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RESEARCH REPORT

Genetics and Breeding

Comparative transcriptome analysis by RNA-Seq of the regulation of low temperature responses in *Dendranthema morifolium*

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Abstract
Cold tolerance is the primary limiting factor affecting the quality and productivity of chrysanthemum (*Dendranthema morifolium*). However, few studies have investigated the molecular mechanisms underlying the low temperature response in chrysanthemum. In the present study, we used RNA-Seq technology to compare the transcript profiles of chrysanthemum leaves exposed to two different temperatures (20 and −8 °C). A total of 13.54 Gb of clean reads were assembled into 71,971 unigenes with an average length of 694 bp, and 33,282 unigenes were annotated identified from five well-known protein databases. Of these, 9579, 24,252, 7123, 22,554, and 32,891 unigenes were separately identified in the COG, GO, KEGG, Swiss-Prot, and NCBI databases, respectively. Differentially expressed genes (1592 upregulated and 718 downregulated) were identified between the control (CK) and low temperature treatment (T) groups. KEGG pathway enrichment analysis identified 20 significantly different pathways. Many genes encoding important transcription factors (e.g. *CBF*/*DREB*, *bHLH*, *MYC*, and *ZAT*) as well as proteins (e.g. CCX, CBP, CML, and MAPK) involved in cold signal transduction were up- or down-regulated in the low temperature treatment group. Genes involved in ABA signal transduction and biosynthesis of unsaturated fatty acids were also identified. The expression profiles of these genes were analyzed during the cold treatment stage. These results provide important information for further studies on gene discovery in chrysanthemum and suggest a potential molecular mechanism for the response to low temperature in this plant species.

Keywords Chrysanthemum · Cold-tolerant · Cold signal transduction

1 Introduction

Chrysanthemum (*Dendranthema morifolium*) is a popular ornamental plant worldwide (Silva et al. 2013). Because the growing market requires cold-tolerant and perpetual-flowering chrysanthemum cultivars, improving cold tolerance is an important goal for breeders. Low environmental temperatures can easily damage chrysanthemum plants, which can cause significant losses in many temperate crops (Chinnusamy et al. 2007; Janská et al. 2010; Yang et al. 2005). In *Arabidopsis*, there are multiple and complex cold-response pathways, and many genes involved in the response to low temperature have been identified (Janská et al. 2010; Yamaguchi-Shinozaki and Shinozaki 2006); however, systematic investigations of the molecular basis of low temperature tolerance in chrysanthemum are lacking.

Cold signal transduction involves a series of physiological, biochemical, and molecular reactions in many plants (Yamaguchi-Shinozaki and Shinozaki 2006). Firstly, the cold stress signal is perceived by plants and the physical state of the membrane plays a role in this cold perception (Beck et al. 2004, 2007). Changes in the plasma membrane lead to an increase in the cytosolic Ca^{2+} ion concentration (Komatsu et al. 2007). Certain protein kinases related Ca^{2+} influx is recognized as key regulators of plasma membrane change (Saijo et al. 2000). Mitogen activated protein kinase (MAPK) functions in low temperature signal transduction and regulates low temperature tolerance (Yang et al. 2011). Three candidate low temperature response genes (*ZmMAPKKK*, *ZmCLC-D* and *ZmRLK*) were shown to be dysregulated under conditions of chilling stress in maize (Yang

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et al. 2011). Secondly, cold-regulated gene expression is regulated by different pathways that can be ABA-dependent or ABA-independent (Beck et al. 2007). Three candidate genes (*CBF*s, *DREB1*s, and *COR*) belonging to the ethylene responsive element binding protein family are regulated after receiving the cold stress signal (Stockinger et al. 1997). *ICE1* encodes a *MYC*-like basic helix-loop-helix protein that activates the expression of many downstream genes, leading to a significantly enhanced tolerance to chilling (Chinnusamy et al. 2003; Stockinger et al. 1997). Thirdly, many metabolites including some with protective effects against cold stress and reactive oxygen species (ROS) are photosynthetically regulated (Heidarvand and Amiri 2010), and the proteins associated with these metabolites including certain cold associated and inducible proteins such as dehydrins (DHNs) (Close 1996; Kosová et al. 2007; Location 2006), antifreeze proteins (AFPs) (Guy 1999; Marilyn et al. 1997), heat shock proteins (HSPs) (Banzet et al. 1998; Guy 1999), cold-shock domain proteins (CSDPs) (Guy 1999; Sommerville 1999), and enzymes such as alternative oxidases and desaturases, are differentially expressed in response to cold stress

In our previous study, we found that − 8 °C was the half lethal temperature in the autumn chrysanthemum, and that 20 °C was the optimal temperature for autumn chrysanthemum cultivation. We measured physiological indexes such as membrane fatty and unsaturated fatty acids in autumn chrysanthemum (Li et al. 2013). However, the molecular mechanism of the autumn chrysanthemum response to cold temperature remains unclear. In the present study, we used RNA-Seq technology to characterize the transcriptomes of the autumn chrysanthemum cultivar 'Jin Long Teng Fei' in response to the half lethal temperature. We aimed to identify candidate genes to improve cold tolerance in chrysanthemum and increase the genetic resources available for hardy breeding strategies for chrysanthemum.

2 Materials and methods

2.1 Plant material and cold treatment

Plant material from the autumn chrysanthemum cultivar 'Jin Long Teng Fei' was collected at Henan Agricultural University. In our previous study, the half lethal temperature of autumn chrysanthemum was − 8 °C, and the optimal temperature was 20 °C (Li et al. 2013); therefore, we choose a treatment of − 8 °C for 10 min. Eighteen plants were separated into two groups, a control group (CK) with nine plants grown and a low temperature treatment group (T) with nine plants that were all initially grown at 20 °C for 1 w, and then followed by a treatment at 8 °C for 2 h and −8 °C for 10 min in the T group. For each group, nine leaves were sampled from three biological replicates (e.g. three plants were one biological replicate, with three leaves taken from each plant). Both groups (CK and T) were immediately frozen below −80 °C until RNA extraction. A total RNA from each replicate was extracted, and three total RNA were mixed in total 30 μg in equal amounts. Each group had a mixed RNA from three replicates that was used for sequencing.

2.2 RNA extraction, library construction, and sequencing

Total RNA was extracted from two groups (CK and T) and subjected to purity, concentration, and integrality testing. Qualified RNA (20 μg for each sample) was selected for library construction. Sequencing libraries were generated using NEBNext®Ultra™ RNA Library Prep Kit for Illumina®(NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase(RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 200–250 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μg USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2500 system.

2.3 Sequence assembly and annotation

RNA sequencing was performed by the Biomarker Biotechnology Corporation (Beijing, China). Raw data were checked according to the quality score and low-quality data were filtered and removed. High quality clean data were acquired and used to assemble unigenes using Trinity software (Stefan et al. 2008). Firstly, sequence reads were broken into shorter reads (K-mer), followed by extension of K-mers to contigs. Secondly, components were assembled to search the pair-end overlaps, and then De Bruijin maps

were generated to acquire transcripts (Grabherr et al. 2011). Finally, unigenes were generated from the transcripts.

Unigene annotation was performed by BLAST with an E-value $< 10^{-5}$ (Altschul et al. 1997). Five databases were used to annotate unigenes, including the National Center for Biotechnology Information (NCBI), non-redundant protein (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and Cluster of Orthologous Groups of proteins (COG).

2.4 Expression calculation and identification of differentially expressed unigenes

Unigene expression was calculated using expectation–maximization (RSEM) software (Li and Dewey 2011). RPKMs (reads per kilobases per million reads) were used to evaluate the expression of the unigenes (Li and Dewey 2011). Differentially expressed genes (DEGs) were identified using the Benjamini–Hochberg method (Benjamini and Hochberg 1995) with a false discovery rate (FDR) of < 0.01 and a fold change > 2 (Hu et al. 2010). The identified DEGs were annotated and enrichment analysis was performed with topGO (Alexa and Rahnenfuhrer 2007) and KEGG terms (Kanehisa and Goto 2000).

2.5 Real-time quantitative PCR

Real-time quantitative PCR was performed to verify the DEG results obtained by RNA-Seq. A total of 2 µg of RNA were extracted from the CK and T groups after quality and integrity testing. The PrimeScript RT Kit (Takara, China) was used to synthesize the first strand cDNA. The tests were performed using the Applied Biosystems 7500 Real Time PCR system with a 20-µl reaction volume containing 1 µl template, 300 mM each primer, and 10 µl SYBR Premix Ex Taq II (Takara, China). The reaction conditions were 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 34 s at 60 °C. Three replicates were performed for each experiment and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with 18 s ribosomal RNA as the reference gene.

3 Results

3.1 Sequence assembly

A total of 13.54 Gb clean data were obtained after data cleaning and quality checks. The control (CK) and low temperature treatment groups (T) yielded 34,971,435 and 32,734,410 clean reads, respectively. The Q30 values exceeded 89% for all samples (Table 1).

To increase the depth of sequencing, CK and T were combined for assembly by Trinity. A total of 376,073,043 bp of clean reads were assembled into 8,120,672 contigs (Table 2). Then, components were constructed using the paired-end overlaps of the contigs, and 238,436 transcripts (N50 = 1336 bp; mean length = 923.41 bp) were assembled by De Bruijn mapping. Finally, a total of 71,917 high-quality unigene sequences (N50 = 1181 bp; mean length = 694.49 bp) were obtained (Table 2). Thus, high quality clean data were provided for further analysis.

Table 1 Summary data of clean reads in the CK and T libraries

	CK	T
Read number	34,971,435	32,734,410
Base number (bp)	6,993,313,151	6,545,636,632
GC content (%)	43.53	43.18
≥ Q30 (%)[z]	89.81	89.62

[z] ≥ Q30 indicates percentage of clean reads with sequencing accuracy over 99.9%

Table 2 Summary data of combinate sequence assembly of the CK and T libraries

Statistics	Contig	Transcript	Unigene
Total number	8,120,672	238,436	71,917
Total length (bp)	376,073,043	220,175,111	49,945,972
N50 length (bp)	47	1336	1181
Mean length (bp)	46.31	923.41	694.49
Annotation counts			33,282
Annotation ratio (%)			46.27

3.2 Gene annotation

The Nr protein, Swiss-Prot, COG, GO, and KEGG databases were used to annotate 33,282 unigenes (46.27%) out of 71,917 unigenes with an E-value threshold of < le-5, indicating that 53.73% (38,936 unigenes) had no annotation in any of the databases due to the lack of genomic and transcriptomic information on chrysanthemum (Table 2).

GO provides three ontologies (cellular components, biological processes, and molecular functions) to analyze genes (Conesa et al. 2005). Based on the blast results, 24,252 unigenes were annotated and covered 56 GO functional categories. Figure 1 shows that the 'cell part' (14,984 unigenes) term in cellular components, the 'catalytic activity' (12,594 unigenes) term in molecular functions, and the 'metabolic process' (16,249 unigenes) term in biological processes were the most dominant for the three ontologies. A high number of unigenes belonging to 'cell' (14,812 unigenes), 'organelle' (12,638 unigenes), 'binding' (11,555 unigenes), and 'cellular process' (15,085 unigenes) were also identified within the sequencing

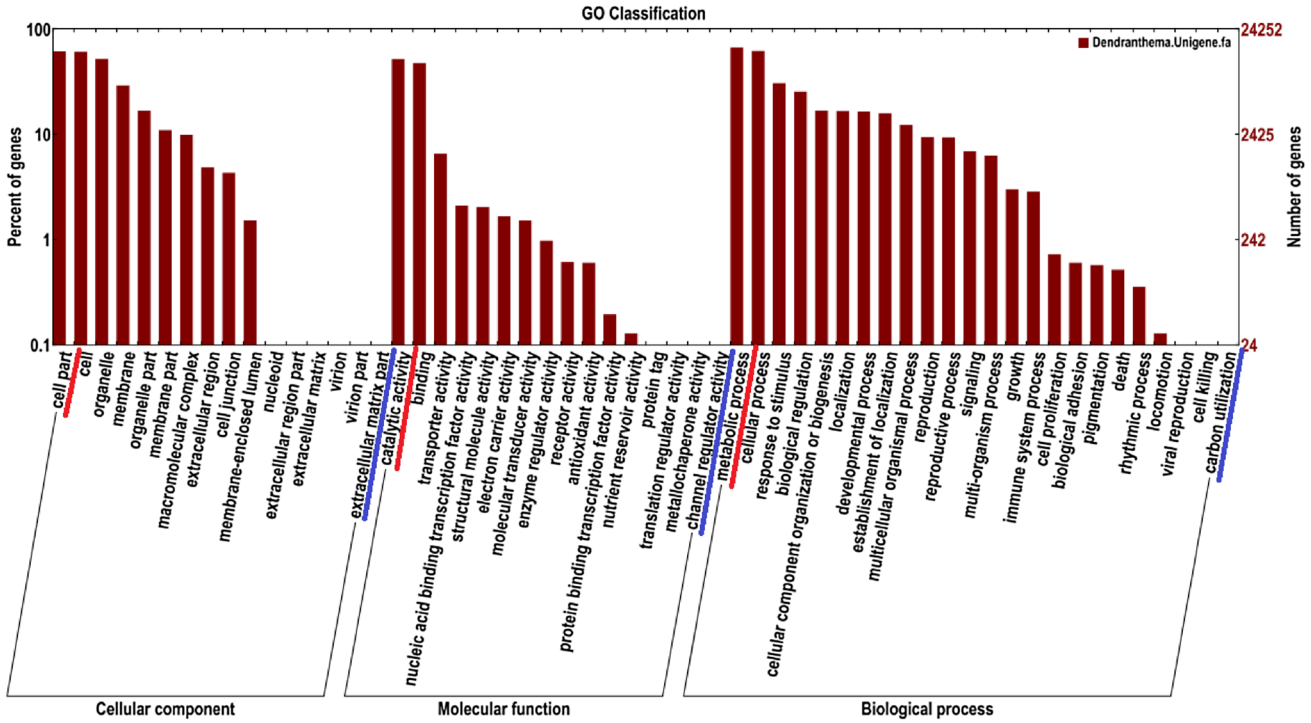

Fig. 1 Map of GO functional categories. Red lines indicate the most dominant category in the ontologies. Blue lines indicate the least dominant category in the ontologies. (Color figure online)

results. In addition, A low number of unigenes from the 'extracellular matrix part' (6 unigenes), 'channel regulator activity' (1 unigenes), and 'carbon utilization' (5 unigenes) categories were identified in each of the three GO ontologies.

COG provides a method to search genes by eukaryotic COG classification or ID, which was useful to identify homologous proteins. Based on the results, 9579 unigenes were annotated covering 25 KOG categories (Fig. 2). The most dominant categories were 'general function prediction only' (2664, 27.81%) followed by 'signal transduction mechanisms' (1259, 13.14%), 'transcription' (1433, 14.96%), and 'replication, recombination and repair' (1569, 16.38%). The least dominant categories were 'extracellular structures' (0, 0%), 'nuclear structure' (4, 0%), and 'cell motility' (13, 0.14%).

KEGG allows the identification of potential pathways associated with the annotated genes in a network. A total of 7123 unigenes were annotated in the database covering 116 pathways. The dominant pathways were 'metabolic pathway' (1925 unigenes) and 'biosynthesis of secondary metabolites' (845 unigenes). In addition, 277 unigenes were mapped to 'plant hormone signal transduction', 68 unigenes to 'phosphatidylinositol signaling system', 55 unigenes to 'biosynthesis of unsaturated fatty acids', and 22 unigenes to 'circadian rhythm-plant'.

3.3 Comparison of transcriptomes between the control group (CK) and the cold treated group (T) in chrysanthemum

A search for DEGs from the database with the proper threshold value (FDR < 0.01 and FC ≥ 2) identified 2310 unigenes, with 1592 upregulated and 718 downregulated genes between CK and T. Similarly, the GO, COG, and KEGG databases were used to annotate DEGs, in which we identified 1833 unigenes, indicating that 477 DEGs (20%) had no match in any of these databases.

The GO annotation showed that 1354 DEGs were enriched in 56 groups as follows: 16 in the 'cellular component' group, 16 in the 'molecular function' group, and 24 in the 'biological process' category. Figure 3 shows the functional categories of DEGs compared to those in all annotated unigenes. A greater difference between DEGs and all annotated unigenes was associated with a higher probability of the categories identified. In the 'metabolic process' category, the most significantly different terms were 'structural molecule activity', 'nutrient reservoir activity', and 'nucleic acid binding transcription factor activity'. In the 'biological process' category, the most significantly different terms were 'cell killing' and 'biological adhesion'. Finally, in the 'cellular component' category the most significantly different terms were

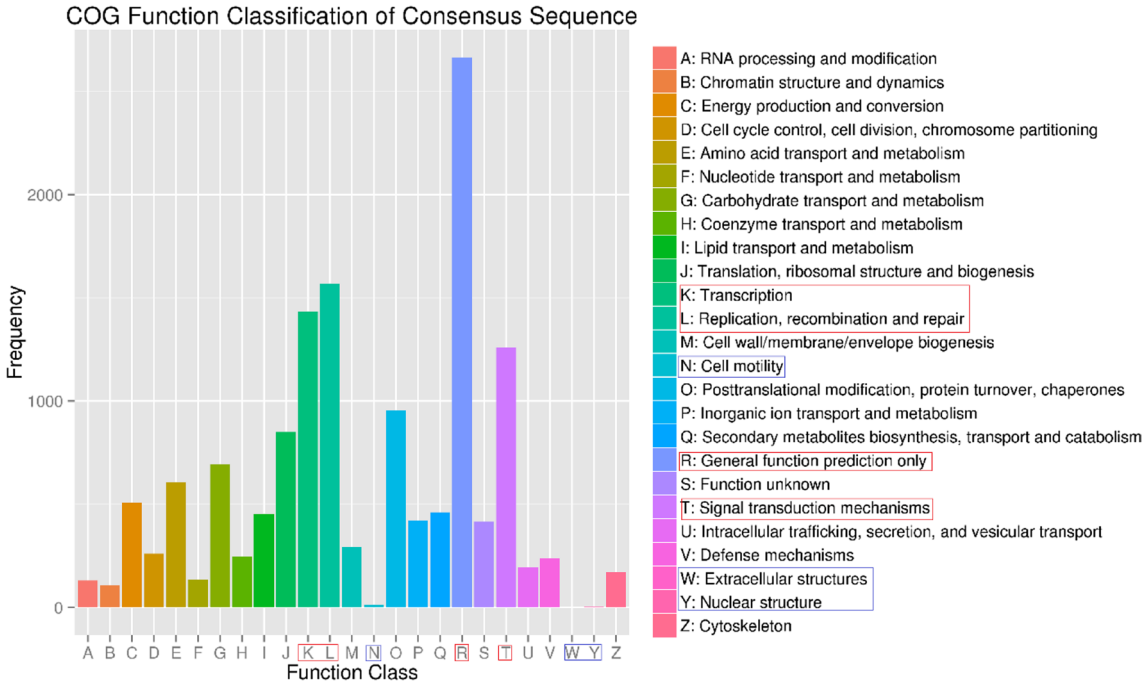

Fig. 2 Map of Cog function classifications. Red rectangles indicate high frequency categories. Blue rectangles indicate low frequency categories. (Color figure online)

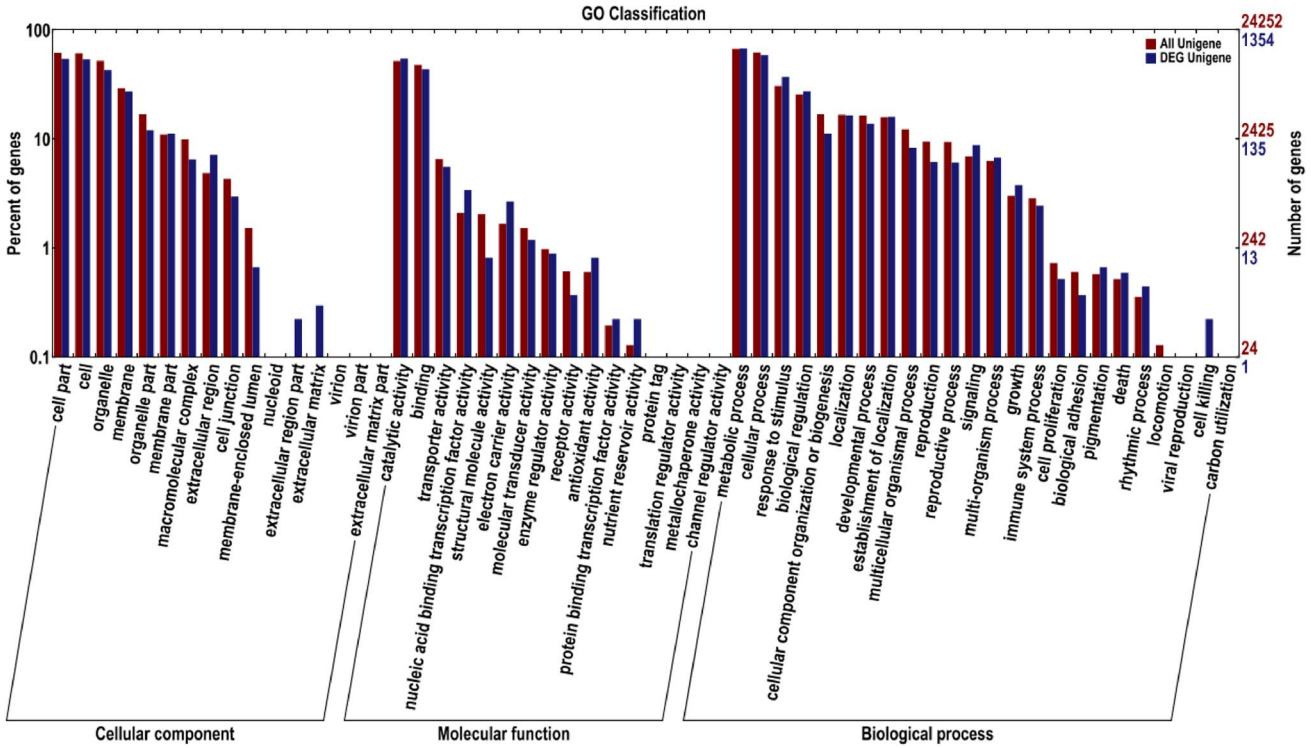

Fig. 3 Map of GO functional categories of DEGs. Red cylinders indicate the unigene number in GO classification of all annotated unigenes. Blue cylinders indicate unigene number in GO classification of DEGs. (Color figure online)

Table 3 Top10 significant terms in GO enrichment analysis of total unigenes

ID	Term	Annotated	Significant	Expected	KS
GO:0045548	Phenylalanine ammonia-lyase activity	13	12	0.73	7.7e−10
GO:0003700	Sequence-specific DNA binding transcript	507	46	28.46	4.3e−08
GO:0003735	Structural constituent of ribosome	352	2	19.76	5.2e−08
GO:0016168	Chlorophyll binding	37	18	2.08	2.5e−07
GO:0016717	Oxidoreductase activity	39	11	2.19	1.3e−06
GO:0003843	1,3-beta-D-glucan synthase activity	60	1	3.37	4.0e−05
GO:0005515	Protein binding	2435	134	136.7	5.6e−05
GO:0016303	1-phosphatidylinositol-3-kinase activity	13	0	0.73	0.00012
GO:0046983	protein dimerization activity	358	27	20.1	0.00028
GO:0016760	cellulose synthase activity	69	10	3.87	0.00039

Significant indicates the number of annotated DEGs. Expected indicates the expected number of DEGs. KS indicates Kolmogorov–Smirnov test value, which shows the significance of the GO term. Lower KS values indicate higher significance

'membrane-enclosed lumen', 'extracellular region part', and 'extracellular matrix'. Enrichment analysis of DEGs annotated on GO was performed by topGO. Ten terms were selected as the top10 categories with significant enrichment on GO (Table 3).

KEGG pathway analysis showed that 336 out of 1836 DEGs were assigned to 50 pathways. Figure 4 shows the dominant pathways, which were as follows: plant hormone signal transduction (28), phenylpropanoid biosynthesis (23), photosynthesis-antenna proteins (21), plant-pathogen

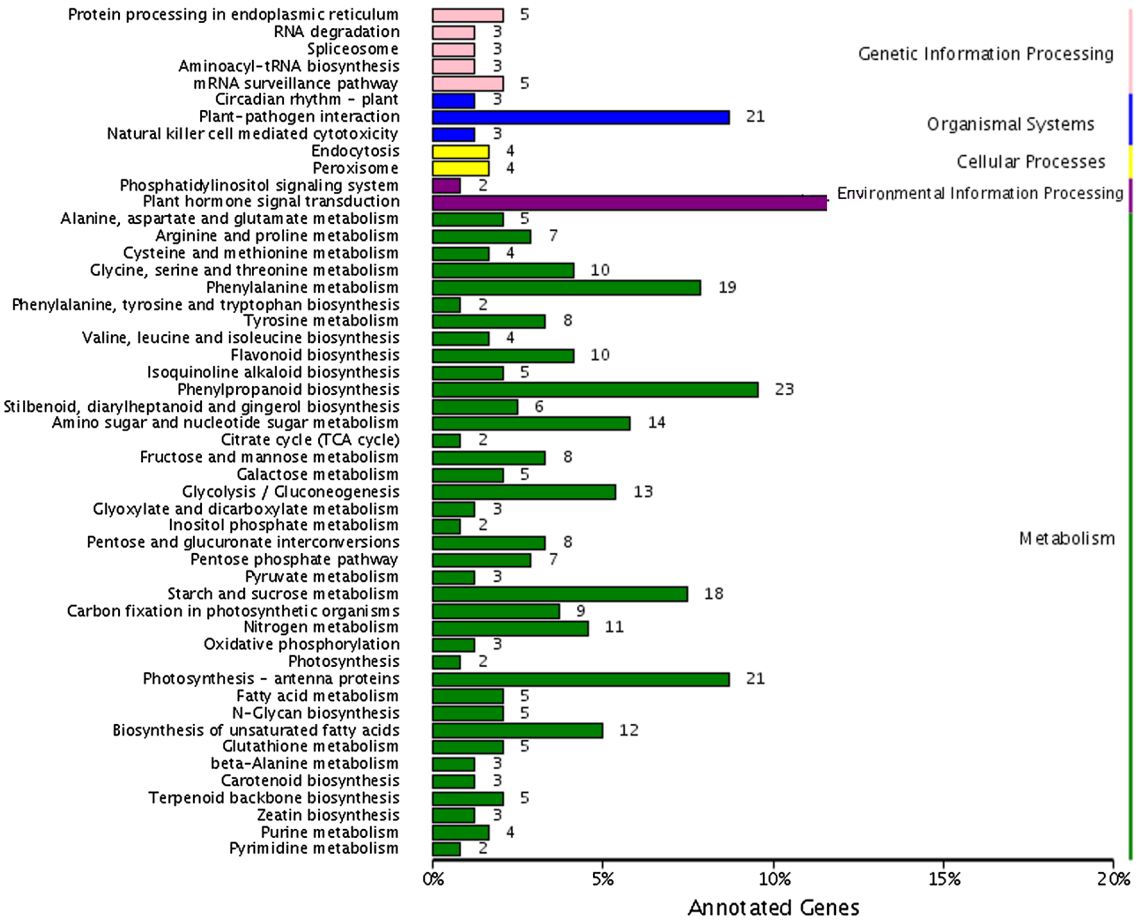

Fig. 4 Unigene numbers in KEGG pathways of DEGs

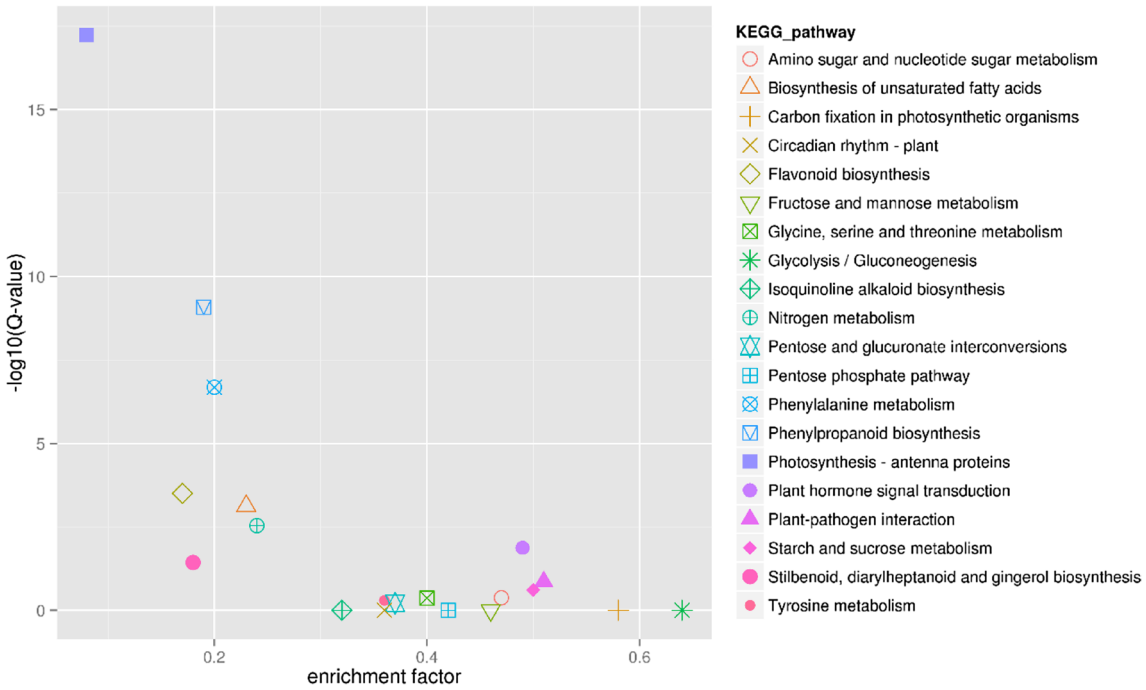

Fig. 5 KEGG enrichment analysis of DEGs. Symbols indicate KEGG pathways. The x-axis indicates the value of the enrichment factor, and the y-axis indicates its significance level

interaction (21), phenylalanine metabolism (19), and starch and sucrose metabolism (18). Enrichment analysis of KEGG pathways was performed to search for significant pathways. The top 20 significant pathways are shown in Fig. 5, including photosysthesis-antenna proteins, phenylpropanoid biosynthesis, phenylalanine metabolism, flavonoid biosynthesis, biosynthesis of unsaturated fatty acids, nitrogen metabolism, plant hormone signal transduction, and stilbenoid, diarylheptanoid and gingerol biosynthesis. These findings indicated that a diverse range of metabolic processes were activated by low temperature treatment in chrysanthemum.

3.4 Identification of genes involved in cold tolerance

Genes with homology to key regulators involved in cold signal transduction in chrysanthemum were searched. In *Arabidopsis*, many cold signal transduction genes were identified using molecular-genetic approaches. Some of the identified genes were involved in Ca^{2+} signaling pathways and the mitogen-activated protein kinase (*MAPK*) cascade, while others encoded important transcription factors. Genes involved in the biosynthesis of unsaturated fatty acids including calcium exchangers (*CCX*), calmodulin-like proteins (*CML*), calcium-dependent protein kinases (*CDPK*), *CBL*-interaction protein kinase (*CIPK*), *bHLH*, *MYB*, and *CBF* were also identified up or down regulated in the cold treatment. Based on the protein annotations of the chrysanthemum transcriptome sequences, a number of genes were identified up or down regulated in the cold treatment, such as homologs of the CBL-interacting protein (*CIPK*) (6), *CDPK* (6), *CCX* (4), calcium-binding proteins (*CBP*) (5), calmodulin-like protein (*CML*) (5), *MAPK* (4), *WRKY* (6) (transcription factor), *MYB* (15), *AP2/ERF* (6), *bHLH* (11), *TCP* (2), Dehydration-responsive element-binding protein *DRBP* (3), and Cys2/His2-type Zinc finger protein (*ZAT*) (7) (Table 4). We also found 11 genes that were similar to genes related to biosynthesis of unsaturated fatty acids.

3.5 Gene expression verified by quantitative RT-PCR

Twenty genes were selected randomly for quantitative RT-PCR to confirm the gene expression profiles from the transcriptomes. These selected genes were all annotated in databases; however, not all genes had detailed predicted gene functions. For the unpredicted genes, we listed Genebank IDs of the most similar genes from other species. The predicted gene functions of these 20 genes are list in Table 5. The templates used for qRT-PCR were those originally used for RNA-Seq. The expression patterns of the 20 genes by RT-PCR were consistent with the profiles by RNA-Seq (Fig. 6).

Table 4 Identified genes involved in cold signal transduction in chrysanthemum leaves

Gene name	Gene ID	CK	T	FDR	log2FC	Regulated
CBL-interacting serine/threonine-protein kinase	c63432	13.76663	32.21735	2.34E−06	1.206674	Up
	c61711	43.3971	202.1011	0	2.200553	Up
	c58475	14.0837	28.65586	0.001099	1.002851	Up
	c55322	56.64816	120.7666	6.29E−05	1.073344	Up
	c55969	10.1276	38.68723	9.99E−16	1.909755	Up
	c52208	4.313614	14.27173	2.05E−10	1.697374	Up
Calcium-dependent protein kinase	c51635	1.40332	5.771268	5.37E−06	1.955841	Up
	c44562	1.161654	4.846047	0.001712	1.923553	Up
	c26187	0.591893	3.263443	1.26E−05	2.306189	Up
	c61916	55.4027	163.2683	2.21E−11	1.540676	Up
	c51894	5.728129	12.58725	0.006816	1.102743	Up
	c60274	2.611269	7.827384	1.47E−05	1.539912	Up
Cation/calcium exchanger	c63334	2.52971	21.03592	0	3.023995	Up
	c57983	4.837151	10.64925	0.000351	1.113571	Up
	c60491	3.791195	16.09581	2.56E−12	2.044478	Up
	c57983	4.464735	11.76755	1.28E−05	1.366858	Up
Probable calcium-binding protein CML	c38992	14.43573	4.494287	9.24E−05	− 1.65704	Down
	c55220	5.640839	12.98063	0.000143	1.176195	Up
	c44460	8.699992	3.273828	0.000264	− 1.40324	Down
	c54316	111.9937	226.9992	0.000459	1.000805	Up
	c58296	473.3442	213.2417	9.11E−06	− 1.16804	Down
Calmodulin-like protein	c51641	288.3391	647.8403	1.52E−05	1.149715	Up
	c52273	57.68313	17.80769	5.95E−13	− 1.71015	Down
	c49042	67.95234	265.2707	0	1.945779	Up
	c62919	14.92551	5.254864	3.80E−09	− 1.51804	Down
	c39396	19.90098	9.445356	0.00012	− 1.08986	Down
Calcium-transporting ATPase	c65177	36.86372	134.317	2.22E−16	1.84689	Up
Mitogen-activated protein kinase kinase kinase	c49604	4.069076	13.2404	3.94E−05	1.644479	Up
	c31942	0	3.403377	4.84E−05	4.219099	Up
	c62108	5.738225	15.97319	8.48E−09	1.454167	Up
	c53182	3.495905	10.617	0.001098	1.537564	Up
Probable WRKY transcription factor	c52480	104.3938	40.75208	1.27E−08	− 1.37403	Down
	c58397	54.12658	11.88581	0	− 2.20043	Down
	c57197	2.232526	7.147268	6.82E−07	1.636973	Up
	c63465	6.823049	16.28116	0.000113	1.226342	Up
	c59653	185.3657	428.5626	4.83E−06	1.190989	Up
	c59212	20.61799	53.73666	1.21E−08	1.362965	Up
Transcription factor MYB	c53832	15.06306	37.36231	4.91E−07	1.289214	Up
	c48650	6.576691	41.19296	0	2.618965	Up
	c63779	70.44154	150.1849	7.47E−05	1.073833	Up
	c55198	2.287005	12.20035	1.19E−14	2.361848	Up
	c57351	16.76411	4.602801	2.44E−12	− 1.87051	Down
	c59545	105.935	51.15486	8.95E−05	− 1.06721	Down
	c56701	0	16.96712	0	8.385638	Up
	c59305	26.42586	58.26775	1.74E−05	1.121684	Up
	c54220	38.23156	80.20519	0.000114	1.050025	Up
	c54539	30.60546	63.46552	0.000176	1.033099	Up

Table 4 (continued)

Gene name	Gene ID	CK	T	FDR	log2FC	Regulated
	c571470	9.08618	21.02093	5.05E−05	1.185612	Up
	c59539	116.4487	287.7996	1.94E−07	1.287141	Up
	c50800	15.85583	45.42817	3.49E−10	1.497955	Up
	c46787	3.567712	9.480694	3.81E−05	1.375301	Up
	c64804	6.8953	2.044231	2.52E−09	− 1.75455	Down
Fatty-acid-binding protein	c60515	2.339427	7.352033	1.81E−07	1.61523	Up
Ethylene-responsive transcription factor (ERF/AP2/WIN)	c61590	39.97382	141.5776	6.66E−16	1.805265	Up
	c52169	107.7588	51.73651	7.98E−05	− 1.07593	Down
	c41878	0.730754	6.001132	1.83E−06	2.774511	Up
	c50643	14.14605	63.92343	0	2.153296	Up
	c52676	0.343537	8.729774	0	4.378462	Up
	c60784	7.86758	51.56781	0	2.674784	Up
Transcription factor bHLH	c61769	16.38288	121.2179	0	2.867035	Up
	c59580	29.35377	84.86024	2.44E−10	1.510811	Up
	c60263	36.90733	17.04744	3.01E−05	− 1.12982	Down
	c56328	29.5017	12.75528	2.41E−06	− 1.22512	Down
	c53028	15.95211	35.00255	2.68E−05	1.113955	Up
	c59542	25.05551	11.09737	7.60E−06	− 1.19	Down
	c59580	36.11793	105.3134	4.20E−11	1.524944	Up
	c57469	2.659418	10.40929	4.16E−12	1.933256	Up
	c62315	5.016267	25.90306	0	2.337799	Up
	c54041	16.55862	40.22545	5.65E−07	1.260293	Up
	c61093	7.051358	23.45796	1.68E−12	1.710785	Up
Transcription factor TCP	c52476	12.92675	30.60397	1.99E−06	1.222839	Up
	c52476	12.37062	26.2322	0.000888	1.060077	Up
Dehydration-responsive element-binding protein	c62475	44.22347	571.9827	0	3.672833	Up
	c58519	0.748243	5.796939	0	2.869715	Up
Cys2/His2-type Zinc finger protein ZAT	c56171	84.81689	32.27094	6.81E−09	− 1.41002	Down
	c56171	84.81689	32.27094	6.81E−09	− 1.41002	Down
	c50673	23.6516	11.2146	0.007145	− 1.08108	Down
	c50191	15.55314	3.983077	3.24E−12	− 1.9654	Down
	c49680	2.203011	8.176453	2.28E−09	1.84951	Up
	c50673	34.36097	13.87006	9.18E−06	− 1.31742	Down
	c44381	0.114049	1.608127	0.001241	3.060951	Up
FAD protein	c46034	7.413476	19.0443	0.007727	1.309181601	Up
	c49792	8.743942	19.68185	0.000127	1.146113543	Up
	c56988	6.025968	21.36764	0.000104	1.75014536	Up
	c53862	67.54629	27.56381	1.03E−07	− 1.30982324	Down
	c61368	16.12848	42.12597	2.55E−08	1.364687003	Up
Delta-9 fatty acid desaturase	c54638	15.47782	4.940894	2.47E−08	− 1.649551748	Down
Omega-6 fatty acid desaturase	c58735	15.77525	34.26371	6.91E−05	1.098143516	Up
	c50602	17.32329	38.37128	0.000304	1.122241689	Up
Acyl-CoA oxidase	c60103	35.71545	72.623	0.00041	1.005415865	Up
Delta12-fatty acid acetylenase	c64350	19.98595	48.64759	4.03E−07	1.263563177	Up

CK indicates control groups; T indicates cold-treated group; FDR indicates a false discovery rate. log2FC indicates log2 Fold Changes. Fold Changes indicates the fold changes of expression of DEGs between CK groups and T groups

Table 5 Prediction functions of 20 selected genes

Number	Gene name	Prediction function
1	C56381	Uncharacterized protein TCM_041386 [*Theobroma cacao*]
2	C52380	MADS-box transcription factor 1 [*Triticum urartu*]
3	C51527	Predicted beta-1,4-mannosylglyco protein [*Populus trichocarpa*]
4	C43479	Uncharacterized protein LOC101292566 [*Fragaria vesca*]
5	C41206	Gty37 protein [*Gerbera hybrid cultivar*]
6	C47152	U-box domain-containing protein 10 [*Vitis vinifera*]
7	C52917	Predicted coatomer protein [*Populus trichocarpa*]
8	C55972	Heme-binding-like protein [*Arabidopsis thaliana*]
9	C35064	Proline-rich protein 4 [*Vitis vinifera*]
10	C60323	Flavine-containing monoxygenase [*Populus trichocarpa*]
11	C38992	Predicted calcium-binding protein CML31-like [*Vitis vinifera*]
12	C48315	Protein phosphatase 2C [*Ilex paraguariensis*]
13	C46077	Predicted chlorophyll a–b binding protein CP26 [*Vitis vinifera*]
14	C50422	Chlorophyll a–b binding protein [*Petunia hybrida*]
15	C50831	Predicted plasma membrane-associated cation-binding protein 1-like [Fragaria vesca subsp. vesca]
16	C55180	Predicted glutamate–glyoxylate aminotransferase 2-like [*Cucumis sativus*]
17	C55555	Protein BPS1 [*Populus trichocarpa*]
18	C52480	Predicted WRKY4 [*Panax quinquefolius*]
19	C61494	Plastid-specific 30S ribosomal protein 1 [*Theobroma cacao*]
20	C58396	Predicted UDP-glucose 4-epimerase protein [*Populus trichocarpa*]

4 Discussion

There is a paucity of research on the molecular mechanisms of cold tolerance in chrysanthemum. Our present findings improve our understanding of the biochemical pathways involved in physiological processes of cold tolerance. In the present study, approximately 67 million reads were obtained and 33,282 out of 71,917 unigenes were annotated against public databases, indicating that 38,635 unigenes (53.7%) had no current annotations. The assembled unigenes in this study were assigned to an extensive range of KEGG pathways, which indicated that various transcripts are involved in the response to low temperature in chrysanthemum.

Comparative analysis of the transcriptomes provided valuable information on candidate genes involved in cold tolerance. There were 2310 DEGs, 1836 of which were annotated. Many DEGs were associated with cold signal transduction (Table 4). The function of Ca^{2+} as a messenger in stress signaling is mediated by three main proteins: CDPKs, CaMs, and CBLs (Miura and Furumoto 2013; Reddy et al. 2011). In *Arabidopsis*, overexpression of Calmodulin (CaM) downregulates the expression of cold-responsive genes (Townley and Knight 2002). *CDPKs* are plant-specific genes that function as important detectors in cold signal transduction (Chinnusamy et al. 2004; Maria and Grazyna 2007). In *Camellla sinensis*, calmodulin and *CDPK* genes are involved in cold signal transduction, similar to the *CBL* gene (Wang et al. 2013). *MAPK*, which is upregulated in response to cold in *Arabidopsis* (Ichimura et al. 2000), is regulated by different transcription factors (Teige et al. 2004; Viswanathan and Jian-Kang 2002; Zhu et al. 2007). In the present study, homologs of *CIPK* (6), *CDPK* (6), *CCX* (4), *CBP* (5), (*CML*) (5), and *MAPK* (4) were identified among the DEGs as significantly up- or down-regulated genes in response to the cold treatment in chrysanthemum. We speculate that these genes may play similar roles in cold signal transduction in chrysanthemum as their homologs in *Arabidopsis* and potentially other species.

Important transcription factors that have roles in the response to cold stress were also found in the DEGs identified in our experimental treatment group. In *Arabidopsis*, *CBF/DREB* controls *COR* in response to cold stress (Chen et al. 2008; Stockinger et al. 1997). *ICE1*, which encodes a bHLH protein that binds specifically to *MYC* in the *CBF/DREB* promoter region and upregulates *DREB1a/CBF3*. This upregulation enhances tolerance to chilling and freezing (Chen et al. 2008). The transcription factor *Zat12* interacts with 24 *COS* genes, of which 15 are cold-repressed genes and nine are cold-induced genes (Chinnusamy et al. 2007; Vogel et al. 2005). We identified homologs of the *CBF/DREB* (6), *bHLH* (11), *MYC* (15), and *Zat12* (7) transcription factors in chrysanthemum that had significant transcriptional changes in treatment sample. We speculate that

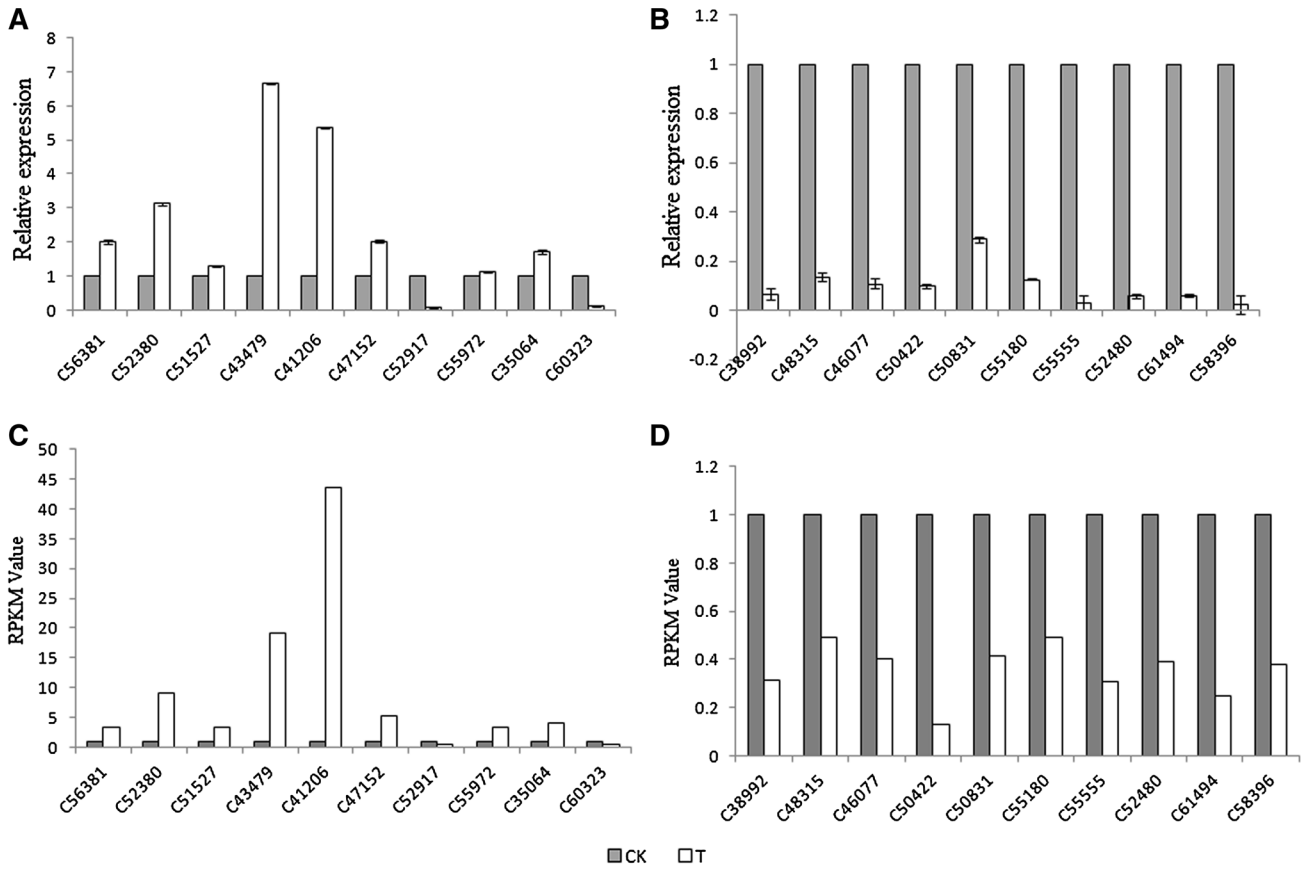

Fig. 6 Expression level of 20 selected genes identified from our analysis of cold tolerance in chrysanthemum. **a** and **b** show the relative expression level of 20 selected genes assessed by RT-PCR. **c** and **d** show the RPKM value of control (CK) and cold temperature (T) treatment groups. The RPKM value had been calculated as log2 and normalized the expression data to the control values

these genes may be involved in cold signal transduction in chrysanthemum.

Since Lyons proposed the hypothesis for the membrane phase transition of cold injury (Lyons and Raison 1971), several studies showed that the proportion and content of unstaurated fatty acids in membrane lipids are closely related to cold tolerance of plants (And and Murata 1996; Murata and Los 1997; Somerville 1995). Due to the activity of fatty acid desaturase, double bonds are introduced into saturated fatty acids in plants and become unsaturated fatty acids. Here, RNA-Seq was used to compare genes related to unsaturated fatty acids in different transcriptomes subjected to subzero and normal growing temperatures. The DEGs related to the biosynthesis of unsaturated fatty acids are shown in Table 4. Most of the associated unigenes were upregulated, which indicated that low temperature could enhance the expression of these unigenes. Only two unigenes were downregulated, *FAD2-7* and delta-9 fatty acid desaturase. Delta-9 fatty acid desaturase is a soluble enzyme that catalyzes the desaturation of stearoyl-ACP, which is converted to glyceride or polyunsaturated fatty acids by the action of other enzymes (Thompson et al. 1991). In extremely cold temperatures, the desaturation products of stearic acid may be mass synthesized and lead to the negative feedback of delta-9 fatty acid desaturase, resulting in the downregulation of the unigene. Further research is required to explain the change in *FAD2-7* unigene transcription. Most DEGs belonged to the *FAD2* category, indicating that the *FAD2* gene was abundantly expressed.

Furthermore, log2FC indicates the logarithm of the expression difference between the two group, which is reflected in the change in scale. The ω-6 fatty acid desaturase showed the greatest change in expression, suggesting that it is involved in the biosynthesis of unsaturated fatty acids under conditions of low temperature. Many unigenes (20%) encoding unknown proteins were identified, which provides valuable information. Certain unknown unigenes were significantly upregulated in the cold-treated group, suggesting that they are involved in regulating the response of chrysanthemum to cold stress. Further studies should focus on the expression of these unknown proteins in response to cold stress in chrysanthemum.

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