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Towards an Improved Vita Fruit (*Passiflora tenuifila* BRS VT): Functional Properties Basis for a Better Post-Harvest

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ABSTRACT. Brazil is a center of diversity for the genus *Passiflora*. *Passiflora tenuifila*, Killip is a wild species popularly called garlic passion fruit. It has nutritional and functional properties as anti-oxidant, anti-inflammatory, anxiolytic and anti-convulsant. These properties are mainly associated with phenolic compounds, flavonoids, bioactive amines, carotenoids, anthocyanins and vitamin C present in various parts of the plant. In order to identify genes related to the biosynthesis of these compounds responsible for nutritional and functional properties and to the ripening of the fruit we have described the seeds and arils *P. tenuifila* transcriptome at two different developmental stages. We found the presence of several genes related to different biosynthetic pathways of secondary compounds, in addition to genes related to the *Myb* transcription factor and the *cytochrome P450* enzyme, among others. We found three genes that can be used as reference genes. We have validated the gene expression levels of 4 genes at different stages of fruit ripening by qPCR: (i) *caffeic acid-o-methyltransferase*, (ii) *leucoanthocyanidin reductase*, (iii) *ACC synthase* and (iv) *4-coumarate-CoA ligase*. This is the first report of transcriptome analysis of *P. tenuifila*.

Key words: Garlic passion fruit; transcriptome; differential expression; qPCR.

INTRODUCTION

Brazil is a center of diversity for the genus *Passiflora*, containing almost one-third of the 520 different species found in the world (Cerqueira-Silva et al., 2014; Faleiro et al., 2008). At least 70 are consumable species (Cunha et al., 2002) and some of them are considered nutritional and functional foods (Costa, 2017; Vectore-Neto, 2015; Sozo, 2014; Bomtempo, 2011; Costa & Tupinambá, 2005).

Passiflora tenuifila, Killip is a wild species of the genus, popularly called garlic passion fruit, due to the odor and flavor of the ripe pulp. It has been widely studied for several parameters such as (i) genetic diversity of accessions in the collection (Almeida et al., 2009); (ii) seed germination (Junghans et al., 2019); (iii) susceptibility to diseases and pests (Pereira et al., 2017; Dianese et al., 2017); (iv) phenology in the Brazilian Cerrado region (Antonini et al., 2019); (v) pollination during fruiting (Soares et al., 2015); (vi) harvest and post-harvest aspects (Rinaldi et al., 2021; Pereira et al., 2017) and (vii) their main nutritional, functional and pharmacological properties (Holanda et al., 2019). Some functional compounds found in *P. tenuifila* were demonstrated to have anti-oxidant, anti-inflammatory, anti-convulsant, anxiolytic, and anti-tremor properties. The pulp extract of *P. tenuifila* confirms these properties without risk of toxicity (Holanda et al., 2020; Bomtempo, 2011; Sozo, 2014; Vector-Neto, 2015). These properties are mainly associated with phenolic compounds, flavonoids, bioactive amines, carotenoids, protoanthocyanins, fibers, and vitamin C present in the plant (pulp, peel of fruits and leaves) (Santos et al., 2021; Holanda et al., 2019).

The objective of this work was to sample the *Passiflora tenuifila* transcriptome to characterize genes related to the biosynthesis pathways of its main nutritional and functional properties or fruit quality attributes.

This is the first report of the transcriptome of *P. tenuifila*. This study generated a wealth of data now available to the science community. We have selected three genes to be used as references in qPCR. Furthermore, we analyzed the gene expression of four genes related to fruit quality, including those involved in the biosynthesis pathways of nutritional and functional properties and related to the ripening of fruits. Those genes can now be used to modulate several harvest and post-harvest treatments aiming its quality increase for commercialization.

MATERIAL AND METHODS

Plant material

Fruits and leaves of *Passiflora tenuifila*, cultivar BRS Vita Fruit (BRSVT) (Faleiro et al., 2018) registered by the Ministry of Agriculture (MAPA) (RNC nº 36579; SNPC nº 20170307 / MAPA) and present in the Collection "Flor da Paixão", hosted and protected at Embrapa Cerrados (Planaltina-DF, latitude 15º35'30", longitude 47º42'30"). *P. tenuifila* plants grew in field conditions. The production of the plants was done at 1007 m in height, climate type AW according to the Köppen classification, and average annual precipitation of 1460 mm, in Distrofic Red-Yellow Latosol soil corrected two years earlier with dolomitic limestone to raise V% = 50. The planting hole fertilizer was done with mineral and organic (20 L of tanned cow dung) supplementation according to soil analysis. The irrigation was applied to reach the field capacity whenever necessary. Three biological

replicates of fruit at 4 distinct developmental stages (E1, E2, E3, E4) (Figure 1) were collected in 2014 (February and December) until 2017 (always December), immediately processed for seed/aryl, peel, or leaves RNA extraction in liquid nitrogen. The remaining powdered tissue was stored in 15ml Falcon tubes at -80ºC. 2014 February samples compose *Mi-seq* and 2014 December samples compose *Hi-seq* sequencing analysis. RNA used to validate gene expression qPCR were extracted from fruit and leaves samples harvested in December 2017 (Figure 1).

Figure 1. *Passiflora tenuifila* fruits at four different development stages (E1, E2, E3, E4) right after harvest in the field.

RNA extraction

Total RNA was extracted from 100 mg of seed/aryl, and peels at four different stages (E1, E2, E3, E4), and leaves using the Plant RNAeasy® kit (Qiagen, Foster City CA, USA) using DNase I treatment in the column according to the manufacturer's instructions. Sample quantity and purity were determined using Nanodrop-2000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was verified by electrophoresis in a 1.0% agarose gel stained with ethidium bromide (0.5 μg mL−1) and in a Bioanalyzer (Model 2100 expert) before sequencing.

Transcriptome Analysis

Quality RNAs from seed/aryl in stages E2 and E3 analyzed in the Bioanalyzer (RIN: 8.0 – 9.8) were sent dehydrated for transcriptome sequencing (RNA-seq) in a pool of three biological repeats, by stage of development (E2 and E3), for *Mi-seq* sequencing analysis (*Illumina*) (2X300, 2 samples, 2 lines) with the MiSeq V3 kit (600 cycles) *Illumina* cat# MS1023003 in the Genomics Lab. sequencing service at the Catholic University of Brasília (UCB). And, in three independent biological replicates, treated with RNA stable (Biomatrica) for RNA-seq *Hi-seq* (*Illumina*) sequencing (2X100, 6 samples, 1 line) with the Truseq RNA sample prep kit (*Illumina*) in the LacTad sequencing service at the University of Campinas (UNICAMP).

Bioinformatics

Bioinformatics analyses were carried out at Embrapa Agricultura Digital, taking into account a strategy of a first backbone assembly using the larger fragments from the *Mi-seq* sequencing (about 300 bp) and, then, complemented with smaller fragments from *Hi-seq* sequencing (fragments of around 100 bp) ensuring that the final assembly covered the complete functional genome. Sequencing analyses followed a procedure established at Embrapa Agricultura Digital where potential undesirable contaminants sequences was removed, followed by quality analysis of

fragments generated in each type of sequencing and statistical and differential expression analysis (Fast QC; Flash Assembler - v. 1.2.11; Trinity, TMM, R) (Grabherr et al., 2011; Haas et al., 2013). Homology searches were performed using Blast (Altschul et al., 1997).

Quantitative Polymerase Chain Reaction (qPCR)

After RNA extraction from seeds/aryl, peel and leaves, quantification and integrity evaluation, a reverse transcription reaction was performed for cDNA synthesis from 500ng of total RNA using the QuantiTect ® Reverse Transcription Kit following the manufacturer's instructions (Qiagen, Foster City CA, USA). The reverse transcribed cDNA was then diluted 1:5 with nuclease-free water and used for qPCR analysis. Six candidate reference genes were chosen among housekeeping genes based on their expression level (low, medium, and high) in *P. tenuifila*. Four genes homologous to genes involved in the biosynthesis pathway (phenolics and flavonoids compounds) were selected as targets. The software WEB Primer 3 (Untergrasser et al., 2012) was used to design the primers with the following parameters: a) annealing temperature of 60°C; b) primer length of 20 bp; c) GC content of 50% and; d) amplicon length from 100–200 bp. 1 to 2% agarose gel electrophoresis verified primer specificity in PCR prior to amplicon capillary electrophoresis on an ABI3730 system with POP7 polymer and BigDye v3.1 sequencing (*Myleus* Biotechnology - www.myleus.com). Amplicon fragments were verified in sequence comparisons using the CLCBio software, v. 7.7.1 (Qiagen, Foster City CA, USA) and in GenBank sequences using the BLASTX algorithm (Altschul et al., 1997). qPCR reactions were performed using an AB7500 system (Applied Biosystems) using SYBR Green Power Up kit (Applied Biosystems) in 96-well optical reaction plates (Applied Biosystems) sealed with ultra-transparent sealing film (Applied Biosystems). Reactions were performed in a total volume of 13µl containing 6.5µl of 2 X Power Up Mix (AB); 0.77 µM of each primer, 2.0 µl of cDNA diluted 1:10 and nuclease-free water. The reaction conditions were 50°C for 2min, 95°C for 2min, followed by 40 cycles of 30s at 95°C, 1:30 min. at 55°C, 62.4°C or 64.5°C (according to primer Tm) and 1 min at 60°C with a recording of the fluorescent signal. The final step was the generation of denaturation curves for each reaction to confirm specific amplification. Standard curves of a set of 10-fold serial dilutions of pooled cDNA samples demonstrated primer specificity for all genes studied prior to qPCR analysis. PCR efficiency (E) was calculated as $E = (10^{-1/slope}) - 1$. An Excel spreadsheet (Microsoft, v. 10) calculated the slope and R^2 for each gene under study. All seed/aryl and leaf samples resulted in triplicates/biological and technical samples. All peel samples resulted in technical/sample triplicates, including the control. The final average values of gene expression obtained with the same melting curve for each sample resulted in the final values of the quantification cycle (Cq).

Data analysis

Normalization analysis of the best reference gene or the best pair of genes for the qPCR was performed using two algorithms: qBase plus (GNorm) (v.2.3; Biogazelle, Belgium) (Hellemans et al., 2007) and BestKeeper software. (Pfaffl et al., 2004).

RESULTS

The *Miseq* RNA sequencing from seed/aryl generated 30 to 60 million sequences with 11 Gb being considered of high quality (Gb>30). *Hiseq* sequencing produced 17 to 60 Gb of sequences with a quality of 95% (Gb>Q30) (Table 1). The second repetition of the stage 2 (E2) sample in *Hiseq* sequencing was the one in which there were fewer sequences.

Table 1. Total number of reads obtained in *Miseq* and *Hiseq* RNA sequencing from seed/aryl in Gb.

Sample		#Reads (Gb > Q30)
Seq Miseq	E2/Stage 2 (pool)	11.3
	E3/Stage 3 (pool)	10.7
Seq Hiseq	1.E2	64.2
	2.E2	17
	3.E2	44.2
	1.E3	63.2
	2.E3	40.6
	3.E3	48.9

Table 2. 10 most Differentially expressed genes (DEGs) in the *P. tenuifila* Transcriptome.

Gene code	pvalue	Fold Change	*Evalue*	Homology/NCBI Acession number*
c31498_g1	5,59E-34	6,868456	0	*CYP749A22-like citocromo P450* (*Populus euphratica*) XP 011015632.1
c63774_g2	1,10E-31	10,88395	$4e^{-105}$	*Myb-related protein 305-like* (*Coffea arabica*) XP 027073189.1
c24493_g2	1,57E-31	10,01619	0	*Glycerol-phosphate-2-O-acyl-transferase-6* (*Tumera sublata*) KAJ_4826684.1
c26267_g2	2,92E-31	7,708059	$3e^{-57}$	*Hypothetical protein TSUD 50780* (*Trifolium subterraneum*) GAU49549.1
c26267_g1	7,41E-31	8,908789	$7e^{-100}$	*Hypothetical protein Tsubulata_040799* (*Turnera subulata*) KAJ4825133.1
c29482_g1	2,20E-28	6,944572	$6e^{-87}$	*Serine-Threonine protein kinase* (*Salix koriyanagi*) KAJ 6695345.1
c9915_g1	6,37E-28	7,415687	$8e^{-136}$	*Hypothetical protein Tsubulata_015173* (*Tumera subulata*) KAJ 4826078.1
c34266_g1	7,24E-28	6,216299	$3e^{-71}$	*Pectinesterase-inhibitor-4-like* (*Populus alba*) XP 034929732.1
c28420_g1	9,93E-28	6,098355	$1e^{-70}$	*Hypothetical protein Tsubulata 006923* (*Tumera subulata*) KAJ 4847747.1
c27277_g1	3,10E-26	8,686877	$7e^{-155}$	*Hypothetical protein Tsubulata 006168* (*Tumera subulata*) KAJ 4835905.1

*Homologies as were taken in NCBI, Blast X on June, 2024

All sequencing-generated data were used to assemble the transcriptome. The obtained results demonstrated that, in general, it was possible to assemble 95,707 different gene partial sequences and 139,628 isoforms of those genes. It was observed that the lowest *p-value* homologous sequence in differential expression analysis between stage 2 (E2) and stage 3 (E3) indicates that it is a cytochrome *P450* (*Evalue* 0, *CYP749A22-like cytochrome P450* (*Populus euphratic*) *a*, NCBI XP_011015632.1) and the second lowest *p-value* is a possible *Myb*-type transcription factor (*Evalue* $3e^{-105}$ *Myb-related protein 305-like* (*Coffea arabica*), NCBI XP_027073189.1) (Table 2).

Differential Expressed Genes (DEGs) analysis also showed that there are 72,884 DEGs, of which 215 with *p-values* bellow $1e^{-10}$, 1.415 with *p-values* bellow $1e^{-5}$, 3.824 with p-values bellow $1e^{-3}$.

Another observation was that the most expressed sequence (which presents more reads) in five libraries is related to the *Cowpea*-aphid-borne mosaic virus polyprotein (*Evalue* 0, hology 92.65%, NCBI QFU 28541.1, in 2024) already described in *P. edulis* (Nascimento et al., 2006), but not described for *P. tenuifila* before this work. The visual observation of collected fruits did not identify any signs of infection.

Enzymes related to the biosynthesis pathway of phenolic compounds were also found in this gene database, such as phenylalanine-ammonium-lyase, transcinnamate-4-mono-oxidase, 5-hydroxyferulic acid-o-methyltransferase, 4-coumarate-CoA ligase, cinnamoyl-CoA reductase, cinnamyl alcohol dehydrogenase 2, coniferin-beta-glucosidase, peroxidase A3a precursor (EC 1.11.1.7), and bifunctional catalase/peroxidase, among others.

From a manually or reversed-blast curated list of ninety-three gene sequences, specific primers were first tested in PCR and their amplicon sequences confirmed through capillary sequencing. Six housekeeping genes (HKG) and four sequences of genes associated with secondary compounds biosynthetic pathways were selected to study differential gene expression during *P. tenuifila* ripening fruits (Figure 2A and Table 3). The length range of the amplicons was 118-195pb.

Among the six candidates to HKG, four selected sequences are homologous to hypothetical proteins *(HPT027299*, *HPT038363*, *HPT036083*, *HPEZV62*). Other candidates are homologous to myotubularin-like phosphatidylinositol-3-phosphatase 1-like-isoform X1 from *Jatropha curcas* (*Evalue* 0; XP_012090071.1) (*PIP*) and aminopeptidase M-like protein from *Hevea brasiliensis* (*Evalue* 0; XM_021817337.2) (*AMP*). Two selected target genes are homologous to *Turnera*

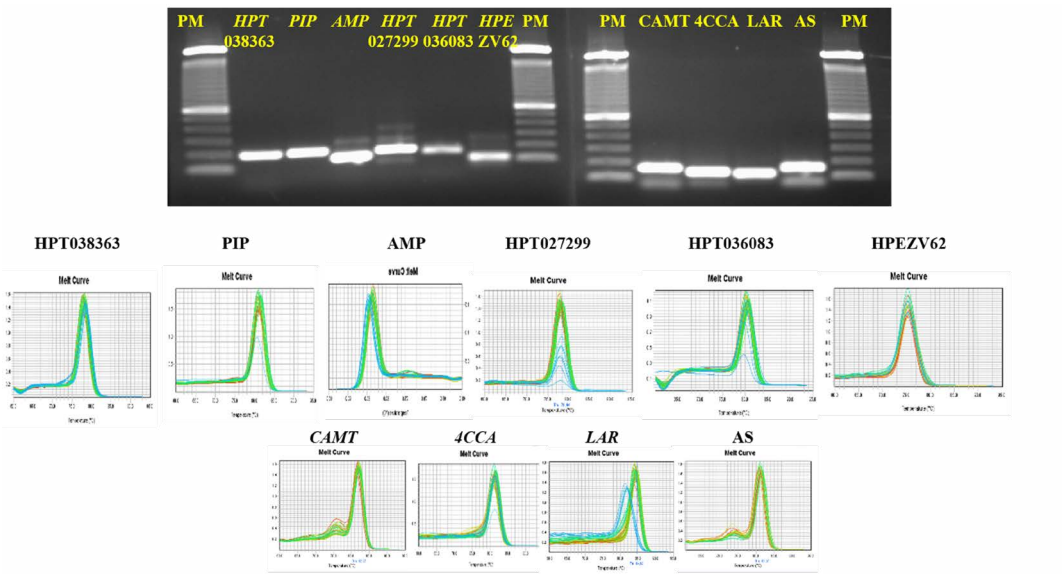

Figure 2. A - Electrophoresis on a 1% gel stained with 0.5 µg.ml-1 ethidium bromide showing the studied amplicon gene sequences. Molecular weight MW-100 bp (Invitrogene®) B- Melting curves resulted from each gene studied in qPCR. Yellowish, and orange curves correspond to seed/aryl and peel samples (different stage samples), respectively. The blue curves are equivalent to leaf samples.

Table 3. Characteristics of genes selected in the transcriptome of *Passiflora tenuifila,* validated in qPCR.

Gene	Type	Homology	*Evalue*/ Identity(%) (%)	NCBI*
HPT027299	R	*hypothetical protein T.subulata_027299* [*Turnera subulata*]	0 /73.66	KAJ_4842432.1
HPT038363	R	*hypothetical protein T.subulata_038363* [*Turnera subulata*]	$2e^{-73}$/ 76.3	KAJ_4827517.1
HPT036083	R	*hypothetical protein T.subulata_036083* [*Turnera subulata*]	0 / 73.4	KAJ_4830704.1
HPEZV62	R	*Casein kinase 1* *[Hibiscus trionum*]	$1e^{-173}$/ 96.63%	GMI_92686.1
PIP	R	*Phosphatidyl inositol 3 phosphatase myotubularin 1 isoform X1* *[Jatropha curcas}*	0 /82.13	XP_012090071.1
AMP	R	*aminopeptidase M1-like* [*Hevea brasiliensis*]	0 /82.83	XM_021817337.2
AS	T	*1-aminociclopropane-1-carboxylate synthase* [*Manihot esculenta*]	0 /83.44	XP_021629594.1
LAR	T	*leucoanthocyanidin reductase 1* [*Populus nigra]*	$3e^{-173}$ 72.83	XP_061986048
CAMT	T	*Caffeic acid 3-O-methyltransferase* 1 [*Turnera subulata*]	0 /91.23	KAJ_4835650.1
4CCA	T	*4-coumarate-CoA ligase 2 [Turnera subulata]*	0 /90.84	KAJ_4830135.1

R=Reference; T=Target; *Homologies taken in NCBI, BlastX on June, 2024

subulata as caffeic acid 3-o-methyl transferase gene (*Evalue* 0; KAJ_4835650.1) (*CAMT*) and 4-coumarateCoA ligase gene from *Turnera subulata* (*Evalue* 0; KAJ_4830135.1) (*4CCA*). ACC synthase gene is homologous to *Manihot esculenta* gene (*Evalue* 0; XP_021629594.1) (*AS*); leucoanthocyanidin reductase from *Populus nigra* (*Evalue* $3e^{-173}$; XP_061986048) (*LAR*). As shown, several sequences were closely related to *Turnera subulata*, which represents a plant from the Passifloraceae family. These results indicate consistency in the *P. tenuifila* transcriptome assembly. Among the selected target gene sequences, the *CAMT* gene was among the most 100 DEGs in the transcriptome. The possible *CAMT* gene constitutes a precursor enzyme of caffeic acid that has been associated with anti-oxidant, neuroprotective, anti-inflammatory, anti-viral, anti-cancer and diabetes properties (Cizmarova et al., 2020). The sequence associated with *LAR* represents a gene in the biosynthesis pathway of leucoanthocyanidins derived from flavonoids biosynthesis (Liu et al., 2021). The possible *4CCA* gene constitutes the main enzyme of the phenylpropanoid biosynthesis pathway and regulates the synthesis of lignin and flavonoids (Li et al., 2020). The *AS* encodes an enzyme of the ethylene biosynthesis pathway, a phytoregulator related to the processes of fruit ripening and senescence.

The calculated efficiency (E) for the genes studied in qPCR ranged from 1.90 to 2.54 with the R^2 ranging from 0.94 to 0.99 (Table 4). The primers for the *HPEZV62*, *HPT036083*, *AS* and *4CCA* genes were the least efficient (Table 4).

Table 4. Parameters of Amplification Efficiency for Analyzed Genes in qPCR.

Gene	*Amplicon Length (bP)*	*Tm(°C)*	*Efficiency*	*Slope*	*R^2*
HPT027299	188	55	1,91	-3,55	0,98
HPT038363	193	55	2,11	-3,1	0,98
HPT036083	159	55	2,54	-2,5	0,96
HPEZV62	103	62,4	2,31	-2,74	0,97
PIP	195	62,4	1,94	-3,47	0,99
AMP	142	62,4	2	-3,32	0,97
AS	153	55	2,4	-2,6	0,94
LAR	118	64,5	1,95	-3,43	0,99
CAMT	158	55	1,90	-3,6	0,99
4CCA	132	62,4	2,3	-2,72	0,94

Table 5. *GNorm* and *Bestkeeper* Algorithms analysis.

Genes/Analysis	*AMP*	*HPT038363*	*HPT027299*	*PIP*	*HPT036083*	*HPEZV62*
qBase GeNorm (M <0.5)	**0,385**	**0,395**	0,405	**0,47**	0,605	ND
Bestkeeper Standard deviation	**0,49**	**0,42**	0,54	**0,67**	1,06	ND
(SD< 1)	**	**0,78***	2,35*	**0,90***	1,22*	ND
CV	**1,91**	**1,36**	2,08	**2,27**	3,41	ND
	**	**2,53***	8,54*	**3,01***	3,92*	ND

*with leaves samples; **leaves samples in AMP were not determined

In fact, the *HPT036083* and *HPEZV62* genes produced more expression differences in the repetitions. The *AS* gene expression pattern appears to occur mainly during the E2 and E4 stages of the seed/aryl and peel of ripening fruit. Finally, the *4CCA* gene shows considerable expression in the peel during E4 compared to all others. All these situations result in an analysis destabilization producing low efficiency of the primers. Another surprising observation for the *LAR* gene expression analysis detected a melting curve slightly different in seeds/aryl and peel compared to leaf samples (blue curves, Figure 2B). This observation may be possibly a product of a differential mRNA transcriptional expression in mature leaf compared to seeds/aryl and peel.

GNorm (Hellemans et al., 2007) and *Bestkeeper* (Pfaffl et al., 2004) were used to find the best genes or pairs of genes to use as HKG. *GNorm* included in the *qBase plus* software (v.2.3) (Figure 3) considers the *M value* among all studied genes. The *M value* is an expression variability parameter based on all studied samples. *M values* must be less than 0.5 (M < 0.5). *Bestkeeper* algorithm calculates an *Index* that is the geometric mean of the crossover point (CP) values of three candidate genes. The best genes selected by *Bestkeeper* are related to the standard deviation (SD) and the percentage of sample variability (V). The standard deviation must be less than 1 (SD < 1) and the variability low. *GNorm and Bestkeeper* analyses were complementary (Table 5). Both algorithms performed the analysis without the *HPEZV62* gene, peel samples at Figure 3.

The E3 stage, and leaves. The *HPEZV62* gene, peel at the E3 stage and leaf samples showed the greatest variability. In fact, when leaf samples were included in the *Bestkeeper* analysis showed increased gene expression variability especially in leaf samples for the *HPT027299* gene (Figure 4).

Taking into account both analyses, we can say that the genes *HPT038363*; *AMP*, and *PIP* are the best choices to use as qPCR reference genes for gene expression studies in *P. tenuifila* fruits

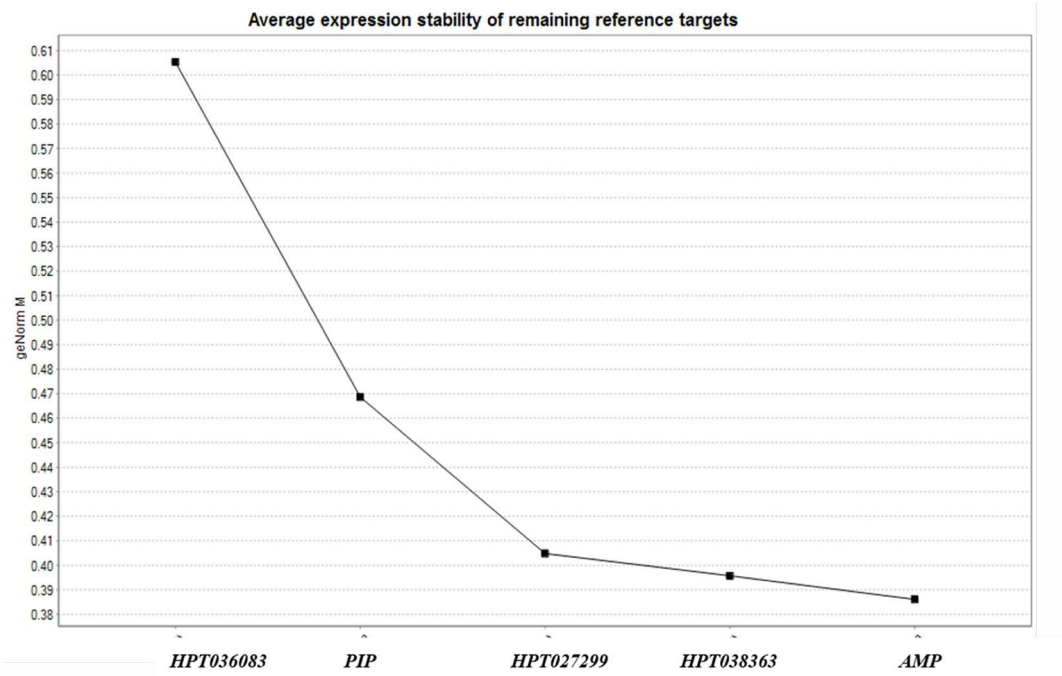

Figure 3. GNorm analysis of candidate reference gene stability.

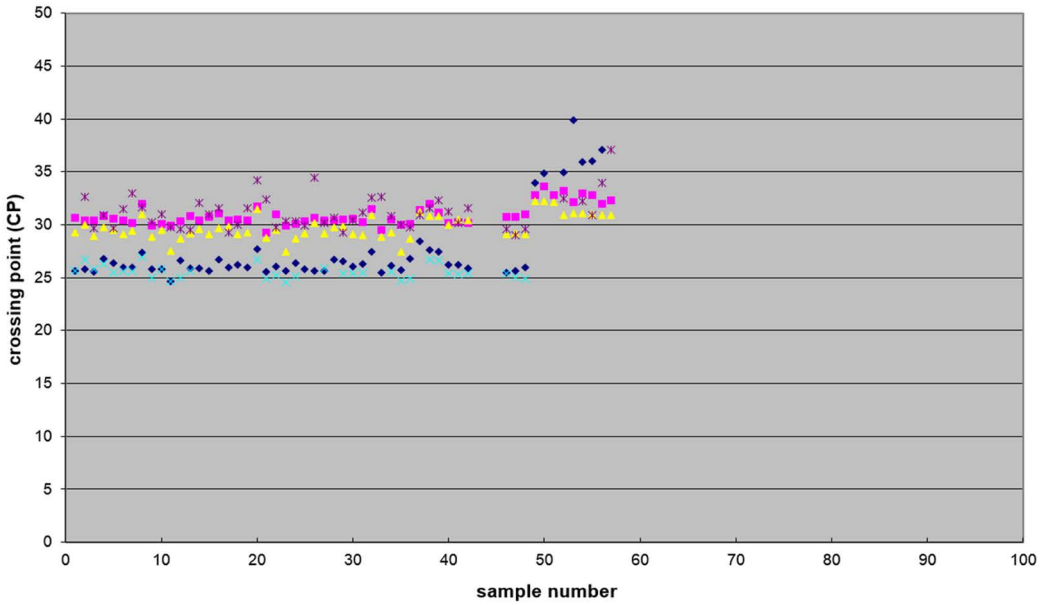

Figure 4. *Bestkeeper* output showing different amplification levels for genes *HPT027299* (dark blue), *HPT038363* (pink), *AMP* (light blue), *PIP* (yellow) and *HPT036083* (brown). Leaves samples correspond to 50 to 60 samples.

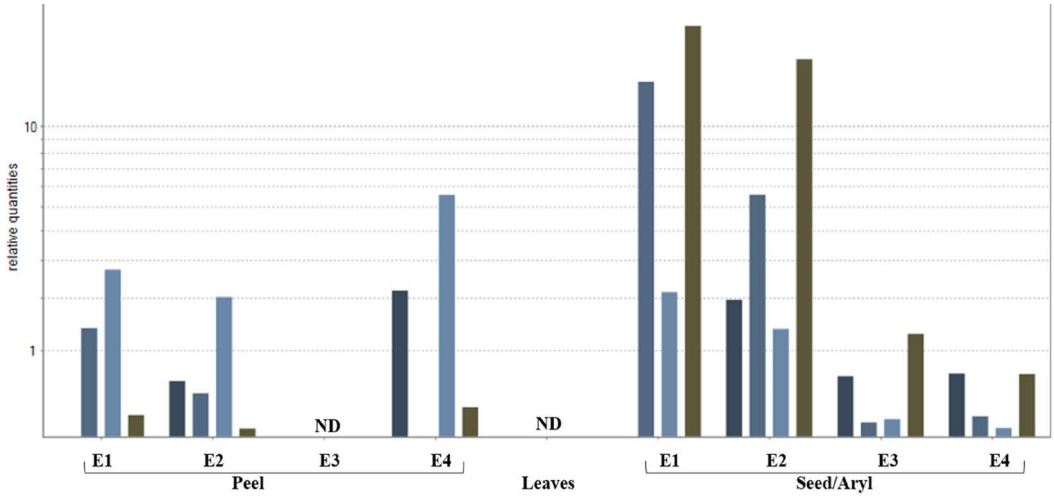

Figure 5. Relative gene quantification in the seeds/aryl and peel at beginning of fruit ripening (E1 and E2), turning to mature (E3) and mature fruit (E4): *AS* (dark blue), *CAMT* (blue), *4CCA* (light blue) and *LAR* (brown) genes in qPCR during *P. tenuifila*. ND-not determined.

(seeds, aryl, and peel) (Figure 3, Table 5). They resulted in the best values of *M* (*GeNorm*), SD, and V (*Bestkeeper*) values. Moreover, genes *HPT038363* and *PIP* are the most stable in leaf samples. Finally, the *HPT036083* gene was the most variable gene during analyses and should not be used as a reference gene.

The relative quantification of the target genes *CAMT*, *4CCA*, *LAR*, and *AS* in *P. tenuifila* fruits (seed/aryl and peel) was carried out considering the *HPT038363*, *AMP*, and *PIP* genes as reference genes. The result demonstrated the high expression of the *CAMT*, *4CCA*, and *LAR* genes at the beginning of the seed/aryl development (stages E1 and E2) (Figure 5). However, considerable expression of the 4CCA gene was even higher in the skin of mature fruits (stage E4) compared to the seed/aryl. The *AS* gene has an expression peak at E2 and another late larger peak at E4 in peel. Peel samples from E3 showed high variability and were not considered. In seed/aryl samples after the *AS* peak at E2, it was observed a small drop at E3 that continues until E4. The *AS* gene at E4 seed/aryl showed an expression higher when compared to the *CAMT* and *4CCA* genes, and almost the same level of *LAR* expression.

DISCUSSION

The most expressed DEGs found in this transcriptome are the cytochrome *p450* and the *Myb* transcription factor genes. Both are related to fruit and seed development processes and secondary compounds biosynthesis. Cytochrome P450s were associated with seed size and development control in *Arabidopsis thaliana*, rice, soybean, tomato, and regulation of fruit size in Sweet Cherry (*Prunus avium* L.) (Qi et al., 2017). The high expression level of the *Cytochrome P450* gene in the developing fruits of the *P. tenuifila* is expected to have similar functions. *Cytochrome P450* is also involved in the oxidative tailoring of triterpenes metabolism and steroids (Malhotra & Franke, 2022). *Myb* transcription factors are related to anthocyanins biosynthesis (Li et al., 2022). So, both highly expressed DEGs found in the results could be related to secondary compounds biosynthesis contributing to *P. tenuifila* anti-oxidative and anti-inflammatory properties.

Today, qPCR is the most used analysis to study gene expression in a wide variety of species. Crucial parameters to develop a good qPCR analysis such as RNA purification and quantification, removal of residual genomic DNA (gDNA) and efficiency of cDNA synthesis are important factors to consider carefully. In this study, we controlled the parameters described above using quality protocols for RNA extraction or cDNA synthesis and verifying RNA spectrophotometrically, using Bioanalyzer or in agarose gels prior to expression analysis. Both RNA extraction and cDNA synthesis protocols consider residual gDNA removal. In addition, we checked all primers in previous PCR amplification analysis and confirmed their amplified sequences through amplicon sequencing. And, finally, performed qPCR efficiencies to each analyzed gene. The use of biological and technical triplicates, three different tissue types and four different developmental stages strengthen our results for stability of studied reference genes. Candidates to qPCR reference genes were normalized using two different algorithms demonstrating consistent results. Finally, the use of three different reference genes in relative gene expression studies minimized errors.

Taking into account gene homologies and gene expression, we observed that the genes included in the pathways of phenolic compounds, flavonols and protoanthocyanidins (*caffeic acid-o-methyltransferase*, *4-coumarate-CoA ligase* and *leucanthocyanidin reductase*) have an expression peak in the early stages (E1, E2) of seed/aryl and peel during fruit ripening (*CAMT*, *4CCA*, *LAR* genes) (Figure 5). Expression of the *4CCA* gene (*4-coumarateCoA ligase*) in peel at the E4 stage was even higher, being the highest expression detected in this case. Based on this, a more relevant presence of flavonoids is expected in the peel in mature fruit skin when compared to the seed/aryl as is phenolic compounds in seeds/aryl. The *AS* gene, being the first enzyme for ethylene biosynthesis, showed two peaks of expression at E2 and E4 when considering peel, and a peak at E2, with less expression at E3, and E4 when considering seed/aryl. This result coincides with color change observed in the fruit skin (and aryl) at stages E2 and E4 (Figure 1). Therefore, *AS* gene expression may represent a genetic marker for *P. tenuifila* fruit ripening. The expression analysis of genes *CAMT*, *4CCA* and *LAR* gathers information about the fruit attributes when considering phenolic compounds or flavonoids biosynthesis pathways responsible to anti-oxidant, anti-inflammatory or other properties already known (Sozo, 2014). Porika et al., 2016 consider the study of gene expression or gene regulation during fruit ripening a strategy to improve the harvest and post-harvest of fruits to increase their quality attributes. Kapoor et al., (2022) consider that quality attributes such as the production of pigments or other nutritional or functional compounds can be a biotechnological development strategy aiming at food biofortification. Furthermore, during different post-harvest treatments, it is important to have a quick and reliable test for fruit quality. In this case, it could be the gene expression analysis of enzymes associated with ethylene biosynthesis or related to functional properties using qPCR.

It was shown that the *AS* and *CAMT* genes were also expressed in at least four other different species of *Passiflora*, such as *P. edulis*, *P. alata*, *P. setacea,* and *P. quadrangularis* (Cordeiro et al., 2017a, b). Based on the presented results, we can point *AS* gene expression as a gene marker for peel and seed/aryl at E2 and E4 stages to study fruit ripening in *Passiflora*.

Caffeic acid and protoanthocyanidins are compounds with antioxidant, anti-inflammatory, anticonvulsants, anxiolytics properties (Holanda et al., 2020, 2019) produced in the initial stages (E1 and E2) of seed/aryl. *P.tenuifila* fruits seem to have a higher content of flavonoids in the peel of the mature fruit and phenolic compounds in seeds at early stages of development. These results also show possible genetic markers to study fruit ripening at least in these five different *Passiflora* species (*P. tenuifila*, *P. edulis*, *P. alata*, *P. setacea* and *P. quadrangularis*). *4CCA*, *CAMT*, and

LAR may also be gene markers for an intense expression of flavonoids, derivatives, or phenolic compounds biosynthesis pathways in peel and seed/aryl of ripe and unripe fruits.

This work represents the first report analysis of *P. tenuifila* seed/aryl transcriptome validated by relative gene expression during fruit ripening using qPCR. This study selected three genes (*HPT038363*, *AMP*, and *PIP*) as reference genes in qPCR. It was also possible to observe a different pattern of gene modulation related to enzymes in the biosynthesis pathways of ethylene, phenolic compounds, or flavonoids during fruit ripening. The use of gene expression as markers related to fruit metabolism, such as ethylene, biosynthesis of phenolic compounds, or flavonoids, can be a strategy to identify quality attributes for the fruit market.

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DATA ACCESS

The sequencing data were deposited at the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1149057 and will be accessed by the time of publication.

ETHICS STATEMENT

The Project has a completely regular legal status with respect to the Federal Special Authorization for the transport and access to genetic resources for bioprospecting purposes (Process No. 02000.001515/2013-85) (DOU No. Sisgen Protocol No. AADBE7C).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

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