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De Novo Transcriptome Sequencing in *Passiflora edulis* Sims to Identify Genes and Signaling Pathways Involved in Cold Tolerance

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Abstract: The passion fruit (*Passiflora edulis* Sims), also known as the purple granadilla, is widely cultivated as the new darling of the fruit market throughout southern China. This exotic and perennial climber is adapted to warm and humid climates, and thus is generally intolerant of cold. There is limited information about gene regulation and signaling pathways related to the cold stress response in this species. In this study, two transcriptome libraries (KEDU_AP vs. GX_AP) were constructed from the aerial parts of cold-tolerant and cold-susceptible varieties of *P. edulis*, respectively. Overall, 126,284,018 clean reads were obtained, and 86,880 unigenes with a mean size of 1449 bp were assembled. Of these, there were 64,067 (73.74%) unigenes with significant similarity to publicly available plant protein sequences. Expression profiles were generated, and 3045 genes were found to be significantly differentially expressed between the KEDU_AP and GX_AP libraries, including 1075 (35.3%) up-regulated and 1970 (64.7%) down-regulated. These included 36 genes in enriched pathways of plant hormone signal transduction, and 56 genes encoding putative transcription factors. Six genes involved in the ICE1–CBF–COR pathway were induced in the cold-tolerant variety, and their expression levels were further verified using quantitative real-time PCR. This report is the first to identify genes and signaling pathways involved in cold tolerance using high-throughput transcriptome sequencing in *P. edulis*. These findings may provide useful insights into the molecular mechanisms regulating cold tolerance and genetic breeding in *Passiflora* spp.

Keywords: passion fruit; cold tolerance; RNA sequencing; DEG; ICE1–CBF–COR

1. Introduction

Passiflora is the largest genus of the Passifloraceae family, with more than 500 species [1]. *Passiflora* species are distributed throughout Latin America, and Brazil and Colombia are the centers of diversity for this genus [2], and many of these species are widely cultivated for their edible fruit, medicinal efficacy, and ornamental properties. In the early 20th century, *Passiflora edulis* Sims as an edible fruit was introduced to China, mainly in Taiwan, Guangdong, Guangxi and Fujian. *P. edulis* is known for its taste, is used in Brazilian traditional folk medicine and is included in pharmacopoeias of several countries [3,4]. Leaf extracts of *P. edulis* are considered to treat alcoholism, anxiety and insomnia [5]. The flower has been used for the treatment of cough and bronchitis, and the seed oil as a lubricant and massage oil [6].

Low temperatures represent a major abiotic constraint to plant growth, productivity and distribution [7]. To ensure optimal growth and survival, plants must respond and adapt to cold stress, by implementing a wide range of biochemical and physiological processes. Currently, the most thoroughly understood cold-signaling pathway is the ICE1–CBF–COR pathway. C-repeat binding factors (CBFs) can activate the expression of numerous downstream cold-responsive (*COR*) genes by binding to the cis-acting elements [8,9]. Overexpression of *AtCBF1* or *AtCBF3* enhanced chilling, drought and salt stress tolerance in many species, including *Brassica* spp. [10], wheat (*Triticum aestivum* L.) [11], tomato (*Solanum lycopersicum* L.) [12] and rice (*Oryza sativa* L.) [13]. Inducer of CBF expression 1(ICE1) belongs to the bHLH transcription factor (TF) family, which control *CBF* genes [14]. ICE1 is affected by ambient temperature and is in an inactive state in a warm environment; however, it is activated upon exposure to cold and induces *CBF* expression. Mitogen-activated protein kinase (MAPK) kinase 2 (MKK2) is activated by cold and controls COR expression to improve plant tolerance to freezing [15]. Recent studies have shown that chilling stress activates *SNF1-related protein kinases 2.6/open stomata 1* (*SnRK2.6/OST1*), and then *SnRK2.6* interacts with and phosphorylates ICE1 to activate the *CBF–COR* gene-expression cascade and increase cold tolerance [14,16]. In *Arabidopsis thaliana* (L.) Heynh, only 12% of *COR* genes were regulated by *CBF* [17], which indicated that other regulatory pathways may also be activated in response to cold stress. The abscisic acid (ABA) signal transduction pathway played a key role in plant cold tolerance, with approximately 10% of the ABA response genes involved in cold stress [18], and ABA signaling pathways may be involved in regulating *COR* expression [19,20].

The passion fruit (*P. edulis*), also known as the purple granadilla, is widely cultivated as the new darling of the fruit market throughout southern China. This exotic and perennial climber is adapted to warm and humid climates, and thus is generally intolerant of cold. Fortunately, we have developed a cold-tolerant *P. edulis* variety ('Pingtang 1', KEDU), which is widely cultivated in the south of Guizhou. From breeding and popularization, this variety has undergone testing of several rounds of extreme low temperature, especially in the 2008 Snow Disaster in the south of China; it showed significantly higher cold tolerance in the field than the other varieties mainly cultivated in Guangdong and Guangxi [21]. However, there is limited information about gene regulation and signaling pathways related to the cold stress response in this species. Here, two transcriptome libraries (KEDU_AP vs. GX_AP) were constructed from the aerial parts of cold-tolerant and cold-susceptible varieties of *P. edulis*, respectively. De novo transcriptome sequencing was carried out to identify genes and signaling pathways involved in cold tolerance. This report is the first to identify genes and signaling pathways involved in cold tolerance using high-throughput transcriptome sequencing in *P. edulis*.

2. Materials and Methods

2.1. Plant Materials

In a previous field investigation in Pingtang County, Guizhou Province, we found that after the 2008 Snow Disaster, several *P. edulis* ('Pingtang 1') survived and that they could survive winter temperatures below −2°C [21]. We carried out conservation and cutting propagation, and in a series of experiments found that 'Pingtang 1' had significantly higher cold tolerance than 'Purple Fragrance 1' [21]. The two varieties of *P. edulis*, cold-tolerant 'Pingtang 1' and cold-susceptible 'Purple Fragrance 1' were planted in Pingtang County, Guizhou Province (25°44′24.77″ N, 106°48′45.44″ E; altitude 884 m). The aerial parts (stem, leaf, flower and fruit) of 'Pingtang 1' and 'Purple Fragrance 1' were sampled for RNA extraction in the winter of 2016 (November 9, temperature of 4–6 °C). All *P. edulis* samples were stored at −80 °C in an ultra-low temperature freezer.

2.2. Total RNA Extraction, cDNA Library Preparation and Transcriptome Sequencing

Total RNA was extracted from samples according to the protocol of RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA integrity was monitored by agarose gel electrophoresis (1%) and

using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The cDNA library of 'Pingtang 1' was named KEDU_AP and that of 'Purple Fragrance 1' named GX_AP. Following the manufacturer's instructions, two cDNA libraries were generated using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Boston, Massachusetts, USA). According to the manufacturer's recommendations, clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA). The prepared libraries were sequenced, and 150-bp paired-end reads were generated (Illumina Hiseq 2500, San Diego, CA, USA). The raw images were transformed using CASAVA base-calling into the FASTQ format of raw reads (raw data). To get clean reads, low-quality reads and adapter sequences were removed using SeqPrep (https://github.com/jstjohn/SeqPrep) and Cutadapt [22].

2.3. Transcriptome Assembly and Function Annotation

Transcriptome assembly was achieved using Trinity version r20140413p1 [23] based on the left.fq and right.fq, with the min_kmer_cov set as 2 by default and all other parameters set as their defaults. For function annotation, the longest transcript of each gene was defined as the 'unigene'. All assembled unigenes were BLASTed in Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KEGG ortholog database (KO) and Gene ontology (GO) databases using BLAST2GO of version 2.5 with a cut-off E-value of 10^{-6} [24].

2.4. Differential Expression Analysis

Gene expression level of all samples was estimated by mapping clean reads to the Trinity transcripts assembly using RSEM version 1.2.15 [25], with the bowtie2 parameter set at mismatch 0. Then, read counts for each gene were obtained from the mapping results. Prior to Differentially expressed genes (DEG) analysis, the read counts were normalized using the edgeR program package with the Trimmed Mean of *M*-values method [26,27]. The DEGseq R package was used to analyze differential expression of two *P. edulis* samples. The *p*-value was adjusted using *q*-value [28]. The significant differential expression threshold was set as q-value < 0.005 and $|\log_2(\text{foldchange})| > 1$ [29]. The identified DEGs were used for GO enrichment analyses, which were performed using the GOseq R package (version 1.10.0), based on the Wallenius non-central hypergeometric distribution [30]. Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis was performed using KOBAS version 2.0.12 [31].

2.5. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from various samples with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with RNAse-free DNase I (Ambion, Austin, Texas, USA). Of the DNase-treated RNA, 1 μg was reverse transcribed using random hexamer primers. The resulting cDNA was diluted, and 2 μL of the diluted cDNA was used. Specific primers (Tm, 58–61 °C) were designed to generate PCR products of 70–150 bp (Table S1). The qRT-PCR was performed on an ABI ViiA 7 Real-time PCR platform. FastStart Universal SYBR Green Master (Rox) was used for qRT-PCR assays according to the manufacturer's protocol. For each sample, three replicates were performed in a final volume of 20 μL containing 10 μL of SYBR Premix Ex Taq (2×), 0.4 μL of 50 × ROX Reference Dye II, 0.4 μL (10 μM) of each primer, 2 μL of cDNA, and 6.8 μL of dH2O. Thermo-cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C annealing and extension for 34 s. All reactions were performed in triplicate. The specificity of the PCR amplification procedures was checked with a heat dissociation protocol after the final cycle of the PCR to ensure that each amplicon was a single product. Relative expression was calculated as the difference in delta-Ct between the target gene and the internal control, *histone H3.3* (*HIS*) gene.

3. Results

3.1. Transcriptome Sequencing and De Novo Assembly

In total, there were 60,881,198 raw reads generated from KEDU_AP and 71,719,162 from GX_AP. The sequencing raw data were submitted to the Short Reads Archive (SRA) database under the accession number SRP106510. Of the raw reads from KEDU_AP, more than 95.87% bases had a q value ≥ 20, and for GX_AP 95.15%. The GC-contents were 44.84% and 45.20% for KEDU_AP and GX_AP, respectively. After removing low-quality reads, 57,840,324 clean reads from KEDU_AP and 68,443,694 from GX_AP were obtained. These were used for de novo assembly.

The Trinity software generated 127,474 transcripts with an average length of 1077 bp and an N50 of 2057 bp (Table 1). In total, 86,880 unigenes were obtained in the range of 201–13,397 bp with an N50 length of 2222 bp. Of these, 20,947 (24.11%) were 200–500 bp, 21,684 (24.96%) were 500–1000 bp, 22,051 (25.38%) were 1–2 kb and the remaining 22,198 (25.55%) were >2 kb (Table 1).

Table 1. Length distribution of unigenes and transcripts.

Nucleotide Length	Transcripts	Unigenes
200–500 bp	60,598	20,947
0.5–1k bp	22,605	21,684
1–2k bp	22,073	22,051
>2k bp	22,198	22,198
Total	127,474	86,880
Min length (bp)	201	201
Mean length (bp)	1077	1449
Max length (bp)	13,397	13,397
N50 (bp)	2057	2222

3.2. Function Annotation and Classification

All the 86,880 assembled unigenes were annotated to the seven databases using the BLAST algorithm (Table 2). In total, 64,067 unigenes were annotated, accounting for 73.74% (Table 2). There were 10,457 (12.03%) unigenes successfully annotated in all seven databases. Analyses showed that 60,028 (69.09%) unigenes had high homology with sequences in the Nr database and 46,093 (53.05%) in the Nt database. The details of other database proportions are shown in Table 2.

Table 2. Unigenes were annotated to the seven databases.

Component	Number of Unigenes	Percentage (%)
Annotated in NCBI non-redundant protein sequences (NR)	60,028	69.09
Annotated in NCBI non-redundant nucleotide sequences (NT)	46,093	53.05
Annotated in KEGG ortholog database (KO)	24,312	27.98
Annotated in Swiss-Prot	47,717	54.92
Annotated in Pfam	45,544	52.42
Annotated in Gene ontology (GO)	46,754	53.81
Annotated in EuKaryotic Orthologous Groups (KOG)	17,742	20.42
Annotated in all databases	10,457	12.03
Annotated in at least one database	64,067	73.74
Total unigenes	86,880	

There were 46,754 unigenes divided into three functional GO categories: biological process (BP), cellular component (CC) and molecular function (MF) (Figure 1). In the BP category, these matched unigenes were annotated to 25 GO terms, with the three top terms being 'cellular process' (27,925), 'metabolic process' (26,692) and 'single-organism process' (21,336). For CC and MF, these unigenes were clustered into 20 and 10 GO terms, respectively, with 'cell' (16,101) and 'binding' (27,248) as the largest subcategories. There were 17,742 unigenes divided into 26 groups for KOG

analysis (Figure 2): the largest group was R (general functional prediction only), followed by O (post-translational modification, protein turnover and chaperon) and then J (translation, ribosomal structure and biogenesis).

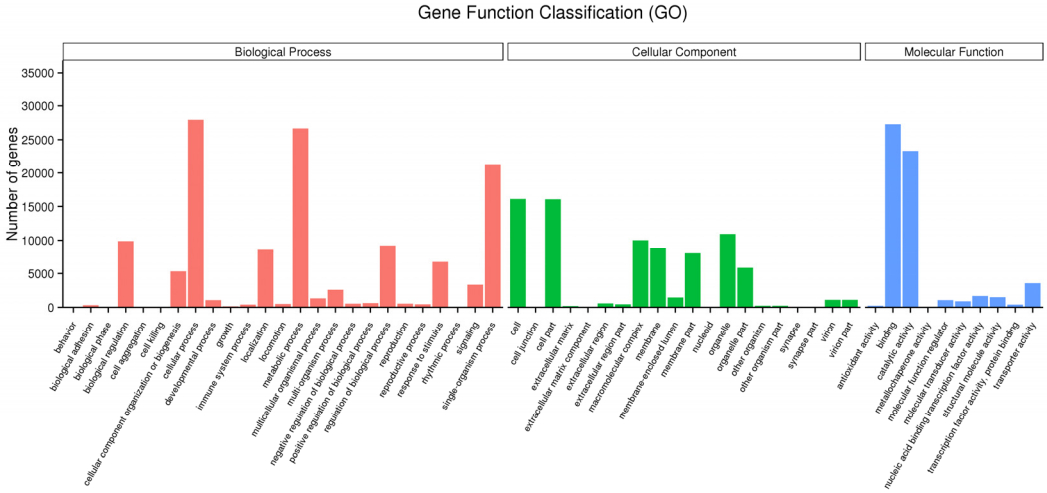

Figure 1. Gene ontology (GO) classification of unigenes. All the annotated unigenes were divided into three functional GO categories: biological process (BP), cellular component (CC) and molecular function (MF).

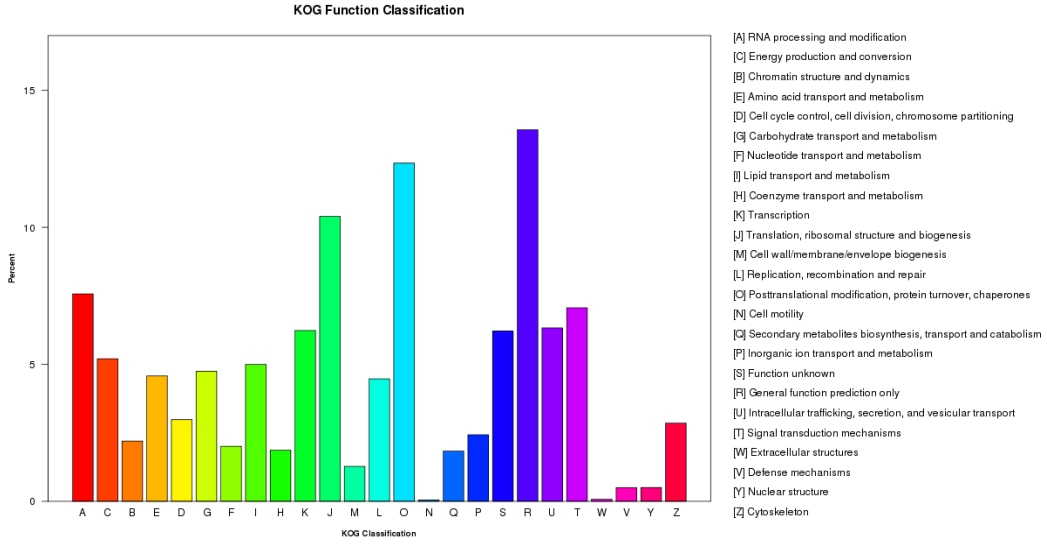

Figure 2. EuKaryotic Orthologous Groups (KOG) annotation of putative proteins. The *x*-axis indicates the names of the 25 groups of KOG. The *y*-axis indicates the percentage of the number of genes annotated to the group out of the total number of genes annotated.

A total of 18,366 unigenes were assigned to 19 metabolic pathways in the KEGG database (Figure 3). These 19 KEGG pathways were clustered into five branches: cellular processes (A) of 1164 unigenes, environmental information processing (B) of 999 unigenes, genetic information processing (C) of 4935 unigenes, metabolism (D) of 10,430 unigenes and organismal systems (E) of 838 unigenes. The metabolic pathways with the largest number of unigenes were 'carbohydrate metabolism' (2101), followed by 'translation' (2087) and 'folding, sorting and degradation' (1528).

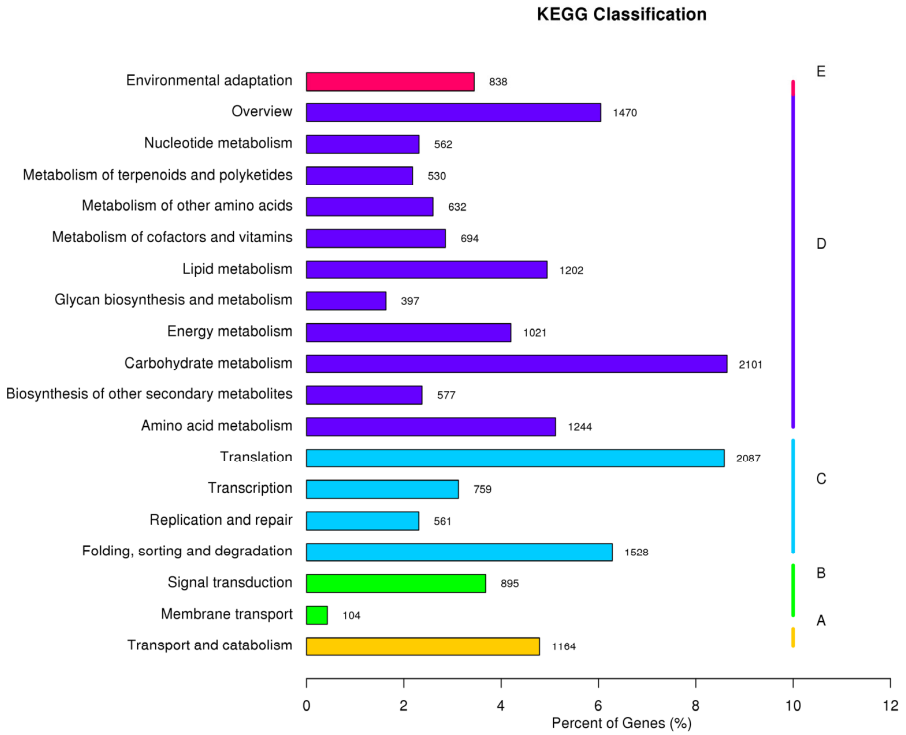

Figure 3. Kyoto encyclopedia of genes and genomes (KEGG) annotation of putative proteins. The *x*-axis indicates the percentage of the number of genes annotated to the pathway out of the total number of genes annotated. The *y*-axis indicates the name of the KEGG metabolic pathway. The genes were divided into five branches according to the KEGG metabolic pathway: Cellular Processes (A), Environmental Information Processing (B), Genetic Information Processing (C), Metabolism (D) and Organismal Systems (E).

3.3. DEG Identification, GO and KEGG Enrichment Analysis

A total of 80,810 unigenes (Fragments per kilobase of exon per million fragments mapped (FPKM) > 0.3) in two groups were identified, with 57,213 unigenes in common. There were 3045 significant DEGs identified between the KEDU_AP and GX_AP libraries. For these DEGs, if $\log_2$Foldchange >1, the DEG was considered as up-regulated but if $\log_2$Fold change <−1, it was considered as down-regulated. The 3045 DEGs included 1075 up-regulated and 1970 down-regulated DEGs (Figure 4).

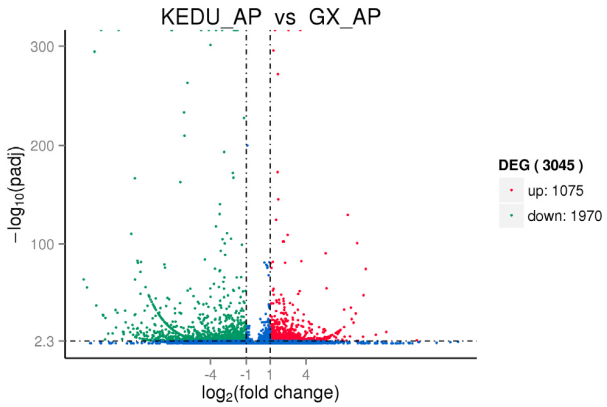

Figure 4. Differentially expressed genes (DEGs) identified between KEDU_AP and GX_AP. The red dots represent significant up-regulated genes and the green dots represent down-regulated genes.

Between the two libraries of enriched GO terms, the analysis showed that 'catalytic activity' and 'single-organism metabolic process' had the highest degree of enrichment (Figure 5). In the KEGG enrichment analysis (Figure 6), the most significantly enriched pathway was 'flavonoid biosynthesis', which mainly consisted of up-regulated DEGs. The second most significantly enriched pathway was 'phenylpropanoid biosynthesis', which also mainly consisted of up-regulated DEGs. Down-regulated DEGs were dominant in the 'photosynthesis-antenna proteins' and 'amino sugar and nucleotide sugar metabolism' (Figure S1). The greatest numbers of DEGs were involved in 'starch and sucrose metabolism', followed by 'phenylpropanoid biosynthesis'.

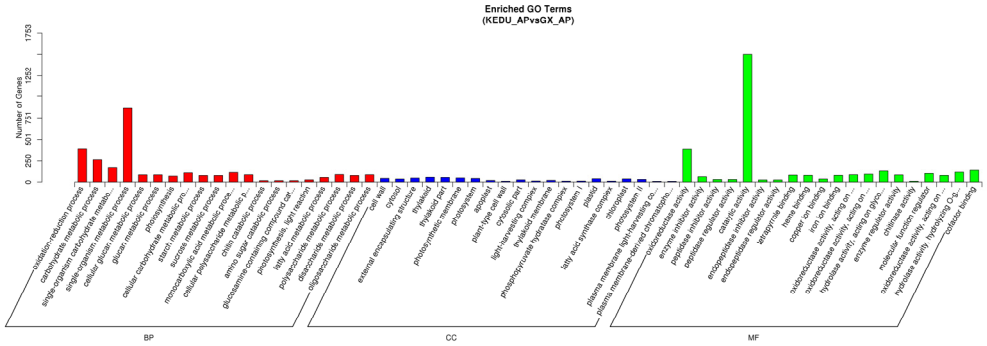

Figure 5. Functional gene ontology classification of DEGs. The *y*-axis indicates the number of DEGs in a category.

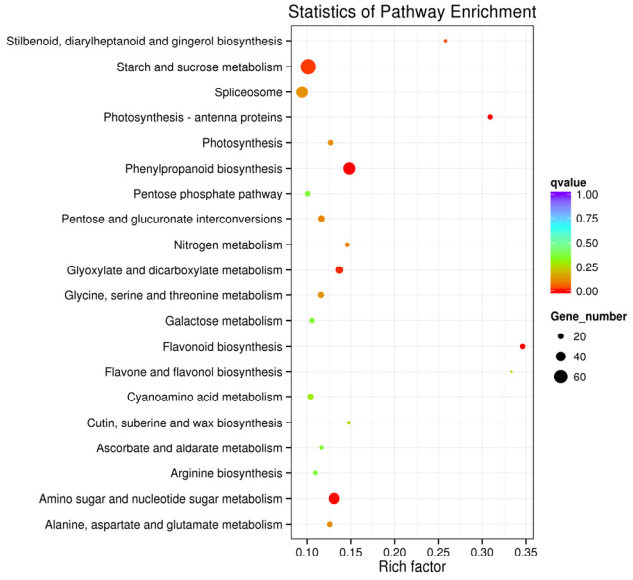

Figure 6. KEGG pathway enrichment of DEGs. The *y*-axis indicates the pathway name, and the *x*-axis indicates the enrichment factor corresponding to the pathway. The *q*-value is represented by the color of the dot. The number of DEGs is represented by the size of the dots.

3.4. DEGs Involved in the Plant Hormone Signal Transduction Pathways

Plant hormones play an important role in plant cold tolerance. A total of 36 DEGs and 677 background unigenes were in the enriched pathway of plant hormone signal transduction (Figure 7). Plant hormone signal transduction contained eight sub-paths. In the auxin signal transduction sub-pathway, there were two up-regulated and nine down-regulated genes. In the cytokinin signal transduction sub-pathway, there were three up-regulated and four down-regulated genes. There was just one up-regulated gene in the gibberellin signal transduction sub-pathway. Only one down-regulated

gene was related to jasmonic acid signal transduction. There were up-regulated and down-regulated genes in the brassinolide and salicylic acid signal transduction pathways, which of them all contained three up-regulated genes and one down-regulated gene. Ethylene and ABA have vital functions in cold-stress signaling. In the ethylene signal transduction sub-pathway, there were two up-regulated genes and one down-regulated gene. In the ABA signal transduction sub-pathway, there were two up-regulated genes and one down-regulated gene for PYR/PYL, two down-regulated genes for PP2C, and one up-regulated and two down-regulated genes for SnRK2.

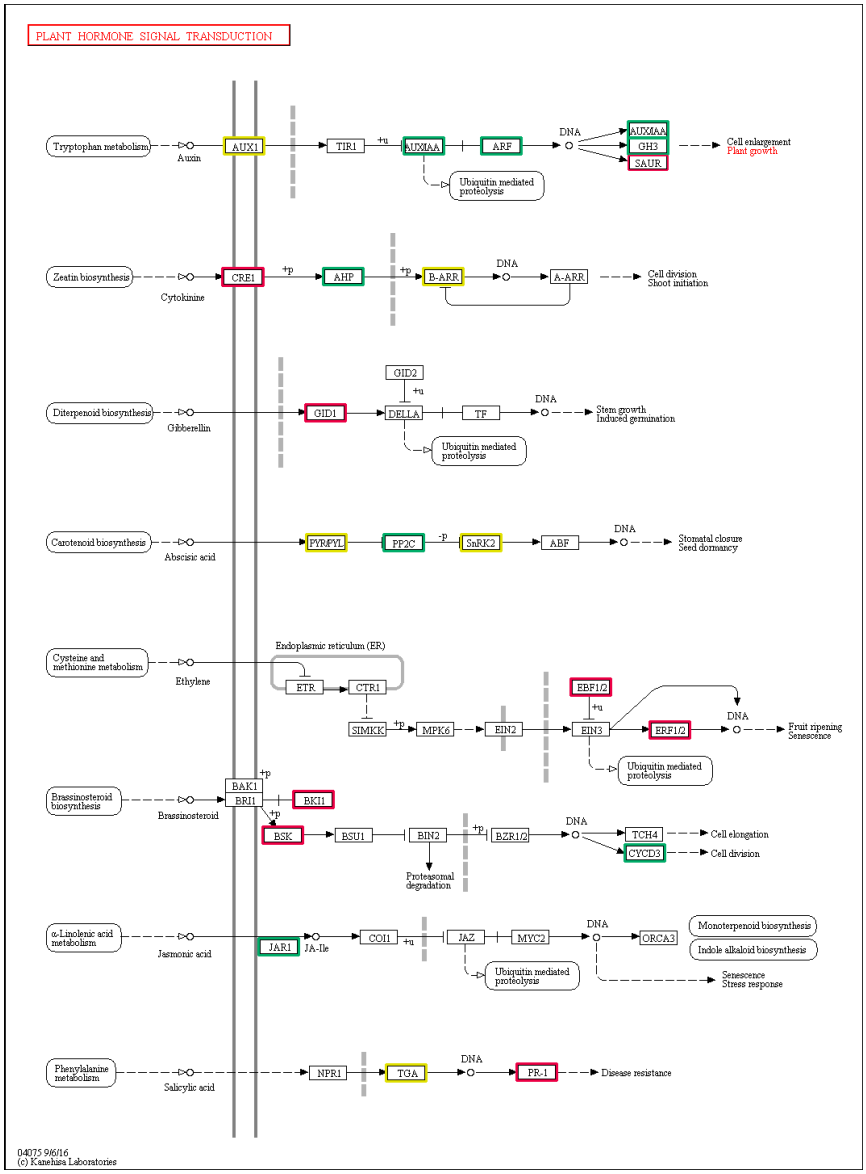

Figure 7. DEGs involved in the plant hormone signal transduction pathway. The enriched KEGG ortholog database (KO) terms are colored according to DEG regulation: red indicates up-regulation, green indicates down-regulation, and yellow indicates both up- and down-regulation.

3.5. Verification by qRT-PCR

In the ICE1–CBF–COR pathway, we identified one ICE1, one COR, two ICE1-like and two CBF genes. These genes were up-regulated in cold-tolerant variety 'Pingtang 1' (KEDU_AP) by qRT-PCR verification (Figure 8). To verify the reliability of the transcriptome data, 11 DEGs were randomly selected and examined using qRT-PCR at the transcriptional level (Figure S2). The expression

patterns of the 11 DEGs were consistent with the transcriptome data (R^2 = 0.81321, p-value = 0.002334). These results indicate that our transcriptomic analysis was highly reproducible and reliable.

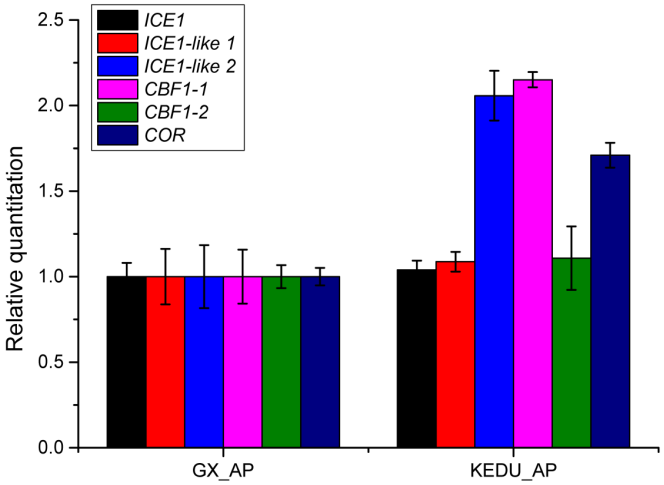

Figure 8. The expression of six genes involved in the ICE1–CBF–COR pathway. *ICE*, Cluster-5862.10114; *ICE-like 1*, Cluster-5862.30769; *ICE-like 2*, Cluster-5862.1181; *CBF1-1*, Cluster-5862.30492; *CBF1-2*, Cluster-5862.29181; and *COR*, Cluster-5862.23126.

4. Discussion

As a key messenger, the cytosolic Ca^{2+} occupies an important role in response to cold stress [32,33]. Research shows that cold-induced Ca^{2+} influx is positively correlated with accumulation of cold-induced transcripts in *Arabidopsis* [34] and alfalfa (*Medicago sativa* L.) [35]. Cold stress increases the cytosolic Ca^{2+} level, and this is then sensed by Ca^{2+} sensor proteins such as calmodulin (CaM) and calcineurin B-like proteins (CBLs) [36,37]. The increased levels of Ca^{2+} in plant cells affect the expression level of CBF and COR genes in the cold signaling pathway [7,38]. In this study, 332 unigenes were annotated as CaMs and CBLs, and included 14 DEGs. Among these DEGs, seven (four CaM and three CBL) genes were up-regulated in KEDU_AP, and seven (six CaM and one CBL) were down-regulated (Figure S3 and Table 3).

Table 3. Identified DEGs, unigene numbers and ratios of DEG/unigene.

Component	Unigenes	DEGs	Ratio (%)
AP2/EREBP	216	8	3.7
WRKY	161	12	7.5
bZIP	181	8	4.4
MYB	387	14	3.6
NAC	221	11	5.0
B-ARR	20	3	15
CaM/CBLs	332	14	4.2
MAPK	93	11	11.8
LEA	38	4	10.5

Plant protein kinases, such as Mitogen-activated protein kinases (MAPKs), play a central role in cellular signaling. MKK2 is a member of the MAPK family, and is upstream of MPK4 and MPK6, which are activated by low temperatures [14]. Previous studies showed that the influx of calcium is involved in cold-stress regulation of MAPKs [39]. The receptor-like kinase CRLK1 may associate calcium signaling with the MAPK cascade by binding to calcium, and CaM interacts with MEK kinase 1 (MEKK1) [40] (Figure 9). We identified 93 MAPK unigenes, which included 11 DEGs: nine up-regulated and two down-regulated (Figure S3 and Table 3).

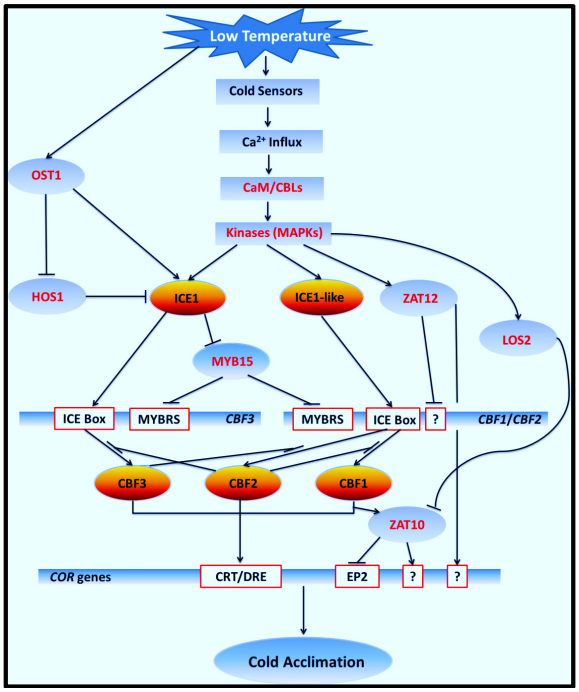

Figure 9. Diagram of cold-responsive regulatory networks [8,14]. The arrows indicate activation, whereas lines ending with a bar indicate negative regulation; the "?" indicates unknown cis-elements. Abbreviations: CaM, calmodulin; CBLs, calcineurin B-like proteins; MAPKs, mitogen-activated protein kinases; ICE1, inducer of CBF expression 1; MYB, V-MYB avian myeloblastosis viral oncogene homolog; MYBRS, MYB transcription factor recognition sequence; *OST1*, *open stomata 1* (*SnRK2.6*); *HOS1*, *high expression of osmotically responsive genes1*; CBF, C-repeat binding factor; *COR*, *cold-responsive genes*; CRT, C-repeat elements; DRE, dehydration-responsive elements; *LOS2*, *low expression of osmotically responsive genes 2*; ZAT10 and ZAT12 are two C2H2 zinc finger transcription factors.

In this work, 56 differentially expressed TFs were identified, and were divided into six TF families: AP2/EREBP, WRKY, bZIP, MYB, NAC and B-ARR (Figure S4 and Table 3). Most of them have been reported to be associated with cold tolerance in plants [41–44]. The TFs of CBFs belong to the AP2/EREBP family [45]. Three CBF genes are known in Arabidopsis, and are induced with plant exposure to cold, and regulate COR gene accumulation [46,47] (Figure 9). Overexpression of OsMYB4 could improve freezing tolerance in Arabidopsis by increasing COR gene expression, and OsMYBS3 and OsMYB2 enhanced cold resistance in rice [48–50]. We identified six genes in the ICE1-CBF-COR pathway of *P. edulis*, and their expression levels in the cold-tolerant variety 'Pingtang 1' were higher than that in cold-susceptible 'Purple Fragrance 1'.

In addition to the TFs, other important genes involved in plant cold tolerance include late-embryogenesis-abundant proteins (LEAs), antifreeze proteins (AFPs), superoxide dismutase (SOD) and proline [51–53]. LEAs are highly hydrophilic and provide protection for plants during cold stress. We also found four differentially expressed LEAs (Figure S3 and Table 3), with a high expression level in KEDU_AP.

In this study, we identified candidate genes and signaling pathways associated with cold tolerance by transcriptome sequencing of cold-tolerant variety 'Pingtang 1' and cold-susceptible 'Purple Fragrance 1'. The heatmap of DEGs showed that many genes related to cold-tolerance had higher expression levels in the cold-tolerant variety, indicating that they played a role in the chilling stress response. This report is the first to identify genes and signaling pathways involved in cold tolerance using high-throughput transcriptome sequencing in *P. edulis*. These findings may provide useful insights into the molecular mechanisms regulating cold tolerance and genetic breeding in *Passiflora* spp.

5. Conclusions

P. edulis is mainly distributed in tropical and subtropical regions and has difficulty surviving at low temperatures. To elucidate the molecular mechanisms of cold tolerance, we collected the aboveground parts of cold-tolerant variety 'Pingtang 1' and cold-susceptible 'Purple Fragrance 1' and subjected them to high-throughput sequencing. In total, 26,284,018 clean reads were obtained, and 86,880 unigenes with a mean size of 1449 bp were assembled. There were 3045 significant differentially expressed genes (DEGs) identified between 'Pingtang 1' (KEDU_AP) and 'Purple Fragrance 1' (GX_AP). To provide reference for the cold-tolerance breeding of *P. edulis*, we screened many DEGs, constructed their expression profiles and analyzed their functions; some potentially vital cold-tolerance genes and transcription factors were identified, and the expression levels of the two varieties were compared and analyzed. As the first report on the high-throughput sequencing of cold-tolerant *P. edulis*, this study should provide novel insights into cold-tolerance genes for *P. edulis* and be a valuable molecular basis for study in *Passiflora spp*.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/11/435/s1, Table S1: Primer sequences; Figure S1: KEGG pathway enrichment of up-regulated and down-regulated DEGs; Figure S2: Comparison of expression results between RNA sequencing (RNA-seq) and qRT-PCR; Figure S3: Heatmap of important DEGs associated with cold-tolerance; Figure S4: Heatmap of differentially expressed transcription factors (TFs) associated with cold-tolerance.

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Abbreviations

Nr	NCBI non-redundant protein sequences
Nt	NCBI non-redundant nucleotide sequences
Pfam	Protein family
COG	Clusters of orthologous groups of proteins
KOG	EuKaryotic Orthologous Groups
Swiss-Prot	A manually annotated and reviewed protein sequence database
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG ortholog database
GO	Gene ontology
FPKM	Fragments per kilobase of exon per million fragments mapped
DEGs	Differentially expressed genes

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