

Comparative transcriptome analysis of two *Gerbera hybrida* cultivars revealed cold tolerance mechanism

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Abstract

Temperature is one of central factors that shape the distribution and growth pattern of *Gerbera hybrida*. The cultivars of this species have vast variation in the morphology and stress adaptation. In the current study, comparative transcriptome analysis of two haploid cultivars with discriminative phenotype, i.e., *Gerbera hybrida Hongjixing* and *Gerbera hybrida Qiuri*, was used to uncover differentially expressed functional unigenes using an Illumina Hiseq2000 sequencing platform. Through high-throughput cDNA sequencing (RNA-seq), we generated 99160 UniGenes from 19.09G primary reads with an average length of 978 bp and an N50 equaling 1620. It was filtered by Trimmomatic and assembled by Trinity. A total of 58127 transcripts were functional annotated by mapping to five protein databases by BLASTX, with topmost differently expressed unigenes (DEGs) directed to transcriptional regulation, secondary metabolism and stress response, etc. Cold treatment (-2°C) of haploid seedlings in intelligent cooled incubator had adverse effects on both cultivars, which was more serious in *Qiuri* which was exhibited by the lower chlorophyll fluorescence after cold treatment, compared to *Hongjixing*. This indicates the better cold tolerance of *Hongjixing*. The qRT-PCR result indicated that besides common pathways, novel pathways like latex biosynthesis potentially contributes to the better cold tolerance of *Hongjixing*. In view of the ubiquity of cold temperature, this study provided new insights into the mechanism dissection and guidance for cold stress condition.

Keywords: Gerbera; cold stress; transcriptome; interspecies.

Abbreviations: RNA-seq_high-throughput cDNA sequencing, DEGs_differently expressed unigenes, qRT-PCR_quantitative reverse transcription-PCR.

Introduction

Gerbera hybrida, a well-known herb of family Asteraceae is famous for its medicinal as well as ornamental values (Krips et al., 2001). This herb has a rich biodiversity with specialized structures such as elaborate inflorescence, fused petals and aborted margins (Laitinen et al., 2005; Teeri et al., 2006) and strong adaptability to the environment (Bu et al., 2015). It is ranked in the top five global cut and pot flower market, together with rose, carnation, chrysanthemum and tulip (Gong and Deng, 2010).

Gerbera hybrida have been used in flower organ differentiation (Laitinen et al., 2005; Meng and Wang, 2004; Zhang et al., 2017), phytohormone regulation (Huang et al., 2017; Li et al., 2015), secondary metabolites of flavor (Deng et al., 2014) and tissue culture (Murashige et al., 1974). However, it has gained little attention with relation to stress tolerance, which could aid in identifying key functional genes and optimizing yield outputs in different environments. *Gerbera hybrida* is mainly planted in greenhouses. A number of modern urbanization attempts have been made to

cultivate this plant in the outfield but it remained a big challenge due to low temperature during winter nights. Our field experience found that different cultivars of *Gerbera hybrida* show large variation in their biotic and/or environmental responses, i.e., some cultivars show growth retardation under low temperature, while others could keep flowering even below 0°C. For example, *Gerbera hybrida Hongjixing* could tolerate the extreme cold temperature of -4°C due to its genetic potential.

RNA-seq is a comprehensive approach to track transcriptome profiling (Wang et al., 2009), which has been widely used to reveal temporal-spatial differences of gene expression (Wang et al., 2011; Wigge et al., 2005). Transcriptome analysis of the inflorescence of *Gerbera hybrid* uncovered novel regulators of flower type (Laitinen et al., 2005). Gene sets abundantly expressed during petal organogenesis and EST-SSR markers were also revealed by transcriptional analysis (Gong and Deng, 2010; Laitinen et al., 2007).

However, no comparison has been made between intra-species so far.

The natural breeding of *Gerbera hybrida* is time-consuming and highly heterozygous because of its long life cycle and cross-pollination (Nagaraju et al., 1998). But haploid breeding with unfertilized pollen is an ideal material for commercial cultivation in *Gerbera*. In this study, two domestically cultivated *Gerbera* cultivars from Yunnan province of China, i.e., *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri, with different cold tolerance, were compared by transcriptome analysis to uncover the cold-tolerant mechanism of both cultivars. This study would provide useful guidance for future farming application practices.

Results

RNA sequencing using Illumina platform and assembly of unigenes

A total of 19.09 GB raw reads were generated through pair-end sequencing on Illumina HiSeq2000 platform, with Q20 percentage value over 97% and moderate GC content for *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri, which indicate the reliability of the quality of raw sequencing data (Table 1). After processing the raw data by Trimmomatic, clean reads were assembled *de novo* into 138,363 contigs and 92,606 unigenes for Hongjixing, 146,762 contigs and 99,813 unigenes for Qiuri. Nucleotide length is an integrated criterion to assess the sequencing and assembly success. The average length of contig count 398 bp with its N50 equaling 810 bp and that of unigene reached 791 bp with its N50 equaling 1505 bp for Hongjixing, while for Qiuri it was 781 bp and 1528 bp as regarding to contig N50 and unigene N50. Size distribution of all unigenes showed that 34.64% of total unigenes had sequence length larger than 1000 bp (Fig.S1).

Functional annotation and classification of unigenes

Function of each unigene was annotated after getting highest sequence similarity by doing BLAST to protein databases. A total of 58127 unigenes could be successfully annotated (Table 2). Nr database gave the highest percentage of annotation number. Further attention was paid to the quality of these annotation results. As shown in Fig.1A, all annotated unigene had e-value less than 10⁻⁵, which is a vendible threshold for sequence blast. E-value distributions indicated that 52.1% of the Nr annotated unigene had high similarity (E-value < 10⁻⁶⁰). The similarity distribution proved that only 9.2% of annotated unigene had similarities less than 40%, and 61.9% had similarities larger than 60% (Fig. 1B). Regarding the species distribution of annotated unigene, *Vitis vinifera* accounted for the largest proportion followed by *Lycopersicon esculentum* and *Amygdalus persica* which occupied approximately 2/3 of the species number (Fig. 1C).

GO ontology is an international standard system for gene function classification, which describes the property of gene and its product with a suit of dynamic controlled vocabulary (Ashburner et al., 2000). Based on previous annotated results, we attached information to GO analysis module in Blast2GO software (<https://www.blast2go.com/>).

Compartmentalized Go term at the macro levels includes biological process, cellular component and molecular function. From the point of category view, biological process own the largest mean percentage in annotated unigenes, while cellular process, metabolic process, cell, cell part and organelle ranked the top 5 in the sublevel classification (Fig.2).

Differential expressed gene (DEG) analysis

Among the assemble outputs, 92960 unigenes were mapped to Qiuri, and 91841 unigenes on Hongjixing, respectively, with 87.1% of the unigenes coexisted in both cultivars (Fig.3A). In order to get insight into the transcriptional pattern of identified sequence, unigene expression levels were characterized by using the reads per kilobase of exon model per million mapped reads (FPKM) algorithm. The FPKM ratio of *Gerbera hybrida* Hongjixing versus *Gerbera hybrida* Qiuri was log10 plotted in Fig.3B. Taking two-fold ratio as significant criterion, we found 19100 significant upregulated and 13931 significant downregulated unigenes in *Gerbera hybrida* Hongjixing.

To unveil the functional significance of transcriptional level, we picked top 30 upregulated unigenes of *Gerbera hybrida* Hongjixing for in-depth exploration (Fig.3C). 20 out of the top 30 conspicuous upregulated unigenes were annotated by Nr database successfully. The gi number of the 20 annotated unigene was converted to Gene ID for online KEGG analysis (<https://biobdnet-abcc.ncifcrf.gov/db/db2db.php>). The KEGG results showed that DEGs mainly participated in carbon metabolism (Unigene45349_All), nucleotide metabolism (Unigene46167_All, Unigene49221_All), secondary metabolism (Unigene48068_All, Unigene49105_All), transcription regulation (Unigene44870_All, Unigene45911_All, Unigene49221_All) and defense related response (Unigene49372_All) (Table 3). Essentially, all these unigenes could relate to multiple abiotic stress responses.

Cold tolerance assay

As cold temperature is a pivotal factor that affects the distribution of *Gerbera hybrida* (Broholm et al., 2008), we treated haploid *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri seedlings under -2 °C for 2 h in a controlled chamber, with the programme documented in (Zhang et al., 2014). Fv/Fm is a reliable indicator to reflect the photosynthetic efficiency of PSII, which is widely used in stress tolerance assays (Li et al., 2006; Zhao et al., 2005; Zhou et al., 2016). Before cold treatment, both Hongjixing and Qiuri showed normal growth, with high photosynthetic potential indicated by blue color (Fig.4A) but retarded growth was observed in both Hongjixing and Qiuri. However, Hongjixing survived better after cold stress, as reflected by high photosynthetic potential (Fig.4B). Leaf margins of Qiuri almost died and only the main stem remained alive.

Expression profile of candidate DEGs in response to cold treatment

To validate the crucial role of DEGs in response to low temperature, we carried out qRT-PCR analysis. After cold treatment, the qRT-PCR results showed that 12 out of 20

Table 1. Quality index of transcriptome sequencing and de novo assembly.

		<i>Gerbera_hybrida</i> Hongjixing	<i>Gerbera_hybrida</i> Qiuri	All
Reads	Total reads	111,868,608	113,041,480	-
	Clean reads	105,625,976	106,538,440	-
	Total clean nucleotides (nt)	9,506,337,840	9,588,459,600	-
Paired-end percentage(%)		83.81%	76.98%	-
Q20 percentage (%)		97.82	97.97	-
N percentage (%)		0	0	-
GC percentage (%)		44.83	44.55	-
Contig	Total number	138,363	146,762	-
	Mean length	398	396	-
	N50	810	781	-
Unigene	Total number	92,606	99,813	99,160
	Mean length	791	802	978
	N50	1505	1528	1620

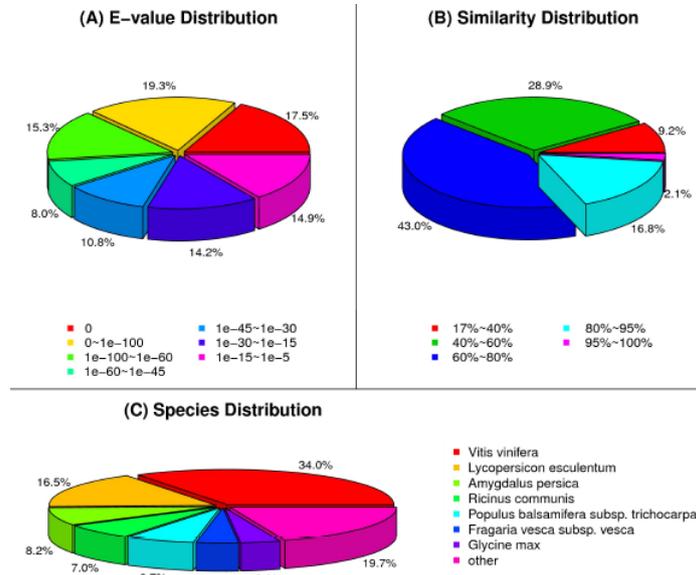


Fig 1. Annotation of unigenes according to Nr database. (A) E-value distribution according to Blast comparison annotation with Nr database. All the annotated unigenes have a significant E-value; (B) Similarity distribution according to Blast comparison annotation with Nr database, around two thirds of unigenes have a similarity higher than 60%; (C) Unigenes in our data have a best hit genes in others species according to Blast comparison annotation with Nr database, *Vitis vinifera* genome offers the most annotation.

annotated DEGs showed significant expression variation between *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri. Among them, Unigene49221, Unigene49372, Unigene50734, Unigene44870, Unigene45349, Unigene45911, Unigene46132, Unigene48047, Unigene48068, Unigene48783 and Unigene49105 of *Gerbera hybrida* Hongjixing had significant upregulation as compared to *Gerbera hybrida* Qiuri (Fig.4), which clearly reflects their potential function in cold tolerance. However, these unregulated DEGs were mainly involved in nucleotide metabolism, transcriptional regulation, secondary metabolite biosynthesis and stress response.

Discussion

Cold tolerance facilitates the planting feasibility and international influence of *Gerbera hybrida*. In our previous breeding practices, Hongjixing was selected as cultivar

candidate for abundant flowers yield and strong stress resistance (Yu, 2014). In Yunnan, cold stress became a major factor preventing the *Gerbera hybrida* for outfield planting due to the temperature difference between day and night during winter. As Hongjixing and Qiuri showed growth tendency in winter, we carried out transcriptome analysis between these cultivars. For *Gerbera hybrida*, we assembled *de novo* transcriptome sequences in Trinity which was based on raw data, because of the non-availability of referenced genome information for *Gerbera hybrida*. In total, 99,160 unigenes were assembled from approximately 105 thousand clean reads, equivalent to 92 million contigs (Table 1). The numbers of unigenes and contigs are almost two times than the ray florets transcriptome analysis of Kuang et al. (2013), which may be ascribed to the tissue-specific expression or sequencing depth. Within these unigenes, 34% were similar to *vitis vinifera* (vitaceae) and 16.5% were homologous to

Table 2. Statistics of the TOP30 unregulated DEGs of *Gerbera hybrida Hongjixing*.

gene-ID	Nr-ID	Nr-annotation	Ratio
Unigene45349	gi 255557953 ref XP_002520005.1	Thermosensitive gluconokinase, putative [<i>Ricinus communis</i>] >gi 223540769 gb EEF42329.1 Thermosensitive gluconokinase, putative [<i>Ricinus communis</i>]	10.36
Unigene46934	--	--	10.07
Unigene50753	gi 312599845 gb ADQ91848.1	late embryogenesis abundant protein group 9 protein [<i>Arachis hypogaea</i>]	9.23
Unigene48047	gi 12585428 sp O82702.1 VATG1_TOBAC	RecName: Full=V-type proton ATPase subunit G 1; Short=V-ATPase subunit G 1; AltName: Full=Vacuolar proton pump subunit G 1 >gi 3717987 emb CAA06756.1 G subunit of Vacuolar-type H ⁺ -ATPase [<i>Nicotiana tabacum</i>]	9.09
Unigene45440	gi 225461754 ref XP_002285533.1	PREDICTED: uncharacterized protein LOC100252018 [<i>Vitis vinifera</i>] >gi 302142861 emb CBI20156.3 unnamed protein product [<i>Vitis vinifera</i>]	8.99
Unigene48058	gi 295844360 gb ADG43176.1	Putative major latex-like protein [<i>Salvia miltiorrhiza</i>]	8.96
Unigene46132	gi 84874520 gb ABC68264.1	Unknown protein, partial [<i>Saussurea involucreta</i>]	8.92
Unigene46878	--	--	8.86
Unigene49372	gi 37778025 gb AAR02572.1	Resistance protein candidate RGC2 [<i>Lactuca sativa</i>]	8.78
Unigene46846	--	--	8.73
Unigene46617	--	--	8.72
Unigene44870	gi 32698524 gb AAP86602.1	Eukaryotic translation initiation factor 4E [<i>Lactuca sativa</i>]	8.67
Unigene50192	gi 225425076 ref XP_002271486.1	PREDICTED: ankyrin repeat-containing protein At3g12360-like [<i>Vitis vinifera</i>]	8.63
Unigene50625	--	--	8.61
Unigene45932	--	--	8.60
Unigene50761	--	--	8.59
Unigene3028	gi 312222661 dbj BAJ33519.1	CACTA transposable element [<i>Dahlia pinnata</i>]	8.45
Unigene49105	gi 1403040 emb CAA62682.1	Chalcone synthase [<i>Gerbera hybrida cultivar</i>]	8.39
Unigene48379	--	--	8.35
Unigene45991	gi 224110490 ref XP_002315535.1	Predicted protein [<i>Populus trichocarpa</i>] >gi 222864575 gb EEF01706.1 predicted protein [<i>Populus trichocarpa</i>]	8.33
Unigene49112	gi 226532506 ref NP_001152411.1	ATFP4 [<i>Zea mays</i>] >gi 195656017 gb ACG47476.1 ATFP4 [<i>Zea mays</i>]	8.25
Unigene48073	--	--	8.22
Unigene47007	gi 356516321 ref XP_003526844.1	PREDICTED: uncharacterized protein LOC100802446 [<i>Glycine max</i>]	8.20
Unigene50734	gi 462405507 gb EMJ10971.1	Hypothetical protein PRUPE_ppa013483mg [<i>Prunus persica</i>]	8.18
Unigene50730	gi 75223179 sp O80362.1 RK10_TOBAC	RecName: Full=50S ribosomal protein L10, chloroplastic; AltName: Full=CL10; Flags: Precursor >gi 3298441 dbj BAA31511.1 chloroplast ribosomal protein L10 [<i>Nicotiana tabacum</i>]	8.13
Unigene46167	gi 462396571 gb EMJ02370.1	Hypothetical protein PRUPE_ppa003624mg [<i>Prunus persica</i>]	8.12
Unigene49221	gi 460377394 ref XP_004234478.1	PREDICTED: DNA-directed RNA polymerases I, II, and III subunit rpabc3-like isoform 1 [<i>Solanum lycopersicum</i>] >gi 460377396 ref XP_004234479.1 PREDICTED: DNA-directed RNA polymerases I, II, and III subunit rpabc3-like isoform 2 [<i>Solanum lycopersicum</i>]	8.10
Unigene48714	gi 462405306 gb EMJ10770.1	Hypothetical protein PRUPE_ppa011099mg [<i>Prunus persica</i>]	8.03
Unigene28	--	--	8.01
Unigene48783	gi 147798869 emb CAN61630.1	Hypothetical protein VITISV_003191 [<i>Vitis vinifera</i>]	7.98

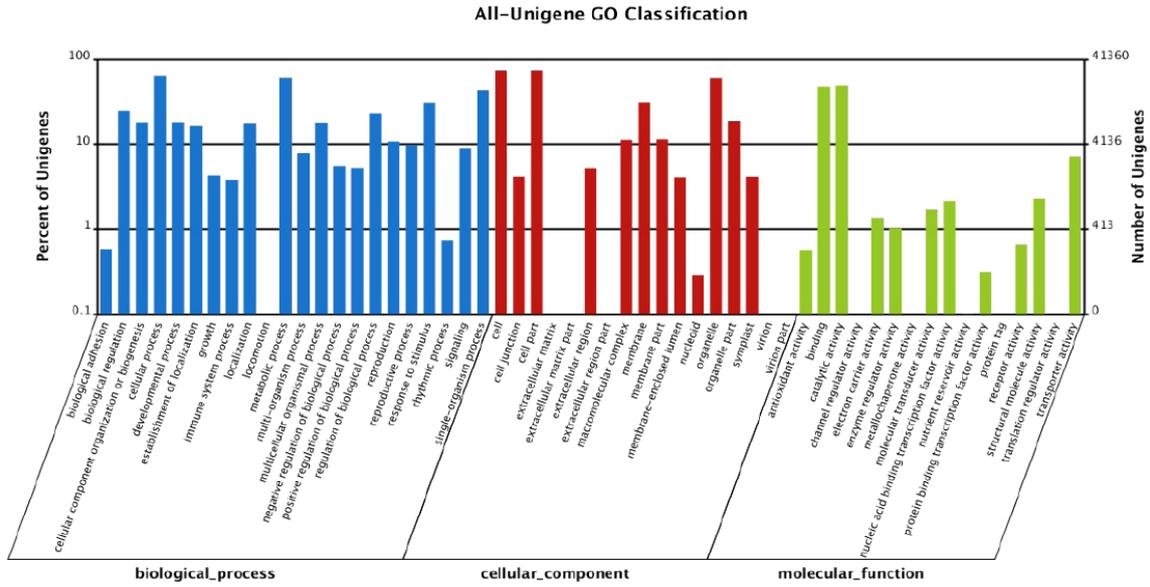


Fig 2. GO functional classification of annotated unigenes according to the transcriptome data.

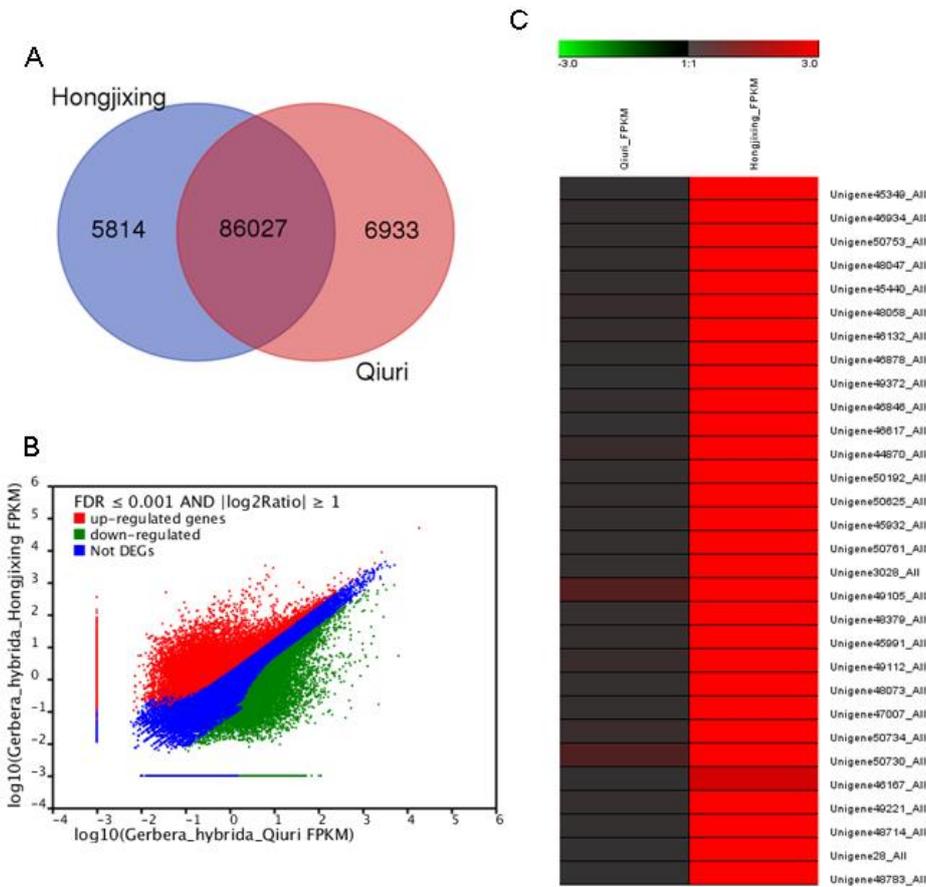


Fig 3. (A) The Venn diagram shows the number of transcripts overlap between *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri. (B) Log conversion of FPKM in both species. Red dots denote significant upregulated unigene in *Gerbera hybrida* Hongjixing, green dots mean significant downregulated unigene in *Gerbera hybrida* Qiuri, blue dots marked insignificant unigenes. (C) The heatmap for the top 30 upregulated DEGs of *Gerbera hybrida* Hongjixing.

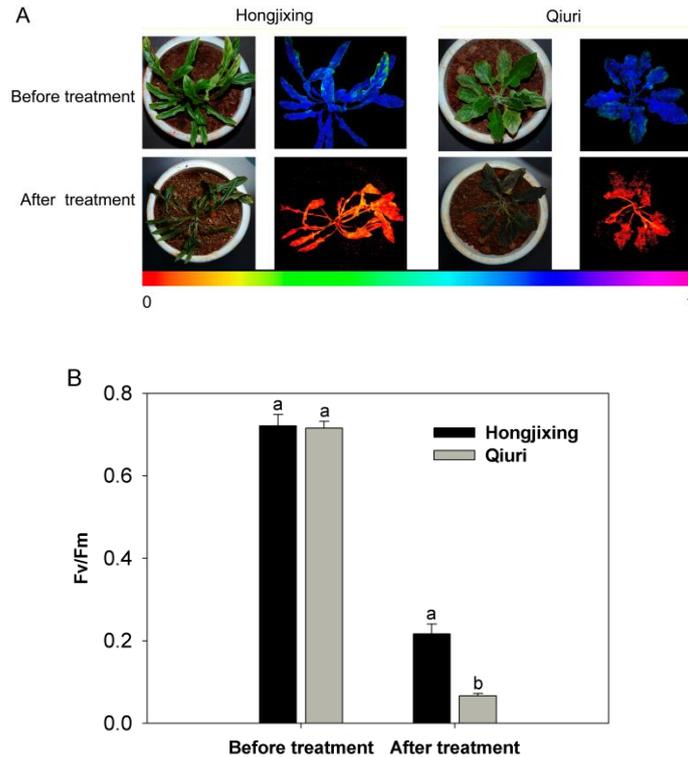


Fig 4. (A) Effect of cold treatment on the phenotype of *Hongjixing* and *Qiuri*. The second and fourth col-group pictures are taken by chlorophyll fluorescence imaging. The pseudo-color code on the bar ranged from 0 (red) to 1 (purple) indicating the maximum quantum yield of PSII. (B) Quantified statistic of Fv/Fm value before and after cold treatment. Each treatment replicated 3 times, with the bar on the column stand for SD.

lycopersicon esculentum (Solanaceae), implying potential relative phylogenetic relationship of these two species with *Gerbera hybrida*, presumably supporting the taxonomy of Basavaraju et al. (2009) and Sarkar (1993).

Comparative transcriptomics is widely used in identifying key points and functional revealing (Koenig et al., 2013; Rossouw et al., 2008), even within species (Davidson et al., 2012). Novel floral fragrance-related genes between two rose plants were revealed by Lewinsohn (2012) through genomic expression data analysis. In our study, more than 13 thousand unigenes expressed differently (\log_2 ratio >1) between *Hongjixing* and *Qiuri*, with 2427 unigenes only expressed in *Qiuri* (data not show). Considering the positive function of the candidate unigenes, we focused on the top 30 upregulated unigenes that co-expressed in both species. Apart from 10 unigenes with none functional annotation, it was presumed that remaining 20 annotated DEGs played crucial role in shaping adaptive strategies in *Hongjixing*, thus further qRT-PCR analysis was applied to validate their expression patterns in response to cold stress. The results showed that seventeen out of the twenty DEGs expressed more in *Hongjixing* than in *Qiuri*. A hypothetical vitis protein with none functional characterization (unigene 48783) and a predicted protein that supposed to functions in RNA degradation (unigene 45911) showed contrasting expression level during cold stress response (Fig.5). Resistant protein (unigene 49372), a putative eukaryotic translation initiation

factor 4E that is responsible for RNA transport (unigene 44870), an unknown protein (unigene 50734) and a Chalcone synthase that is involved in flavonoid biosynthesis (unigene 49105) hold expression difference at nearly hundred times. Chalcone synthase related enzyme was reported to mediate flavonoid biosynthesis, which is a kind of defense compounds responsible for stress stimulus (Dao et al., 2011; Fini et al., 2011; Richard et al., 2000; Winkel-Shirley, 2002; Yang et al., 2002). A gluconokinase in pentose phosphate pathway (unigene 45349) was thought to counter against oxidative stress and assist photosynthesis under stress condition (Abdeen et al., 2010; Kruger and von Schaewen, 2003; Lejay et al., 2008; Savitch et al., 2001). This is in line with the higher chlorophyll fluorescence (Fv/Fm) of *Hongjixing* (Fig.5). A putative unigene latex-like protein (unigene 48058) was also supposed to cause a better cold tolerance of *Hongjixing*, which was extracted from *Calotropis procera* and reported for protection against inflammation and oxidative stress (Kumar and Roy, 2007), but its biological function of engagement in cold response have never been reported before. Two unigenes (Unigene44870 and Unigene45991) involved in RNA regulation were found to be active in cold tolerance. Based on the cognition that genes with similar expression patterns usually exhibit functional correlation (Ren et al., 2014), we speculated that some of these upregulated unigenes comprised in the same cold tolerance pathway.

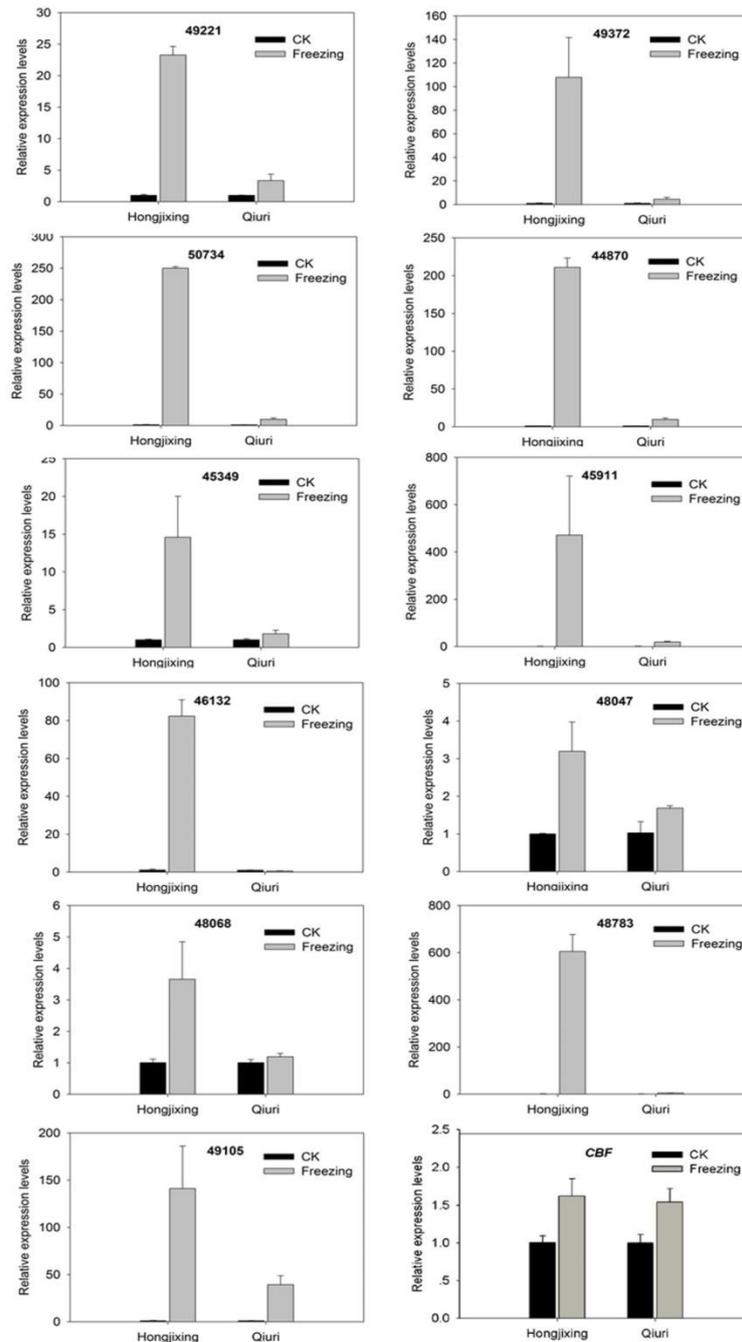


Fig 5. Relative expression levels of 12 DEGs in response to cold stress. DEGs, differentially expressed unigenes. CK, control treatment with both cultivars grow under normal growth condition. Each treatment replicated 3 times, with the bar on the column stand for SD.

The ICE-CBF-COR pathway is well conserved component that contribute to cold tolerance across numerous species (Chinnusamy et al., 2007; Cook et al., 2004; Jiang et al., 2011; Thomashow et al., 2001). However, for the well-known fatal CBF factor that is involved in cold-tolerance pathway (Chinnusamy et al., 2007; Novillo et al., 2004; Novillo et al., 2007), we did not observe significant expression difference between Hongjixing and Qiuri. Regarding cold tolerance via novel secondary metabolites (ie, latex) such as production or other unknown pathways, we speculated that Hongjixing outfit other cultivars (like

Qiuri). Nevertheless, none of these key candidates could be functionally characterized in vivo,

efficient approaches are required to identify the biological-functions of these eminent genes.

Materials and methods

Plant materials

Haploid seedlings of two *Gerbera hybrida* cultivars were successfully regenerated by culturing the unfertilized pollen of diploid parent. Plants were grown in a phytotron with

constant temperature of 22°C and photoperiod of 16h light/8h dark. After two months under the normal condition, healthy seedlings with uniform growth were sampled, frozen in liquid nitrogen and prepared to RNA-seq analysis.

Cold treatments

To test the difference between the cold-tolerance between the two *Gerbera hybrida* cultivars, two-month-old seedlings with uniform growth were subjected to cold treatment of -2°C for 2h in an intelligent cooled incubator (Rumed 3301). Samples were taken before and after treatments, all treatments were replicated 3 times.

RNA extraction

Two cultivars of *Gerbera*, *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri were used in this study. Five cultured plants of haploid *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri were collected from tissue cultured sprouts. It was quick-frozen in liquid nitrogen. Total RNA of these samples was extracted using TRIZOL method (Zhou et al., 2016). The concentration and quality of RNA was evaluated by spectrophotometer NanoDrop ND-1000 at 260/230 and 260/280.

cDNA library construction

Sequencing library was constructed following the manufacturer's instruction of Illumina (San Diego, CA, USA). The mRNA with poly-A tail was enriched from total RNA by Magnetic Oligo (dT) beads and pulled down with Tris-HCl buffer. The eluted RNA was subjected to sonication, to break it into smaller fragments of 100-500 bp. Fragmented mRNA was then reverse transcribed into cDNA using SuperScript cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). Double stranded cDNA fragments were purified using QiaQuick PCR extraction kit. These fragments were then fused with adapter which served as templates during PCR amplification. cDNA library was constructed with these PCR products and loaded into the flow cells.

Sequencing, de novo assembly and annotation

The cDNA library was end-paired sequenced from the adapter-fused fragments on Illumina HiSeqTM 2000 according to the manufacturer's instructions. Raw reads were first filtered for the quality control of the raw data, using the default parameters set in the Illumina, i.e., unknown nucleotides (N>5%). The adapters, fragment with size less than 10 bp and low-quality reads were discarded, then subjected to reads trimming in Trimmomatic to get clean reads. Clean reads were assembled *de novo* by Trinity. The Trinity make up of three modules and concatenate clean reads automatically, to form Contigs and unigenes (Grabherr et al., 2011). These reads were mapped again to the contigs, until paired-end reads do not connect with any contigs at either end; these fully extended contigs were called unigenes. The unigenes were further subjected to redundancy removal, which is delimited by 94% identity within fragments larger than 100 bp.

Non-redundant unigenes were submitted to Nr, Swiss-Prot, KEGG or COG databases for homolog search and

annotation comparison based on BLAST algorithm, with threshold e-value $\leq 10^{-5}$. Meanwhile, unigenes were mapped to nucleotide database Nt used to consolidate the BLASTX results. When a unigene was unmapped to any aforementioned databases, ESTScan were used to predict the coding sequences. Gene ontology (GO) annotation and functional classification was carried out in Blast2GO and WEGO software.

Differential expressed candidate unigene screen and its pathway analysis

Expression pattern of unigenes were compared between *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri based on FPKM ratio. Variation larger than 2 times and $FDR \leq 0.001$ was considered as criterion to screen differential expressed unigene. More stringent standards were used to refrain false and ambiguous annotations by rechecking it online by BLASTX in Nr database. For better understanding the biological property of these differential unigene, we investigated corresponding metabolic pathway that were involved in by the KEGG analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of screened unigene

Better cold tolerance of *Gerbera hybrida* Hongjixing in winter motivated us to explore its underlying mechanism in response to cold/freezing treatment. Expression profiles of the top 30 differentially expressed unigene were investigated both in *Gerbera hybrida* Hongjixing and *Gerbera hybrida* Qiuri, then both cultivars (one-month-old haploid sprouts) were exposed to -2°C for two hours and then it was recovered at room temperature for one week. Samples were collected at indicated time and frozen in liquid nitrogen. The total RNA was isolated by TRIZOL, 2 μ g total RNA was reverse-transcribed to cDNA as mentioned above. This product was used as template in real time PCR. Primers of these unigene were located in the un-conserved region for specificity and listed in Table S1.

Statistical analysis

This study use fragments per kilobase of exon model per million mapped (FPKM) to normalize the expression levels of transcripts. The identification of differently expressed unigenes (DEGs) was filtered by the ratio of log-transformed FPKM and false discovery rate (FDR). For testing the effect of cold stress on the expression of candidate DEGs, qRT-PCR results were compared between before and after cold treatment with one way ANOVA.

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