Proteogenomic analysis of pitaya reveals cold stress-related molecular signature

Junliang Zhou¹, Zhuang Wang¹, Yongya Mao¹, Lijuan Wang¹, Tujian Xiao¹, Yang Hu²,³, Yang Zhang⁴ and Yuhua Ma¹

¹ Guizhou Institute of Pomological Sciences, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou, China
² Zhejiang Academy of Forestry, Hangzhou, Zhejiang, China
³ Zhejiang Provincial Key Laboratory of Biological and Chemical Utilization of Forest Resources, Hangzhou, Zhejiang, China
⁴ Fudan University, Institutes of Biomedical Sciences, Shanghai, Shanghai, China

ABSTRACT

Pitayas (Hylocereus spp.) is an attractive, highly nutritious and commercially valuable tropical fruit. However, low-temperature damage limits crop production. Genome of pitaya has not been sequenced yet. In this study, we sequenced the transcriptome of pitaya as the reference and further investigated the proteome under low temperature. By RNAseq technique, approximately 25.3 million reads were obtained, and further trimmed and assembled into 81,252 unigene sequences. The unigenes were searched against UniProt, NR and COGs at NCBI, Pfam, InterPro and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and 57,905 unigenes were retrieved annotations. Among them, 44,337 coding sequences were predicted by Trandecoder (v2.0.1), which served as the reference database for label-free proteomic analysis study of pitaya. Here, we identified 116 Differentially Abundant Proteins (DAPs) associated with the cold stress in pitaya, of which 18 proteins were up-regulated and 98 proteins were down-regulated. KEGG analysis and other results showed that these DAPs mainly related to chloroplasts and mitochondria metabolism. In summary, chloroplasts and mitochondria metabolism-related proteins may play an important role in response to cold stress in pitayas.

INTRODUCTION

Pitaya (Hylocereus spp.), a member of the Cactaceae family, has been attracting attention worldwide owing to their attractive, highly nutritious and commercially valuable fruits (Luo et al., 2014; Hua et al., 2016), which can be found in the “small exotic fruits” category in the fruit market. Two types of pitayas are commercially produced on a large-scale: the white-fleshed pitaya (Hylocereus undatus) and the red-fleshed pitaya (Hylocereus polyrhizus) (Song et al., 2016a, 2016b). The Hylocereus genus used to grow at the subtropical and tropical regions of the Americas and now pitaya crops are mainly grown in countries such as Colombia, Mexico, Costa Rica, Nicaragua and Vietnam (Ortiz & Takahashi, 2015). The pitaya plant has also attracted horticultural interest because it is
highly drought resistant, enabling pitayas to be grown in areas stricken by drought (Rodriguez-Jimenez et al., 2014; Jimenez et al., 2012). In South China, the pitaya plantations are thriving, particularly in the karst regions, such as the Yunan and Guizhou provinces.

In the past decades, pitaya research studies have mainly focused on the biochemistry of the betalains synthesized by pitaya, including their purification and identification (Stintzing, Schieber & Carle, 2002; Wybraniec et al., 2009; Sogi et al., 2010), their physical and chemical properties (Woo et al., 2011), and their antioxidant and radical-scavenging capacity (Garcia-Cruz et al., 2013). Metabolite profiling of red-fleshed (H. polyrhizus) and white-fleshed (H. undatus) pitayas has tentatively identified several betalain biosynthesis-related compounds (Suh et al., 2014). Several key genes in the betalain biosynthesis pathway have also been identified using transcriptomic analysis (Hua et al., 2016). The HuCAT3 gene of pitaya, which encodes catalase, has been isolated and characterized and its expression profile under abiotic stress has been analyzed (Nie et al., 2015). However, the genomic resources available for pitaya are still scarce. More genetic data needs to be generated to aid further studies, such as investigations of pitaya resistance to abiotic and biotic stresses, and for crop breeding.

High-throughput-omics techniques like genomics, transcriptomics or proteomics have recently been widely adopted by plant biologists for studying the plants under various environmental stress (Meena et al., 2017). Especially, high-throughput RNA sequencing (RNA-Seq) technology is a powerful and cost-efficient tool for transcriptome analysis (Ansorge, 2009; Sa et al., 2013; Yu et al., 2014; Wang et al., 2010). For gene expression profiling, especially in those organisms that are non-model organisms and lack genomic sequences, RNA-Seq is a particularly suitable technology. For example, Illumina sequencing technology offers millions of sequence reads from a single instrument run, and only takes a few days to generate a huge amount data (Crawford et al., 2010). It has been shown that the sequencing data from a single illumina run can generate enough read coverage for de novo transcriptome assembly and gene discovery and differential expression profiling analysis (Hudson, 2008). However, mRNA is biological intermediate product, which cannot substantially reflect protein expression level. Recently, tandem mass spectrometry coupled with high performance liquid chromatography have provided a way for obtaining global proteome data and their expression, named label-free proteomics methods. Label-free proteomics have been successfully applied in many plants such as Arabidopsis thaliana (Niehl et al., 2013) and Nicotiana attenuata (Weinhold et al., 2015), and non-model plants like Zingiber zerumbet (Mahadevan et al., 2015) and Piper nigrum (Mahadevan et al., 2016). Label-free proteomics is a high-throughput technique, which has several advantages including handling of proteins without gels, in-solution digestion by trypsin and easy use of internal peptide standards. Label-free proteomics is also applicable to identification of novel proteins and studying non-model organism proteomes which have very limited genomic information (Mahadevan et al., 2016).

In this study, we used Illumina sequencing technology to sequence the transcriptome of pitaya. The unigenes were annotated using six public databases (UniProt, Pfam, InterPro, KEGG and NR and COGs at NCBI), and served as the background database for
label-free proteomics of pitaya. Here, we focused on differentially abundant proteins (DAPs) between control samples and cold treatments.

**MATERIALS AND METHODS**

**Plant material and RNA extraction**

The pitaya cultivar “Tiegusu” was used in this study. “Tiegusu” has light-green flowers and is one of most widely grown commercial cultivars in China. The pitaya plants were grown in a greenhouse at the Guizhou Academy of Agricultural Sciences (Guizhou, China) under a temperature range of 23–28 °C and natural light. To identify as many transcripts involved in cold stress as possible, RNA was extracted from 12 samples of six different tissues (young roots, tender shoots, stems, flower buds, new stems and fruits) from plants that had been grown at 0 °C (cold treatment) for 3 days or grown at a normal temperature (25 °C treatment). A TRIZol® reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from each sample and RNase-free DNase I (TaKaRa) was used to treat the samples by following the manufacturer’s protocol. Equal amounts of total RNA from each treatment were pooled together for cDNA library construction and Illumina deep sequencing.

**cDNA library preparation and Illumina sequencing for transcriptome analysis**

RNA-Seq was performed at Shenting Genomics Institute (Hangzhou, China) using an Illumina HiSeq™ 2000 (Illumina Inc., San Diego, CA, USA). Briefly, poly (A)+ mRNA was isolated from the pooled total RNA sample using Oligo (dT) magnetic beads. The mRNA was fragmented into short fragments using a fragmentation buffer to avoid priming bias. A SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) with a random hexamer-primer (Illumina) was used to synthesize double-stranded cDNA. The synthesized cDNA was then subjected to end-repair and phosphorylation, and the repaired cDNA fragments were 3′-adenylated with Klenow exo- (3′–5′ exo minus, Illumina). The ends of the 3′-adenylated cDNA fragments were ligated by Illumina paired-end adapters. The ligation products were purified on 2% agarose gel to select the appropriate templates for downstream enrichment. The cDNA fragments (approximately 200 bp) were excised from the gel. After end reparation and ligation of the adaptors, the products were amplified by PCR using PCR primers PE 1.0 and 2.0 (Illumina) with fusion DNA polymerase and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Finally, the cDNA library was constructed using 200 bp insertion fragments. The library was validated using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), then sequenced using Illumina HiSeq™ 2000 (Illumina Inc., San Diego, CA, USA). The raw data was deposit at [www.iprox.org](http://www.iprox.org) with accession project ID: IPX0001296002.

**Data filtering, de novo assembly and annotation**

The raw reads were cleaned by removing adaptor sequences, empty reads and low-quality reads using Trimmomatic (v0.32). The cleaned reads (a minimal sequence length of 200 bp
with identity value above 95%) were assembled by the de novo software Trinity (v2.0.6) with default parameter. Then the TGICL and Phrap Clustering tools were used to obtain sequences that could not be extended at either end. The obtained sequences were defined as unique transcripts (or unigenes) (Grabherr et al., 2011; Pertea et al., 2003; Vogel et al., 2006). To obtain annotation of transcripts, all unigene sequences were searched against NCBI nr (non-redundant protein) database using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov/) with cutoff of E-value $10^{-5}$. Functional annotation was performed using Gene Ontology (GO) and analyzed using the Blast2go (http://www.blast2go.org) software (Conesa et al., 2005). The COG and KEGG Orthology (KO) annotations were performed using the A. thaliana and Oryza sativa genome sequences as reference data in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012; Wixon & Kell, 2000; Ogata et al., 1999). HMMER (http://hmmer.org) was used to obtain a domain-based annotation with Pfam (http://Pfam.sanger.ac.uk) database (Finn et al., 2016). All unigene sequences were further scanned by TransDecoder (v2.0.1) to identify Coding sequence (CDS). In totally, 44,337 CDS were predicted.

**Protein extraction and profiling**

Each replicate of pitaya samples was pulverized with mortar and pestle to a fine powder in liquid nitrogen, respectively. About 1 g of sample was used for protein extraction using a filter-assisted sample preparation method. Briefly, the sample was suspended in five mL protein extraction buffer (0.5 M Tris–HCl (pH 7.5), 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, and 40 mM dithiothreitol (DTT)) for 10 min at room temperature. After that, equal volume (five mL) of Tris-phenol was added. After 30 min of shaking, the suspension was centrifuged at 8,000×g and 4 °C for 5 min. After centrifuging, the upper phenolic phase was collected for further extraction by adding an equal volume of extraction buffer to the supernatant. Then, four volumes of 0.1 M ammonium acetate in methanol were added and kept the mixture overnight at −20 °C for protein precipitation, then centrifuging at 4 °C, 8,000×g for 10 min and discarded the supernatant. The pellet was washed thrice at 4 °C with ice-cold acetone. Finally, the pellet was dried for 2 h in a vacuum drier. After drying, the rehydration solution (100 μL; 8 M (w/v) urea, 0.1 M (w/v) Tris, and 10 mM DTT) was used to solubilize the pellet. The concentration of protein was determined by Bradford method.

In the centrifuge tube, the deposit was buffer exchanged with 8 M urea containing 10 mM DTT and 100 mM Tris–HCl. Then, the deposit was alkylated with 55 mM iodoacetamide. The urea concentration in the extract was then diluted to 1 M using the Tris–HCl buffer (pH 7.6). Protein samples were digested by trypsin (enzyme to substrate ratio = 1:20) in a thermomixer (1,000 rpm) overnight at 37 °C. Nano LC-1DTM plus system (Eksigent, Dublin, CA, USA) combined with AB Triple TOF 5600 MS (Foster City, CA, USA) were used to analysis digested peptides. Firstly, eight μL crude polypeptide was injected using a full sample loopin. Crude polypeptide was then desalted on a ChromXP trap column (NanoLC TRAP Column, 3 μm C18-CL, 120 A, 350 μm × 0.5 mm; CA, USA) and then eluted into a second analytical column (Nano LC C18 reversed-phase column.
(3C18-CL, 75 μm × 15 cm; CA, USA)) using a linear gradient formed by mobile phases A (5% ACN and 0.1% FA) and mobile phases B (95% ACN and 0.1% FA) for 120 min gradient at a flow rate of 300 nL/min. AB Triple TOF 5,600 system was operated in data-dependent acquisition mode to automatically switch between TOF–MS and product ion acquisition using Analyst Software (TF1.6). β-Galactosidase digestion was used to calibrate every two samples by 10 min for elution and 30 min for identification.

Proteome data processing
Raw MS files from AB Triple TOF 5600 were processed by MaxQuant version 1.5.2 (http://www.maxquant.org). MS/MS spectra were searched against the transcriptome derived database above mentioned. Precursor mass and fragment mass were identified with an initial mass tolerance of 6 ppm and 20 ppm, respectively. The search included variable modifications of methionine oxidation and N-terminal acetylation, and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set to seven amino acids and a maximum of two mis-cleavages was allowed. MS runs were analyzed with the “match between runs” option. For matching, a retention time window of 20 s was selected. Proteins matching to the reverse database were filtered out. The false discovery rate was set to 0.01 for peptide and protein identifications. The raw data of proteome were all deposit at http://www.iprox.org with accession project ID: IPX0001296001.

RESULTS
Illumina paired-end sequencing and de novo assembly of reference pitaya transcripts
In this study, from one plate (8 lanes) in a single sequencing run, a total of 25.3 million reads were obtained, generating approximately 4.2 giga base pairs (Gbp) of raw data (Table 1). After the removal of adaptor sequences, low-quality reads (Q-value < 25) and ambiguous reads, 21.3 million high-quality clean reads (3.1 Gbp, 84.4% of the raw data) remained. The quality of the clean reads data was assessed based on the base-calling quality scores. The scores obtained using Illumina’s base-caller Bustard. Phred-like quality scores at the Q30 level were obtained for 97.23% of the clean reads data. All the high-quality reads were assembled into 110,330 isotigs (81,252 unigenes) with a maximum size of 200 bp (Fig. 1A). The isotigs were more abundant than those previously reported for a pitaya transcriptome dataset by Hua et al. (2016). The greater number of isotigs obtained in our study may be the result of trying to acquire the most comprehensive coverage possible by sequencing RNA extracted from six different types of pitaya plant tissues. The assembled isotigs have an average contig length of 934 bp (868 bp, unigene) and an N50 of 1,445 bp (1,373, unigene) (i.e., 50% of the assembled bases were incorporated into contigs of 1,445 bp or longer). Although a large proportion of the contigs (48.77%) were between 200 to 500 bp, we obtained 62,413 contigs (51.23%) that were more than 500 bp in length (Fig. 1A). Most of the highly expressed unigenes were approximately 2,500 bp in length (Fig. 1B).
Functional annotation and classification of the pitaya transcripts

In order to acquire complete functional information, sequence-based alignments of the unigenes were performed against different public databases, including the NCBI non-redundant protein database (NCBInr), Swiss-Prot/UniProt, KEGG pathway, GO and KOG cluster using the BLASTX algorithm with a significant E-value threshold of $< 10^{-5}$.
The distribution of the log10 (e-value) of the NCBInr BLAST and Swiss-Prot searches were normally, and the center of distribution were all at approximately 10^{-50} (Fig. 2A), which indicated that the annotation had a high degree of similarity with known sequences. The Hidden Markov Model method was used to search both the InterPro and Pfam databases for Domain/family searches, and BLASTX was used to search alignments against the Clusters of Orthologous Groups (COGs) database at NCBI, with the E-value thresholds also set at ≤ 1 e^{-5} (Table S1). Out of 56,388 hits in the Nr database, 43,127 unigenes also had hits in the Swiss-Prot database (1,487 unigenes only had hits in Swiss-Prot) (Fig. 2B). The number of sequences that were annotated by searching the GO, eggNOG, KEGG and Pfam databases are shown in Fig. 2C. In total, 53,462 sequences were assigned to 23 of the EuKaryotic Orthologous Group (KOG) categories (Fig. 3). The KOG tool is a eukaryote-specific version of the COG tool for identifying orthologous and paralogous proteins (26). The KOG database also provides information about the classification of gene...
products, including their evolutionary relationships (Tatusov et al., 2003; Koonin et al., 2004). Based on the assumption that every protein evolved from an ancestor protein, pitaya isotigs were compared with known sequences in the KOG database to predict and classify their possible functions. Among the 23 KOG categories, “S: Function unknown” was the largest group (12,569 isotigs; 23.51% of all isotigs), followed by “R: General function prediction only” (6,608; 12.36%) and “O: Posttranslational modification, protein turnover, chaperones” (4,395; 8.2%). “N: Cell motility” was the smallest group (25; 0.04%), followed by “D: Cell cycle control, cell division, chromosome partitioning” (65; 0.12%), “B: Chromatin structure and dynamics” (225; 0.42%) and “V: Defence mechanisms” (234; 0.43%) (Fig. 3). To retrieve function of pitaya, we mapped the annotated sequences to GO and canonical KEGG pathways (Kanehisa et al., 2008). The results showed that the most highly represented GO term was the “GO:0006950, response to stress,” with 451 members, and “path:ko04626, plant–pathogen interaction” was enriched at KEGG pathways, with 114 members (Figs. 4D and 5).

**Proteomics characterization of pitaya under cold stress**

In order to analyze the mechanism of pitaya response to cold stress, proteomics approach was used to identify and determine the abundance of proteins in all of tissues. The proteins of those samples (cold treatment vs. control) were extracted and each group treatment conducted three biological replicates. iBAQ values derived from MaxQuant software were used to represent the abundance of identified protein. Hierarchy clustering analysis, a quality-control measure based on expression profiles among replicates, indicates that the results are highly reproducible (Fig. 6). A total of 1,712 non-redundant proteins were identified (including those samples with 24 h cold treatment and control; Table S2).
Figure 4  GO and KEGG pathway enrichment analysis of the pitaya transcriptome. Gene number of subcellular location (A), molecular function (B) and biological process (C) for all identified RNA in pitaya. KEGG pathway mapping result was shown in (D).

Figure 5  Bee swarm graphs of the enriched KEGG pathway and the FPKM value of the genes within the pathway. The x-axis represents the different KEGG pathways, the y-axis represents the FPKM value and the hexagons represent genes.

Comparison of the abundance profiles of proteins between control and cold treatment of pitaya

In this study, the label-free quantitative proteomic analysis characterized the differences in protein synthesis between cold treatment and control. We used volcano plot to show differential expressed proteins. Compared with the control group, the up-regulated and
down-regulated proteins (fold change ≥ 2, p ≤ 0.05) in cold treatment group were 18 and 98, respectively (Fig. 7; Table S3). Total 116 DAPs were subjected into Blast2GO and sorted by major enrichment of biological processes (Fig. 8), including proton transport, ATP hydrolysis coupled proton transport, glycolytic process, carbon fixation, ATP synthesis coupled proton transport, ATP metabolic process, proteolysis, metabolic process, ion transport, and intracellular protein transport. All DAPs were successfully annotated with KEGG pathways, sorted by enrichment score (Fig. 8), including AMPK signaling pathway (path:ko04152), fructose and mannose metabolism (path:ko00051), pentose phosphate pathway (path:ko00030), glycolysis/gluconeogenesis (path:ko00010), starch and sucrose metabolism (path:ko00500), carbon fixation in photosynthetic organisms (path:ko00710), ascorbate and aldarate metabolism (path:ko00053), pentose and glucuronate interconversions (path:ko00040), amino sugar and nucleotide sugar metabolism (path:ko00520), carbon metabolism (path:ko01200). It was found that proteins under ATP related GO catalogues were decreased, which indicated that the cold stress reduced the activity of energy generate, storage or release, which was one of the key aspects of life activity.

**Overview and analysis of differentially abundant proteins related to cold stress**

Compared with the control group, there are 18 proteins were increased expression abundance after 24 h cold treatment. The expression level of VPS15 Serine threonine-kinase, TRPA2 alpha chloroplastic tryptophan synthase and PPDK1 chloroplastic phosphate dikinase were significantly higher than other proteins. However, majority of
the DAPs were down regulated and the 20 most decreased DAPs were listed in the Table S3. Among them, it is interesting that a number of them are chloroplastic or mitochondrial related proteins. Of which, 6 DAPs, including Ketol-acid reductoisomerase, Glutamate decarboxylase, Malate dehydrogenase, Pentatricopeptide repeat-containing protein, Phosphoglycolate phosphatase and Monodehydroascorbate reductase, are related to chloroplastic proteins, and 5 DAPs, including Aldehyde dehydrogenase family 2, Monodehydroascorbate reductase, Glycine dehydrogenase, and Probable mitochondrial-processing peptidase are related to mitochondrial proteins. Some studies provide evidence that chloroplastic and mitochondrial proteins play an important role in response to cold stress.

**DISCUSSION**

The RNA-Seq technology is an efficient technology for characterizing transcriptomes of the non-model organisms. In this study, the isotigs were more abundant than those previously reported for a pitaya transcriptome dataset by Hua et al. (2016), indicating that the Illumina paired-end sequencing project generated a substantial fraction of pitaya genes, so it can be used for next analysis. Interestingly, more and more transcriptome studies have found that the number of up-regulated proteins response to cold stresses was
significantly more than down-regulated proteins in wheat (Winfield et al., 2010), Lotus japonicas (Calzadilla et al., 2016), and Arabidopsis (Fowler & Thomashow, 2002). Many tropical plants adopted in temperate climate including pitaya cannot live in the cold climate. Here, we identified several DAPS refer to cold stress in pitaya and further analysis showed that chloroplasts and mitochondria metabolism-related proteins may play a vital role in response to cold stress in pitayas.

Serine threonine-kinase is one of the protein kinases composed of two-component signal transduction systems (Zschiedrich, Keidel & Szurmant, 2016). The two-component

---

(A) Biological Process

(B) KEGG

Figure 8 GO and KEGG pathway enrichment analysis of the pitaya proteome. Enrichment score of biological process (A) and KEGG pathway (B) for all identified proteins.

Full-size DOI: 10.7717/peerj.8540/fig-8
systems are the signal transduction system which most have been investigated at present and play the major roles in regulating cell activities in many eukaryotes. Due to increasing experimental data, it has been discovered that more and more co-regulation and crosstalk regulations among signal transduction systems. And the two-component systems are found that to be very important in corresponding to abiotic stresses (Liu et al., 2015; Tang et al., 2017). The role of serine/threonine protein kinases (STPKs) in the cold response was much studied in Synechocystis (Zorina et al., 2014). A screening of a collection of STPK mutants identified it as a possible transcriptional regulator for lower temperatures adaption (Zorina et al., 2014). TRPA2 tryptophan synthase and PPDK1 phosphate dikinase are chloroplastic enzymes. The tryptophan (Trp) biosynthetic pathway leads to the production of many secondary metabolites with diverse functions, Arabidopsis tryptophan pathway enzymes have been shown inducing by abiotic stress to allow for increased biosynthesis of secondary metabolites (Zhao, Williams & Last, 1998), which may be the reason of TRPA2 tryptophan synthase increased under cold stress as well. PPDK reversibly interconverts pyruvate, ATP, and orthophosphate with phosphoenolpyruvate (PEP), AMP, and pyrophosphate (PPI) and provides diverse functions in various plant tissues (Lappe et al., 2018). There is a study suggested that PPDK1 was associated with the antioxidant systems (Xu et al., 2018). However, the role of TRPA2 and PPDK1 in cold stress remain unclear yet.

Many metabolic reactions of plant were take place in the chloroplast in plants, however the metabolic balance in chloroplasts is easily disturbed by environmental stresses. Thereby, the reprogram of specific cold-stress proteins in the chloroplast is important for plants adaptation to cold stress (Artus et al., 1996), such as increasing the stability of chloroplast membranes during freezing (Steponkus et al., 1998), modification of photosystem II photochemical properties (Hurry & Huner, 1992) and of ROS-scavenging systems (Bowler & Van Montagu, 1992), resulting in the reduction of sensitivity to photoinhibition at low temperature. In addition, mitochondrial also can produce ROS and many metabolites, which may serve as retrograde signals to adapt cold responses (Ng et al., 2014). A defect in the mitochondrial complex I also enhances ROS accumulation and causes the mutant plants to have reduced expression of cold-responsive genes and to exhibit chilling and freezing sensitivity (Lee et al., 2002). Similarly, mutations in CHY1, which encodes a peroxisomal beta-hydroxyisobutyryl (HIBYL)-CoA hydrolase needed for valine catabolism and fatty acid beta-oxidation, also cause ROS accumulation and impair cold-responsive gene expression and freezing tolerance (Dong et al., 2009). Here, our data also showed It is majority of chloroplastic or mitochondrial related proteins were down-regulated, including Ketol-acid reductoisomerase, Glutamate decarboxylase, Malate dehydrogenase, Pentatricopeptide repeat-containing protein, Phosphoglycolate phosphatase, Monodehydroascorbate reductase, Aldehyde dehydrogenase family 2, Monodehydroascorbate reductase, Glycine dehydrogenase, and Probable mitochondrial-processing peptidase. Under cold stress, most aldehyde dehydrogenase gene superfamily members showed decreased expression in grape and Arabidopsis (Zhang et al., 2012). Similar proteomic studies showed that some key enzymes
involved in Krebs cycle (malate dehydrogenase) and many photosynthesis-related proteins (ATP synthase subunits) were down-regulated in wheat exposed to cold stress (Li et al., 2015). In addition, the accumulation of stress defense proteins including Cu/Zn superoxide dismutase, ascorbate peroxidases were significantly increased in bread wheat exposed to cold stress (Han, Kang & Guo, 2013). Many previous studies are consistently showed that GO terms related to photosynthesis and CO\textsubscript{2} fixation are down-regulated as well (Sanz-Saez et al., 2010; Kazemi-Shahandashti & Maali-Amiri, 2018; Yu et al., 2018). More and more evidence showed that chloroplast and photosynthesis are affected when plants subjected to cold stress. The similar results of the reduction of photosynthesis, which we found in pitaya, were also reported in maize. The accumulation of chlorophyll in actively glowing rice leaves was significantly inhibited in cold stress (Glaszmann, Kaw & Khush, 1990). This reduction of photosynthesis might relate to the phenomenon that low temperature conditions cause a reduction in maximum quantum yields for CO\textsubscript{2} uptake, as well as reduce the photochemical efficiency of photosystem II and then result to decrease the rate of light saturated photosynthesis (Kratsch & Wise, 2000; Renaut, Hoffmann & Hausman, 2005). A proteomics study of Thellungiella halophila based on two-dimensional electrophoresis demonstrated that half of differential abundant proteins stimulated under cold stress were identified to related to chloroplast physiology and function (Gao et al., 2009), which also suggesting that partial of cold stress tolerance regulation is going through chloroplast function or metabolism. A comparable analysis of rice seedling proteome also gave similar results (Hashimoto & Komatsu, 2007), further corroborating our pitaya proteomics data.

CONCLUSION

The RNA-Seq technology is an efficient technology for characterizing transcriptomes of the non-model organisms. In this study, the Illumina pared-end sequencing project generated a substantial fraction of pitaya genes can be used as the reference and further investigated the proteome. Label-free proteomic analysis study of pitaya identified 116 DAPs associated with the cold stress in pitaya, of which, 18 proteins were up-regulated and 98 proteins were down-regulated. KEGG analysis and other results showed that these DAPs mainly related to chloroplasts and mitochondria metabolism. In summary, chloroplasts and mitochondrial metabolism-related proteins may play an important role in response to cold stress in pitaya.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was supported by National Natural Science Foundation of China (No. 31560547) and Talents of Guizhou science and technology cooperation platform ([2017]5603). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Grant Disclosures
The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31560547.
Talents of Guizhou Science and Technology Cooperation Platform: [2017]5603.

Competing Interests
The authors declare that they have no competing interests.

Author Contributions
- Junliang Zhou conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Zhuang Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yongya Mao conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Lijuan Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Tujian Xiao conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yang Hu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yang Zhang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yuhua Ma conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability
The following information was supplied regarding data availability:
All RNAseq and proteomic data files are available from the Integrated Proteome Resources: IPX0001296002.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.8540#supplemental-information.
REFERENCES

Ansorge WJ. 2009. Next-generation DNA sequencing techniques. New Biotechnology 25(4):195–203 DOI 10.1016/j.nbt.2008.12.009.

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF. 1996. Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. Proceedings of the National Academy of Sciences of the United States of America 93(23):13404–13409 DOI 10.1073/pnas.93.23.13404.

Bowler M, Van Montagu D. 1992. Inze superoxide dismutase and stress tolerance. Annual Review of Plant Physiology and Plant Molecular Biology 43(1):83–116 DOI 10.1146/annurev.pp.43.060192.000503.

Calzadilla PI, Maiale SJ, Ruiz OA, Escaray FJ. 2016. Transcriptome response mediated by cold stress in Lotus japonicus. Frontiers in Plant Science 7(203):374 DOI 10.3389/fpls.2016.00374.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21(18):3674–3676 DOI 10.1093/bioinformatics/bti610.

Crawford JE, Guelbeogo WM, Sanou A, Traoré A, Vernick KD, Sagnon N, Lazzaro BP. 2010. De novo transcriptome sequencing in Anopheles funestus using Illumina RNA-Seq technology. PLOS ONE 5(12):e14202 DOI 10.1371/journal.pone.0014202.

Dong C-H, Zolman B-K, Bartel B, Lee B-H, Stevenson B, Agarwal M, Zhu J-K. 2009. Disruption of Arabidopsis CHY1 reveals an important role of metabolic status in plant cold stress signaling. Molecular Plant 2(1):59–72 DOI 10.1093/mp/ssn063.

Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A. 2016. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Research 44(D1):D279–D285 DOI 10.1093/nar/gkv1344.

Fowler S, Thomashow MF. 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14(8):1675–1690 DOI 10.1105/tpc.003483.

Gao F, Zhou Y, Zhu W, Li X, Fan I, Zhang G. 2009. Proteomic analysis of cold stress-responsive proteins in Thellungiella rosette leaves. Planta 230(5):1033–1046 DOI 10.1007/s00425-009-0903-6.

García-Cruz L, Valle-Guadarrama S, Salinas-Moreno Y, Joaquin-Cruz E. 2013. Physical, chemical, and antioxidant characterization of pitaya (Stenocereus pruinosus) fruits. Plant Foods for Human Nutrition 68(4):403–410 DOI 10.1007/s11130-013-0391-8.

Glaszmann JC, Kaw RN, Khush GS. 1990. Genetic divergence among cold tolerant rices (Oryza sativa L.). Euphytica 45(2):95–104.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnrirke A, Rhind N, Di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29(7):644–652 DOI 10.1038/nbt.1883.

Han Q, Kang G, Guo T. 2013. Proteomic analysis of spring freeze-stress responsive proteins in leaves of bread wheat (Triticum aestivum L.). Plant Physiology and Biochemistry 63:236–244 DOI 10.1016/j.plaphy.2012.12.002.

Hashimoto M, Komatsu S. 2007. Proteomic analysis of rice seedlings during cold stress. PROTEOMICS 7(8):1293–1302 DOI 10.1002/pmic.200609921.
Hua QZ, Chen CJ, Zhe C, Chen PK, Ma YW, Jingyu W, Jian Z, Guibing H, Jietang Z, Yonghua Q. 2016. Transcriptomic analysis reveals key genes related to betalain biosynthesis in pulp coloration of Hylocereus polyrhizus. Frontiers in Plant Science 6:1179.

Hudson ME. 2008. Sequencing breakthroughs for genomic ecology and evolutionary biology. Molecular Ecology Resources 8(1):3–17 DOI 10.1111/j.1471-8286.2007.02019.x.

Hurry VM, Huner NP. 1992. Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiology 100(3):1283–1290 DOI 10.1104/pp.100.3.1283.

Jimenez G, Gomez G, Perez AM, Blanco-Metzler A. 2012. Estimation of glycaemic index of peach palm (Bactris gasipaes) cooked fruits and chips, and pitahaya (Hylocereus spp.) pulp. Archivos Latinoamericanos De Nutricion 62(3):242–248.

Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y. 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Research 36(Suppl. 1):D480–D484 DOI 10.1093/nar/gkm882.

Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Research 40(D1):D109–D114 DOI 10.1093/nar/gkr988.

Kazemi-Shahandashti S-S, Maali-Amiri R. 2018. Global insights of protein responses to cold stress in plants: signaling, defence, and degradation. Journal of Plant Physiology 226:123–135 DOI 10.1016/j.jplph.2018.03.022.

Koonin EV, Fedorova ND, Jackson JD, Jacobs AR, Krylov DM, Makarova KS, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Rogozin IB, Smirnov S, Sorokin AV, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA. 2004. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. Genome Biology 5(2):R7 DOI 10.1186/gb-2004-5-2-r7.

Kratsch HA, Wise RR. 2000. The ultrastructure of chilling stress. Plant Cell and Environment 23(4):337–350 DOI 10.1046/j.1365-3040.2000.00560.x.

Lappe RR, Baier JW, Boehlein SK, Huffman R, Lin Q, Wattebled F, Settles AM, Hannah LC, Borisjuk L, Rolletschek H, Stewart JD, Scott MP, Hennen-Bierwagen TA, Myers AM. 2018. Functions of maize genes encoding pyruvate phosphate dikinase in developing endosperm. Proceedings of the National Academy of Sciences of the United States of America 115(1):E24–E33 DOI 10.1073/pnas.1715668115.

Lee B-H, Lee H, Xiong L, Zhu J-K. 2002. A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. Plant Cell 14(6):1235–1251 DOI 10.1105/tpc.010433.

Li X, Hao C, Zhong J, Liu F, Cai J, Wang X, Zhou Q, Dai T, Cao W, Jiang D. 2015. Mechano-stimulated modifications in the chloroplast antioxidant system and proteome changes are associated with cold response in wheat. BMC Plant Biology 15(1):219 DOI 10.1186/s12870-015-0610-6.

Liu ZX, Li HC, Wei YP, Chu WY, Chong YL, Long XH, Liu ZP, Qin S, Shao HB. 2015. Signal transduction pathways in Synechocystis sp. PCC, 6803 and biotechnological implications under abiotic stress. Critical Reviews in Biotechnology 35(2):269–280 DOI 10.3109/07388551.2013.838662.

Luo H, Cai Y, Peng Z, Liu T, Yang S. 2014. Chemical composition and in vitro evaluation of the cytotoxic and antioxidant activities of supercritical carbon dioxide extracts of pitaya (dragon fruit) peel. Chemistry Central Journal 8(1):1 DOI 10.1186/1752-153X-8-1.

Mahadevan C, Jaleel A, Deb I, Thomas G, Sakuntala M. 2015. Development of an efficient virus induced gene silencing strategy in the non-model wild ginger-Zingiber zerumbet and
investigation of associated proteome changes. *PLOS ONE* 10(4):e0124518
DOI 10.1371/journal.pone.0124518.

Mahadevan C, Krishnan A, Saraswathy GG, Surendran A, Jaleel A, Sakuntala M. 2016. Transcriptome-assisted label-free quantitative proteomics analysis reveals novel insights into piper nigrum—phytophthora capsici phytopathosystem. *Frontiers in Plant Science* 7(739):785 DOI 10.3389/fpls.2016.00785.

Meena KK, Sorty AM, Bitla UM, Choudhary K, Gupta P, Pareek A, Singh DP, Prabha R, Sahu PK, Gupta VK, Singh HB, Krishanani KK, Minhas PS. 2017. Abiotic stress responses and microbe-mediated mitigation in plants: the omics strategies. *Frontiers in Plant Science* 8(868):172 DOI 10.3389/fpls.2017.00172.

Ng S, De Clercq I, Van Aken O, Law SR, Ivanova A, Willems P, Giraud E, Van Breusegem F, Whelan J. 2014. Anterograde and retrograde regulation of nuclear genes encoding mitochondrial proteins during growth, development, and stress. *Molecular Plant* 7(7):1075–1093 DOI 10.1093/mp/ssu037.

Nie Q, Gao G-L, Fan Q-J, Qiao G, Wen X-P, Liu T, Peng Z-J, Cai Y-Q. 2015. Isolation and characterization of a catalase gene "HuCAT3" from pitaya (*Hylocereus undatus*) and its expression under abiotic stress. *Gene* 563(1):63–71 DOI 10.1016/j.gene.2015.03.007.

Niehl A, Zhang ZJ, Kuiper M, Peck SC, Heinlein M. 2013. Label-free quantitative proteomic analysis of systemic responses to local wounding and virus infection in *Arabidopsis thaliana*. *Journal of Proteome Research* 12(6):2491–2503 DOI 10.1021/pr3010698.

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. 1999. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* 27(1):29–34 DOI 10.1093/nar/27.1.29.

Ortiz TA, Takahashi LSA. 2015. Physical and chemical characteristics of pitaya fruits at physiological maturity. *Genetics and Molecular Research* 14(4):14422–14439 DOI 10.4238/2015.November.18.5.

Pertea G, Huang X, Liang F, Antonescu V, Sultana R, Karamycheva S, Lee Y, White J, Cheung F, Parvizi B, Tsai J, Quackenbush J. 2003. TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. *Bioinformatics* 19(5):651–652 DOI 10.1093/bioinformatics/btg034.

Renaut J, Hoffmann I, Hausman J-F. 2005. Biochemical and physiological mechanisms related to cold acclimation and enhanced freezing tolerance in poplar plantlets. *Physiologia Plantarum* 125(1):82–94 DOI 10.1111/j.1399-3054.2005.00554.x.

Rodriguez-Jimenez B, Dominguez-Ortega J, Ledesma A, Cava-Sumner B, Kindelan-Recarte C. 2014. Generalized urticaria due to yellow pitahaya (*Selenicereus megalanthus*). *Journal of Investigational Allergology and Clinical Immunology* 24(2):124–125.

Sa KJ, Choi SH, Ueno M, Park K-C, Park YJ, Ma KH, Lee JK. 2013. Identification of genetic variations of cultivated and weedy types of *Perilla* species in Korea and Japan using morphological and SSR markers. *Genes & Genomics* 35(5):649–659 DOI 10.1007/s13258-013-0117-1.

Sanz-Saez A, Erice G, Aranjuelo I, Nogues S, Irigoyen JJ, Sanchez-Diaz M. 2010. Photosynthetic down-regulation under elevated CO₂ exposure can be prevented by nitrogen supply in nodulated alfalfa. *Journal of Plant Physiology* 167(18):1558–1565 DOI 10.1016/j.jplph.2010.06.015.

Sogi DS, Sharma S, Oberoi DPS, Wani IA. 2010. Effect of extraction parameters on curcumin yield from turmeric. *Journal of Food Science and Technology* 47(3):300–304 DOI 10.1007/s13197-010-0047-8.

Zhou et al. (2020), *PeerJ*, DOI 10.7717/peerj.8540
Song H, Chu Q, Yan F, Yang Y, Han W, Zheng X. 2016a. Red pitaya betacyanins protects from diet-induced obesity, liver steatosis and insulin resistance in association with modulation of gut microbiota in mice. *Journal of Gastroenterology and Hepatology* **31**(8):1462–1469 DOI 10.1111/jgh.13278.

Song H, Zheng Z, Wu J, Lai J, Chu Q, Zheng X. 2016b. White pitaya (*Hylocereus undatus*) juice attenuates insulin resistance and hepatic steatosis in diet-induced obese mice. *PLOS ONE* **11**(2):e0149670 DOI 10.1371/journal.pone.0149670.

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF. 1998. Mode of action of the COR15a gene on the freezing tolerance of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **95**(24):14570–14575 DOI 10.1073/pnas.95.24.14570.

Stintzing FC, Schieber A, Carle R. 2002. Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear (*Opuntia ficus-indica* (L.) Mill. by high-performance liquid chromatography–electrospray ionization mass spectrometry. *Journal of Agricultural and Food Chemistry* **50**(8):2302–2307 DOI 10.1021/jf011305f.

Suh DH, Lee S, Do Heo Y, Kim Y-S, Cho SK, Lee S, Lee CH. 2014. Metabolite profiling of red and white pitayas (*Hylocereus polyrhizus* and *Hylocereus undatus*) for comparing betalain biosynthesis and antioxidant activity. *Journal of Agricultural and Food Chemistry* **62**(34):8764–8771 DOI 10.1021/jf5020704.

Tang G, Wang S, Lu D, Huang L, Li N, Luo L. 2017. Two-component regulatory system ActS/ActR is required for *Sinorhizobium meliloti* adaptation to oxidative stress. *Microbiological Research* **198**:1–7 DOI 10.1016/j.micres.2017.01.005.

Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA. 2003. The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4**(1):41 DOI 10.1186/1471-2105-4-41.

Vogel JP, Gu YQ, Twigg P, Lazo GR, Laudencia-Chingcuanco D, Hayden DM, Donze TJ, Vivian LA, Stamova B, Coleman-Derr D. 2006. EST sequencing and phylogenetic analysis of the model grass *Brachypodium distachyon*. *Theoretical and Applied Genetics* **113**(2):186–195 DOI 10.1007/s00122-006-0285-3.

Wang X-W, Luan J-B, Li J-M, Bao Y-Y, Zhang C-X, Liu S-S. 2010. De novo characterization of a white fl Y transcriptome and analysis of its gene expression during development. *BMC Genomics* **11**(1):400 DOI 10.1186/1471-2164-11-400.

Weinhold A, Wielsch N, Svatos A, Baldwin T. 2015. Label-free nanoUPLC-MS<sup>E</sup> based quantification of antimicrobial peptides from the leaf apoplast of *Nicotiana attenuata*. *BMC Plant Biology* **15**(1):18 DOI 10.1186/s12870-014-0398-9.

Winfield MO, Lu C, Wilson ID, Coghill JA, Edwards KJ. 2010. Plant responses to cold: transcriptome analysis of wheat. *Plant Biotechnology Journal* **8**(7):749–771 DOI 10.1111/j.1467-7652.2010.00536.x.

Wixon J, Kell D. 2000. Website review: the Kyoto encyclopedia of genes and genomes-KEGG.. *Yeast* **17**(1):48–55 DOI 10.1002/1097-0061(20000930)17:3<225::AID-YEA34>3.0.CO;2-5.

Woo KK, Wong FNF, Chua HSC, Tang PY. 2011. Stability of the spray-dried pigment of red dragon fruit *Hylocereus polyrhizus* (Weber) Britton and rose as a function of organic acid additives and storage conditions. *Philippine Agricultural Scientist* **94**(3):264–269.

Wybraniec S, Stalica P, Jerz G, Klose B, Gebers N, Winterhalter P, Spórna A, Szaleniec M, Mizrahi Y. 2009. Separation of polar betalain pigments from cacti fruits of *Hylocereus*...
pyroclouthis by ion-pair high-speed countercurrent chromatography. *Journal of Chromatography A* **1216**(41):6890–6899 DOI 10.1016/j.chroma.2009.08.035.

Xu M, He D, Teng H, Chen L, Song H, Huang Q. 2018. Physiological and proteomic analyses of coix seed aging during storage. *Food Chemistry* **260**:82–89 DOI 10.1016/j.foodchem.2018.03.129.

Yu H, Ji R, Ye W, Chen H, Lai W, Fu Q, Lou Y. 2014. Transcriptome analysis of fat bodies from two brown planthopper (*Nilaparvata lugens*) populations with different virulence levels in rice. *PLOS ONE* **9**(2):e88528 DOI 10.1371/journal.pone.0088528.

Yu Y, Lv Y, Shi Y, Li T, Chen Y, Zhao D, Zhao Z. 2018. The role of phyto-melatonin and related metabolites in response to stress. *Molecules* **23**(8):1887 DOI 10.3390/molecules23081887.

Zhang Y, Mao L, Wang H, Brocker C, Yin X, Vasiliou V, Fei Z, Wang X. 2012. Genome-wide identification and analysis of grape aldehyde dehydrogenase (ALDH) gene superfamily. *PLOS ONE* **7**(2):e32153 DOI 10.1371/journal.pone.0032153.

Zhao JM, Williams CC, Last RL. 1998. Induction of *Arabidopsis tryptophan* pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. *Plant Cell* **10**(3):359–370 DOI 10.1105/tpc.10.3.359.

Zorina AA, Bedbenov VS, Novikova GV, Panichkin VB, Los DA. 2014. Involvement of serine/threonine protein kinases in the cold stress response in the cyanobacterium *Synechocystis* sp. PCC 6803: functional characterization of SpkE protein kinase. *Molecular Biology* **48**(3):390–398 DOI 10.1134/S0026893314030212.

Zschiedrich CP, Keidel V, Szurmant H. 2016. Molecular mechanisms of two-component signal transduction. *Journal of Molecular Biology* **428**(19):3752–3775 DOI 10.1016/j.jmb.2016.08.003.