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Research Article

Temporal transcriptome analysis provides molecular insights into flower development in red-flesh pitaya

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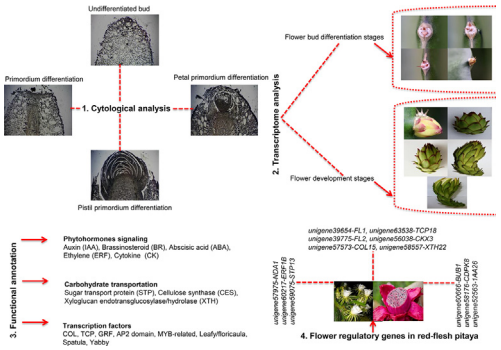
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Pitaya is an important economic fruit worldwide due to its numerous health benefits. A systematic understanding of the mechanism underlying flower development is essential to obtain higher fruit yield and quality. However, the genetic mechanism of flower development is not yet investigated in pitaya. Herein, a transcriptome analysis was performed to determine the transcriptional changes during flower development in red-flesh pitaya by utilizing nine different stages of flower development.
Result: A total of 95,412 unigenes were generated with a mean length of 913 nt, and N50 value of 1878 nt. Comparative transcriptomic analysis showed many differentially expressed genes (DEGs) among the flower growth stages. Furthermore, an array of key DEGs were enriched in hormone signaling, transcription, carbohydrate transport, and energy production pathways. In particular, *indole-3-acetic acid, abscisic acid, ethylene-responsive transcription factor, constans-like, teosinte branched 1/cycloidea/proliferating cell nuclear antigen factor, apetala1-like, agamous-like MADS-box protein, sepallata, growth-regulating factor, putative axial regulator yabby, leafy/floricaula homolog,* and *MYB* gene-associated transcription factors displayed altered expression, suggesting their critical roles in floral organ development of pitaya. Besides, genes related to sugar synthesis, transportation, and utilization mediate flower growth regulation in pitaya. Eleven genes were selected to perform qRT-PCR analysis to verify the results of the RNA sequencing.

Abbreviations: DEG, differentially expressed gene; FPKM, fragments per kilobase of exon model per million reads mapped; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; qRT-PCR, quantitative real-time PCR; TF, transcription factor.

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Transcriptome
Unigenes

Conclusions: Our results provide important insights into the transcriptional regulation of pitaya flower development. These data resources will be a cornerstone for future research that aims at exploring the genetic control of flower development in pitaya.

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1. Introduction

Hylocereus polyrhizus is an edible ornamental fruit plant, originating from Latin America, and widely cultivated in tropical regions [1,2]. It is commonly known as red-fleshed pitaya or dragon fruit and belongs to the family Cactaceae, and genus *Hylocereus* [3,4]. There are 14 different types of species of pitaya but red-flesh and white-flesh (*H undatus*) pitayas are commercially cultivated on a large scale in China, Thailand, Philippines, Israel, Malaysia, Mediterranean regions, and Southeast Asia [5,6,7]. In fact, red-flesh pitaya has more consumer preference than white-flesh pitaya because of its attractive color and nutritive characteristics [2]. Vietnam followed by China is the largest producer and European union countries are the main exporters of pitaya in the world [8]. At present, pitaya is proficiently grown in Guangxi, Guangdong, Hainan, Yunnan, Guizhou, Fujian and Taiwan provinces of China [7]. Pitaya has the ability to tolerate stressful environments like drought, heat, and soil infertility by absorbing carbon through crassulance acid metabolism [9,10,11]. Furthermore, pitaya is an excellent nutritional source of vitamin C, organic acids, sugars, polyphenols [5], phosphorus, calcium, and antioxidants [12]. It has many health-promoting and medicinal values such as the positive impact on digestion, metabolism, vision, immunity, antioxidants, anti-mutation [13], inhibition of diabetes, cardiovascular diseases, cancer, and liver diseases [14,15] Due to these properties, it is an important species to the scientists all over the world.

Flowers develop into fruits; so they are important organ of plants. In pitaya, flowers grow at the side of the stem, with 3–5 flowers on each stem [16,17]. Flower in pitaya is spectacular, hermaphrodite, blooms at night, pollinated in daylight, and requires long sunshine [3,17]. Pitaya needs long days to induce flower. During the short days of winter season it requires supplementary light or temperature to induce flowers [3]. Previous studies have reported that the regulatory mechanism of flowering in fruit plants is complex and involves multiple pathways or gene interaction [18,19]. To date, several flower-related genes have been identified, of which primary genes such as *Flowering locus C* (*FLC*), *Flowering locus T* (*FT*), *Suppressor of Overexpression of Constans 1* (*SOC1*), *Leafy* (*LFY*), *Apetala1* (*AP1*), and *Fruitfull* (*FUL*) are well identified and characterized to induce flowering in *Arabidopsis thaliana* [20,21]. Genetic variants related to phytohormones including auxin, jasmonic acid, gibberellic acid, brassinosteroid, and ethylene have fundamental roles in flower growth and development in plants through signaling and homeostasis [22,23,24,25]. Furthermore, the circadian clock pathway genes including *Constans like* (*CO*), *Late elongated hypocotyl* (*LHY*), and *Circadian clock-associated 1* (*CCA1*) are the critical regulators of flowering time [26,27]. The results of previous research works stated that *Cycling DoF Factor 1* (*CDF1*), *MADS-box*, and *Teosinte branched 1/cycloidea/proliferating cell nuclear antigen factor* (*TCP*) like transcription factor families facilitate the phenomenon of flowering through modification of regulatory genes [28,29,30]. However, functional characterization of genes involved in flower growth and development is not yet cleared in many fruit plants.

In recent years, high-throughput techniques specifically RNA sequencing is widely utilized to explore gene expression profiles in plant species that lack a reference genome [31,32]. The comprehensive transcriptome analysis is an efficient, powerful, and cost-effective method to identify regulatory genes with specific traits in plants [33,34]. Great progress has already been achieved to clear the genetic mechanism of flower development in fruits like Chinese jujube [35], blueberry [36], pineapple [37], sweet cherry [38], almond [39], olive [40], and kiwifruit [41]. Previous transcriptomic studies in red-fleshed pitaya were mainly focused on betalain biosynthesis [2,42], disease resistance [43], stress responses [7], and pulp color formation [44]. However, genetic resources related to flower development are very limited. Molecular research regarding floral bud, sepal, petal, ovule, and ultimately flower development needs to be conducted to uncover the genetic mechanism of reproductive growth in pitaya. This study was conducted to explore the genetic mechanism of flower development in red-fleshed pitaya. There are nine different stages out of which four are used for flower bud differentiation and five for flower development to perform transcriptomic analysis. Furthermore, key regulatory genes were also identified for flower development and bud differentiation. Our results provide useful insights into the genetic bases of flower development in red-fleshed pitaya.

2. Materials and methods

2.1. Plant material and methods of cytological observation

The plant material used in our study was red-fleshed pitaya variety Guihonglong 1. The test material was 4-year-old pitaya plants obtained from the Guangxi Modern Agricultural Technology Demonstration Park, China. For cytological observation, the flower bud tissues of different periods were dissected under the SZM series stereo microscope (Shangrao Ogilvy Electronic Technology Co., Ltd.) to observe the differentiation state of the flower bud growth. Pictures were taken and recorded. For the other part, we first cut flower buds to a length of about 1 cm, and collected fresh flower buds and placed them in FAA fixative solution (50% ethanol:-formaldehyde:glacial acetic acid = 89:6:5 volume ratio) for more than 24 h for paraffin section preparation. Specific steps for paraffin sectioning are as follows: (1) Dehydration: pour off the fixative solution, add 50% ethanol, let stand for 30 min at room temperature; repeat once, let stand for 20 min at room temperature. Soak in 70% ethanol overnight. Continue dehydration the next day, and the alcohol concentration gradient and time are as follows: 80% ethanol for 2 h, 95% ethanol for 2 h, absolute ethanol for 2 h, and 1.5 h (twice). (2) Transparent: 1/2 absolute ethanol + xylene mixture for 2 h, pure xylene for 2 h, 1.5 h (twice). Finally, add a small amount of xylene (submerge the material) and crushed wax, and put it in a 40°C incubator overnight (without opening the lid). (3) Wax immersion: Adjust the temperature of the thermostat to 60°C and time it for 1 h; change to pure wax for 4 h; pure wax (3 times) for 2 h each. (4) Embedding: Pour the liquid wax with the material into the stacked paper slot, and quickly put it

into ice water to solidify the wax, preventing the generation of air bubbles and uneven wax condensation. (5) Sectioning: trim the paraffin block and slice it with a German SLEE semi-automatic rotary paraffin microtome CUT5062 (Beijing Labixin Technology Development Co., Ltd.), to a thickness of 10 μm. (6) Patch and microscopic examination: Apply a little sticky tablet on the glass slide, put the cut wax tape into warm water, fish it on the glass slide, and then use the biological microscope SMART (Aote Optical Instrument Co., Ltd.) Determine its shape and cutting effect, and finally place the slices in a 38°C incubator for 3 h. (7) Dewaxing: xylene for 30 min, xylene for 5 min, 1/2 absolute ethanol + 1/2 xylene mixture for 5 min, absolute ethanol for 5 min, absolute ethanol for 5 min, 95% ethanol for 5 min, 80% ethanol for 5 min. (8) Microscopic examination: The tissue sections were placed under a fluorescence microscope Olympus BX53 (Shanghai Fulai Optical Technology Co., Ltd.) for observation and photomicrographs.

2.2. Sample preparation and RNA sequencing

During the flower bud differentiation and flowering period, nine different flower tissue stages were selected to collect samples in three biological replicates. These stages were flower bud undifferentiated stage (denoted as F1,2,3), flower primordium differentiation stage (F4,5,6), petal primordium differentiation stage (F7,8,9), pistil primordium differentiation stage (F10,11,12), young bud stage (F13,14,15), small bud stage (F16,17,18), medium bud stage (F19,20,21), large bud stage (F22,23,24), and flower opening stage (F25,26,27). The samples were harvested, then immediately frozen in liquid nitrogen, and stored in an ultra-low temperature refrigerator at −80°C before further use. In total, all 27 samples with three technical repeats were used to extract total RNA. High-quality RNA was isolated with TRIZOL® reagent (Life technologies, Carlsbad, CA, USA). Purified RNA was obtained by using RNase-free DNase I (TaKaRa, Kyoto, Japan). The quality and quantity of RNA were tested with Bioanalyzer Agilent 2100 (Agilent technologies, USA). After library construction and quality check, *de novo* pair-end RNA sequencing on the Illumina HiSeq Ten X platform (Illumina Inc., San Diego, CA, USA) was performed following the recommended protocol.

2.3. Unigene assembly, annotation, and analysis of DEGs

Before assembly, the adaptors, ambiguous bases, and low-quality reads were filtered to obtain high-quality clean reads. Trinity with default parameters was used to assemble high-quality reads into unigenes sequences [45]. Unigenes' functional annotations were retrieved and compared with the NCBI Non-redundant nucleotide and protein database (Nr), Swiss-Prot, Clusters of Orthologous Groups of proteins database (COG), Pfam, EuKaryotic Orthologous Group (KOG), Kyoto Encyclopedia of Genes and Genomes database (KEGG), and Gene Ontology (GO) by using blastx with an e-value $<1 \times 10^{-5}$ [46]. The transcript expression level was measured by the FPKM parameter. DEGs were projected with R package DESeq2 [47]. Genes with log2 fold change ≥ 1 and ≤ -1 and a false discovery rate < 0.05 were considered DEGs among different samples. All DEGs were subjected to KEGG enrichment analysis according to Kanehisa et al. [48].

2.4. Quantitative RT-PCR verification of the transcriptome data

To validate our RNA seq data, we selected 11 genes and performed qRT-PCR analysis. Gene specific primer pairs were designed using Primer Premier 5.0 and listed in Table S1. The cDNAs were produced with iScript cDNA Synthesis Kit (BIO-RAD). The iTaq Universal SYBR Green Supermix 50 ml (BIO-RAD) kit was used to prepare a reaction mixture in three technical replicates for each sample. qRT-PCR running parameters and data analysis were followed as per descriptions of Dossa et al. [49]. In our qRT-PCR analysis, *Actin(1)* was the reliable reference gene and served as a quantification control as previously verified in pitaya [50].

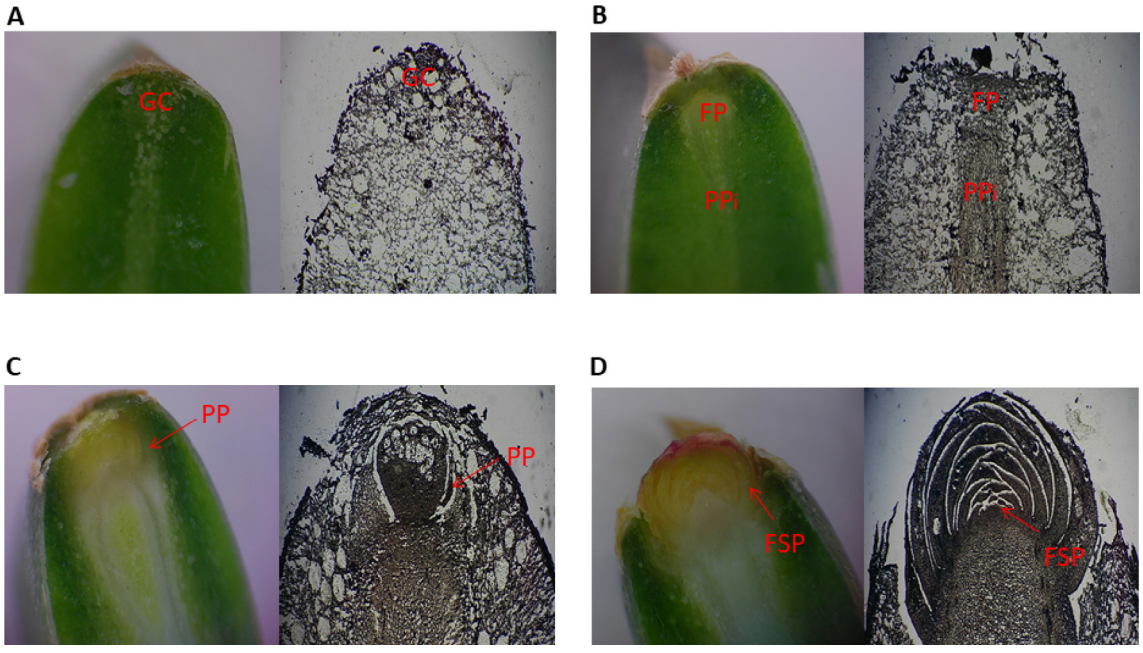

Fig. 1. Morphological and cytological observations during different flower bud differentiation stages in pitaya (A) undifferentiated stage (B) flower primordium differentiation stage (C) Petal primordium differentiation stage (D) pistil primordium differentiation stage. Here, GC: Growth cone, FP: Floral primordia, PPi: Primary pith, PP: Petal primordium, FSP: Female and stamen primordia.

3. Results

3.1. Morphological and cytological observations of flower bud differentiation stages

We performed cytological observations to determine the right sampling stages of flower bud differentiation in pitaya. The paraffin sections showed that pitaya flower bud differentiation needs to undergo a series of morphological changes from the initial stage to the end stage. Based on the characteristics of each stage, it can be divided into four major stages. The first stage is undifferentiated (vegetative growth stage). At this stage, the difference between flower buds and leaf buds is not obvious, and the two cannot be distinguished from the internal structure and external morphology. Moreover, the growth cone at the bottom of the spine begins to expand, the width and height increase at the same time, and the top of the plant gradually changes. It becomes round, bulge in a semi-circular shape and grows upwards, and the number of cells increases (Fig. 1A). The second stage is flower primordium differentiation stage. During this stage, the upper end of the protruding growth cone becomes wider and hypertrophic, bulges upward, and takes a hemispherical shape. Later, the growth point continues to increase, and the primordial meristem cells in the lower end of the growth cone will develop into the primary marrow (Fig. 1B). Throughout the process of flower bud differentiation, the flower primordium is differentiated at the top of the growth cone and it always grows vertically upwards. The formation of flower primordium marks the plant's transition from the vegetative growth state to the reproductive growth state. After this period, pitaya plants begin to undergo the differentiation process of flower organs. The petal primordium differentiation is the third stage. During this stage, the apical meristem expands further, becomes longer and wider, the internal cells continue to elongate, and the growth cone edge produces small protrusions, which are the petal primordia (Fig. 1C). The small protrusions formed on both sides continue to elongate and expand. It appears like half-moon-shaped, curved toward the center, and protecting the entire flower primordium. The last stage of flower bud differentiation is the pistil primordium differentiation. During this stage, flower buds rapidly differentiate and develop, further expand and become longer and wider. The petal primordium splits faster, continues to elongate and widen, and at the same time bends inward, forming a new bulge on its inner side, which is the pistil primordium (Fig. 1D).

3.2. Transcriptome sequencing, unigene assembly, and annotations

In order to have a high resolution gene expression pattern during flower development, nine different stages were selected to execute transcriptome analysis. The stages were comprised of four flower bud differentiation and five flower developmental stages. Hereafter stages were named as flower bud undifferentiated stage (F1,2,3), flower primordium differentiation stage (F4,5,6), petal primordium differentiation stage (F7,8,9), pistil primordium differentiation stage (F10,11,12), young bud stage (F13,14,15), small bud stage (F16,17,18), medium bud stage (F19,20,21), large bud stage (F22,23,24), and flower opening stage (F25,26,27) (Fig. 2). RNA sequencing was performed from 27 different floral bud samples (nine stages with three replicates each). The average number of clean reads was 26,240,914 in this project (Table 1). The percent-

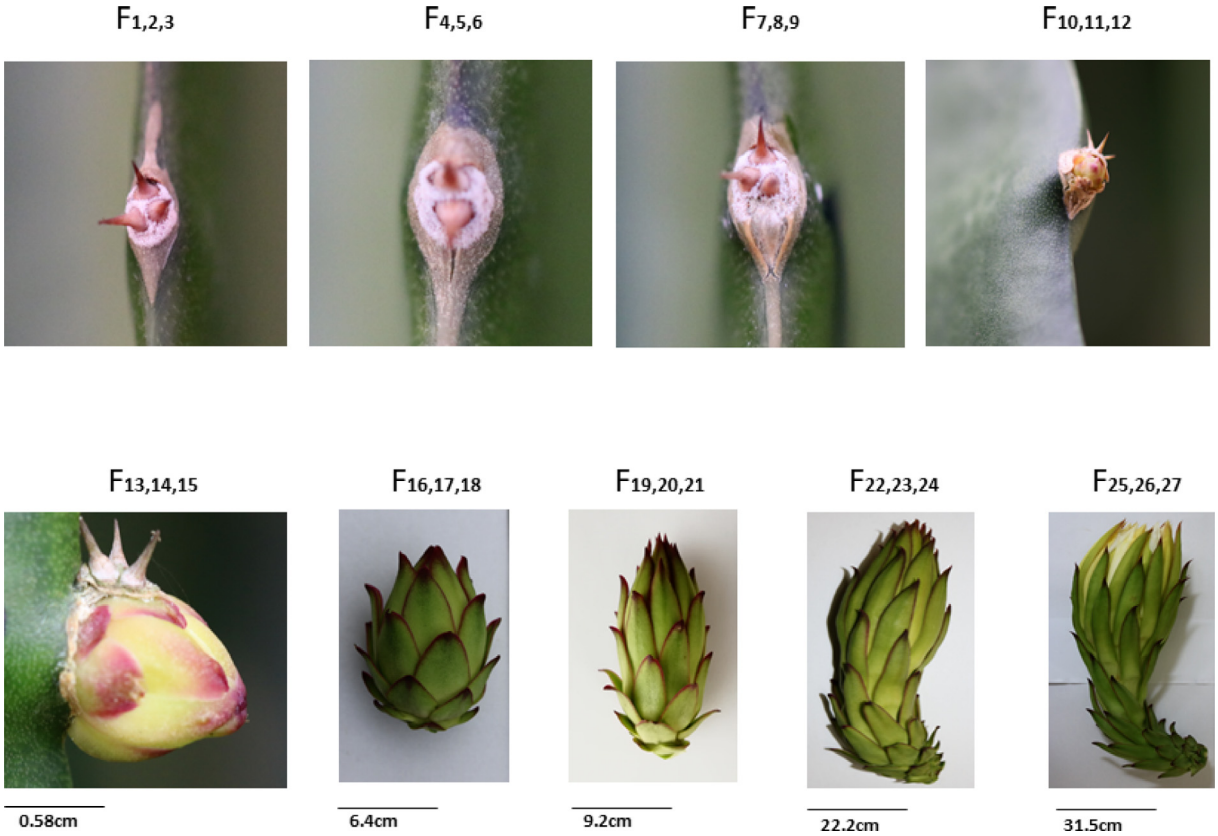

Fig. 2. Nine different flower developmental stages of pitaya were used for transcriptomic analysis. F1,2,3: flower bud undifferentiated stage; F4,5,6: flower primordium differentiation stage; F7,8,9: petal primordium differentiation stage; F10,11,12: pistil primordium differentiation stage; F13,14,15: young bud stage; F16,17,18: small bud stage; F19,20,21: medium bud stage; F22,23,24: large bud stage; F25,26,27: flower opening stage. Here numeric in subscript represents three biological repeats of each stage. Measurements represent mean value of repeats.

Table 1
Overview of the transcriptome sequencing and quality check for 27 sequenced libraries of pitaya fruit.

Samples	Clean Reads	Mapped Reads	GC (%)	Q30 (%)
F01	29891383	84.6	48.5	92.9
F02	29708790	84.6	48.7	92.8
F03	28395269	86.4	50.8	93.1
F04	25425301	85.1	49.3	92.6
F05	26206166	84.9	49.8	92.9
F06	23551340	84.8	49.5	92.2
F07	29730930	85.0	48.8	92.5
F08	25974523	83.4	48.7	92.1
F09	23045666	84.5	48.2	92.6
F10	27076767	83.4	46.8	92.8
F11	22539466	83.0	47.0	92.1
F12	24864527	82.1	46.6	92.8
F13	23872308	82.6	46.8	92.8
F14	21600703	82.9	46.5	92.5
F15	24673375	82.4	46.6	92.4
F16	25663130	85.0	50.2	92.6
F17	29002658	84.3	50.0	92.9
F18	25545244	84.4	50.5	93.0
F19	30968950	81.6	48.8	92.9
F20	25292445	81.6	49.5	93.1
F21	21664937	81.4	49.1	92.6
F22	23396894	82.2	48.7	92.4
F23	25918717	81.8	48.0	93.1
F24	28820376	81.4	49.0	92.8
F25	33543857	81.7	50.1	93.1
F26	26991114	82.8	50.0	92.8
F27	25139851	82.4	50.1	93.0

age of mapped reads was on average 82%. The average Q30% and GC% scores were over 93 and 49%, respectively. These results show the high quality of sequencing data, suitable for downstream analysis. A total of 87,136,072 bases were assembled with the Trinity software. The total number of unigenes assembled was 95412, with a mean length of 913 nt and N50 length value of 1878 nt (Table S2).

The length of unigenes ranged from 200 to more than 3000 nt (Fig. 3A). Due to the unavailability of a whole reference genome of *H. polyrhizus*, the annotation analysis was performed for the assembled unigenes using different public databases. It was found that 31244, 18564, and 18,483 unigenes were annotated in the Non-redundant, in Pfam, and SwissProt, respectively (Fig. 3B). Most of the unigenes had matched sequences with *Beta vulgaris* (24.5%) followed by *Chenopodium quinoa* (19.8%) (Fig. S1). The expression levels of each unigene were determined by fragments per kilobase per million reads (FPKM) value. According to the principal component analysis (PCA), the PC1 and PC2 explained more than 90% of the total variation among all samples (Fig. 4A). Distinct clustering in this study revealed differences between tissues from each stage and fewer variations within the three biological replicated samples of each stage. Furthermore, a strong correlation (>0.90) was observed between the three biological replicates of each stage (Fig. 4B). In contrast, a low correlation was observed between different stages. These findings indicate significant differences between each stage.

3.3. Dynamic change of the transcriptome during flower development in pitaya

Here, we analyzed dynamic changes in transcriptome at the different flower developmental stages of pitaya fruit. Differentially expressed genes (DEG) between each stage were screened with log2 (fold change) value >1 or <−1. The total numbers of DEGs for each comparison together with regulation patterns (Up/down) are shown in Fig. 5. The comparative analysis among flower buds at the undifferentiated stage (F1,2,3) and flower primordium differentiation stage (F4,5,6) showed 205 DEGs, 115 of which were up-regulated and 90 down-regulated in the flower primordium differentiation stage. The lowest number of DEGs (79) was observed in comparison of pistil primordium differentiation stage (F10,11,12) and young bud stage (F13,14,15). This indicates a weak transcriptomic change among these stages. The comparison of the transcriptomic difference between other stages showed significant differences. For instance, the comparison of the large bud stage (F22,23,24) and flower opening stage (F25,26,27) resulted in 7017 DEGs. Similar results were found between the petal primordium differentiation stage (F7,8,9) and the pistil primordium differentiation stage (F10,11,12). A high number of DEGs among different flower development stages is valuable to understand gene expression regulation associated with flower development in pitaya fruit.

3.4. Pathway enrichment analysis of DEGs during flower development in pitaya

To explore the biological mechanism of DEGs involved in the different stages of flower development in pitaya, KEGG enrichment

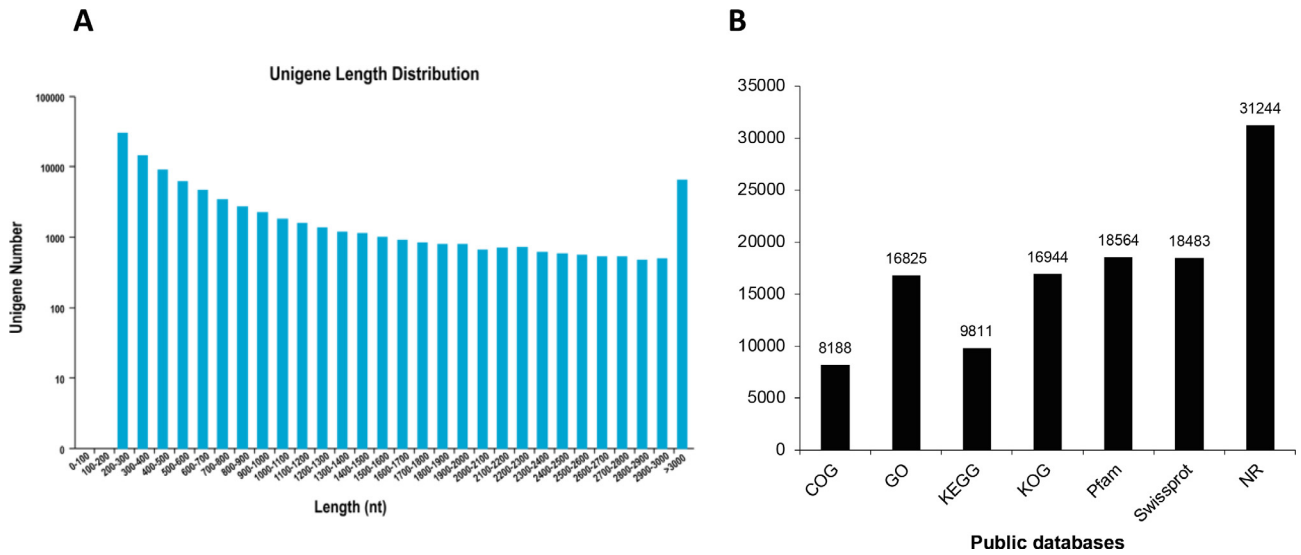

Fig. 3. Length distribution and functional annotations of unigenes. (A) Overall length distribution of sequence (B) Statistics of functional annotations for unigenes in different databases.

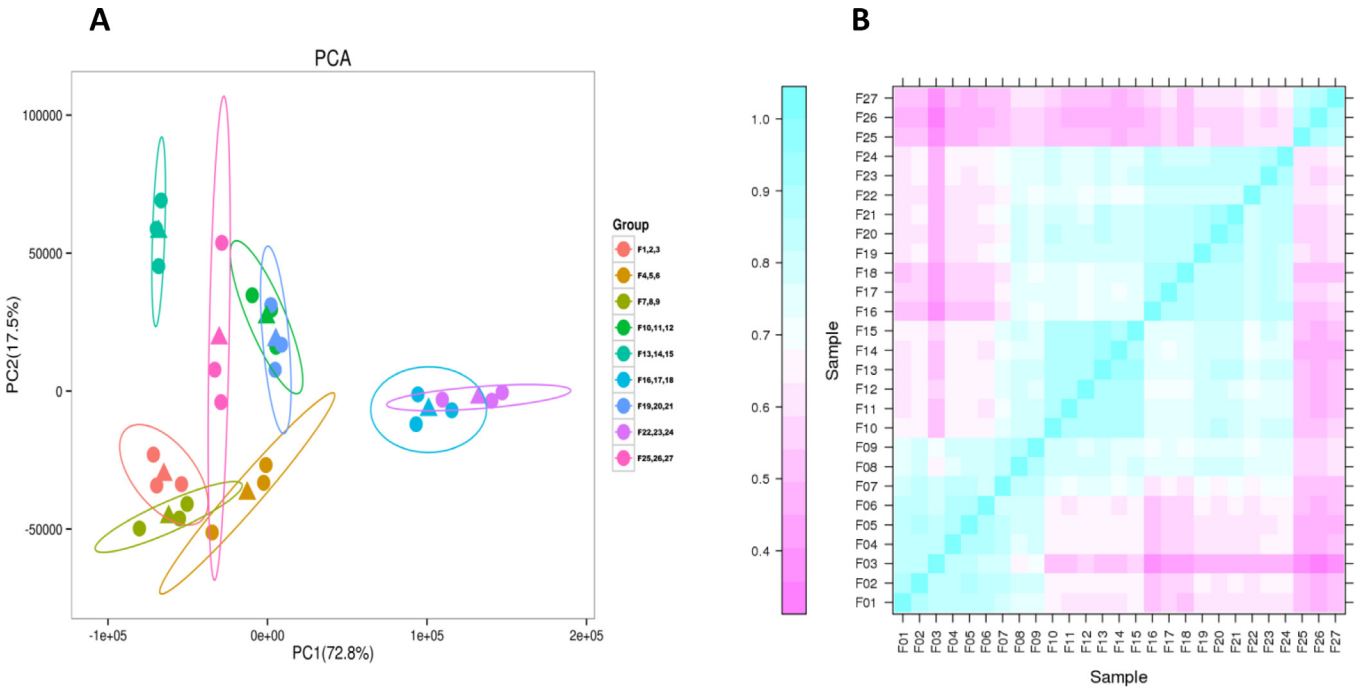

Fig. 4. Principal component analysis and correlation of all samples (A) Principal component analysis (B) Correlation analysis among sample.

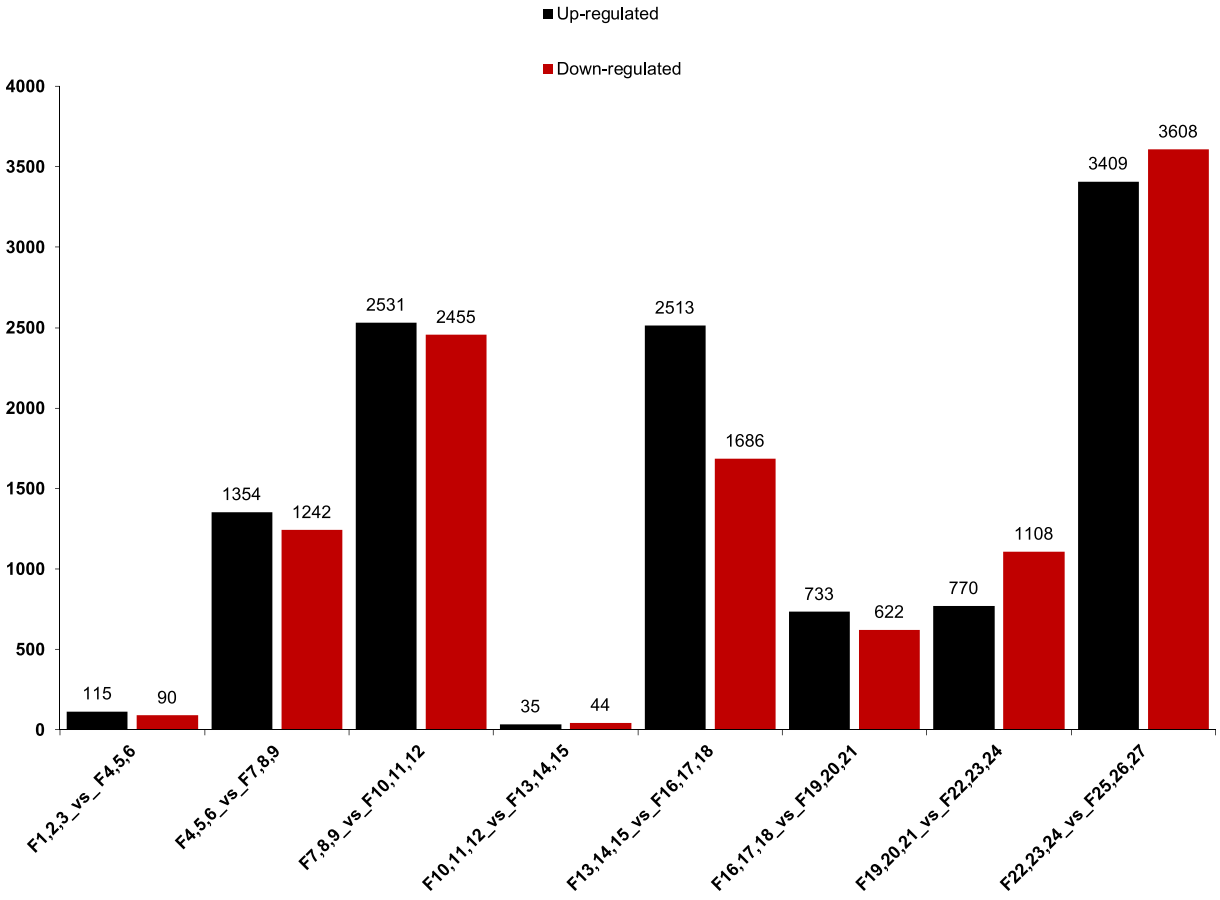

Fig. 5. Gene statistics for DEGs in different flower development stages of pitaya fruit.

analysis (*p-value* < 0.05) for each stage was performed in this study (Fig. 6). Functional enrichment analysis showed that DEGs involved in pathways such as protein processing in endoplasmic reticulum and endocytosis had more significance in comparison to flower bud undifferentiated stage (F1,2,3) and flower primordium differentiation stage (F4,5,6). The majority of DEGs were

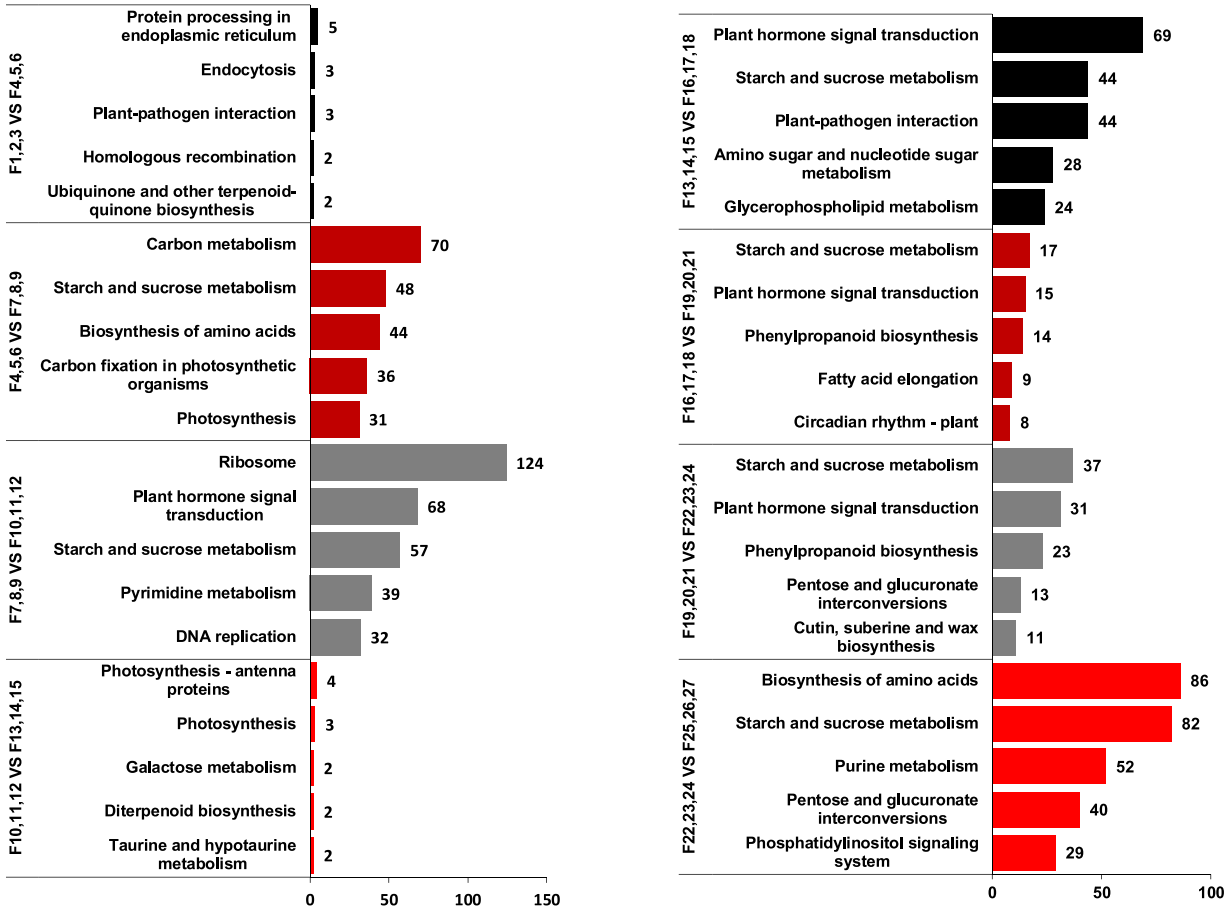

Fig. 6. Most enriched pathways for DEGs in different flower development stages of pitaya fruit.

enriched in sugar metabolism, carbon metabolism, and biosynthesis of amino acids among flower primordium differentiation stage (F4,5,6) and petal primordium differentiation stage (F7,8,9). In the comparison of petal primordium differentiation stage (F7,8,9) and pistil primordium differentiation stage (F10,11,12), ribosome, sugar metabolism, and plant hormones signal transduction pathways had higher significance value and number of DEGs. Pathway categorization revealed the majority of DGES were enriched in photosynthesis-related pathways in comparison to the pistil primordium differentiation stage (F10,11,12) and young bud stage (F13,14,15). Interestingly, plant hormone signal transduction and sugar metabolism were core representative pathways among comparisons including young bud stage (F13,14,15) vs small bud stage (F16,17,18), medium bud stage (F19,20,21) vs small bud stage (F16,17,18) medium bud stage (F19,20,21) vs large bud stage (F22,23,24), and large bud stage (F22,23,24) vs flower opening stage (F25,26,27). The assimilation, distribution, and consumption of nutrients are vital for proper reproductive growth in plants. Thus, genes involved in the biological process related to these pathways may contribute to the flower development in pitaya.

3.5. Gene expression changes during flower bud differentiation stages

Floral bud initiation plays an important role in the biological process of flower development and ultimately governs the destiny of fruit set in plants. The development of floral bud is controlled by many genes, environmental, and physiological aspects [51]. To explore gene regulation during flower bud formation in pitaya, comprehensive comparisons were performed between four different development stages of flower bud differentiation. Flower bud at the undifferentiated stage (F1,2,3) was used as a reference time point and compared with the flower primordium differentiation stage (F4,5,6), petal primordium differentiation stage (F7,8,9), and pistil primordium differentiation stage (F10,11,12). The comparative results revealed large genetic differences in the comparison of flower bud undifferentiated stage (F1,2,3) vs pistil primordium differentiation stage (F10,11,12) (Table S3). On the other hand, both flower bud undifferentiated stage (F1,2,3) and flower primordium differentiation stage (F4,5,6) had a lower number of DEGs. Distribution of DEGs showed less overlapped and more stage-specific DEGs among different stages of flower bud differentiation. This higher portion of stage-specific DEGs is associated with complex gene regulatory changes during floral bud formation. Thus, further excavation identified 47 core genes for flower bud differentiation which exhibited significant altered expression patterns at various stages of flower bud differentiation (Fig. 7). The most representative pathways of core DEGs were carbohydrate transport, hormone signaling, energy production, and transcription factor. Genetic evidence indicated that phytohormones facilitate mechanisms of flower differentiation, formation, and opening in plants [52,53]. In this study, phytohormone-associated genes such as auxin-induced protein 22D-like (*unigene50766-AUX22D*) and auxin-induced protein 22A-like (*unigene54409-AUX22*) were down-regulated with advance floral

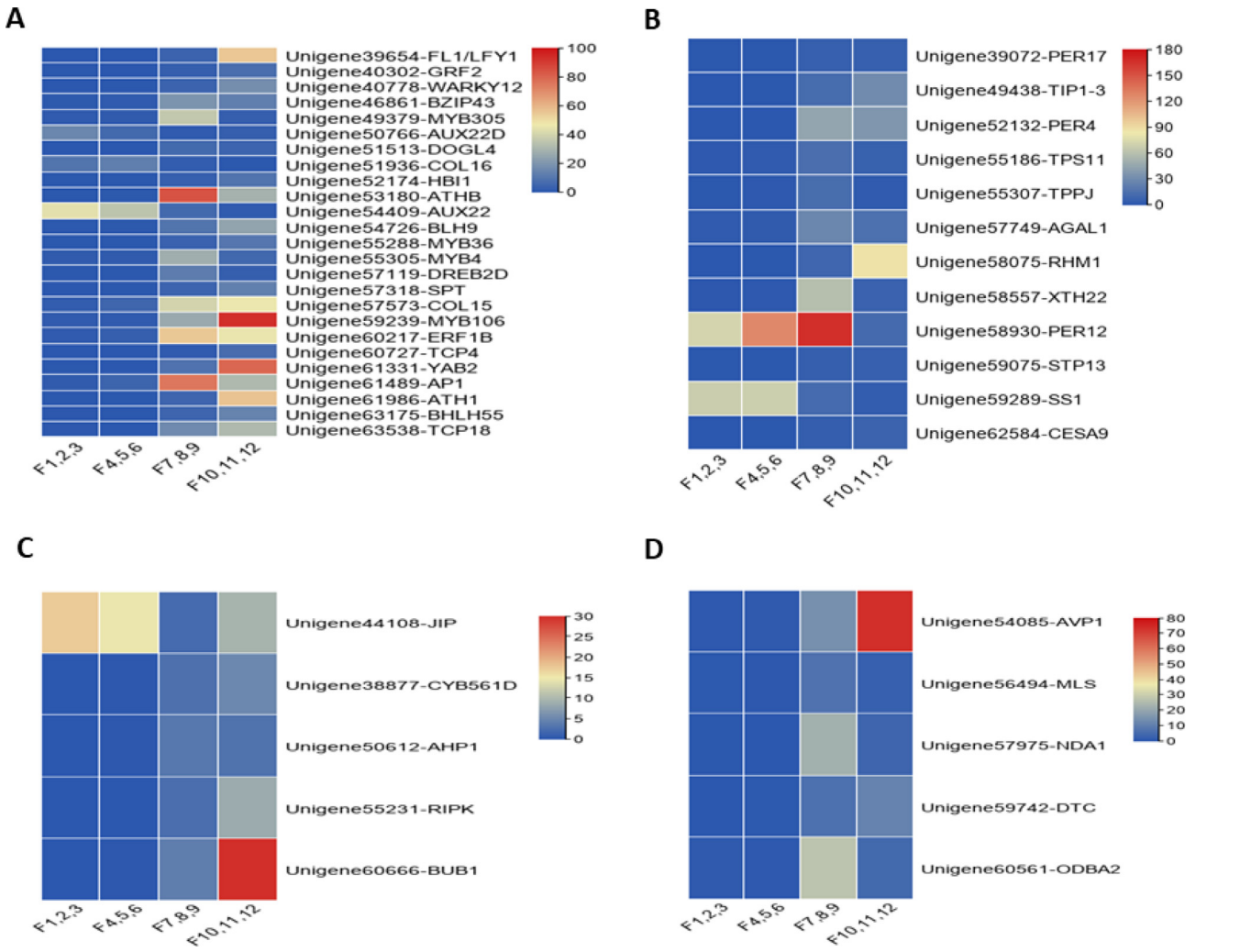

Fig. 7. Expression profile of key genes involved in flower bud differentiation of pitaya (A) Main Transcription factors (B) Carbohydrate transportation (C) Signal transduction (D) Energy production.

bud differentiation process. Only, *AP2* domain/ethylene-responsive transcription factor 1B (*unigene60217-ERF1B*) showed up-regulation during the petal primordium differentiation stage (Fig. 7A).

Interestingly, many genes involved in carbohydrate transportation were observed with a relatively high expression during petal primordium differentiation stage e.g., sugar transport protein 13-like (*unigene59075-STP13*), cellulose synthase A catalytic subunit 9 (*unigene62584-CESA9*), xyloglucan endotransglucosylase/hydrolase protein 22 (*unigene58557-XTH22*), peroxidases (*unigene52132-PER4*, *unigene58930-PER12*, *unigene39072-PER17*). Conversely, the expression profile of starch synthase 1 (*unigene59289-SS1*) was reduced at the petal primordium differentiation stage (Fig. 7B). Besides these, five genes involved in signal transduction (*unigene44108-JIP, unigene38877-CYB561D, unigene50612-AHP1, unigene55231-RIPK, unigene60666-BUB1*) displayed significant altered expressions at different stages of flower bud differentiation (Fig. 7C). Energy production in the form of sugar and protein is a prerequisite for rapid floral bud establishment. Our analysis found five genes (*unigene54085-AVP1*, *unigene56494-MLS*, *unigene57975-NDA1*, *unigene60561-ODBA2*, *unigene59742-DTC*) related to energy assimilation function exhibited up-regulation at petal primordium differentiation stage (Fig. 7D). In particular, our results identified that genes related to transcription factors such as constans-like (*unigene57573-COL15*, *unigene51936-COL16*), teosinte branched 1/cycloidea/proliferating cell nuclear antigen factor (*unigene60727-TCP4*, *unigene63538-TCP18*), putative axial regulator yabby (*unigene61331-YAB2*), growth-regulating factor (*unigene40302-GRF2*), apetala1-like MADS-box (*unigene61489-AP1*), leafy/floricaula homolog (*unigene39654-FL1*), *MYB*-related (*unigene49379-MYB305*, *unigene55288-MYB36*, *unigene59239-MYB106*), spatula (*unigene57318-SPT*), and *AP2* domain/dehydration-responsive element-binding protein 2D (*unigene57119-DREB2D*) most likely performed a critical role in emergence time, morphology, and differentiation mechanisms of flower bud in pitaya (Fig. 7A).

3.6. Gene expression changes during flower development

The flower is produced in plants during the transition from vegetative to reproductive growth. To clear gene regulation during flower development in pitaya, comparisons were made among five different development stages of a flower by utilizing the pistil primordium differentiation stage (F10,11,12) timepoint as reference. It was determined that very fewer gene expression differences (79 total DEGs) existed between the comparison of pistil primordium differentiation stage (F10,11,12) and young bud stage (F13,14,15) stage. In contrast, comparisons of pistil primordium

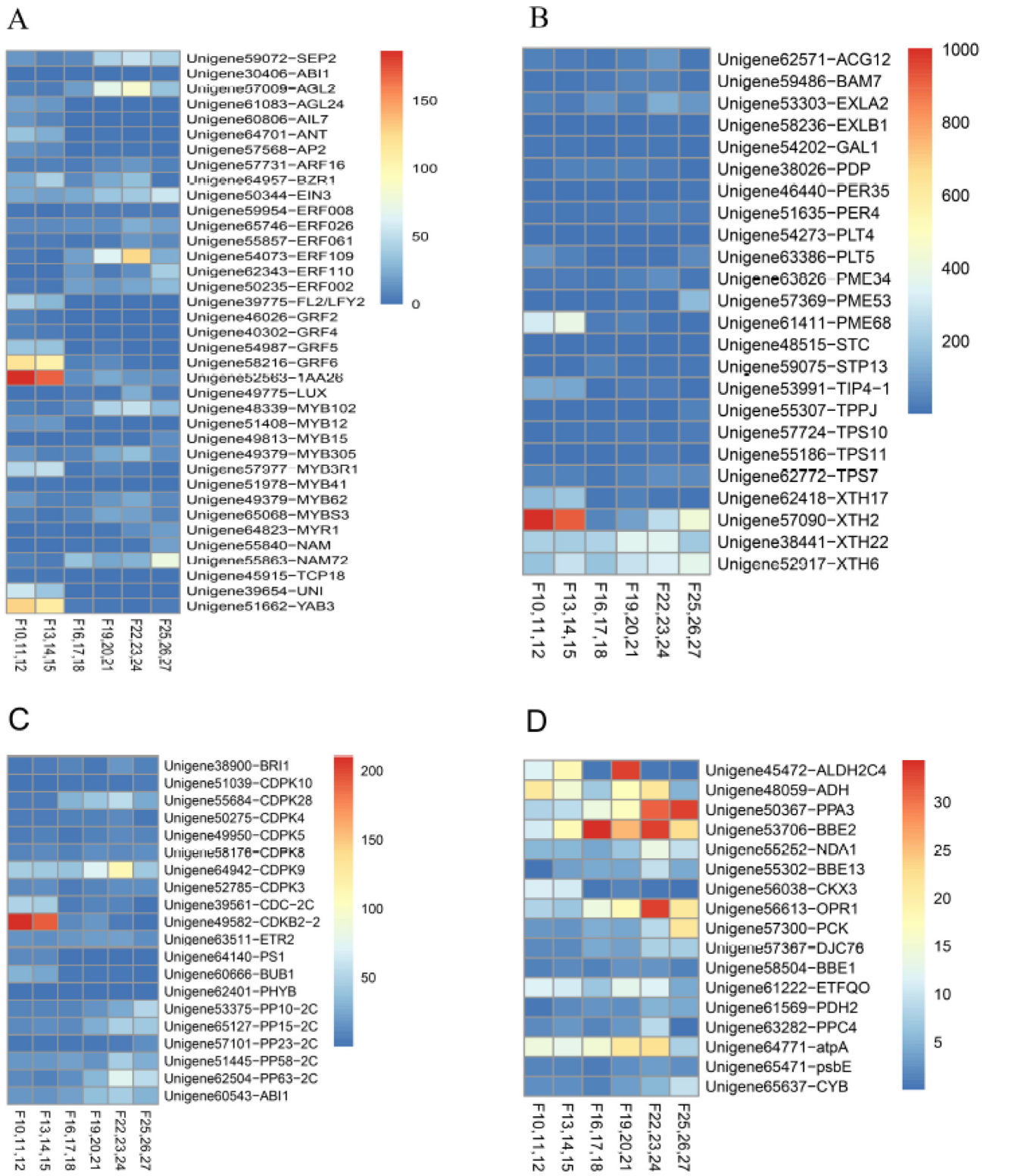

Fig. 8. Expression profile of key genes involved in flower development of pitaya (A) Main Transcription factors (B) Carbohydrate transportation (C) Signal transduction (D) Energy production.

differentiation stage (F10,11,12) with other stages such as small bud stage (F16,17,18), medium bud stage (F19,20,21), large bud stage (F22,23,24), and flower opening stage (F25,26,27) had a higher portion of the total number of DEGs (Table S3). For example, the pistil primordium differentiation stage (F10,11,12) vs flower opening stage flower opening stage (F25,26,27) showed 10,283 total number of DEGs, 4968 of which were up-regulated and 5315 down-regulated in the pistil primordium differentiation stage. The distribution of DEGs revealed 1492 overlapped DEGs among the last four stages of flower development. This higher portion of overlapped DEGs is further sorted by selecting those genes with highly modified expressions during all flower developmental

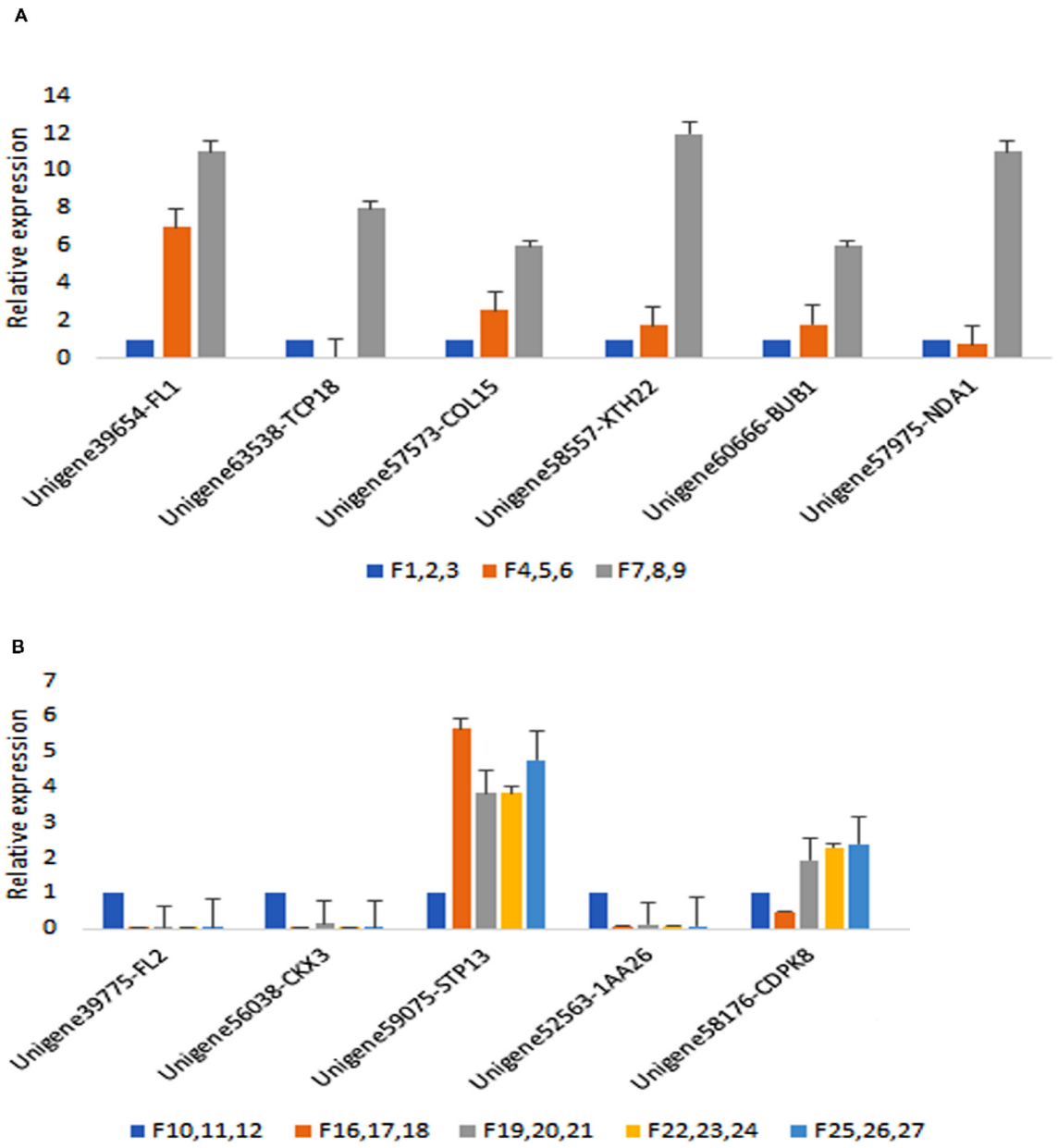

Fig. 9. qRT-PCR of 11 key genes involved in flower development of pitaya (A) Floral bud differentiation stages (B) Flower development stages.

stages. In this way, 75 core genes involved in function related to carbohydrate transport, signal transduction, energy production, and transcription were identified (Fig. 8). Hormonal genes associated with auxin (IAA), brassinosteroid (BR), abscisic acid (ABA), ethylene, and cytokinin had differential expression during different flower growth stages in this study. One abscisic acid insensitive unigene *30406-ABI1* together with IAA unigene*57731-ARF16* were observed with higher expression during large bud and flower opening stages. Similarly, seven ethylene (*unigene50344-EIN3*, *unigene59954-ERF008*, *unigene65746-ERF026*, *unigene55857-ERF061*, *unigene54073-ERF109*, *unigene62343-ERF110*, *unigene50235-ERF002*) functional-related genes showed up-regulation in these two stages of flower development (Fig. 8A). Three genes named *unigene52563-1AA26*, *unigene64957-BZR1*, and *unigene56038-CKX3* related to IAA, BR, and cytokinin, respectively, were upregulated during pistil primordium differentiation and young bud stages than lateral stages of flower development.

Carbohydrate transportation is critical for floral transition in plants. Our results detected that one sugar *unigene48515-STC* and four trehalose-phosphate functional genes named *unigene55307-TPPJ*, *unigene57724-TPS10*, *unigene55186-TPS11*, and *unigene62772-TPS7* showed upregulation during large bud and flower opening stages (Fig. 8B). Conversely, *unigene59075-STP13* and *unigene53991-TIP4-1* involved in the mechanism of sugar transportation and trehalose-phosphate, respectively, were down regulated at these two stages. The expression profile of carbohydrate transporter genes such as expansin-like (*unigene53303-EXLA2*, *unigene58236-EXLB1*), petal death protein (*unigene38026-PDP*) peroxidase, (*unigene46440-PER35*, *unigene51635-PER4*) polyol transporter (*unigene54273-PLT4*), pectin methylesterase (*unigene63826-PME34*, *unigene57369-PME53*), and xyloglucan endotransglucosylase (*unigene38441-XTH22*, *unigene52917-XTH6*) revealed their pivotal role in late stages of flower bud formation (Fig. 8B). Calcium-dependent protein kinases (CDPKs) produce complex signal transduction networks, act as an essential plant nutrient, and regulate plant growth including pollen tube growth in plants [54,55]. In this study, seven CDPK signal transduction pathway genes such as *unigene51039-CDPK10*, *unigene55684-*

CDPK28, *unigene50275-CDPK4*, *unigene49950-CDPK5*, *unigene58176-CDPK8*, *unigene64942-CDPK9*, and *unigene52785-CDPK3* were observed with higher expression during stages of the large bud and flower opening (Fig. 8C). Furthermore, one brassinosteroid (*unigene38900-BRI1*) and one abscisic acid insensitive (*unigene 60543-ABI1)* had undulating expression changes during flower development stages. The expression profile of five protein phosphatase genes (*unigene53375-PP10-2C*, *unigene65127-PP15-2C*, *unigene57101-PP23-2C*, *unigene51445-PP58-2C*, *unigene62504-PP63-2C*) involved in signal transduction showed up-regulation at the flowering stage as compared to pistil primordium differentiation and young bud stage stages (Fig. 8C).

Serval enzymatic genes involved in energy production pathways showed modified expression during different stages of flower developments. For example, two dehydrogenase unigenes *45472-ALDH2C4* and *48059-ADH* were detected with lower expression at flower opening, whereas three reductase unigenes *55252-NDA1*, *56613-OPR1*, *61222-ETFQO*, one ATP synthase subunit alpha (*unigene 64771-atpA*), and three berberine bridge enzyme-like unigenes (*53706-BBE2*, *55302-BBE13*, *58504-BBE1*) had up-regulated expression during stages of the large bud and flower opening but lower expression at pistil primordium differentiation and young bud stages (Fig. 8D).

Transcription factors (TFs) regulate the complex network of flower development in plants either by themselves or by interacting with other TFs, genes, and proteins [50]. This study identified that key flower development TFs including developmental protein sepallata 2 (*unigene59072-SEP2*), agamous-like MADS-box protein (*unigene57009-AGL2*, *unigene61083-AGL24*), *AP2*-like ethylene-responsive transcription (*unigene60806-AIL7*, *unigene64701-ANT*), floral homeotic protein apetala 2 (*unigene57568-AP2*), floricaula/leafy homolog 2 (*unigene39775-FL2*), growth-regulating factor (*unigene46026-GRF2*, *unigene40302-GRF4*, *unigene54987-GRF5*, *unigene58216-GRF6*), *Myb*-related (*unigene48339-MYB102*, *unigene51408-MYB12*, *unigene49813-MYB15*, *unigene49379-MYB305*, *unigene57977-MYB3R1*, *unigene51978-MYB41*, *unigene49379-MYB62*, *unigene65068-MYBS3*, *unigene64823-MYR1*), No apical meristem (*unigene55840-NAM*, *unigene55863-NAM72*), *TCP* (*unigene45915-TCP18*), protein unifoliata (*unigene39654-UNI*), and axial regulator yabby (*unigene51662-YAB3*) are essential and govern genetic mechanism of flower development in pitaya (Fig. 8A).

3.7. qRT-PCR analysis

In this study, 11 putative candidate genes involved in flower development were chosen to confirm the efficacy of RNA-seq data by real-time qRT- PCR analysis. We selected six genes (*unigene39654-FL1*, *unigene63538-TCP18*, *unigene57573-COL15*, *unigene58557-XTH22*, *unigene60666-BUB1*, and *unigene57975-NDA1*) from the first three flower bud differentiation stages (Fig. 9A). Five genes such as *unigene39775-FL2, unigene56038-CKX3*, *unigene59075-STP13*, *unigene52563-1AA26*, and *unigene58176-CDPK8* were chosen from late flower development stages (Fig. 9B). It was observed that gene regulation patterns as calculated by RNA-seq data were similar to qRT-PCR analysis, thereby, certifying the precision of our RNA-seq data analysis in pitaya flower.

4. Discussion

Pitaya is a long-day plant, requires sunshine or light to bloom, and cannot flower in the winter season. Regardless of the economic significance of pitaya, the genetic basis of reproductive growth including floral organ development has been poorly researched. Understanding the regulatory network of flower development is important to increase commercial level production worldwide. This study identified key regulatory transcripts of flower development through transcriptomic data of samples collected at different flower growth stages in pitaya.

4.1. Transcription factors associated with hormone signaling regulate flower development in pitaya

TFs are regulatory proteins that bind to the promoter region to activate or repress the transcription of a specific gene. In this way, TFs regulate gene expression changes and ultimately play a critical role in the growth and development of plants [56]. This study precisely identified several key genes that belong to *AUX/IAA*, *ARF*, *LFY*, *ANT*, *AIL*, *bZIP*, *BZR*, and *ERF* transcription gene families, suggesting their potential roles in floral bud differentiation and flower development of pitaya. Hormones are key chemicals naturally produced by plants. Generally, they affect vegetative and reproductive growth in plants. Furthermore, their concentration, signaling, and homeostasis regulate flower development, fruit set, and stress conditions in plants [3,57]. Our comprehensive analysis revealed that TFs related to IAA, ABA, BR, and ethylene are fundamental for flower development in pitaya. IAA is an active ingredient of flower induction and differentiation in Arabidopsis [52]. Biosynthesis and signaling of IAA are interconnected with TFs such as *ARFs* and *AUX/IAA*. Moreover, functional activation of *AUX/IAA* and *ARFs* caused floral bud initiation and floral organ development in sweet cherry and Arabidopsis [38,58]. In other species such as jatropha, IAA controls sex determination, increases female to male ratio, and enhances seed weight [59]. Higher expression of IAA-related transcripts at the undifferentiated flower bud stage proposes their dynamic role for floral bud generation in pitaya. In detail, degradation of *ARF* activates transcription of floral fate target gene *LFY* and main regulators of floral meristem outgrowth including *ANT* and *AIL* to induce flower primordium [60]. Upregulated expression of *LFY*, *ANT*, and *AIL* at undifferentiated flower bud stage in pitaya hypothesizes that they are fundamental in flower induction. Hormone ABA induces bud dormancy, increases plant abiotic stress tolerance, and controls flowering time [61,62]. Various studies have suggested that ABA not only delays floral transition but also inhibits plant male sterility and sexual reproduction [63,64]. Furthermore, ABA induced expression of *OsABF1* transcript in rice suppresses phenomena of flowering initiation [65]. Flower in pitaya is hermaphrodite. Therefore, modified expression of ABA-related TFs at flowering stage predicts their inhibitory effect on sexual reproduction in pitaya. Phytohormones BR and jasmonic acid regulate a wide range of plant growth and development including floral transition, stamen development, pollen grain maturation, and male fertility [66]. A highly active form of *BZR1* in inflorescence meristem and sepal primordial reduced expression of organ boundary identity transcripts [67]. Lower expression of *BZR1* at sepal differentiation stage may link with enhanced expression of organ boundary identity transcripts in pitaya. In addition, higher expression of *NAM*-related TFs' family genes at flowering stage in pitaya may be crucial for floral organ identity and development as already determined in *Medicago truncatula* [68].

Ethylene is believed to perform essential roles in flower senescence [69], feminism [70], fruit set [71], and fruit ripping [72] in plants. *ERFs* belong to *AP2* domain type TFs, improve plant stress tolerance, and are involved in plant growth mechanisms mediated by ABA, gibberellins, cytokinins, and BRs [73]. Higher expression of several *ERF*-related TFs during large floral tube and flowering stages in pitaya may be vital to induce fruit development together with ABA and BRs. Previous research has shown that the exogenous application of phytohormones specifically jasmonate and salicylic acid alters balances of IAA, ABA, and cytokinin, delays bloom

in fruits to escape extreme winter, and ultimately increases fruit yield [74,75,76]. Our research observed altered expression of IAA, jasmonate, and ethylene responsive genes during flower development in pitaya. Functional quantification of these hormonal genes for pitaya flower development can be dynamic to obtain economical fruiting in winter season. The results of our research observed modified expression of several *CDPKs* and type *2C PPs* genes. Calcium acts as a second messenger for various phytohormones and regulates several cellular and physiological signal transduction in higher plants [77]. Altered expression of *CDPK3*, *4*, *8*, and *10* in coordination with ABA maybe reduced deleterious effects of abiotic stresses including cold in pitaya as already stated in Arabidopsis [78,79]. Another important secondary signal transduction in plants is magnesium or manganese depends on type *2C PPs* genes [80]. Members of subclass A of *PPs* interact with the *ABI1* and *ABI2* to regulate stress signaling and plant development [81]. The higher expression of several type *2C PPs* genes and *ABI1* gene in the flowering stage of pitaya suggests their roles in flower development. Together, this study identified many key genes linked with hormones and secondary signaling molecules. How these genes interact to induce flower in pitaya however needs further investigation.

4.2. CO and ABCE model-related genes formed a key regulatory network of flower development in pitaya

The circadian clock genes related to *CO* are important for photoperiodic flowering, stabilized in light, and switch vegetative growth to flowering in plants [26,33]. Our comparative analysis revealed higher expression profile of *COL6* and *COL15* at the floral bud differentiation stage perhaps controls flowering time in pitaya. In particular, *CO* genes together with late elongated hypocotyl and circadian clock-associated 1 reduced expression of flowering locus genes to initiate flowering signal in the apical meristem during long-day conditions [82]. Additionally, *TCP* binds with *CO* to control flowering time in Arabidopsis [83]. In this study, two TF genes such as *TCP4* and *TCP18* together with the *CO* gene's up-regulated expression suggest their role in vegetative to reproductive transition. Extensive research on molecular mechanism of flower development has revealed specific floral organ identity genes typically called the ABCE model in fruits like apple [84], peach [85], sweet cherry [38], pineapple [37], and olive [40]. Our analysis identified five ABCE model genes with modified expression during different stages of flower growth in pitaya. Two *AP1* and *AP2* from class A, three *AGL2*, *AGL24*, and *SEP2* from class E were in our results confirming that the model is applicable in pitaya. Previous research showed that overexpression of *API* gene from sweet cherry caused early flowering in Arabidopsis [86]. *AP2* is an important regulator of floral transition and seed development [87]. Floral transcriptomes revealed in pineapple that *AP1* and *AP2* play roles in sepal development and *SEP2* is important for the growth of petal, stamen, gynoecium, and ovules. The upregulation of these genes in pitaya suggest similar roles during flower development. *AGL24* upregulated expression at the sepal differentiation stage indicates that it is necessary for the expression of reproductive programs including floral bud organogenesis in pitaya.

The *YABBY* genes performed specific functions in the leaf, carpel, and ovule [88]. In our analysis, *YAB2* and *YAB3* showed higher expression at bud differentiation and sepal stages of flower in pitaya, respectively. These two genes together with filamentous flower genes are required for normal flower growth in Arabidopsis [89]. The *MYB* transcripts are a large family of proteins, functionally diverse, and involved in the ABA response mechanism in plants [90]. This study identified many MYB-related TFs with significant differential expression during flower development in pitaya, predicting their function in floral growth. Especially, *MYB4* and *MYB305* functioning in response to UV light showed high expression at floral bud differentiation [91]. *MYB15*, *MYB41*, and *MYB102* had modified expression during different flower growth stages and particularly *MYB15* may control cold stress in pitaya [92]. *GRFs* are small plant-specific TFs, higher expression in actively growing tissues, control leaf morphology, shoot apical meristem, and flower development in plants [93,94]. According to our results, *GRF2*, *4*, *5*, and *6* showed upregulated expressions in the sepal differentiation stage, suggesting their functions in floral organ development. Many recent studies have already stated that *GRF*-related transcripts mediate flower development. In particular, *GRF1* and *GRF6* regulate flowering time and flower organ development in rice [95,96]. Furthermore, *GRF* genes are important for stress and hormonal regulation [97]. In short, our study identified putative candidate TFs' regulatory network of flower development in pitaya. However, further functional experimentation will be vital to improve our understanding.

4.3. Role of carbohydrate and energy-related genes in flower development of pitaya

Carbohydrate in the form of sugar is a major plant nutrient. Its synthesis and transportation are fundamental for plant growth and development. Leaves are the main source of sugar synthesis and then are transported to other growing tissues including reproductive organs. In our study, carbohydrate transportation and metabolic genes such as *CESA9*, *PMEs*, *SS1*, *STP13*, *TPSs*, *PERs*, and *XTHs* had significant differential expression during floral bud and flower growth in pitaya. Plant cell wall key components are cellulose, pectin, and xyloglucan. *CESA*-related genes mainly control cellulose biosynthesis and ultimately regulate pollen tube growth and embryogenesis in Arabidopsis [98]. While, *PMEs* cause cell wall thickening due to pectin and inhibit pollen tube growth [99]. *XTHs* are enzymes that generally function for cell wall expansion. Several *XTH*-associated genes are supposed to be involved in flower opening and petal abscission in roses [100]. Starch enzymatic-related genes such as *SS* regulate net carbon availability and balancing in plants through an increasing rate of synthesis or decreasing the rate of breakdown [101]. In plants, a high constitutive level of sugar transportation genes including *STP13* improves the capacity to absorb glucose resulting in higher abiotic stress resistance [102]. Trehalose is a soluble sugar and has been found in plants in minor quantities. Interestingly, the *TPS* gene controls agronomic traits including inflorescence architecture in maize [103]. *PER*-related genes are expressed in a wide range of tissues, mediate physiological processes including stress signaling, and IAA metabolism [104]. However, their role in flower development is still not explored. Regarding the key genes involved in energy production and transportation, our analysis identified that genes such as ATP synthase, reductases, and oxidases, and dehydrogenases probably participate in the flower growth in pitaya through coordination with other regulatory genes.

5. Conclusions

We reported a comprehensive transcript profiling at different development stages of pitaya flower. At the gene expression level, TFs related to hormone and floral organ identity mainly mediate flower development in pitaya. Genes involved in nutrient assimilation and transportation have critical functions in flower growth. Our results identified many putative candidate genes for the phenomenon of pitaya flower development. However, further research for functional characterization of these candidate genes is needed. In addition, further transcriptome data from different genotypes and more stages of flower development could be generated and combined with our report in order to build a comprehensive gene

co-expression regulatory network for flower development in pitaya.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

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Data availability

The raw RNA-seq data have been submitted to NCBI SRA under the project number PRJNA725049 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA725049).

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