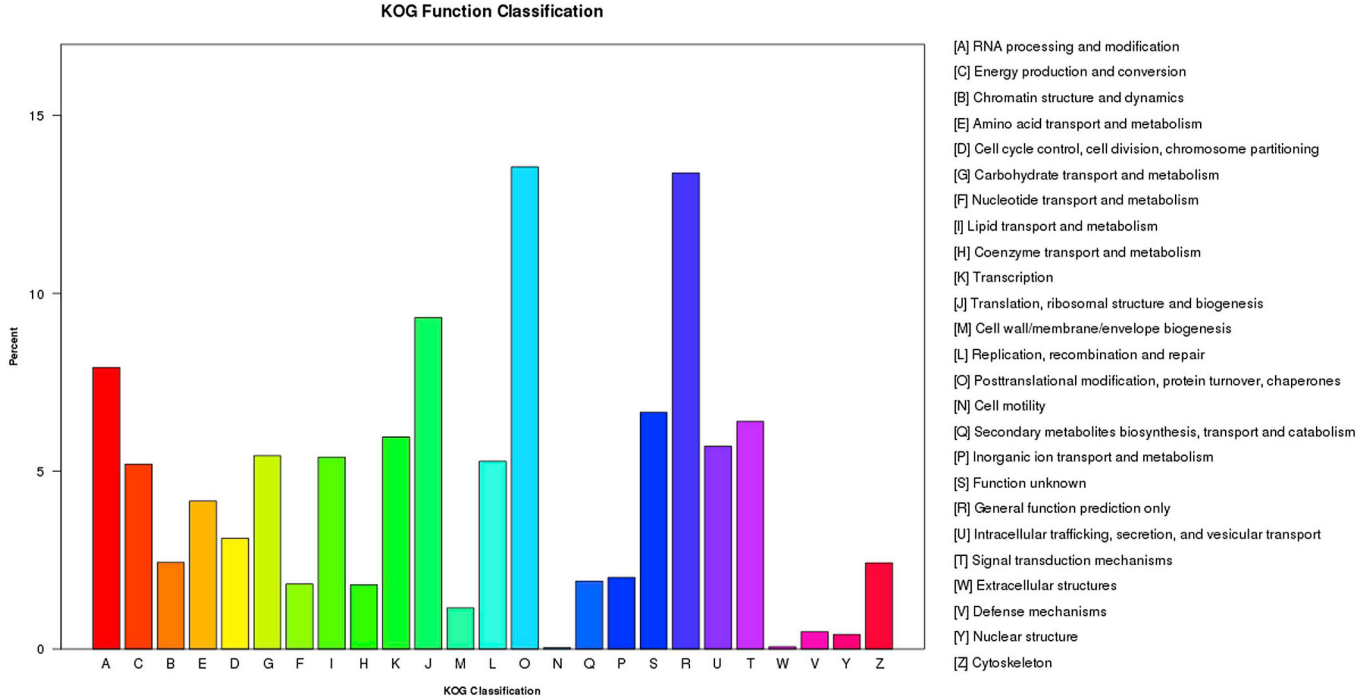

Fig. 2. KOG annotation of putative proteins. In total, 29199 genes were assigned to KOG classifications and divided into 26 specific categories. The x-axis represented 26 groups of KOG. The y-axis indicated the percentage of the number of genes annotation under the group in the total number of genes annotation.

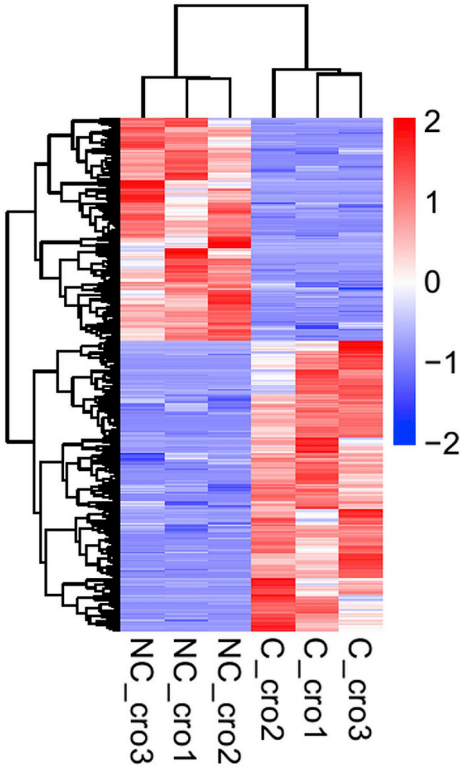

Fig. 3. Hierarchical clustering of the differentially expressed genes. Different colors denoted different expression levels, with blue representing low expression levels and red representing high expression levels. The values beside the colors represented $\log_2$(FPKM + 1). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Continuous cropping altered the physiological and biochemical traits of *C. tangshen*

It is well documented that continuous cropping affected the morphological, physiological, biochemical and molecular characteristics of plants. In this study, we investigated the effect of continuous cropping on selected physiological and biochemical traits of *C. tangshen*. MDA, a product of membrane lipid peroxidation, is generally used as a stress marker (Polle et al., 1997). Here, C-cro significantly increased the MDA content of *C. tangshen* compared with the NC-cro group, suggesting a higher level of C-cro induced lipid peroxidation in *C. tangshen* leaves. By contrast, compared to the NC-cro group, plants grown under C-cro treatment exhibited significantly lower chlorophyll content (Chl a, Chl b and total Chl). Our results were consistent with the previous findings in potato (*Solanum tuberosum* L.) (Liu et al., 2017) and *Rehmannia gltinosa* (Zhang et al., 2011), which reported the significant reduction in chlorophyll content and photosynthetic activity under continuous cropping condition. It has been reported that antioxidant enzymes play a pivotal role in plants stress tolerance. According to the study by Zhang et al. (2010), continuous cropping significantly inhibited the activities of antioxidant enzymes such as SOD and CAT in *Angelica sinensis* (Oliv.) Diels. In this study, continuous cropping reduced the activity of SOD but enhanced the activity of CAT in *C. tangshen*. Similar results were reported in *R. gltinosa* in response to continuous cropping treatment (Zhang et al., 2011).

To investigate the molecular mechanisms of continuous cropping response in *C. tangshen,* we also performed a genome-wide transcriptome profiling by RNA-seq approach. The results indicated that 60 unigenes were related to “reactive oxygen species metabolic process” GO term (Table S3). In addition, the ‘Peroxisome’, which comprised 645 unigenes, was the highly represented KEGG pathway under ‘Transport and catabolism’ subcategory (Table S4). Furthermore, 20 *SOD* coding and four *CAT* coding *uni*genes were identified (Additional file 17), but none of them were significantly differentially expressed between C-cro and NC-cro, which was inconsistent with enzyme activity change. Similar results were reported in tomato (*Lycopersicum esculentum* L.) under different temperature conditions (Soydam Aydin et al., 2013). The result might reveal the lack of correlation between the change in mRNA level and protein product (Dale and Schantz, 2002). In addition, the protein stability might affect the activities of enzymes (Privalov, 1990), or probably the *SOD* and *CAT* expression regulation occurred at the post-transcriptional level. However, the overall physiological and biochemical analyses results revealed that continuous cropping was a serious problem for the growth and development of *C. tangshen*.

4.2. Global transcriptional patterns of *C. tangshen* in response to continuous cropping

Generally, continuous cropping practice affects the yield and quality of many crops and is considered as an adverse factor for agricultural production. However, plants molecular mechanism in response to continuous cropping remains unknown. But, with the development of high-throughput sequencing technology, several continuous cropping-responsive micro-RNAs have been identified in *R. glutinosa* (Yang et al., 2011). Recently, Wang et al. (2015a) found two up-regulated and 13 down-regulated *MYB* genes in the root of *R. glutinosa* Libosch in response to continuous cropping. In addition, the transcriptional change in response to autotoxins (benzoic acid) was uncovered in *Panax ginseng* using transcriptome sequencing method (Wu et al., 2015). These studies provide the background information to further elaborate the molecular mechanisms underlying plants response to continuous cropping.

In this study, we observed that continuous cropping was a serious threat to the growth and development of *C. tangshen.* Here, the transcriptome profile of *C. tangshen* in response to continuous cropping was unraveled. Six *C. tangshen* leaf cDNA libraries were constructed and sequenced using the Illumina HiSeq platform. Consequently, 24,526 and 20,416 unigenes specific to NC-cro and C-cro group, respectively, and 96,312 commonly expressed unigenes were identified. In addition, we obtained 762 differentially expressed genes including 430 up-regulated and 332 down-regulated in response to continuous cropping. However, these DEGs were not fully annotated and enriched, because of the specificity of the plant species and the limitation of the available databases. Nevertheless, for the first time, we demonstrated continuous cropping induced transcriptional changes in *C. tangshen,* which could enrich the transcriptome data of the plant. The study could also provide useful evidence for other plant species in response to continuous cropping. In this study, we found some DEGs related to TFs, photosynthesis and plant hormone signal transduction, which might be partially responsible for the physiological and phenotypic changes in response continuously cropping.

4.3. DEGs related to transcription factors

Transcription factors (TFs) play important roles in plant growth and abiotic stress response. The MYB is a major superfamily of TFs affecting plant growth and development under stress environment (Wang et al., 2015a). For instance, an MYB-like TF (KUA1) modulated peroxidase expression and ROS homeostasis and determined the leaf cell expansion and the final size of organs in *Arabidopsis thaliana* (Lu et al., 2014). Recently, Wang et al. (2015a) found 15 differentially expressed *MYB* genes (13 remarkably down-regulated) in *R. glutinosa* in response to continuous cropping, suggesting the involvement of *MYB* in response to continuous cropping. Similarly, we identified three significantly down-regulated *MYB* unigenes in response to continuous cropping. Consequently, we presumed that the suppression of *MYB* could result in small organ size under continuous cropping stress in *C. tangshen* by

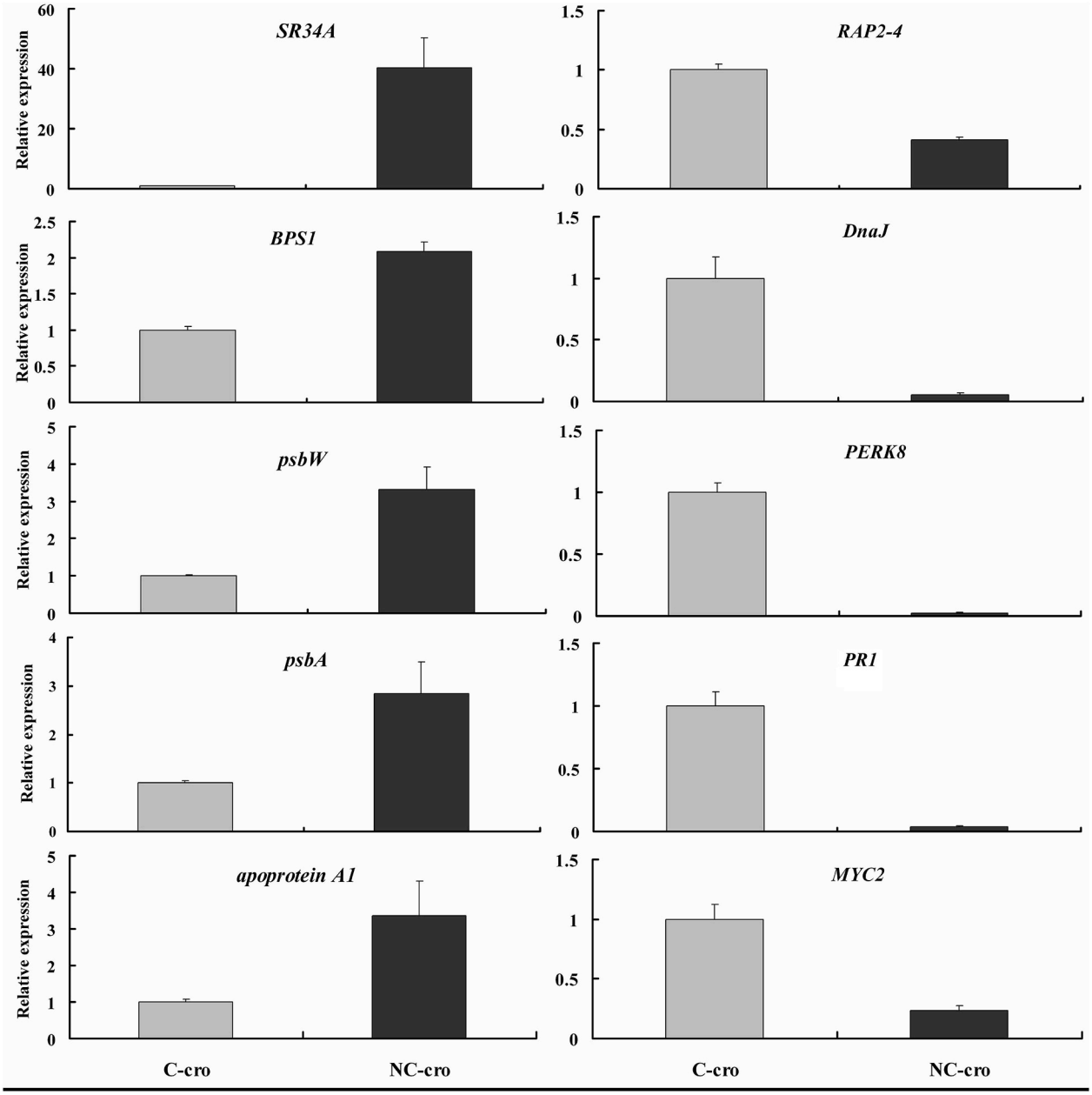

Fig. 4. Q-PCR analysis of 10 selected DEGs in leaves of C-cro and the NC-cro plants. *UBC* gene was used as the reference gene for normalization of gene-expression data. Three independent experiments and three technical replicates were performed.

modulation the expression of peroxidases and ROS homeostasis.

The bZIP family is one of the largest groups of TFs that control the crucial developmental and physiological processes of the plant (Castro et al., 2017). Recent studies have shown that many *bZIP* genes are involved in pathogen defense, osmotic stress and salt stress response in different plant species (Amorim et al., 2017; Castro et al., 2017; Mianlengeh et al., 2018; Liu et al., 2019). Likewise, bHLH TFs also take part in diverse plant biological processes and abiotic stress responses (Chen et al., 2015; Hu et al., 2015). In the present study, four *bZIP* and five *bHLH* differentially expressed genes were identified in *C. tangshen*, suggesting that TFs like bZIP and bHLH play an important role in the continuous cropping response. They also accounted for almost one half of the identified differentially expressed TFs genes, and further investigation is needed to elucidate their functions in *C. tangshen* under continuous cropping stress.

4.4. DEGs associated with photosynthesis metabolism

Photosynthesis, a highly integrated and regulated process, is sensitive to environmental change and abiotic stress (Ensmingera et al., 2006). In this study, several DEGs related to 'photosynthesis' metabolism were identified in *C. tangshen*, and were down-regulated in C-cro compared to the NC-cro group. From those DEGs, *psbA* and *psbW* were related to photosystem II (PSII), and *psaA* was associated with photosystem I. *PsbA* encodes for D1, which is a core protein in the PSII reaction center, and responsible for photosynthetic electron transport from Q_A to Q_B (Roffey et al., 1994). But, abiotic stresses usually suppress the transcription and translation of *psbA* gene (Nishiyama et al., 2004), and D1 content also negatively affected by various stresses (Wang et al., 2018).

PsbW is associated with PSII protein complexes, and play an important role in stabilizing PSII structure and photosynthesis (García-Cerdán et al., 2011). *PtopsbW* was found to be crucial in *Populus*

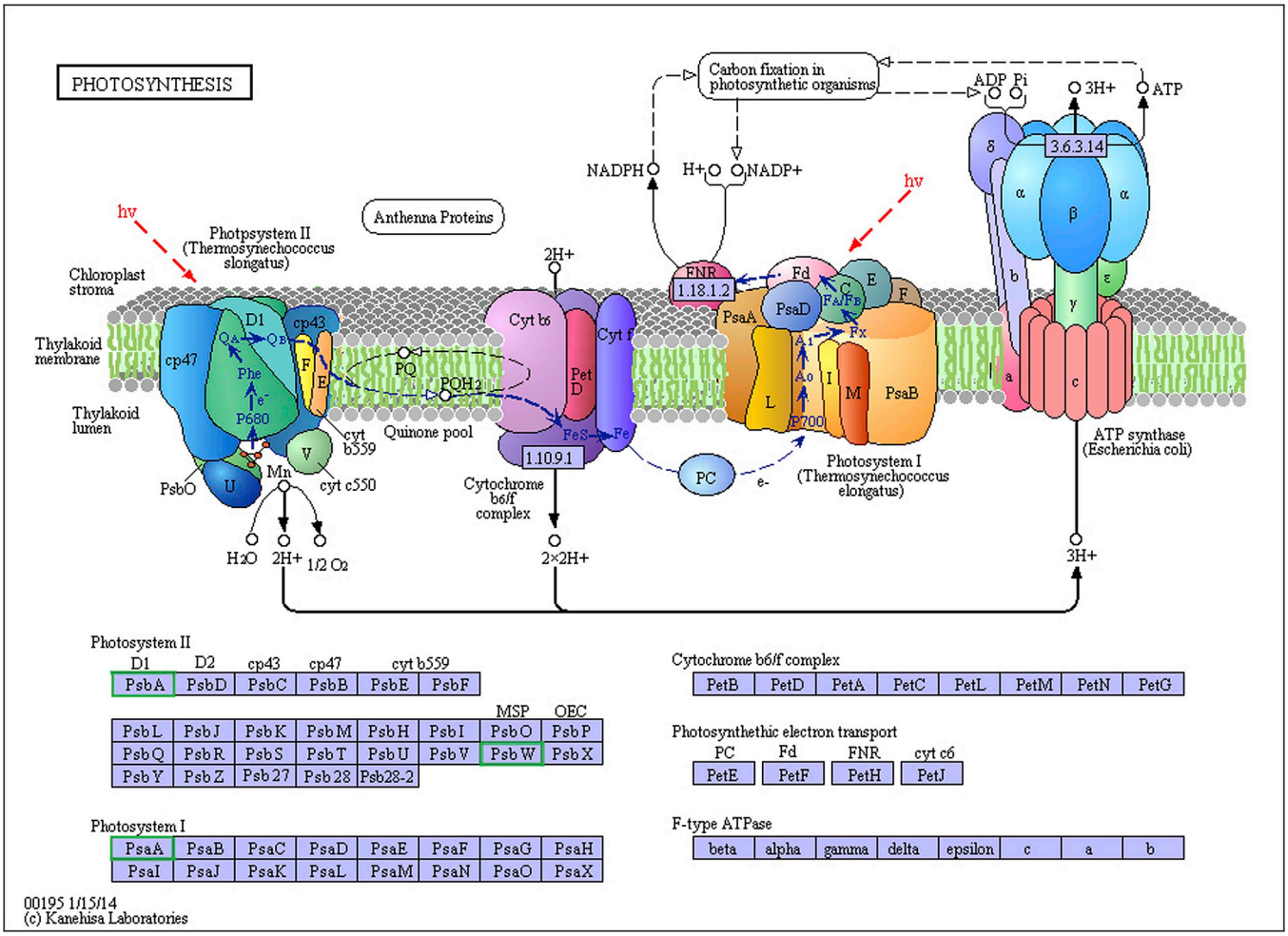

Fig. 5. Differentially expressed genes (DEGs) related to photosynthesis pathway in C-cro vs NC-cro treatment. Green frame denoted down-regulated gene, while black frame denoted gene showing no differential expression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tomentosa plant growth and development and particularly might be associated with chloroplast development (Wang et al., 2017). It has been indicated that *psbW* was significantly down-regulated in tall fescue (*Festuca arundinacea*) following Pb treatment (Li et al., 2017). On the other hand, *PsaA* coding for P700 protein of photosystem I, is an important photosynthetic reaction center gene that is generally down-regulated by heat stress in the plant (Danilova et al., 2018).

Similar with previous reports, we observed that continuous cropping down-regulated the expression of *psbA*, *psbW* and *psaA* genes in *C. tangshen* (Fig. 5), which could be as a result of several physiological responses such as the readjustment of energy homeostasis and rearrangement of transcription-translation machinery (Caldana et al., 2011). Furthermore, the down-regulation could ultimately decrease the chlorophyll content and the repair rate of photosynthetic proteins and result in *C. tangshen* growth inhibition under continuous cropping condition.

4.5. DEGs related to plant hormone signal transduction

Continuous cropping inhibited various hormone signaling pathways involved in cell division, cell enlargement, plant growth and defense system in *C. tangshen* (Fig. 6). *Gretchen Hagen3* (GH3) is a supervisor gene family in the fluctuation of auxin, and function in balancing auxin pools (Fu et al., 2011). The expression of *GH3* is generally associated with plant development and morphogenesis such as shoot, root, and hypocotyl (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5853644/; Nakazawa et al., 2001).

It has been indicated that *GH3* was suppressed under low auxin level (Hagen and Guilfoyle, 1985). Meanwhile, the over-expression of *GH3* gene enhanced plants resistance to pathogens (Jagadeeswaran et al., 2007; Nobuta et al., 2007). In our study, the *GH3* gene was down-regulated in response to continuous cropping, which may indicate lower auxin concentration in the continuously cropped *C. tangshen*.

It is well established that, cytokinin (CK) as an essential phytohormone, regulates various biological processes (Mok and Mok, 2001; Argueso et al., 2009). In *Arabidopsis thaliana*, CK signaling involves a two-component system containing three cytokinin receptors (CRE1, AHK2, and AHK3) and Arabidopsis Histidine Phosphotransfers (AHPs) (To and Kieber, 2008; Kim and Jeon, 2013). CRE1 is a histidine kinase that can trigger a cascade of phosphorelay reactions after cytokinin perception. Previous studies indicated that many members of the *CRE1* family play an important role in root growth, leaf development and meristem activity (Higuchi et al., 2004; Nishimura et al., 2004). In addition, higher expression of *CRE1/AHK4* was required for programmed cell death in cultured Arabidopsis cells (Vescovi et al., 2012). Besides, the cytokinin *CRE1* pathway affected root development and environmental stresses resistance (Laffont et al., 2015). In this study, for the first time, *CRE1* gene was significantly up-regulated in *C. tangshen* in response to continuous cropping, but whether this is attributed to the change in CK content or not is still unknown. In addition, the entire physiological role of *CRE1* was yet to be uncovered in the continuously cropped *C. tangshen*. In Arabidopsis, the expression of all the three *AHP* (*AHP2*, *AHP3*, and *AHP5*) genes was down-regulated by

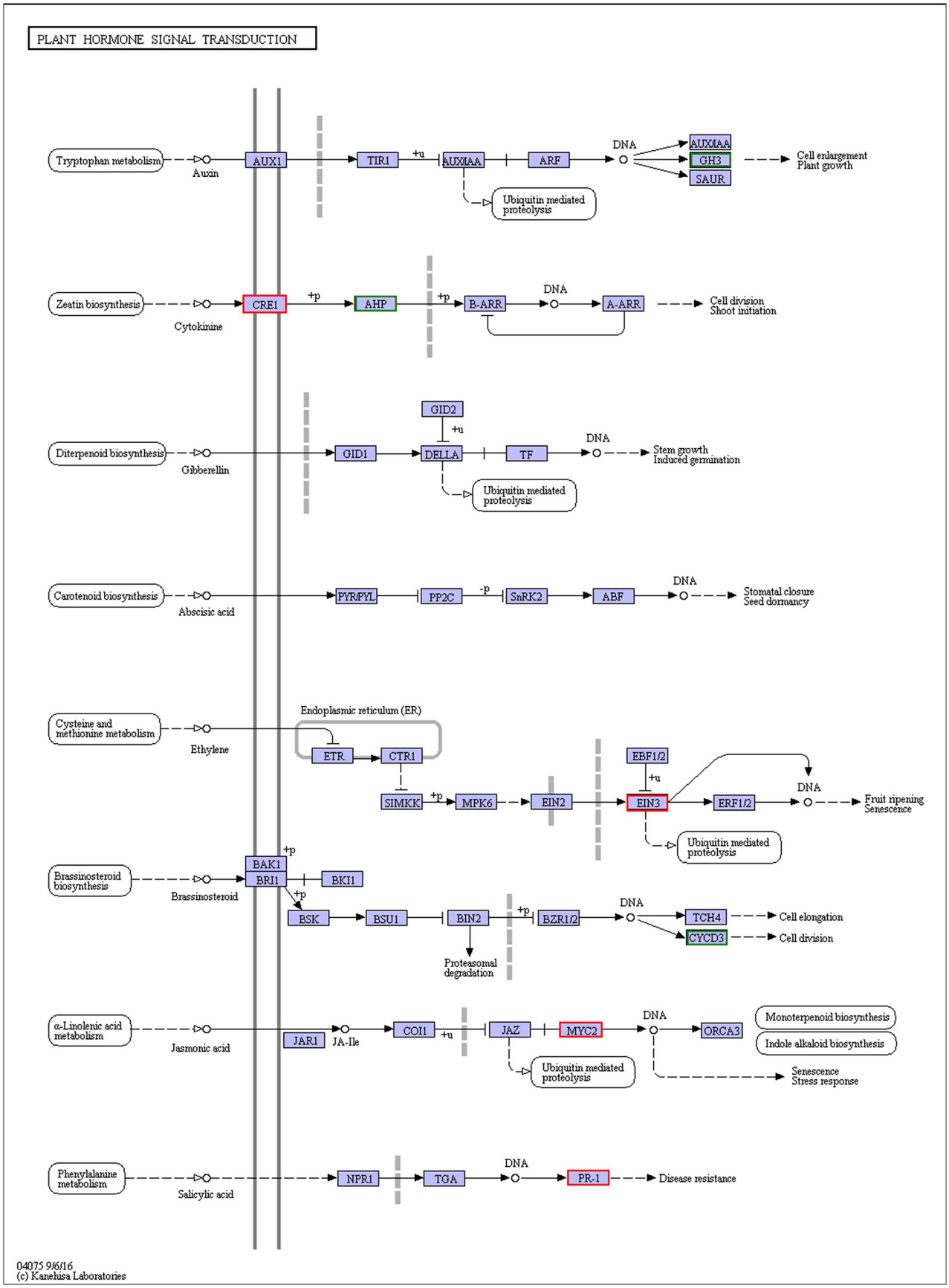

Fig. 6. Differentially expressed genes (DEGs) related to plant signal transduction in C-cro vs NC-cro treatment. Down-regulated gene was denoted by green frame, up-regulated gene was denoted by red frame, while gene showing no differential expression was denoted by black frame. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dehydration, which probably resulted from the drought stress-induced reduction of endogenous CK level (Nishiyama et al., 2013). Similarly, in our study, the expression of *AHP* gene was reduced by continuous cropping in *C. tangshen*, which might finally affect cell division and shoot meristem initiation. In addition, the *AHP* expression change was inconsistent with that of *CRE1*, suggesting the complicated regulation of *CRE1* pathway in *C. tangshen*.

Cyclin-dependent kinases (CDK) control the plant cell division through binding with a positive regulatory cyclin subunit (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4507761/; Gonzalez et al., 2012). The *D-type cyclin* (*CYCD*) gene family plays a critical role in promoting cell division when perceiving mitogenic signals such as auxin and cytokinin (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4507761/; Riou-Khamlichi et al., 1999; Dewitte et al., 2007). In Arabidopsis, *CYCD3* subgroup genes were rate-limiting factors for cambial cell proliferation and secondary growth. Moreover, *CYCD3* subgroup regulated the balance between cell division and cell expansion in shoot lateral organs (Dewitte et al., 2007), and is necessary to promote the cambial cell cycle (Collins et al., 2015). Its lower expression could lead to a significant decline in the diameter of the stem and hypocotyl by decreasing mitotic activity in the cambium (Collins et al., 2015). In the present study, the expression of *CYCD3* gene was suppressed in response to continuous cropping, which might result in reduced mitotic activity in the cambium. Therefore, it was comprehensible that the continuously cropped *C. tangshen* plants displayed thinner stems and lower height.

MYC2 is a master transcription factor in the Jasmonate (JA) pathway, which differentially regulates the expression of JA responsive genes (Takagi et al., 2016). It also acts as a positive regulator of both JA-mediated resistances to insect pests and oxidative stress (Dombrecht et al., 2007). However, *MYC2* gene represses those genes associated with defense responses against pathogens (Anderson et al., 2004; Dombrecht et al., 2007). Recently, *MYC2* has been suggested to function redundantly in activating JA-induced leaf senescence (Qi et al., 2015). In Arabidopsis, it could accelerate the transcription of a key enzyme gene that participates in chlorophyll degradation during plant senescence (Zhu et al., 2015). In the present study, for the first time, *MYC2* was found up-regulated in *C. tangshen* in response to continuous cropping, which might decline the transcription of genes related to defense response against pathogens. Based on our observations, leaf senescence and violet root rot induced by pathogenic fungi were indeed more severe in continuously cropped *C. tangshen*, and supports the above assumption.

Ethylene-insensitive3 (*EIN3*) is a major regulator in the ethylene pathway. It is indicated that *EIN3* participated in the regulation of ethylene-induced chlorophyll degradation (Qiu et al., 2015), and could promote the leaf senescence progression in Arabidopsis (Li et al., 2013). In addition, the transcription of *EIN3* was activated during leaf senescence in sorghum (*Sorghum bicolor* (L.) Moench) (Wu et al., 2016). At the same time, *EIN3* family genes were also associated with the plant defense against pathogen attack and environmental stimuli. For example, *EIN3* played a negative regulatory role in Arabidopsis disease resistance (Chen et al., 2009). *TaEIL1*, a wheat homolog of *AtEIN3*, also negatively modulated the defense response to wheat-stripe rust fungus (Duan et al., 2013). In our study, the expression of *EIN3* was significantly enhanced in response to continuous cropping. Therefore, the physiological changes in the continuously cropped *C. tangshen* might be also partially associated with the up-regulation of *EIN3* gene.

The pathogenesis-related protein 1 (PR-1) is another important defense protein, and the *PR-1* gene expression has been considered as a marker for salicylic acid-mediated disease resistance (Breen et al., 2017). In addition, *PR-1* genes respond to abiotic stimuli and play an important role in plant growth or development (Memelink et al., 1990). It has been shown that PR-1 proteins strongly accumulated in the senescing leaves of tobacco plants (Fraser, 1981). In the present study, like *MYC2* and *EIN3*, *PR-1* gene was also significantly up-regulated in response to continuous cropping. Collectively, our results demonstrated the involvement of JA, ethylene and salicylic acid signaling pathways in the regulation of leaf senescence and susceptibility to pathogen attack. Meanwhile, *MYC2*, *EIN3* and *PR-1* gene might play an intricate and highly orchestrated regulatory role during the physiological processes in the continuously cropped *C. tangshen*.

In summary, for the first time, the RNA-Seq technology was applied to unravel the molecular mechanisms of *C. tangshen* in response to continuous cropping. The results revealed that some genes related to TFs, photosynthesis metabolism pathway and the plant hormone signal transduction pathway (cell division, cell enlargement, plant growth, and defense response, etc) were significantly altered. In addition, transcriptome profiling results were consistent with the phenotypic and physiological changes such as chlorophyll content and fungous disease resistance reduction. The study could help for a better understanding of the molecular mechanism of continuous cropping obstacles in *C. tangshen*. Moreover, our findings will be beneficial for overcoming continuous cropping problems in *C. tangshen* cultivation.

Author contributions

LH and WM (Wang Mo) conceived the study and designed the experiments. HY, ZM and ZW cultivated and prepared the plant materials, AL, YJ and LH carried out the experiments, YJ and WH analyzed the data. LH wrote the manuscript. WM (Misganaw Wassie) revised the manuscript. All authors read and approved the final paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.001.

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