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Transcriptomic analysis of the development of *Opuntia albicarpa* fruits in relation to carbohydrate metabolism and flavonoid biosynthesis

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Abstract. *Opuntia albicarpa* is an endemic species of Mexico with cultural and economic importance. The opuntia fruits, named prickly pear or tunas, are rich in glucose, fructose, and sucrose. The objective of this study was to contrast morphological changes in the development and ripening of the fruits, and detect genes involved through transcriptomics to identify some important metabolic pathways. Total RNA from fruits of three developmental stages was sequenced in paired-end mode with Illumina technology. High-quality reads were assembled de novo and annotated against the Swissprot protein database, and differentially expressed genes between the three stages were determined. The de novo ensembled transcriptome was represented by 198,958 transcripts (41.95% GC content). A total of 22,460 and 39,683 sequences were annotated with Blastx and Blastp, respectively. In addition, 10,106 genes were identified from Blastx and 11,781 genes with Blastp. In the differential expression analysis for the comparison 60 days after flowering vs. the start of ripening, 1572 transcripts were upregulated and 412 downregulated, in 60 days after flowering vs. ripened fruit, there was an upregulation of 1265 transcripts and 454 downregulated, and at the start of ripening vs ripened fruit, 96 transcripts were upregulated and 745 downregulated. The pathways of carbohydrate metabolism and flavonoid biosynthesis during the development and ripening of fruits were analyzed to quantify the genes involved. The results can be used for genetic and molecular studies on the genus Opuntia and better understand its biology.

Keywords: *De novo assembly, Gene expression, Prickly pear, Ripening fruit, Metabolic pathways*

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Introduction

The genus Opuntia (nopal or prickly pear) is mainly distributed in desert and semi-desert areas of the southern United States of America, Mexico, South America; as well as in different parts of the world (Granados-Sánchez and Castañeda-Pérez, 2003). This genus comprises between 150 and 180 recognized species (Majure *et al.*, 2012). In Mexico, the fruits and stems of wild species, in the process of domestication and cultivated are consumed (López-Gutiérrez *et al*., 2015; Ruiz-Juárez *et al*., 2024). Plants of the genus Opuntia serve as sources of fruits and vegetables for food, medicine, cosmetic purposes, fodder, and as a source of natural colorants; in addition, due to its great importance in the development of desert agriculture, in recent years, the beneficial potential for health of some of its bioactive compounds such as phenols, flavonoids and betalains has been reported (Mendez-Farroñan, 2017).

The taxonomy of Opuntia has been based on morphological characters; it was subsequently enriched with biochemical, physiological and cytogenetic descriptors (Valdez-Cepeda *et al.*, 2003; Reyes-Agüero *et al.*, 2009). Their characterization with molecular markers has made it possible to know more efficiently the existing phenetic and genetic diversity, support its best taxonomic location and contribute to improvement programs (Illoldi-Rangel *et al.*, 2012). The molecular markers represent reliable tools for the characterization and differentiation of Opuntia varieties, since they are not influenced by the environment (Valadez-Moctezuma *et al.*, 2015). But little has been studied about its transcriptome.

The white fruit (tuna), also called "tuna Blanca of Alfajayucan", "tuna Blanca San Martín" or "tuna Reyna" (*O. albicarpa* Scheinvar) presents an ovate fruit 6 to 9 cm long and 6 to 6.5 cm wide, externally greenish-whitish-yellowish and with a 5-7 mm wide shell (Scheinvar, 1999). Fruit growth lasts up to 132 days after flowering and the ripening process begins at 99 days (Martín del Campo *et al.*, 2019). Analysis of gene expression during fruit development can provide valuable clues about the biological functions of the corresponding genes (Chen *et al.*, 2019). It is important to know the genetic dynamics involved in prickly pear fruit ripening to identify differentially expressed genes (DEG), KEGG pathways, and GO terms. In addition, with the differential expression of genes during the ripening process of fruits resolved with transcriptomics, metabolic pathways related to specific metabolic processes can be analyzed.

Transcriptomics is the study of transcriptome of an organism; that is, the total number of transcripts expressed at the time of sampling. The information content of an organism is recorded in the genes of its genome and expressed through transcription (Lowe *et al.*, 2017). Transcriptomics is an essential area to interpret the functioning of the transcriptional process of genes, which depends on intra or extracellular stimuli that trigger signalling cascades in which genes are expressed or repressed (Sims *et al.*, 2014). RNA-seq is a tool that allows to evaluate the transcriptional state of an organism, organ or tissue. It is typically used to compare different treatments, conditions, or stages of development in search of a specific response to the stress or stimulus received (Van Verk *et al.*, 2013). The objective of this study was to contrast morphological changes in the development and ripening of the fruits, and detect genes involved through transcriptomics to identify important metabolic pathways.

Material and Methods

Sampling

The sampling of *O. albicarpa* fruits was carried out from April to July 2021 in 5-year-old commercial orchards in the municipality of San Martín de las Pirámides, State of Mexico (19° 41' 59.9" N 98 ° 48' 31.6" W). Three plants with a good phenotype and free from damage caused by biotic or abiotic factors were selected. Three fruits were collected from each plant during five stages of development (T1: anthesis, T2: 30 days after anthesis, T3: 60 days after anthesis, T4: beginning of ripening, and T5: ripe fruit, Figure 1). Three replications were considered from each stage of development. The fruits were transported in a cooler to the laboratory, where they were washed with Extran® detergent from the Merck® brand and rinsed with water. Using a scalpel, longitudinal cuts were made in the fruits, which were then wrapped in aluminium foil and preserved in liquid nitrogen until processing.

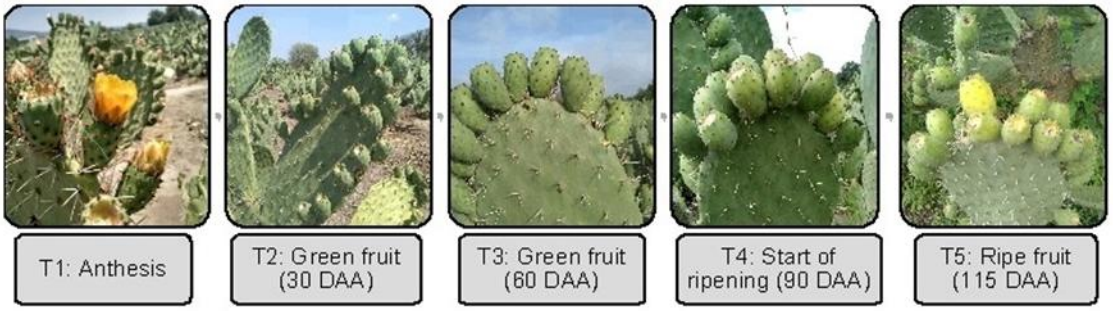

Figure 1. Phenology of the development and maturation of prickly pear fruits (*Opuntia albicarpa* Scheinvar), DAA=Days after anthesis.

Morphological evaluation

For the morphological evaluation, 25 fruits of each stage of development were selected, to which the total fresh weight of the shell and loculus; equatorial diameter, polar diameter and volume were measured. The fresh weights (g) were measured with a digital scale (Sartorius® model PT1200, precision 0.01 g); the equatorial (cm) and polar (cm) diameters were measured by a vernier and the volume with a graduated cylinder. Analysis of variance (ANOVA) was performed with a completely randomized design where time was the source of variation, and Tukey's means comparison tests (0.05); these were done with the Excel plugin XLSTAT®.

RNA extraction and sequencing

For RNA extraction, three grams of total tissue from the fruit were ground in a sterile mortar using liquid nitrogen (Rivas-Garcia *et al*., 2024). Total RNA was extracted with the PureLink® RNA Mini Kit (Invitrogen, USA) following the manufacturer's instructions. The quality and concentration of RNA for each sample were determined using a Nanodrop® UVS-99 UVISDrop spectrophotometer. The libraries were synthesized with the TruSeq® Stranded mRNA LT Sample Prep kit. Sequencing was performed on the NovaSeq6000® (Illumina) system in paired-end mode, with a read length of 101 base pairs.

Data filtering and de novo assembly

Quality control analysis of raw reads was performed with FastQC® v0.72 (Andrews, n.d.) and MultiQC® v1.9 (Ewels *et al.*, 2016). Low quality (phred quality score < 30) and short (length < 60 bp) reads were removed using the Trimmomatic® v0.38.0 program (Bolger, *et al.*, 2014). As there is no reference genome for the genus's Opuntia, the assembly of the transcription sequences (de novo transcriptome) of the readings of the T3, T4 and T5 libraries was performed with Trinity® v2.9.1., applying the default parameters and a contig length of 200 bp (Grabherr *et al.*, 2011). The reads from libraries T1 and T2 were not used due to not meeting the desired quality. A set of representative non-redundant sequences was obtained with CD-HIT-EST® v1.3. (Fu *et al.*, 2012) using a 98% similarity threshold to eliminate potential duplicate transcripts. The Busco® v5.3.2 program (Simão *et al.*, 2015) was used for the evaluation of the quality of the genome assembly, in this analysis the linnage Viridiplantae (green plants and terrestrial algae) was selected. The quality control (FastQC, MultiQC and Trimmomatic) and the transcriptome assembly (Trinity, CD-HIT-EST and Busco) was carried out on the Galaxy web platform (usegalaxy.org) (Afgan *et al.*, 2018).

Functional Annotation

The TransDecoder® v5.5.0 tool (Haas *et al.*, 2013) was used to identify coding regions (with at least 100 amino acids) within the sequences of the transcripts; the input file was the representative assembly. The de novo assembly, and the peptide sequences (Transdecoder result) were aligned against the SwissProt database (56,7483 sequences) (The UniProt Consortium 2021) using Diamond Blastp and Diamond Blastx for an e-value < 10-3. TransDecoder and Diamond was carried out on the Galaxy web platform (usegalaxy.org) (Afgan *et al.*, 2018). In addition, EggNOGG v5.0 (http://eggnog-mapper.embl.de/) (Huerta-Cepas *et al.*, 2019), a tool that uses pre-calculated orthologous groups (OGs) and phylogenies from the EggNOGG database, serves to transfer functional information from detailed data on orthologs; it was used to annotate the ensemble, in which Gene Orthology term, Enzyme Commission (EC), KEGG-Ko (molecular functions represented in terms of functional orthologs), KEGG-pathway (collection of route maps that represent the knowledge of interaction, reaction and molecular relationship networks). The output of peptides from TransDecoder was used as input file and the parameters defaulted by the program.

Differential expression and gene orthology (GO)

Align Reads and Estimate Abundance v2.9.1 software was used to estimate the abundance of isoforms and genes. The abundance estimates from the samples were combined in a single file with the Build Expression Tool Matrix v2.9.1 (Grabherr *et al.*, 2011). To determine the differentially expressed genes between the three times of fruit maturation, the Limma V3.48.0 program was used (Law *et al.*, 2014). Three pairwise comparisons were evaluated: T3 vs T4, T3 vs T5 and T4 vs. T5. The previous tools are available in the Galaxy web platform (usegalaxy.org) (Afgan *et al*., 2018). Differentially expressed transcripts (DET) with |logFC>2| and with adjusted P-value < 0.01 were analyzed in KOBAS-I (http://kobas.cbi.pku.edu.cn/) (Bu *et al.*, 2021) to identify significantly enriched KEGG pathways and GO terms (e-value ≤ 0.0001), using the *Arabidopsis thaliana* genome as a comparison database.

KEEG pathways and GO terms analysis of DET

DET were extracted and explored in the “Amino sugar and nucleotide sugar metabolism”, “Ascorbate and aldarate metabolism”, “Fructose and mannose metabolism”, “Starch and sucrose metabolism”, “Galactose metabolism” and “Flavonoid biosynthesis” pathways; as well as in the GO terms “Sucrose metabolic process”, “Glucose metabolic process”, “Carbohydrate metabolic process” and “Flavonoid biosynthetic process”. Heatmap plots were made with the Heatmap2 Version 3.1.1 tool in Galaxy web platform (usegalaxy.org) (Afgan *et al.*, 2018), to map the DET over the three fruit stages in the libraries using the Z transformation of the normalized TMM values.

Results

Morphological evaluation

In the flowering stage (T1), the fruit presented a green color and its flowers, yellow petals and pink sepals, as well as an abundant number of spines. The loculus represented 28.19% of the total weight of the fruit, and the shell does not separate from the loculus. In the stage 30 days after flowering (T2), the fruit presented a green color, and the flower was completely detached. The weight of the loculus represented 22.83% of the total weight, and the shell has not yet separated from the loculus. In the stage 60 days after flowering (T3), the fruit was green; the weight of the loculus was 22.62% with respect to the full weight and from this stage the peel separates from the loculus. In the early stage of

ripening (T4), the fruit had a green/yellow color; the loculus weight corresponded to 57.11% of the total weight. In the ripe fruit stage (T5), it presented a lemon color, and the loculus weight represented 62.13% of the total weight. The ANOVA indicated that there were significant statistical differences in the morphological variables evaluated depending on the stage of development. The fruit registered a continuous increase in size, which in T5 reached a polar diameter of 7.30 cm, an equatorial diameter of 5.004 cm and a volume of 92.00 cm -3. From stage T3, loculus weight increased rapidly, while shell weight decreased in T4 and increased again in T5 (Figure 2).

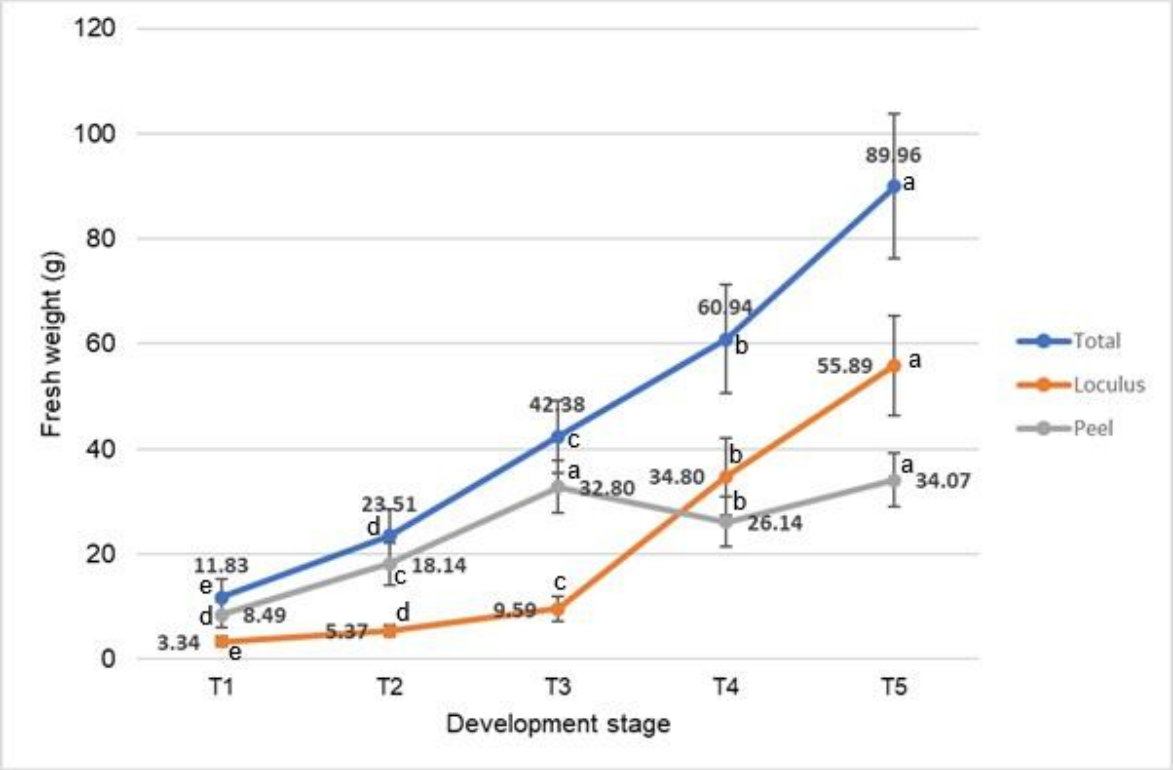

Figure 2. Changes in fresh weight of locule and peel during the development of *Opuntia albicarpa* Scheinvar fruits. Means with different letters are significantly different (Tukey, p≤0.05).

Data filtering and de novo assembly

A total of 704,150,890.00 raw paired-end reads with a length of 101 bp were obtained from fruits 60 days after flowering (T3), onset of ripening (T4) and ripe fruits (T5) of *O. albicarpa*. The number of reads ranged from 68,799,748 (library T3R1) to 87,957,182 (library T3R2). Raw sequence files were uploaded to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra/) under BioProject accession numbers PRJNA803369 (SAMN25649427-35). After quality control (Phred score > 30), a total of 631,639,944 (89.67%) high-quality reads survived, ranging from 61,328,136 (library T3R1) to 79,921,230 reads (library T5R5). The percentage of duplicate reads ranged from 66.10% (library T4R1) to 90.90% (library T3R3) with a mean of 82.27%. The de novo transcriptome assembly of *O. albicarpa* produced 240,169 transcripts. Using CD-HIT-EST to remove duplicates and cluster representative sequences resulted in 198,958 transcripts. For representative sequences, the total GC content was 41.95%; the average transcript length was 1,003 bp; the N50 was 1,734 bp, and the transcript lengths ranged from 181 to 10,434 bp. The general alignment rate ranged between 87.26 (T5R3) and 98.32 (T3R3) with an average of 95.97%, which indicates a good

quality of the assembly. The Busco® program revealed the presence of 97.6% of the 425 orthologous genes (from 57 species) searched for in the viridiplantae dataset, of which 415 orthologous genes were complete (94 were single copies, and 321 were duplicates); 10 were fragmented and any missing orthologous genes were reported; this indicates a good quality of the assembly.

Functional Annotation

Prediction of coding regions using the Transdecoder program resulted in 116,693 PEPs and 398,706 ORFs. The Diamond-Blastx analysis generated 22,460 sequences, of which 21,842 that correspond to plants were identified; while with Blastp, 39,686 sequences were obtained, of which 38,587 correspond to plants. 20,846 transcripts were identified that were present in both Blastx and Blastp (Figure 3A). The most represented plant species in Blastp were: *Arabidopsis thaliana* (78.03%), *Oriza sativa* subsp. Japonica (4.70%), *Nicotiana tabacum* (1.44%), *Solanum lycopersicum* (1.23%), *Spinaca oleracea* (1.22%), *Oriza sativa* subsp indica (0.94%), *Glycine max* (0.79%), *Solanum tuberosum* (0.76%), *Pisum sativum* (0.66%), *Vitis vinifera* (0.59%), *Zea mays*, (0.49%), *Petunia hybrida* (0.46%), *Beta vulgaris* (0.29%) and *Ricinus communis* (0.27%). By eliminating isoforms, 10,106 and 11,781 genes were identified from Blastx and Blastp data (Figure 3B). In total, 12,565 genes were detected.

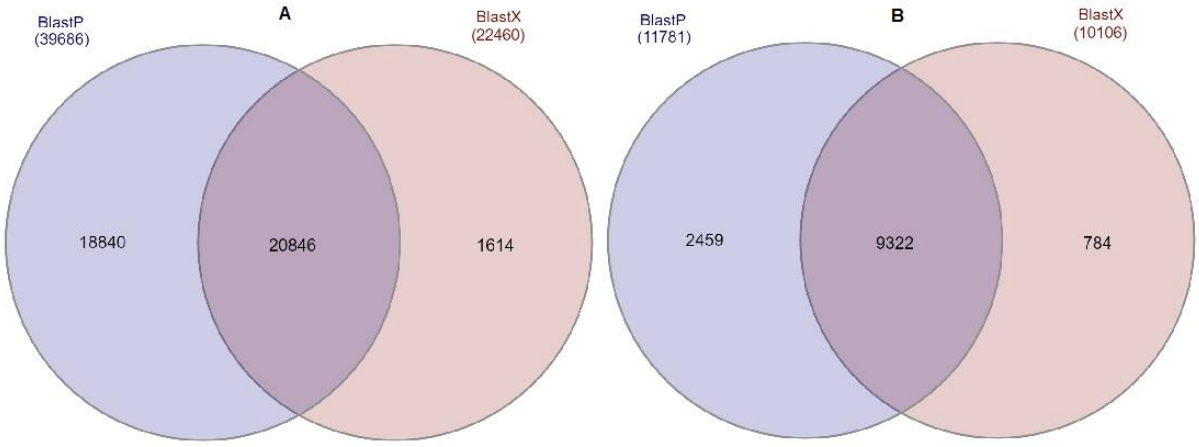

Figure 3. A) Venn diagram with the number of transcripts annotated with Diamond BlastX and BlastP against the UniProtKB/Swiss-Prot database. B) Venn diagram with the number of genes annotated with Diamond BlastX and BlastP.

Analysis performed in EggNOG-mapper® indicated that 37,352 transcripts were associated with at least one GO term and 22,816 transcripts with at least one KEGG pathway. Overall, a total of 11,055 GO terms were identified (25.51% of the total 43,335 in the GO database). In addition, a total of 395 routes from the KEGG map (70.53% of the total 560 pathways in the KEGG database) and 3,978 KOs (15.60% of the total 25,503 in the KEGG Orthology database) were identified.

Differential expression and gene orthology (GO)

Analysis of differentially expressed transcripts (DETs) with log absolute > 2 and adjusted P-value < 0.01 was performed using aligned reads from the de novo assembled transcriptome. A total of 2,059 DETs were identified between T3 and T4, with 1,636 upregulated and 423 downregulated transcripts. For the comparison of T3 vs. T5, 1,797 DETs were identified, with 1,317 transcripts significantly

upregulated and 480 transcripts downregulated. Between T4 and T5, 841 DET were detected with 83 upregulated and 758 downregulated transcripts. The Figure 4 shows the number of DETs of each comparison, and those that are shared with each other, eight transcripts were identified that share the three comparisons made; these correspond to OP063301 (BOR4), OP109553 (ABR1), OP131956 (LOC101215231), OP134022 (TDT), OP151319 (TIFY9), OP151321 (TIFY9) and OP184469 (PMA4).

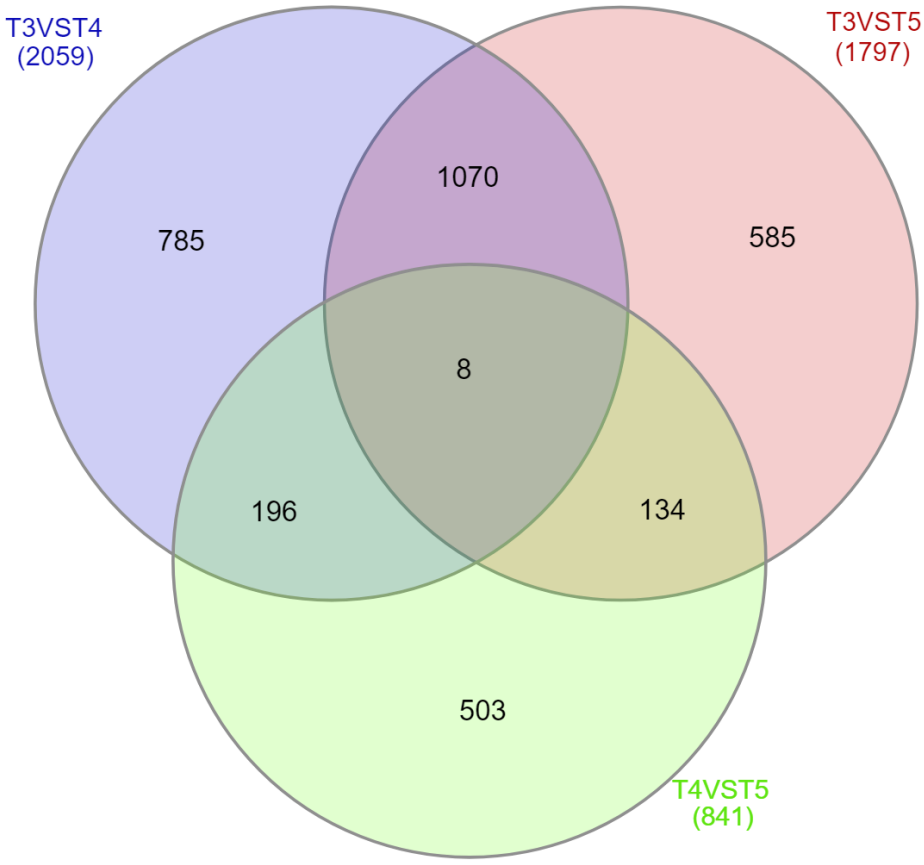

Figure 4. Differentially expressed transcripts in the comparisons of maturation times (T3vsT4, T3vsT5 and T4vsT5) of *Opuntia albicarpa* fruits.

Functional annotation was determined from DET using KOBAS enrichment of KEGG pathways and GO terms. For the comparison between T3 and T4, 27 significantly enriched KEGG pathways were recovered (corrected P value < 0.01), seven of them classified as "Carbohydrate metabolism", the most enriched KEGG pathways are indicated in Table 1. In addition, 221 GO terms were significantly enriched (corrected P value < 0.01). These GO terms are placed in the category of "Biological Process" (BP, 118 GO terms), followed by "Molecular Function" (MF, 67 GO terms) and "Cellular Component" (CC, 31 GO terms). The most enriched GO terms were for BP "response to wounding", "response to cold" and “lignin biosynthetic process”; for the MF category, the most was “protein binding”; and for the terms CC were the "plasma membrane", "cell wall", "cytosol", "secretory vesicle”, “cytoplasm” and “plant-type cell wall”.

Table 1. Top 10 KEGG enriched pathways for *Opuntia albicarpa* Scheinvar.

T3 vs T4	Corr. *P*-value	T3 vs T5	Corr. P-value	T4 vs T5	Corr. P-value
Metabolic pathways	$1.59(e^{-57})$	Metabolic pathways	$7.41(e^{-39})$	Metabolic pathways	$3.53(e^{-15})$
Biosynthesis of secondary metabolites	$4.98(e^{-43})$	Biosynthesis of secondary metabolites	$5.87(e^{-26})$	alpha-Linolenic acid metabolism	$6.09(e^{-11})$
Biosynthesis of amino acids	$1.69(e^{-17})$	Carbon metabolism	$5.62(e^{-13})$	Biosynthesis of secondary metabolites	$4.14(e^{-10})$
Cysteine and methionine metabolism	$7.61(e^{-12})$	Glycolysis / Gluconeogenesis	$2.78(e^{-09})$	Cysteine and methionine metabolism	$7.45(e^{-07})$
Carbon metabolism	$2.16(e^{-11})$	Carbon fixation in photosynthetic organisms	$8.41(e^{-08})$	Amino sugar and nucleotide sugar metabolism	$9.17(e^{-06})$
Phenylpropanoid biosynthesis	$3.31(e^{-09})$	Glyoxylate and dicarboxylate metabolism	$9.94(e^{-08})$	MAPK signaling pathway - plant	0.000158
Plant hormone signal transduction	$3.86(e^{-08})$	Plant hormone signal transduction	$1.17(e^{-07})$	Plant hormone signal transduction	0.001039
Amino sugar and nucleotide sugar metabolism	$1.18(e^{-07})$	Phenylpropanoid biosynthesis	$4.71(e^{-07})$	Arginine and proline metabolism	0.002218
Glycolysis / Gluconeogenesis	$1.58(e^{-07})$	Photosynthesis	$1.24(e^{-06})$	Other types of O-glycan biosynthesis	0.003007
Alpha-Linolenic acid metabolism	$2.50(e^{-07})$	Flavonoid biosynthesis	$1.85(e^{-05})$	Biosynthesis of amino acids	0.007635

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For the comparison of T3 vs. T5, 23 significantly enriched KEGG pathways were recovered (corrected P value < 0.01), six of them classified as "Carbohydrate metabolism", the most enriched KEGG pathways are indicated in Table 1. In addition, 193 GO terms were significantly enriched (corrected P value < 0.01). These GO terms are in "Biological Process" (BP, 100), followed by "Cellular Component" (CC, 37) and "Molecular Function" (MF, 55). The most enriched GO terms were for BP "response to cold" and "response to salt stress"; for the MF category, the most was “protein binding”; and for the terms CC were the "cell wall", "plasma membrane", "cytosol", "secretory vesicle", "plant-type cell wall", "chloroplast" and "extracellular region".

For the comparison between T4 and T5, 10 significantly enriched KEGG pathways (corrected P value < 0.01) were retrieved, most of them classified as “Metabolism-global and general maps”, the most enriched KEGG pathways are shown in the Table 1. In addition, 100 GO terms were significantly enriched (corrected P value < 0.01). These GO terms are in the category of "Biological Process" (BP, 47 GO terms), followed by "Cellular Component" (CC, 18 GO terms) and "Molecular Function" (MF, 34 GO terms). The most enriched GO terms CC were "cell wall" and "plasma membrane"; and for the category BP the most enriched were "response to wounding", "response to cold", "jasmonic acid biosynthetic process", “protein phosphorylation” and “response to fungus”.

KEEG pathways and GO terms analysis of DET

After KEGG pathways and GO terms were selected, which were significantly enriched and, which are important during prickly pear fruit development, such as pathways of carbohydrate metabolism, flavonoid biosynthesis, among others that are described below:

Carbohydrate metabolism pathways

Of the 15 carbohydrate metabolism pathways reported in the KEGG database, the comparison T3 vs. T4 represented 7 significantly enriched pathways (P value < 0.01): “Amino sugar and nucleotide sugar metabolism”, “Glycolysis / Gluconeogenesis”, “Glyoxylate and dicarboxylate metabolism”, “Galactose metabolism”, “Pyruvate metabolism”, “Pentose phosphate pathway” and “Ascorbate and aldarate metabolism”; the comparison T3 vs T5 represented six significantly enriched pathways: “Glycolysis/Gluconeogenesis”, “Glyoxylate and dicarboxylate metabolism”, “Pyruvate metabolism”, “Galactose metabolism”, “Amino sugar and nucleotide sugar metabolism” and “Starch and sucrose metabolism” ; while the comparison T4 vs T5 represented only the “Amino sugar and nucleotide sugar metabolism” pathway.

Figure 5 shows the heatmap representing the low expression, and over expression of genes involved in the " Starch and sucrose metabolism " pathway. On the left side of the Figure 5, there are groups representing genes with a similar expression pattern, and in the lower part of the heat map the development stages, and their repetitions are grouped. Genes that were under-expressed in T3 and T4 and over-expressed in T5 were located: BGLU12 (OP006394; EC 3.2.1.21), TPPA (OP039270; EC 3.1.3.12), TPS7 (OP128468; EC 2.4.1.15), At5g51830 (OP038818, EC 2.7.1.4), TPS5 (OP128465, EC 2.4.1.15), TPPG (OP039271, EC 3.1.3.12) y BAM3 (OP064455; EC 3.2.1.2). Another group of genes had a high expression in T3, and in T4 and T5, their expression tended to decrease: At1g50460 (OP035155; EC 2.7.1.1), ADG2 (OP060556; EC 2.7.7.27), PGMP (OP001310, OP001312; EC 5.4.2.2), WAXY (OP150334, OP150335, OP150339; EC 2.4.1.242), At1g64390 (OP179564, OP179569; EC 3.2.1.4), At1g11820 (OP050809; EC 3.2.1.39), TPS9 (OP037249; EC 2.4.1.15), SPS

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(OP193530; EC 2.4.1.14), BGLU44 (OP170752; EC 3.2.1.21), SS1 (OP085395; EC 2.4.1.21), SS (OP048062; EC 2.4.1.13), DPEP (OP169611; EC 2.4.1.25), TPS5 (OP178383; EC 2.4.1.15), At3g59480 (OP163884; EC 2.7.1.4) and INV*DC4 (OP028592; EC 3.2.1.26). In addition, genes that had a low expression in T3 and high expression in T4 and T5 were located. INV*DC4 (OP028593; EC 3.2.1.26) and BAM2 (OP120782; EC 3.2.1.2).

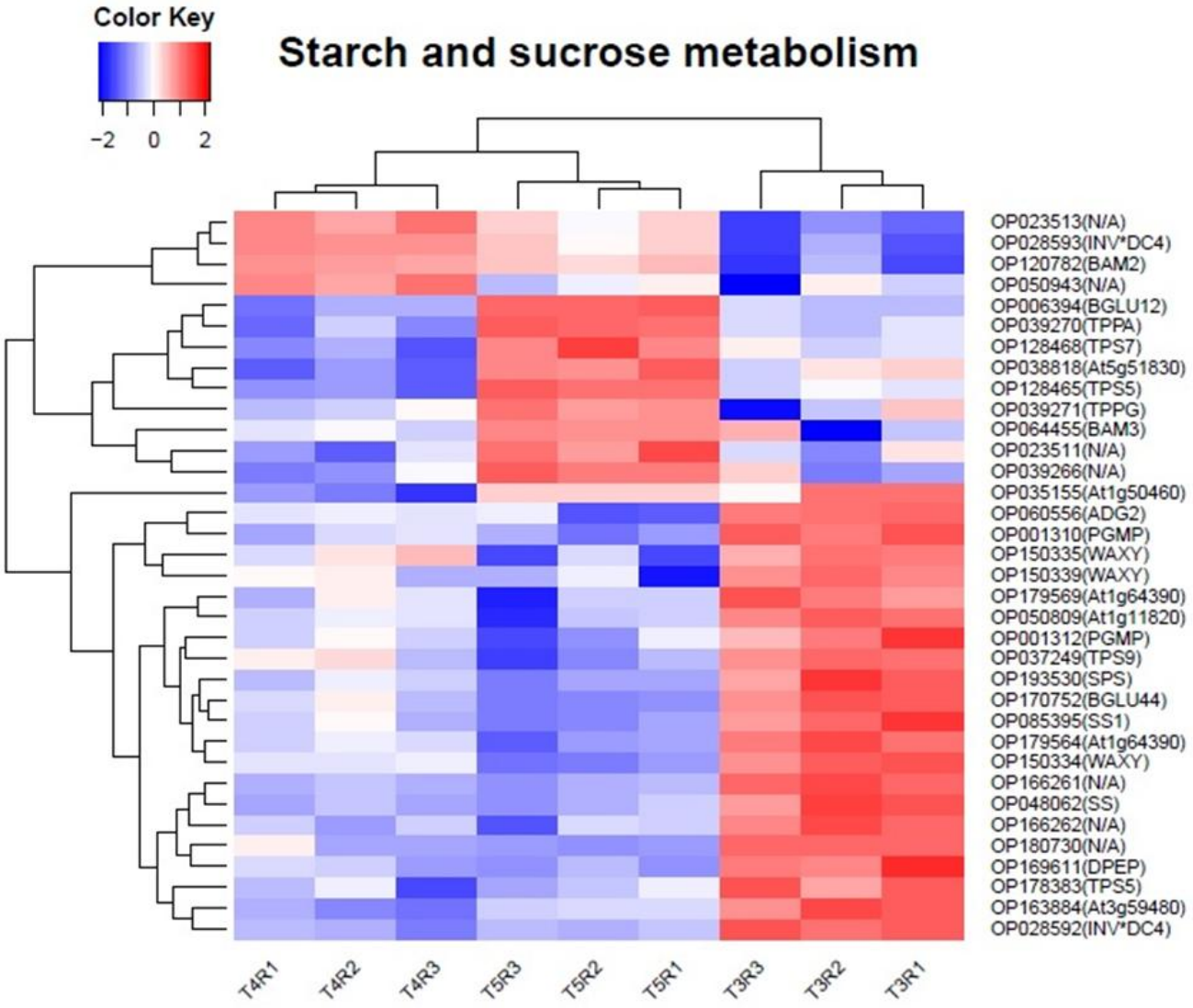

Figure 5. Heatmap of the most differentially expressed transcripts of the KEGG pathway "Starch and sucrose metabolism" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

In the “Galactose metabolism” pathway (Figure 6) the genes detected were under-expressed in T3 and over-expressed in T4 and T5: PFK6 (OP085151; EC 2.7.1.11) AGAL3 (OP173410; EC 3.2.1.22), GALM (OP086472; EC 5.1.3.3) and INV*DC4 (OP028593; EC 3.2.1.26). Other genes presented high expression in T3 and low expression in T4 and T5: PGMP (OP001310, OP001312; EC 5.4.2.2), PFK3 (OP085149; EC 2.7.1.11), GOLS2 (OP101592, OP101593; EC 2.4.1.123), INV*DC4 (OP028592; EC 3.2.1.26), At1g50460 (OP035155; EC 2.7.1.1), AGAL1 (OP017428; EC 3.2.1.22) and RFS (OP087924; EC 2.4.1.82).

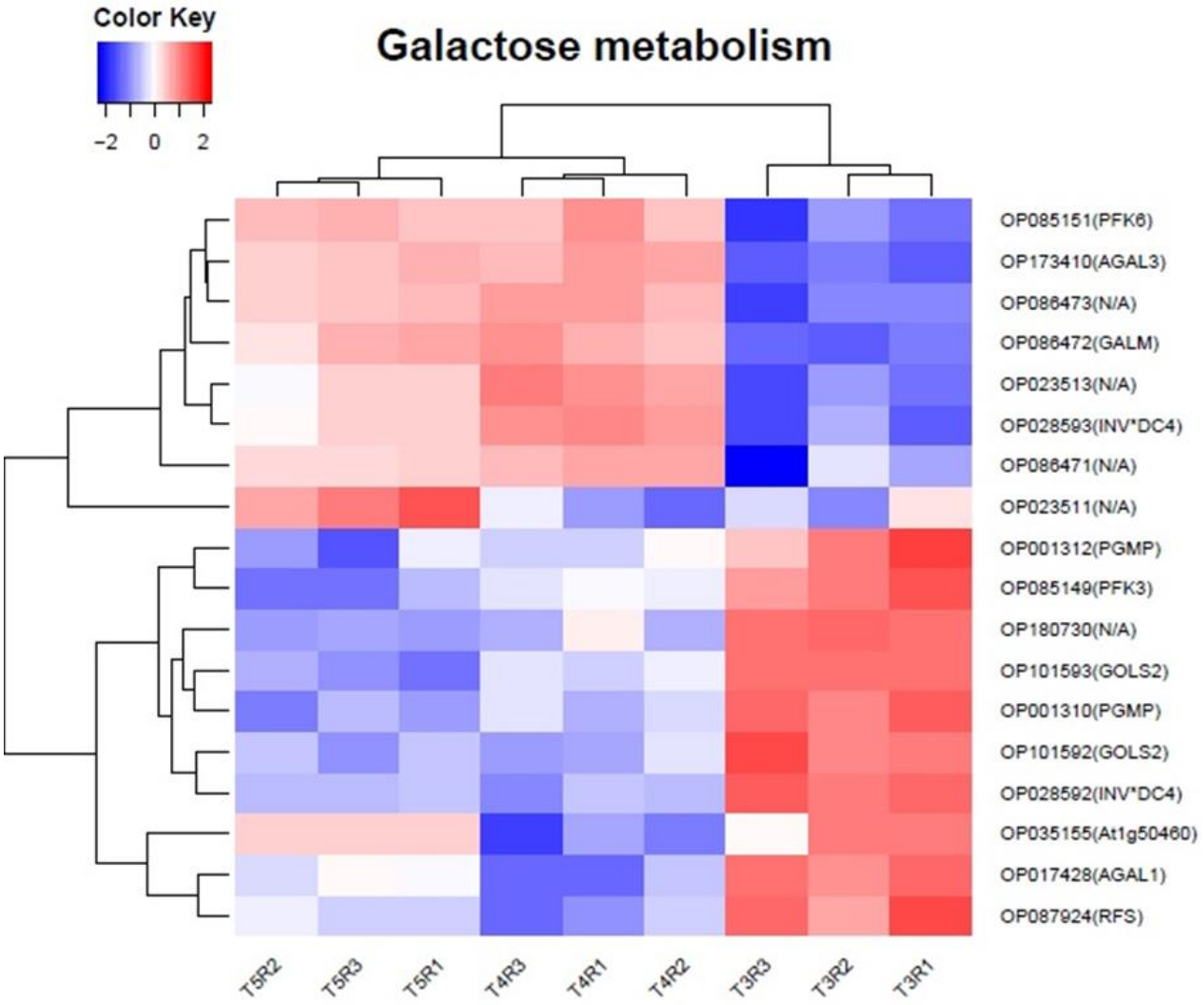

Figure 6. Heatmap of the most differentially expressed transcripts of the KEGG pathway "Galactose metabolism" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

The “Fructose and mannose metabolism” pathway (Figure 7) presented genes that were in low expression in T3 and in high expression in T4 and T5: PFK6 (OP085151; EC 2.7.1.11), FBA3 (OP075106; EC 4.1.2.13), and XYLA (OP152586, OP027977; EC 5.3.1.5). Another group of genes were over-expressed in T3 and under-expressed in T4 and T5: FBA2 (OP064628, OP064630; EC 4.1.2.13), CFBP1 (OP089913; EC 3.1.3.11), PFK3 (OP085149; EC 2.7.1.11), TPI (OP132697; EC 5.3.1.1), and At3g59480 (OP163884; EC 2.7.1.4); and two genes presented high expression in T3, low expression in T4 and high expression in T5: At5g51830 (OP038818; EC 2.7.1.4) and At1g50460 (OP035155; EC 2.7.1.1).

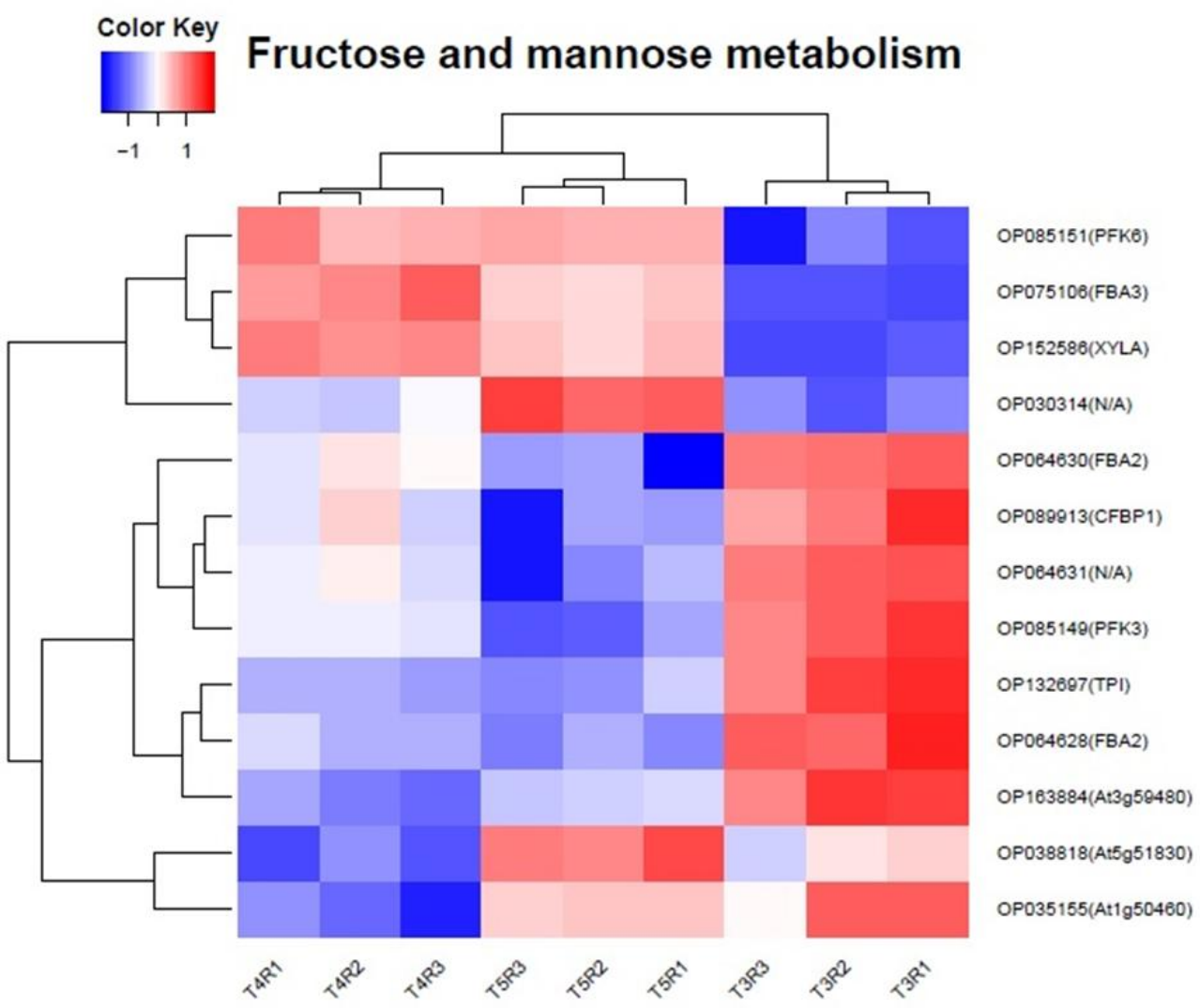

Figure 7. Heatmap of the most differentially expressed transcripts of the KEGG pathway "Fructose and mannose metabolism" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

Flavonoid biosynthesis pathway

In this pathway, genes whose expression was low in T3 and high in T4 and T5 were detected: CHI (OP000217; EC 5.5.1.6) and CYP75B2 (OP040720; EC 1.14.14.82). Genes over-expressed in T3 and under-expressed in T4 and T5 were also found: CYP98A2 (OP160423; EC 1.14), CCOAOMT (OP023778, OP015161; EC 2.1.1.104) and CYP73A16 (OP073728; EC 1.14.14.91) and FHT (OP028058; EC 1.14.11.9). In addition, the FHT gene (OP095543; EC 1.14.11.9) had a high expression in T3, low in T4 and high in T5 (Figure 8).

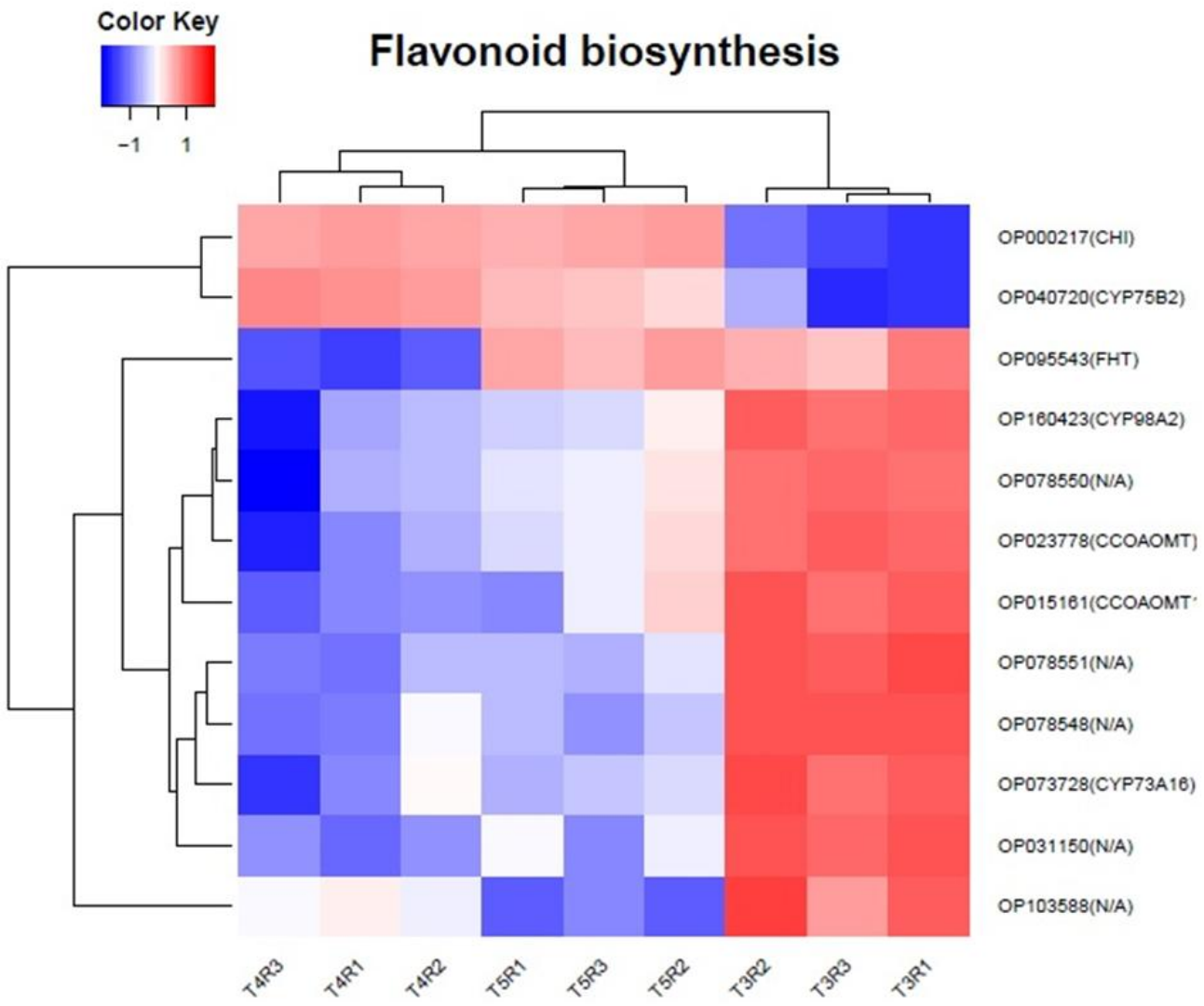

Figure 8. Heatmap of the most differentially expressed transcripts of the KEGG pathway "Flavonoid biosynthesis" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

GO terms of carbohydrates

In the GO term “Carbohydrate metabolic process” genes were identified that were under-expressed in T3 and over-expressed in T4 and T5: LOC100243180 (OP076516; EC 3.2.1.15), OsI_025867 (OP164596; EC 3.1.1.31), CARSR12 (OP165749; EC 3.2.1.23), OLE9 (OP060704; EC 3.2.1.39) and VIT_00031543001 (OP013616; EC 3.2.1.39). There were genes that had a high expression in T3, low in T4 and high in T5: GPDHC1 (OP094718; EC 1.1.1.8), BG1 (OP025911; EC 3.2.1.39), A6 (OP161044; EC 3.2.1.39), GPDHC1 (OP094718; EC 1.1.1.8), At3g47520 (OP091027; EC 1.1.1.37) and SETH3 (OP118935; EC 5.3.1.13). Another group of genes had a high expression in T3 and low in T4 and T5: At1g11820 (OP050809; EC 3.2.1.39), LOC100243180 (OP117834; EC 3.2.1.15), At2g27500 (OP093322; EC 3.2.1.39), BGLU44 (OP170752; EC 3.2.1.21), PGMP (OP001310; EC 5.4.2.2) and CIT (OP002961; EC 2.3.3.16) (Figure 9).

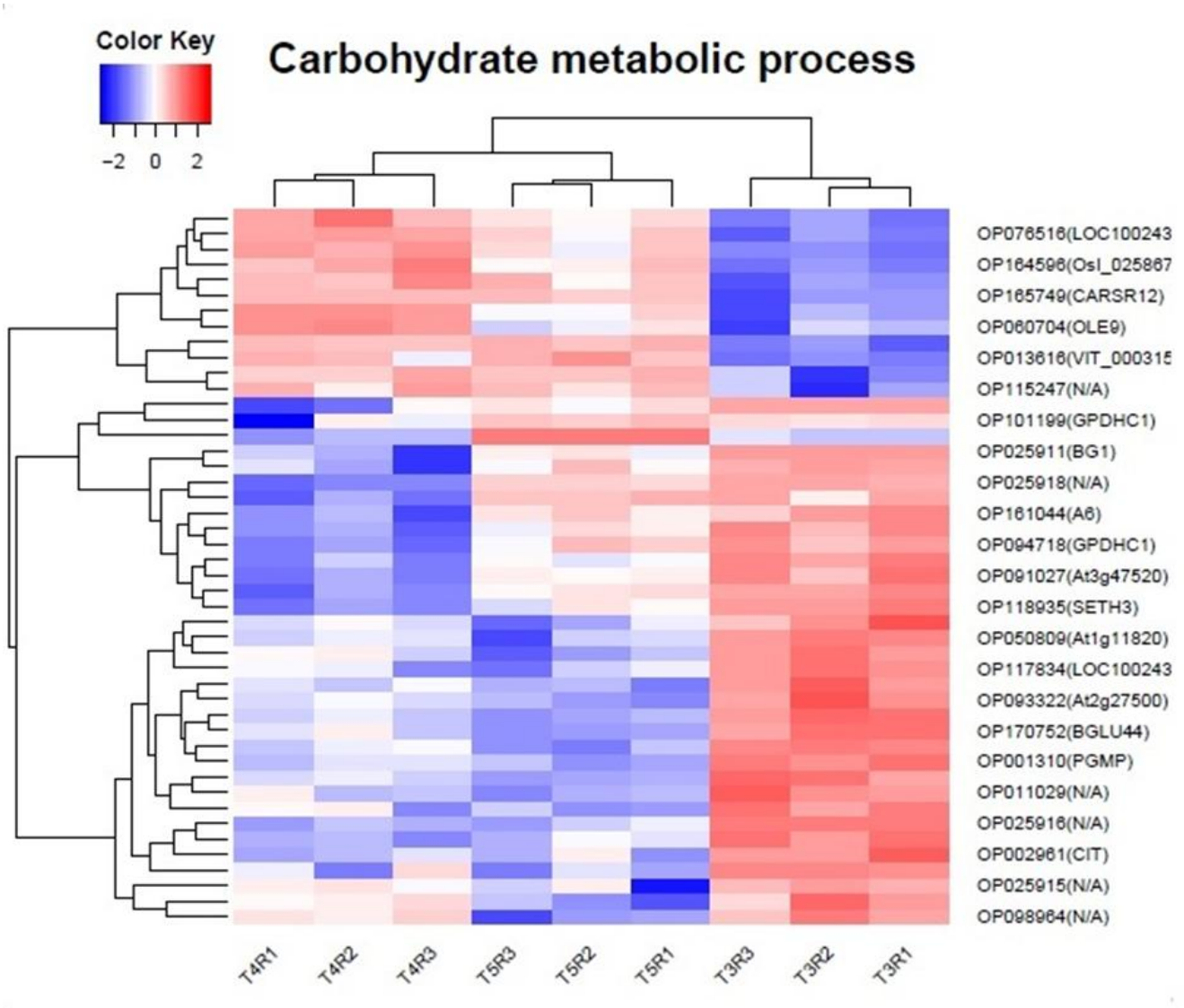

Figure 9. Heatmap of the most differentially expressed transcripts of the GO term "Carbohydrate metabolic process" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

In the GO term “Sucrose metabolic process” genes were identified and that were over-expressed in T3 and decreased in T4 and T5: CFBP1 (OP089913, EC 3.1.3.11), INV*DC4 (OP028592; EC 3.2.1.26) and SS (OP048062; EC 2.4.1.13). Likewise, the SUC3 gene (OP161877; EC N/A) increased its expression at T5.

The GO term “Glucose metabolic process” presented genes with low expression in T3 and a high expression in T4 and T5: GALM (OP086472; EC 5.1.3.3) and VTC2 (OP175145; EC 2.7.7.69). Other genes presented over expression at T3 and under expression at T4 and T5: G6PDH (OP046817; EC 1.1.1.49), G6PD (OP146929; EC 1.1.1.49), GAPA (OP113842, OP113839; EC 1.2.1.13), GAPB (OP113840; EC 1.2.1.13) and DPEP (OP169611; EC 2.4.1.25).

GO term flavonoid biosynthetic process

Here genes over-expressed in T3 and under-expressed in T4 and T5 were detected: DTX35 (OP033464; EC N/A), CYP98A2 (OP160423; EC 1.14.-) and FHT (OP095543; EC 1.14.11.9). Other genes had a low expression at T3 and high expression at T4 and T5: HO1 (OP129445; EC 1.14.14.18), CHI (OP000217; EC 5.5.1.6) and CYP75B2 (OP040720; EC 1.14.14.82) (Figure 10).

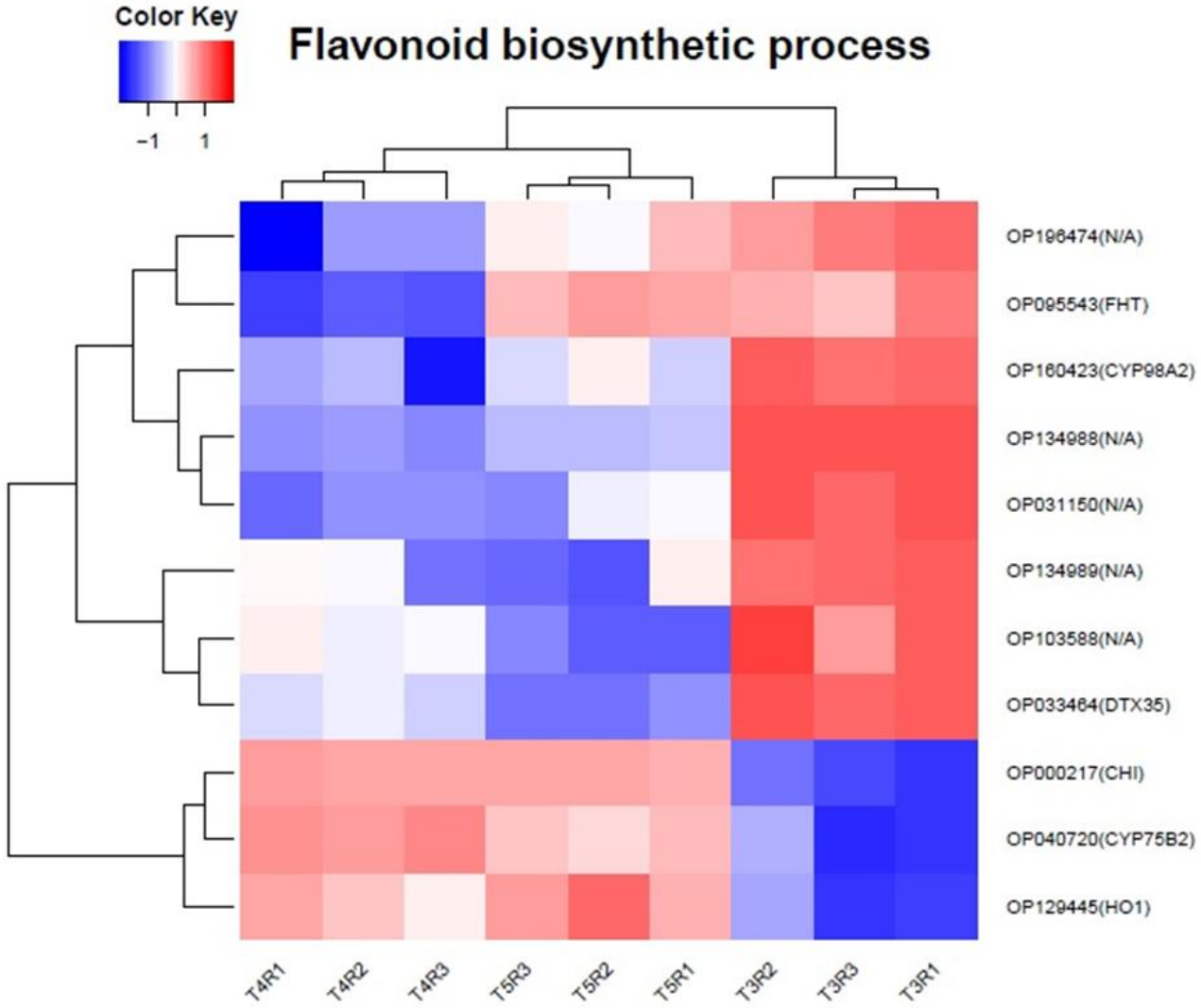

Figure 10. Heatmap of the most differentially expressed transcripts of the GO term "Flavonoid biosynthetic process" of *Opuntia albicarpa* fruit development. The heatmap graph was constructed based on the Z-score of the reads from the nine libraries.

Discussion

Although the Alfajayucan prickly pear (*O. albicarpa* Scheinvar) cultivation has economic and cultural importance in Mexico, there is little research on the genomic/genetic resources of this species, and no transcriptomic information was available during fruit development until the present research. The results on the genetic changes that occurred during the maturation of Opuntia fruits provide a valuable resource to better understand the biology of the genus.

Morphological evaluation

Clear and progressive morphological changes were identified during the development and maturation of the fruit. In the early stages (T1–T3), the fruit maintains a relatively low percentage of locule weight in relation to total weight. In stages T4 and T5, an increase in locule weight is observed, accompanied by a decrease in shell weight, which indicates that during the maturation process of prickly pear, the greatest accumulation of the edible part (locule) occurs.

Data filtering and de novo assembly

The acquisition of over 704 million raw reads and the filtering of 89.67% of the reads demonstrates that the sequenced material was of high quality, ensuring that the assembly of the transcriptome of *O. albicarpa* and subsequent analyses are reliable. The type of RNA sequencing used in this study was paired-end, which, according to Fullwood *et al.* (2009) is the most efficient and accurate approach to demarcate gene transcription unit boundaries and complements other methods for transcriptome studies.

The total of raw reads and clean reads obtained in the present study are consistent with that reported by Zhou *et al.* (2020) for pitahaya for being a crassulacean acid metabolism (CAM) plant, where the total raw and clean readings for each sample ranged from 46,688,418 to 62,038,332 and from 45,033,096 to 60,311,044 respectively, although this is a function of the experimental conditions. The high percentage of duplicate reads (66.10–90.90%) can be associated with the over-representation of very abundant transcripts and may also reflect biases in library construction or the differential expression of highly active genes during the fruit development stages. The high percentage of alignment (87.26–98.32%) between the reads and the assembly indicates that the obtained sequences faithfully represent the transcriptome of *O. albicarpa*.

The assembly obtained in *O. albicarpa* of 240,169 transcripts, reduced to 198,958 after the removal of redundancies using CD-HIT-EST, indicates an effective filtering process that improves the representation of unique sequences. Comparing the assembly obtained with other studies on CAM plants, Cervantes-Pérez *et al.* (2018) in *Agave salmiana* obtained an assembly with 170,844 transcripts from root, leaf and callus data; Gross *et al.* (2013) obtained 20,4530 contigs in *Agave tequilana* and 128,869 contigs in *Agave deserti*; Mallona *et al.* (2011) identified a total of 43,066 contigs with average lengths of 612 bp in *Opuntia ficus-indica*. By other hand, Wu *et al.* (2020) identified 65,317 and 91,638 genes in red-fleshed pitaya and white-fleshed pitaya respectively, in four stages of development; Wu *et al.* (2022) obtained an assembly of 95,412 unigenes with a mean length of 913 bp and an N50 length value of 1878 bp from nine pitahaya flower development stages; Zhang *et al.* (2022) obtained an assembly of 261,468 transcripts and 150,568 unigenes in six stages of pitahaya fruit development. Wang *et al.* (2020) detected 16,157 genes expressed in pineapple fruits. Valadez-Moctezuma *et al.* (2022) with the MACE (Massive Analysis of cDNA Ends) technology obtained 8,383 (GC: 40.1%), 7,890 (GC: 40.11%) and 5,300 (GC: 39.9%) transcripts for the de novo assembly of *O. ficus-indica*, *O. robusta* and *O. joconostle*, respectively.

Functional Annotation

The peptides and open reading frames detected with TransDecoder indicate a high degree of complexity in the analysed transcriptome. Functional annotations through Blastx and Blastp revealed that a significant proportion of sequences correspond to plant species, confirming the biological

relevance of the obtained data. Comparative analyses between Blastx and Blastp suggest a robust identification of conserved genes. From the annotation results, only 11.29% and 34.01% of the transcripts had matches in the UniProtKB/Swiss-Prot database for Blastx and Blastp, respectively. In this regard, Cervantes-Pérez *et al*., (2018) obtained in *Agave salmiana* 40,644 (24%) sequences that presented an open reading frame (ORF) and identified 12,209 putative genes; Zhou *et al.* (2020) identified 44,263 annotated unigenes in pitahaya; Gross *et al.* (2013) obtained in *Agave tequilana* and *Agave deserti* sequences that encode around 35,000 proteins; Mallona *et al.* (2011) identified for *O. ficus-indica* 29,835 contigs that produced BLAST hit scores against sequences from one CAM species (69.3 %).

Likewise, Zheng *et al.* (2021) predicted 28,992 protein-coding genes using protein homology and RNA-seq data in dragon fruit; Wu *et al.* (2022) found that 18,564 and 18,483 unigenes were annotated in Pfam and SwissProt for developing pitahaya flowers; Zhang *et al.* (2022) identified 40,461 annotated unigenes in the NCBI non-redundant protein database (26.9% of total unigenes) in dragon fruit; Valadez-Moctezuma *et al.* (2022) identified that around 22.2-23.7% (1,174-1,986) of the transcripts had matches in the UniProtKB/Swiss-Prot database.

Among the most represented species in the transcriptome of *O. albicarpa*, *Arabidopsis thaliana* stands out (78.03%), reflecting the high homology of the identified genes with well-studied model plants. The large number of KEGG pathways, GO and KO terms enriched in our EggNOG-mapper analysis revealed that the *O. albicarpa* fruit transcriptome included functionally diverse genes. Likewise, indicates that the studied transcriptome contains most of the elements necessary to model the metabolic and functional networks of the species, allowing for future research on biosynthetic pathways and gene regulation. In this regard, Valadez-Moctezuma *et al.* (2022) obtained 282 KEGG pathways and 2,793 GO terms for *O. ficus-indica*, *O. robusta*, and *O. joconostle*; Zheng *et al.* (2021) found 21,655 (74.7%) proteins in dragon fruit that were annotated by eggNOG, of which 10,093 were assigned to GO terms and 9,920 to KEGG pathways.

Differential expression and gene orthology (GO)

The analysis of differential transcript expression (DETs) between the stages of fruit development revealed dynamic changes in gene regulation. Between T3 and T4, positively regulated transcripts were identified, suggesting the activation of genes associated with early ripening processes. In contrast, a higher proportion of negatively regulated transcripts was observed between T4 and T5, indicating the predominance of gene repression mechanisms, possibly stabilizing the metabolic and structural processes of the fruit towards its final stage. This coincides with the report of Yan-Jun *et al.* (2018) who identified in *Lycium ruthenicum* that the development period with the most dynamic transcriptional changes was between S1 (green maturity stage) and S2 (skin color change), with more than half of the DEGs (63.8%). Likewise, Wu *et al.* (2020) found 44,109 differentially expressed genes (DEG) between red-fleshed and white-fleshed pitaya during four stages of development, among which, most DEGs were obtained in R2_vs_W2 (25 days after pollination), with 11,317 DEG upregulated and 4,788 downregulated. On the other hand, Wu *et al.* (2022) found 1,492 overlapping DEG between the last four stages of pitahaya flower development.

Eight shared transcripts were detected in the three comparisons: Boron transporter 4 (BOR4), Ethylene-sensitive transcription factor ABR1 (ABR1), L-ascorbate oxidase-like (LOC101215231),

Tonoplast dicarboxylate transporter (TDT), TIFY protein 9 (TIFY9), and Plasma membrane ATPase 4 (PMA4); suggesting the existence of central regulators that could play critical roles in controlling ripening and responding to environmental stimuli.

Relative to functional annotation from DET using KOBAS, the number of significantly enriched KEGG pathways and GO terms were higher in comparisons that included T3, consistent with the high number of differentially expressed transcripts. In addition, according to the GO terms categorization, the largest number of GO terms corresponds to the category of biological processes (54.63% in T3 vs T4, 53.89% in T3 vs T5 and 52.34% in T4 vs T5). In this regard, Valadez-Moctezuma *et al.* (2022) for the comparison of fruits of *O. ficus-indica* vs. *O. robusta* recovered 14 KEGG pathways and 73 significantly enriched GO terms (corrected P value < 0.01); for the comparison of *O. ficus-indica* vs *O. joconostle* fruits, they detected 11 KEGG pathways and 64 significantly enriched GO terms and for the comparison of *O. joconostle* vs *O. robusta* fruits, they recovered 21 KEGG pathways and 106 significantly enriched GO terms, where most GO terms were classified as BP. In addition, of the main KEGG pathways enriched for *O. albicarpa* (Table 1), several of them coincide with those reported by Valadez-Moctezuma *et al.* (2022) in *O. ficus-indica*, *O. robusta*, *O. joconostle*: “Metabolic pathways”, “Biosynthesis of secondary metabolites”, “Carbon metabolism”, “Carbon fixation in photosynthetic organisms” and “Glycolysis/Gluconeogenesis”.

KEEG pathways and GO terms analysis of DET

The analysis of KEGG pathways and GO terms in *O. albicarpa* allowed the identification of key processes that regulate fruit development, mainly those associated with carbohydrate metabolism and flavonoid biosynthesis. These results show the complex dynamics of gene regulation during fruit ripening and highlight how certain genes are activated or repressed at specific stages to ensure proper fruit formation and metabolite accumulation.

Carbohydrate metabolism pathways

In carbohydrate metabolism, it was observed that multiple pathways showed significant enrichment among stages T3, T4, and T5. This indicates that the mobilization and transformation of sugars is a central process in fruit development. The presence of genes with specific expression patterns with BGLU12, TPS7, BAM3, PGMP and SPS reflects the temporal coordination of carbohydrate metabolism, optimizing the synthesis and storage of sugars according to the stage of development. According to Martín del Campo *et al.* (2019) during the ripening process of the *O. albicarpa* fruit the content of total soluble solids and reducing sugars increase progressively until 125 days after flowering; Zenteno-Ramirez *et al.* (2015) in *O. albicarpa* prickly pear juice quantified 14.55 $mg.mL^{-1}$ of total sugars, of which glucose (8.14 $mg.mL^{-1}$), fructose (6.39 $mg.mL^{-1}$) and sucrose (0.01 $mg.mL^{-1}$) were the most abundant. Likewise, Reyes-Ocampo *et al.* (2019) performed a simple extraction of *O. ficus-indica* mucilage and obtained the composition of arabinose (12.54 %), fucose (1.6 %), galactose (8.89 %), galacturonic acid (13.27 %), glucose (12.24 %), mannose (8.31 %), rhamnose (20.26 %) and xylose (6.85 %). Of the eight pathways of carbohydrate metabolism significantly enriched during the development and ripening of *O. albicarpa* fruits, Valadez Moctezuma *et al.* (2022) reported the pathways "Amino sugar and nucleotide sugar metabolism" and "Galactose metabolism" in *O. ficus-indica*, *O. robusta*, *O. joconostle*, but their analysis was carried out only in cladodes and practically mature fruits, not during growth of the maturation process.

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Wu *et al.* (2022) reported the enrichment of the KEGG pathways of sugar metabolism during pitaya flower development; also reported some genes in carbohydrate transport that were also detected in our study (TPS7, AGAL1, SS1). Zhang *et al.* (2022) found that glucose was the predominant sugar in ripe pitaya fruit and that VAI, NI and SS are crucial enzymes for sugar accumulation. They also obtained seventeen genes involved in the "Starch and sucrose metabolism" pathway. Cortes-Sierra *et al.* (2015) identified differential gene expression patterns in cassava varieties for some starch genes, of which, in the present study, Sucrose synthase (SS), Beta-amylase (BAM2 and BAM3) and Granule-bound starch synthase (WAXY=GBSS) were identified.

Zhang *et al.* (2022) identified 41 DEGs for the "Starch and sucrose metabolism" pathway and 22 DEGs for the "Galactose metabolism" pathway in pecan nut fruits; of which most were quantified in the present study: Phosphoglucomutase (PGMP), Beta-glucosidase (BGLU12, BGLU44), Beta-fructofuranosidase (INV*DC4), Hexokinase-3 (At1g50460), Trehalose-phosphate synthase (TPS5, TPS7 y TPS9), Probable fructokinase-7 (At5g51830), Trehalose-phosphate phosphatase (TPPA, TPPG), Glucan endo-1,3-beta-glucosidase 1 (At1g11820), Glucose-1-phosphate adenylyltransferase (ADG2), Granule-bound starch synthase 1 (WAXY), Probable fructokinase-4 (At3g59480), Endoglucanase (At1g64390) and Sucrose-phosphate synthase (SPS).

In the “Fructose and mannose metabolism” pathway, Hai-Shun *et al.* (2020) reported five differentially expressed genes in *Lolium perenne*, of which Fructose-bisphosphate aldolase (FBA2 and FBA3) and 6-phosphofructokinase (PFK3 and PFK6) were identified in our study.

Flavonoid biosynthesis pathway

The biosynthetic pathway of flavonoids showed a similar pattern of differential regulation. Genes such as CHI, CYP75B2, CYP98A2, and FHT could be linked to the production of flavonoid precursors, antioxidant compounds, and pigments that give color to the fruit during ripening. The enriched flavonoid biosynthesis pathway for *O. albicarpa* was also reported by Valadez-Moctezuma *et al.* (2022) for the comparison of fruits/cladodes of three opuntia species, where the chalcone-flavonone isomerase (CHI) gene was differentially expressed, which is part of the biosynthetic pathway for all classes of flavonoids. Fernandez-Lopez *et al.* (2010) identified the flavonoids myricetin and luteolin, quercetin, isorhamnetin and kaempferol in opuntia fruits; of the genes found in the present study, Flavonoid 3'-monooxygenase (CYP75B2) participates in the synthesis of quercetin.

GO terms of carbohydrates

The GO terms such as 'carbohydrate metabolic process', 'sucrose metabolic process', and 'glucose metabolic process', show a control of gene expression to adjust the availability and flow of sugars. The temporal regulation of specific genes allows the fruit to efficiently synthesize, transform, and store carbohydrates, which is crucial for ripening.

GO term flavonoid biosynthetic process

Similarly, the term GO associated with flavonoid biosynthesis reflects a stepped regulation of genes, coordinating the production of secondary metabolites in fruit development and ripening.

Conclusions

The results on the *Opuntia albicarpa* transcriptome provide a complete and precise basis that can be used for genetic and molecular studies on the genus´ Opuntia and thereby better understand its biology and physiology. This study provides the first transcriptomic characterization of the fruit of *O. albicarpa*, offering a key resource to understand the transcriptional changes during its developmental stages. The high-quality transcriptome assembly and the high alignment rate confirm the reliability of the obtained data. The functional annotation revealed a complex transcriptome, with conserved genes and metabolic pathways that allow for modelling the biological processes of the species. Gene expression is dynamic: genes related to early ripening are activated between T3 and T4, while gene repression predominates between T4 and T5, stabilizing the final processes of the fruit. Key genes involved in carbohydrate metabolism and flavonoid biosynthesis were identified, which promotes the accumulation of sugars, pigments, and antioxidant compounds during ripening. Together, the results show how gene regulation and metabolic pathways are intricately integrated to ensure the development, quality, and functional composition of the fruit.

ETHICS STATEMENT

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF SUPPORTING DATA

Not applicable

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.V.M. and J.J.C.M.; methodology and validation, E.V.M. M.G.J.O, R.O.J.L. and L.R.J.J.; investigation, J.J.C.M. and E.V.M.; writing—original draft preparation, review and editing; supervision, E.V.M., M.G.J.O, R.O.J.L. and L.R.J.J.; funding acquisition, E.V.M.

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