

Molecular cloning and characterization of cDNAs encoding cytosolic malate dehydrogenase and vacuolar (H⁺)-ATPase in *Annona cherimola* and their expression during postharvest ripening

Manuela Gouveia^{1,*}, Mónica Rodrigues^{1,3}, Lucília Teixeira¹ and Nereida Cordeiro²

¹ Competence Centre of Life Sciences, University of Madeira, 9000390 Funchal, Portugal

² Competence Centre for Exact Sciences and Engineering, University of Madeira, 9000390 Funchal, Portugal

Received 7 May 2015; revised 15 March 2016; accepted 20 March 2016

This study aims to investigate the expression of two cherimoya genes putatively related to fruit ripening. Two full-length cDNAs encoding cytosolic NAD-dependent malate dehydrogenase (*AccytMDH*) and vacuolar (H⁺)-ATPase c subunit (*AcVHA-c*) were isolated from *Annona cherimola* using the reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The *AccytMDH* codes for a 332 amino acid polypeptide with a predicted molecular mass of 35.6 kDa. The deduced amino acid sequence for *AccytMDH* shared high identity with other plant homologous malate dehydrogenase proteins. The *AcVHA-c* encodes a proteolipid subunit of the V-type proton ATPase with 166 amino acids (16.7 kDa). Comparison of the deduced amino acid sequence from *AcVHA-c* revealed four transmembrane domains highly conserved among plant counterparts. The expression of *AccytMDH* and *AcVHA-c*, assessed by semi-quantitative RT-PCR showed that there is an increase in the accumulation of transcripts during postharvest ripening, although not correlated by a significant upsurge of titratable acidity they might contribute to organic acid accumulation and translocation during postharvest ripening of cherimoya in association with other enzymes and carriers. By using *AccytMDH* and *AcVHA-c* as molecular targets new strategies can be exploited to get a clear picture in the ripening of cherimoya.

Keywords: Cherimoya, *Annona cherimola*, fruit ripening, gene expression, malate dehydrogenase, vacuolar ATPase

Introduction

During ripening of fleshy fruit chemical modifications, either by enzymatic or non-enzymatic processes, leads to remarkable changes in color, aroma, flavor and decrease in pulp firmness¹. Cherimoya (*Annona cherimola* Mill.) is a soft subtropical fruit which is preferably consumed fresh. It is often considered one of the best tasting fruit in the world due to the pleasing blend of sugars, mainly glucose², organic acids and secondary metabolites stored within the vacuole³. The major drawback of this climacteric fruit is the rapid ripening process (within 5 d at 20°C), that quickly soften the fruit and reduce postharvest life. Therefore, research programs have been developed to shed light on the ripening mechanisms in order to find molecular targets to improve cherimoya quality and storing⁴⁻⁶.

Malic acid, an important element of fruit organoleptic traits, is the predominant organic acid in

several fruit such as peach⁷, apple⁸ and loquat⁹, though it declines during maturation, unlike to what was observed for cherimoya¹⁰. The accumulation of malic acid during fruit ripening is controlled by several factors in intertwined metabolic processes¹¹ being attributed to cytosolic malate dehydrogenase (MDH) the responsibility for much of the malate synthesized. Some MDH isoforms have been identified in plants based upon coenzyme specificity, subcellular localization and physiological function¹². Sweetman *et al*¹³ suggested that differential regulation of individual MDH isoforms could be involved in malate synthesis and degradation depending on the isoform present, cellular conditions and the availability of substrates. The accumulation of malic acid during cherimoya ripening is driven mainly by the activity of MDH¹⁴. This enzyme facilitates the reversible conversion of oxaloacetate and malate using either NAD or NADP as a cofactor to maintain the equilibrium between these two organic acids¹³ and by extension regulates the cytoplasmic pH¹⁵.

The mechanism by which organic acids and soluble sugars are translocated to pulp cells vacuoles is not fully understood but as proton pumps mediate the

* Author for correspondence:

Tel: +351-291-705-387; Fax: +351-291-705-149
mgouveia@uma.pt

³ Present address: Instituto Nacional de Investigação Agrária e Veterinária, I.P. Quinta do Marquês, 2780-159 Oeiras, Portugal

acidification of intracellular compartments they seem to play an important role during fruit enlargement and accumulation of sugars and organic acids^{7,16,17}. Vacuolar (H⁺)-ATPases (V-ATPase) and vacuolar (H⁺)-inorganic pyrophosphatase are proton pumps that produce the proton motive force essential to generate an electrochemical gradient across the vacuolar membrane which is required as a driving force for antiporters of sugar and secondary metabolites transporters¹⁸. The V-ATPase, found in all eukaryotic cells, is a membrane-bound multi-subunit enzyme complex. It is highly conserved among species and comprises two large domains: the peripheral (V₁), a hydrophilic catalytic complex responsible for ATP hydrolysis and the integral (V₀), a hydrophobic transmembrane H⁺ channel. The complex contains 13 different subunits, eight (A – H) from the V₁ domain and five (a, c, c', c'', d) from the V₀ domain^{19,20} that form a ring-like structure in the lipid bilayer and operates by a rotary mechanism coupling proton transport across membranes to ATP hydrolysis. The number of subunits in the ring remains uncertain. For several fruit such as pear, grape berry and tomato V-ATPase is the main proton pump at the mature stage and during ripening as gene expression and activity increases with fruit maturation^{16,21-23}. All subunits are vital to assemble the V-ATPase and suppression of any subunit might reveal important insights into its role^{17,20}.

Only recently full-length coding sequences from *A. cherimola* related to browning⁴, cell wall enzymes⁵ and chilling response⁶ have been characterized. However, none of the cDNAs reported in this article nor the changes in gene expression during postharvest ripening of cherimoya have been investigated so far. As molecular tools might be useful to pursue the molecular and biochemical mechanisms to get a better insight into sugar and organic acid accumulation during cherimoya ripening, we isolated and characterized full-length cDNAs encoding cytosolic NAD-dependent malate dehydrogenase (*AccytMDH*) and vacuolar (H⁺)-ATPase c subunit (*AcVHA-c*) from cherimoya fruit and investigated their expression patterns during postharvest ripening.

Materials and Methods

Plant Material

Cherimoyas (*A. cherimola* Mill. cv. 'Madeira') were harvested (early January) at mature green stage from trees in a commercial orchard in Faial (Madeira

Island, Portugal). Fruit were treated as described before² and stored at room temperature (20 – 22°C) in a dimly lit place. Every day after harvest, 3 to 4 fruit were randomly chosen, peeled, sliced, quick-frozen in liquid nitrogen and stored at – 80°C until required.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 300 mg of fresh frozen material using a mixture of 600 µL of extraction buffer (0.2 M sodium acetate pH 5.0, 10 mM EDTA, 1% SDS) and 600 µL of phenol pre-warmed at 65°C. The same volume of chloroform : isoamyl alcohol (24 : 1) was added before vortexing. After centrifugation the aqueous phase was extracted with 1 volume of chloroform : isoamyl alcohol. Total RNA was precipitated in 8 M LiCl at – 20°C for 2 h, followed by centrifugation at 12000 g for 10 min at 4°C. The RNA pellet was successively washed once with 2 M LiCl, and twice with 80% ethanol. The RNA was dried at room temperature and resuspended in sterile ultrapure water. Before cDNA synthesis the RNA was treated with DNase using the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. The cDNA synthesis was performed in 20 µL with 5 µg of total RNA, oligo (dT)₁₂₋₁₈ (Invitrogen) and AMV-RT (Invitrogen), according to supplier's protocol.

Cloning Partial cDNA Fragments of MDH and V-ATPase

Partial cDNAs encoding malate dehydrogenase and vacuolar H⁺-ATPase were obtained by RT-PCR. Degenerate primers were designed from highly conserved amino acid sequences from homologous in plant species. For malate dehydrogenase nucleotide primers (sense: 5'-ATGGAGYTGKATGCTGC-3' and antisense: 5'-GGTTVACATCAGGRTAGTG-3') were designed from the amino acid sequences MELVDAA and QYPDVNH, respectively. The amplification of vacuolar (H⁺)-ATPase was obtained with oligonucleotides (sense: 5'-GCNTGYGARAAN TGGGGYTT-3' and antisense: 5'-TGGAANAGNA RGATGAKGCC-3') designed from the amino acid sequences ACENWGF and GILLFQ of ACC oxidase, respectively.

The RT-PCR reactions were carried out in a total volume of 50 µL containing 5 µL cDNA (10X diluted), PCR buffer (10 mM Tris-HCl, pH 8.3), 2.5 mM MgCl₂, 0.2 mM dNTPs, 50 ng/µL of each primer and 1 U *Taq* DNA polymerase (Invitrogen). The PCR profile consisted of 94°C, 3 min followed by 35

cycles each of 94°C, 30 s; 45°C *MDH*, 48°C *V-ATPase*, 30 s; 72°C, 30 s; and a final extension of 72°C, 5 min. All PCR reactions were carried out in a Perkin Elmer 2400 thermocycler.

The PCR products with the expected sizes were purified by using high pure PCR product purification kit (Boehringer) or QIAquick gel extraction kit (Qiagen). After purification, DNA fragments were ligated to pGEM-T Easy vector (Promega). The ligation mixture was used to transform *E. coli* DH5 α competent cells. Transformants were selected in LB-agar plates containing ampicillin, IPTG and X-Gal for white/blue screening. Recombinant plasmids were extracted by using QIAprep spin miniprep kit (Qiagen) and the insert confirmed by DNA sequencing.

RACE Procedures and DNA Sequencing

The 5'- or 3'-rapid amplification of cDNA ends (RACE) was performed according to the supplier's protocol (5'/3' RACE kit, 2nd Generation, Roche). Gene specific primers were designed based on the nucleotide sequence of the cDNA fragments obtained previously by RT-PCR using the OligoExplorer 1.2. The 5'- and 3'-RACE of *AcMDH* cDNA was obtained using the primer AcMDH5R-SP3 CCTTGGGAACCCACCAACCA and AcMDH-3R GTTGAGGCATGCACTGGTG, respectively. To obtain the 5'- and 3'- RACE of *AcVHA-c* primers AcvHATPase 5R-SP3 ACA CACCGGCCAT AACGACTG and AcvHATPase-3R AGGCCTGAGC TCGTGATGAA, respectively, were used. The PCR reactions and the thermal cycling were as described by the manufacturer. PCR products were run on a 1% agarose gel containing ethidium bromide (0.5 μ g/mL) in 1X TAE buffer and the corresponding DNA fragments were cloned into pJet 1.2/blunt (Fermentas) according to the manufacturer's recommendations. Plasmids from positive clones were purified using GeneJet plasmid miniprep kit (Fermentas). In all plasmids, cloned cDNA fragments were sequenced on both directions by a commercially AB 3739XL capillary analyzer (Macrogen Europe, The Netherlands) using specific oligonucleotides to the vector.

Sequence Analysis

The nucleotide sequences of the cloned fragments were compared against databases using BLASTX and BLASTP programs at the National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov>)^{24,25} to search for

homology against nucleotide and amino acid sequences. NCBI ORF Finder was used to detect the open reading frame. The deduced amino acid sequences were aligned with the corresponding sequences of other plants using ClustalW2 (<http://www.ebi.ac.uk>)²⁶. The physicochemical properties of the deduced protein were determined by the ProtParam program (<http://web.expasy.org>)²⁷. The Predict Protein (<http://www.predictprotein.org>)²⁸ and TMPred Server²⁹ were employed to foresee the features of the protein. The sequence data of *AccytMDH* and *AcVHA-c* described in this study have been deposited at the NCBI under accession number KJ508880 and KJ508881, respectively.

Semi-Quantitative RT-PCR Analysis

To study the expression of *AccytMDH* and *AcVHA-c* during postharvest ripening semi-quantitative RT-PCR was performed in parallel reactions on each RNA sample using the following gene specific primers, optimal cycle numbers and annealing temperature: for *AccytMDH* 5'-GTTGAGGCATGCACTGGTG-3' and 5'-CACTGACTTGACATTTAGCC-3', 30 cycles, 58°C; for *AcVHA-c* 5'-AGGCCTGAGCTCGTGATGAA-3' and 5'-CCAACAATCAGACCATATAGA-3', 28 cycles, 55 C and for *ubiquitin* 5'-ATGCAGATYTTTGTG AAGAC-3' and 5'-ACCACCACGRAGACGGAG-3', 35 cycles, 50°C³⁰. *Ubiquitin* was used as a reference gene. At each day after harvest, total RNA was extracted as described above and used as template (1.5 μ g) to synthesize the first-strand cDNA in 20 μ L reaction. The corresponding gene products were amplified by RT-PCR as described above, visualized with the BioCaptMW version 99.03 system (Vilber Lourmat, France) and quantified using the Bio-Profil Bio-Gene V99.01 software (Vilber Lourmat, France). The relative expression level for each target gene was determined by calculating the ratio of the target gene intensity to the *ubiquitin* intensity signal obtained from the same cDNA sample.

Titrateable Acidity Determination

Titrateable acidity was performed according to Association of Official Analytical Chemists (AOAC)³¹. Briefly, the juice obtained from 100 g of fruit was diluted with deionized water and titrated to pH 8.2 with 0.1 N sodium hydroxide. This parameter was determined twice per fruit and the results expressed as citric acid equivalents per 100 g of fresh weight.

Statistical Analysis

Data were expressed as means ± standard deviation (SD). At least three replicates were performed for semi-quantitative RT-PCR. The Kolmogorov-Smirnov ($P > 0.05$) was carried out to verify the normality of data. The one-way analysis of variance (ANOVA) was used to test for differences in gene expression and titratable acidity. Significant differences were further analyzed by Scheffé or Tukey's multiple comparisons test ($P < 0.05$). All statistical analysis was performed using SPSS (Statistical Package for Social Sciences) version 15.0 for Windows.

Results

Isolation and Sequence Analysis of AccytMDH

The partial sequence of malate dehydrogenase from *A. cherimola* was amplified by RT-PCR using degenerate primers designed from highly conserved amino acid sequences from MDH homologous in plant. Following PCR reactions, a specific amplified fragment was cloned and sequenced. The sequence analysis showed a 427 bp fragment with over 84% identity at the DNA level to other malate dehydrogenase as *Prunus persica* (gb: AF367442⁷) and *Solanum tuberosum* (gb: DQ294258). Based on the sequence of the cDNA fragment, a set of specific primers were designed to obtain the 5'- and 3'-regions. The full-length cDNA of *AccytMDH* gene revealed to contain 1336 nucleotides excluding the poly(A) tail (Fig. 1). This sequence showed an open reading frame of 999 bp starting with an initiation codon ATG at position 85 and ending with a termination codon TAA at position 1083. Therefore, the coding region was found to be flanked by 5' and 3' untranslated sequences of 84 and 253 bp, respectively. The canonical polyadenylation signal (AATAAA), was located 173 bp upstream from the poly(A) tail but several polyadenylation signals predominant in *Arabidopsis*³² were also present in the 3' untranslated region. The protein encoded by this cDNA comprised 332 amino acids with a predicted molecular mass of 35.6 kDa and a deduced isoelectric point of 5.92. Multiple alignment of the obtained AccytMDH with malate dehydrogenase proteins from other plant sources available from GenBank revealed that it is highly conserved among different species (Fig. 2). The deduced amino acid sequence of AccytMDH shared similarities with those from *Prunus persica* (94%), *Malus domestica* (92%) and *Arabidopsis thaliana* (90%). The highest conserved

region of the protein was the N-terminal as among the first 32 amino acids, 30 residues were identical (94%) in the seven species shown in Figure 2. Furthermore, highly conserved motifs for the NAD-binding (TGAAGQI) and for the catalytic activity (IWGNH), located near the putative active site, were also present in the AccytMDH. Further search on protein structure showed that AccytMDH sequence form α-helices and β-sheet structures representing 49% and 22% of the protein, respectively.

```

ggccacagctgcggtttctgctctcgtcttttccctctccctttgtagggtttgattcct
ggaactctcttccatccatcaatggcgaagaaccagttcgagttctcgttacagga
      M A K E P V R V L V T G
gcoctgggacaaattgggtatgctcttctccaatgattgcaagaggtgatgctgggt
A A G Q I G Y A L V P M I A R G V M L G
cctgaccagcctgtaattttacacatgcttgacattgcaaccagctgctgaggccctgaa
P D Q P V I L H M L D I A P A A E A L N
ggagtaaaaatggagttgggtgatgctgcaattctctcttaagggtggtggtgctaca
G V K M E L V D A A F P L L K G V V A T
actgacgtttgttggagcatgcaactggttcaatattgctgttatggttgggtgggttccc
T E V V E A C T G V N I A V M V G G F P
aggaaagaaggtatggaaagaaagatggtgatgctccaaaatgctctatctacaagct
R K E G M E R R D V M S K N V S I Y K S
caggcactctgcaattagagaagcagctgcaaacctgcaaggttcttctgttctgta
Q A S A L E K H A A A N C K V L V V A N
ccagctaacactaatgcaattgattttgaaagagtttctccatccattctctgagaaaa
P A N T N A L I L K E F A P S I P E K N
ataacatgcttgacacgggttgatcacaataggcacttggctcagatctcagagagc
I T C L T R L D H N R A L G Q I S E R L
aatgtgcaagctcagtgatgtgaaasatggtatcatttggggaaatcattcttcaacac
N V Q V S D V K N V I I W G N H S S Y Q
tatcctgctgctccaccatgcccactctcaaaaaccccaatggcgaagagctctgtaagg
Y P D V N H A T L K T P N G E K S V K E
ctcattgctgatgaaatgggttggaaaggagaattcattacaactgccaacaacagaggt
L I A D D E W L K G E F I T T V Q Q R G
gctgcaataaaggcagcgaagctctcaagctcttctgctgcaagttctctgcttgc
A A I I K A R K I S S A L S A A S S A C
gatcatattctgattgggtgctgggaacccctgaggcacttgggtctccatgggtgtg
D H I R D W V L G T P E G T W V S M G V
tactctgatggttcttacaatgtaccogctggactaatatattcttcccagtcacatgc
Y S D G S Y N V P A G L I Y S F P V T C
tgcaatggagaatggactatcgtccaaggactttcaattgatgaatttctgaggaataaa
C N G E W T I V Q G L S I D E F S R N K
ctggagcaactgcaagcggagctgaccgaggaagccttagcttattcattcctttct
L D A T A A E L T E E K A L A Y S C L S
taaacctccatccatattggccactatgcccagtgaaatttctatgcatgcaaggtaac
*
agatctatggtttccaaaataaagctatcgacttctctgtagcctggaggagccttc
atgctcatctttgtcaalctgctgtgattttattctgtagctttttaccattgcccac
tttaccataggagattttgttttgacgtttttgaaacotttattgactatgaagttatacta
agaccacttatatgggaaaaaaaaaaaaaa
    
```

Fig. 1 — Nucleotide and deduced amino acid sequence of full-length AccytMDH cDNA from *A. cherimola*. Deduced amino acid in a one letter code is under the nucleotide sequence. The asterisk corresponds to stop codon. Putative active site is boxed. Polyadenylation signals are underlined. The arrows show the sequence of specific primers used for semi-quantitative RT-PCR.

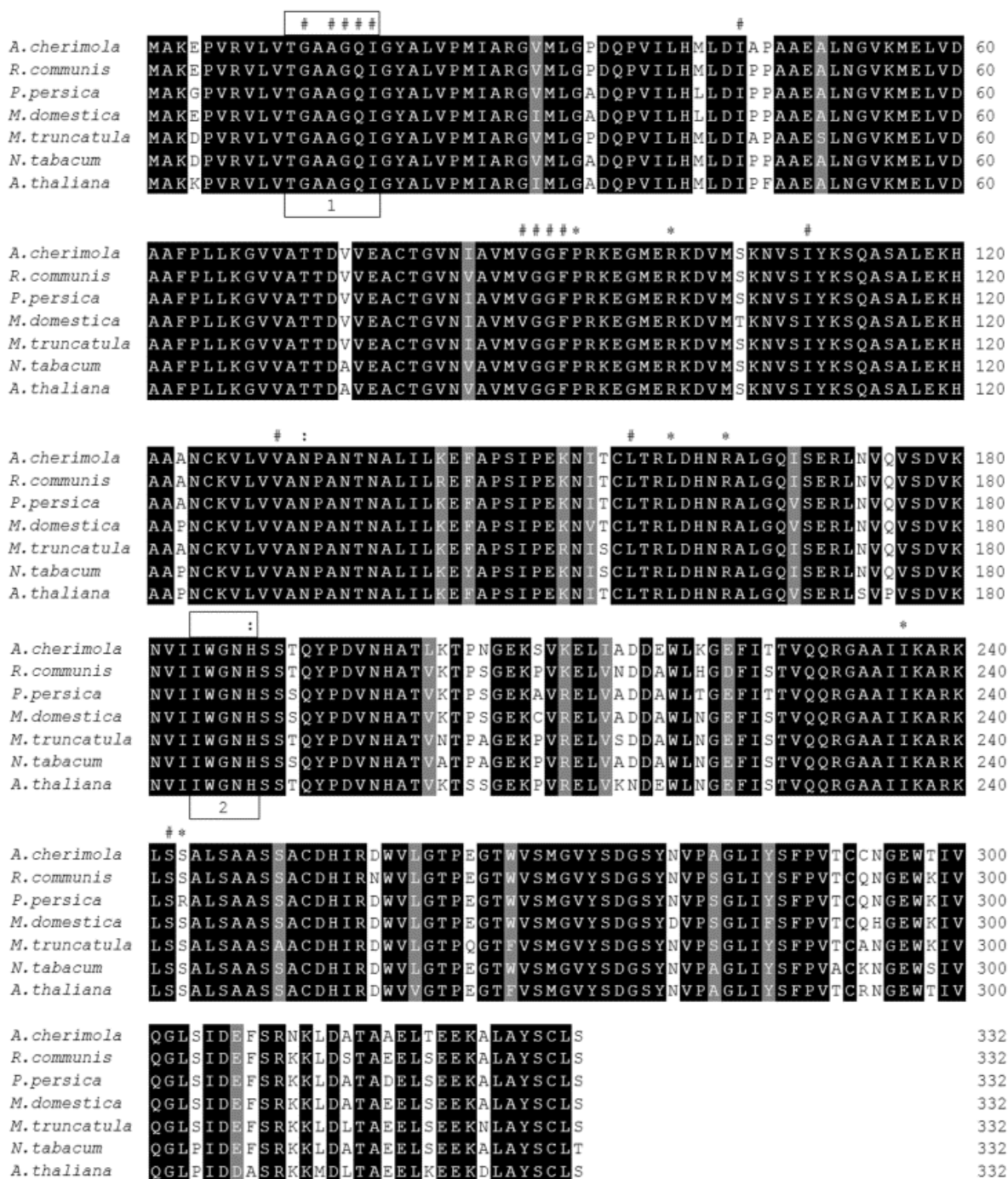


Fig. 2 — Alignment of the deduced amino acid sequence of AcctyMDH with MDH from *Ricinus communis* (gb: XP_002533463), *Prunus persica* (gb: AAL11502), *Malus domestica* (gb: ABB36659), *Medicago truncatula* (gb: XP_003590025), *Nicotiana tabacum* (gb: CAC12826) and *Arabidopsis thaliana* (gb: AAM65532). Conserved and similar amino acids are shaded in black and grey, respectively. The amino acids that binds to NAD (#) malate (*) or both (:). Conserved motifs for NAD-binding (1) and catalytic activity (2) are boxed.

Isolation and Sequence Analysis of *AcVHA-c*

Degenerate oligonucleotides designed from highly conserved amino acid sequences of 1-aminocyclopropane carboxylic acid oxidase

homologous in plant, unexpectedly amplified by RT-PCR a cDNA fragment (346 bp) homologous to the vacuolar ATP synthase 16 kDa proteolipid subunit. This cDNA sequence was used as anchor

to design specific oligonucleotides and a 784 bp full-length cDNA was obtained from rapid amplification of cDNA ends. The open reading frame (498 bp) was flanked by 36 bp at the 5' untranslated region (UTR) and 247 bp at the 3' UTR. This region exhibit at least six polyadenylation signals common in plants³² (Fig. 3). The *AcVHA-c* cDNA encodes a polypeptide of 166 amino acids, with an estimated molecular mass of approximately 16.7 kDa, an isoelectric point of 8.64 and an instability index of 33.49, thus it is a stable protein. The *AcVHA-c* showed only alpha helices (78% of the protein) and revealed four highly conserved transmembrane (TM) domains corresponding to amino acid residues 16-33, 57-76, 99-116 and 134-154 (Fig. 3).

```

ctctatttcgattccttcaaacctaatcccgcagaaatgtcttctgctggcttcagcagc
                                     M S S A G F S S
gatgaaactgctccgttcttcggattccttggcgtgctgctgcccctgcttcttctcctgc
D E T A P F F G F L G A A A A L V F S C
atggggcctgctgctacgggactgccaaagacgagggtcgggtgttgctccatgggagttatg
M G A A Y G T A K S G V G V A S M G V M
aggcctgagctcgtgatgaagtcgaatcgtcccagtcgttatggccggtgtgttgggtatc
R P E L V M K S I V P V V M A G V L G I
tacggcttgattattgctgttattataagcactgggatcaacccaagggcgaatcgtat
Y G L I I A V I I S T G I N P R A K S Y
taccttttcgatgggatgcccatttggctcaggtctcgcctgtgggcttgccgggttg
Y L F D G Y A H L S S G L A C G L A G L
tcagcagggatggcaattggaattgttggatgctggtgtcagggtcaatgctcagcag
S A G M A I G I V G D A G V R A N A Q Q
ccaaagctattcgtgggtatgattctgatcctcctcttgcagaagcacttgcctatatat
P K L F V G M I L I L I F A E A L A L Y
ggctcgtatttgggatcattctctcctctcagctgggtcaatctcgggcagactgagaa
G L I V G I I L S S R A G Q S R A D *
atcacgctcaaggcattttgggaacgcttggctgctgcatagaacattgattgtaac
cgaattagctgctactatggtttatgatggttcaaggagctgctctctgtggagtctcttg
agatttttattcccctctctctcttccacaacagttggctcaatttctctctcggcctc
tctccattggagagatggttatgaacaatgtaagctcgtatatggagtatctatatctttt
gcc
    
```

Fig. 3 — Nucleotide and deduced amino acid sequences of full-length *AcVHA-c* cDNA from *A. cherimola*. Amino acids in bold correspond to the putative transmembrane domains as predicted by secondary structure and amino acid similarity (Rost *et al*, 2004). The asterisk indicates the stop codon. Polyadenylation signals are underlined. Sequences of the specific oligonucleotides used for semi-quantitative RT-PCR are indicated by horizontal arrows.

These TM (TM1, TM2, TM3 and TM4) are over represented by hydrophobic residues alanine, glycine, leucine and valine, and it was predicted to span the membrane four times (Fig. 4). A BLAST search of GenBank showed that *AcVHA-c* shared over 97% identity with that of *Prunus persica* and *Citrus unshiu* and was highly conserved among plant species, except the N-terminal of the protein (Fig. 5). In addition, TM4 contained the putative N, N'-dicyclohexylcarbodiimide binding site represented by the amino acid glutamate (E¹⁴³) critical for proton transport.

Expression Analysis and Changes in Titratable Acidity during Postharvest Ripening

The relative expression of *AccytMDH* and *AcVHA-c* over the course of postharvest ripening was detected by semi-quantitative RT-PCR analysis using *ubiquitin* as a reference gene. The transcript levels of the target genes, *AccytMDH* and *AcVHA-c*, remained almost constant over the first three days after harvest and slightly increased, although not significantly ($P > 0.5$), as postharvest ripening progresses (Fig. 6). The expression profile did not parallel with titratable acidity as a drop was observed on day 2 followed by a sharp rise ($P < 0.5$) on the third day after fruit harvest (Fig. 7). At this time point cherimoyas were ripen and eatable. On the following days fruit continued to ripe concomitantly with a steadily increase ($P > 0.5$) in titratable acidity.

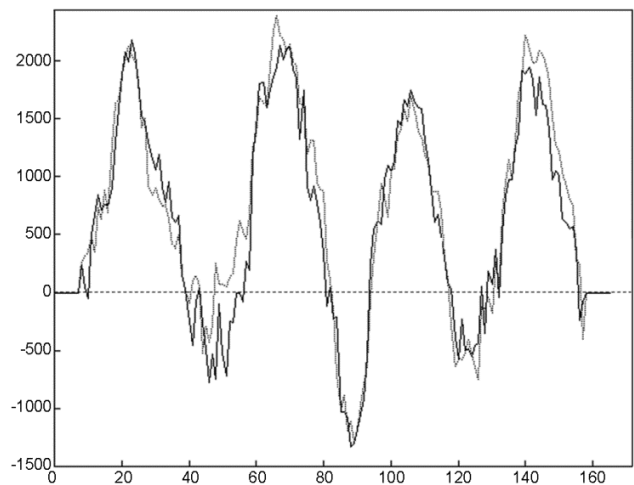


Fig. 4 — Four membrane-spanning domains suggested by hydropathy profile of *AcVHA-c*. Data were analyzed by TMpred Server. Hydrophobic domains are those above the dashed line.

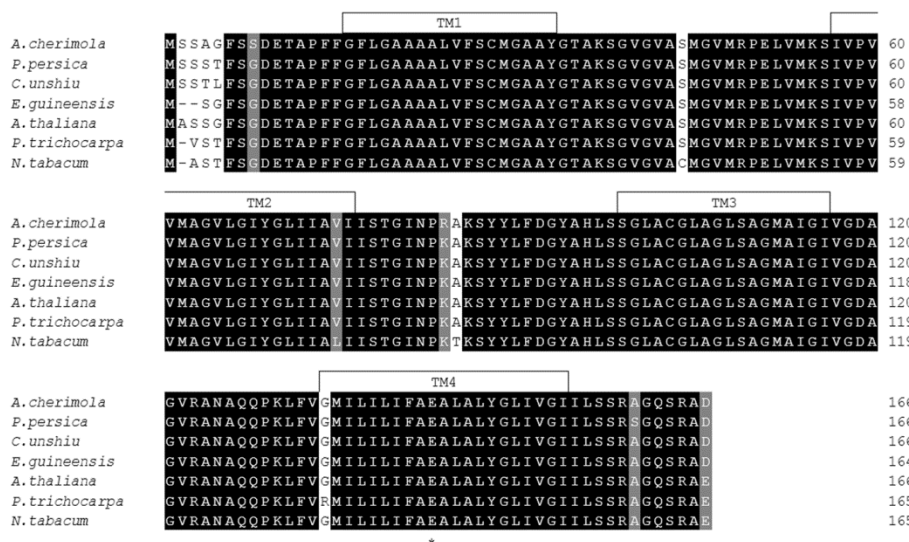


Fig. 5 — Alignment of the deduced AcVHA-c with *Prunus persica* (gb: EMJ01586), *Citrus unshiu* (gb: BAA75516), *Elaeis guineensis* (gb: ACF06516), *Arabidopsis thaliana* (gb: AAD38803), *Populus trichocarpa* (gb: ABK93797) and *Nicotiana tabacum* (gb: CAA65063). Conserved and similar amino acids are highlighted in black and grey, respectively. The four putative transmembrane domains TM1, TM2, TM3 and TM4 are indicated. The asterisk identifies the glutamic acid residue (E) in TM4 which is essential for proton translocation.

Discussion

In cherimoya the balance between soluble sugars and mild acidity granted by malic acid are extremely important organoleptic traits that contribute to influence perception of sweetness and fruit quality. It is assumed that organic acids and sugars are transported and accumulated into the vacuoles of mesocarp cells using the electrochemical potential of proton pumps. However, molecular data on fruit acid metabolism is limited in cherimoya. Hence, two full-length cDNAs designated *AccytMDH* and *AcVHA-c* associated to malate metabolism and transportation, respectively, were isolated from *A. cherimola* fruit. Our analysis showed that *AccytMDH* encodes a cytosolic NAD-dependent MDH and the comparison with other published homologous showed it was highly conserved either at nucleotide or deduced amino acid sequences^{7,32-34}. Moreover, highly conserved motifs for the NAD-binding or catalytic activity usually present in all cytosolic MDH^{12,34,35} were also present in the *AccytMDH*. The secondary structure analysis revealed that *AccytMDH* was mainly constituted with α and β helices, which were intertwined with turn and random coil, essential for substrate and coenzyme binding and stabilization, and therefore for MDH activity.

Sequence analysis indicated that *AcVHA-c* codes for a 16 kDa proteolipid subunit which is the principal integral membrane protein that forms the proton

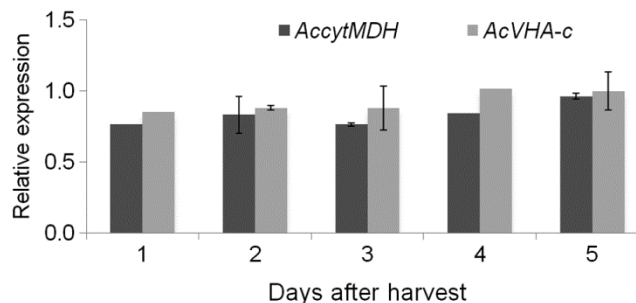


Fig. 6 — The relative expression of *AccytMDH* and *AcVHA-c* during postharvest ripening was analyzed by semi-quantitative RT-PCR. Data represent the mean value of at least three fruit. Vertical bars correspond to standard deviation.

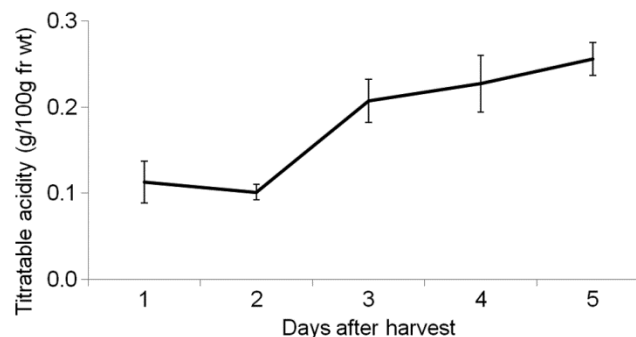


Fig. 7 — Evolution of titratable acidity in cherimoya fruit after harvest and storage at 20–22°C. Vertical bars indicate standard errors.

channel of the vacuolar ATP synthase through which protons are translocated across tonoplast. Alignment of the deduced amino acid sequence demonstrated that AcVHA-c was highly similar to their

counterparts' proteins known in plant species. Actually, it is known that the subunits comprising the (V_0) ring structure are the most highly conserved²³. Furthermore, *AcVHA-c* showed four conserved transmembrane domains computationally predicted²⁸, which is typical of this subunit¹⁹. In the TM4 the amino acid glutamate (E¹⁴³) was present and as reported by others^{20,36} it seems to be vital for proton translocation activity through the V_0 domain as it is the site where N, N'-dicyclohexylcarbodiimide, a H⁺ pore blocker, inhibits the activity of ATPase by reacting with the carboxyl group of the residue.

The genes isolated in this study, *AccytMDH* and *AcVHA-c*, allowed the analysis on to what extent they are involved on malate metabolism and transportation during cherimoya ripening. Both genes are expressed throughout postharvest ripening showing an increase on the transcript levels at the later stages of this process, although a strict relationship between gene expression and titratable acidity was not found. Similar results were also obtained for peach⁷, apple³⁷ and pear³⁸, where the expression levels of several genes engaged in organic acid metabolism and transportation were not correlated with changes in organic acid content. Therefore, it is suggested that in cherimoya apart from *AccytMDH* other key enzymes or regulatory processes affect the increase in titratable acidity. It is noteworthy that malate metabolism is controlled by synthesis, transportation and degradation and that cytosolic NAD-dependent MDH is regarded as part of a shuttle system for the exchange of substrates and reducing equivalents between the cytoplasm and cellular organelles¹⁵. In addition, malate can be exported to the mitochondria to be used as a respiratory substrate¹³.

The vacuolar (H⁺)-ATPases seem to operate by a rotatory mechanism coupling proton transport across tonoplast to ATP hydrolysis, being required to mediate the translocation of sugars and organic acids into the vacuole. In ripe fruit such as pear¹⁶ and grape berries²² V-ATPase is the major proton pump that provides the driving force for active transport of metabolites and possesses a strong acidification capacity maintaining low pH values³⁹. In cherimoya V-ATPase might play an important role as proton pump to translocate organic acids and sugar to the vacuole. In agreement with this hypothesis the expression of *AcVHA-c* increased during fruit ripening contributing to the need to maintain the accumulation of these products, which undergoes a

continuous accumulation during cherimoya postharvest ripening. Nevertheless, further investigations are needed to establish a clear relationship between gene expression and malate accumulation in cherimoya. By using *AccytMDH* and *AcVHA-c* as molecular targets new strategies can be exploited to get a clear picture in the ripening of cherimoyas to further improve fruit quality and storing.

Titrate acidity and organic acids, mainly malic acid, are assumed to contribute to the pleasing taste and aroma of cherimoya. Consequently, the characterization of molecular targets associated to malate metabolism and transportation might be useful to investigate the molecular mechanism of cherimoya ripening. With this study we have provided the cloning and molecular characterization of full length-cDNAs from *A. cherimola* that code for cytosolic NAD-dependent malate dehydrogenase (*AccytMDH*) and vacuolar (H⁺)-ATPase c subunit (*AcVHA-c*), respectively. The expression of these genes and the titratable acidity increased during postharvest ripening, but a correlation was not found between them. Nevertheless, *AccytMDH* and *AcVHA-c* might contribute to organic acid accumulation and translocation during postharvest ripening of cherimoya in association with other enzymes and carriers. This background information could be worthwhile to be exploited in order to get a clear picture in the ripening of cherimoya.

References

- 1 Giovanni J J, Genetic regulation of fruit development and ripening, *Plant Cell*, 16 (2004) 170-180.
- 2 Cordeiro N, Sousa L, Freitas N & Gouveia M, Changes in the mesocarp of *Annona cherimola* Mill. 'Madeira' during postharvest ripening, *Postharvest Biol Technol*, 85 (2013) 179-184.
- 3 Pareek S, Yahia E M, Pareek O P & Kaushik R A, Postharvest physiology and technology of *Annona* fruits, *Food Res Int*, 44 (2011) 1741-1751.
- 4 Prieto H, Utz D, Castro A, Aguirre C, González-Agüero M *et al*, Browning in *Annona cherimola* fruit: role of polyphenol oxidase and characterization of a coding sequence of the enzyme, *J Agric Food Chem*, 55 (2007) 9208-9218.
- 5 Li C R, Shen W B, Lu W J, Jiang Y M, Xie J H *et al*, 1-MCP delayed softening and affected expression of *XET* and *EXP* genes in harvested cherimoya fruit, *Postharvest Biol Technol*, 52 (2009) 254-259.
- 6 González-Agüero M, Cifuentes-Esquivel N, Ibañez-Carrasco F, Gudenschwager O, Campos-Vargas R *et al*, Identification and characterization of genes differentially expressed in cherimoya (*Annona cherimola* Mill) after exposure to chilling injury conditions, *J Agric Food Chem*, 59 (2011) 13295-13299.
- 7 Etienne C, Moing A, Dirlwanger E, Raymond P, Monet R *et al*, Isolation and characterization of six peach cDNAs

- encoding key proteins in organic acid metabolism and solute accumulation: involvement in regulating peach fruit acidity, *Physiol Plant*, 114 (2002) 259-270.
- 8 Yao Y X, Li M, Liu Z, You C X, Wang D M *et al*, Molecular cloning of three malic acid related genes *MdPEPC*, *MdVHA-A*, *MdcyME* and their expression analysis in apple fruits, *Sci Hort*, 122 (2009) 404-408.
 - 9 Yang L T, Xie C Y, Jiang H X & Chen L S, Expression of six malate-related genes in pulp during the fruit development of two loquat (*Eriobotrya japonica*) cultivars differing in fruit acidity, *Afr J Biotechnol*, 10 (2011) 2414-2422.
 - 10 Alique R, Zamorano J P, Calvo M L, Merodio C & De la Plaza J L, Tolerance of cherimoya (*Annona cherimola* Mill.) to cold tolerance, *J Amer Soc Hort Sci*, 119 (1994) 524-528.
 - 11 Etienne A, Génard M, Lobit P, Mbeguié-A-Mbégué D & Bugaud C, What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells, *J Exp Bot*, 64 (2013) 1451-1469.
 - 12 Ding Y & Ma Q H, Characterization of a cytosolic malate dehydrogenase cDNA which encodes an isozyme toward oxaloacetate reduction in wheat, *Biochimie*, 86 (2004) 509-518.
 - 13 Sweetman C, Deluc L G, Cramer G R, Ford C M & Soole K L, Regulation of malate metabolism in grape berry and other developing fruits, *Phytochemistry*, 70 (2009) 1329-1344.
 - 14 Muñoz T, Sanchez-Ballesta M T, Ruiz-Cabello J, Escribano M I & Merodio C, The acid metabolism of *Annona* fruit during ripening, *J Hort Sci Biotechnol*, 79 (2004) 472-478.
 - 15 Scheibe R, Malate valves to balance cellular energy supply, *Physiol Plant*, 120 (2004) 21-26.
 - 16 Suzuki Y, Shiratake K & Yamaki S, Seasonal changes in the activities of vacuolar H⁺-pumps and their gene expression in the developing Japanese pear fruit, *J Japan Soc Hort Sci*, 69 (2000) 15-21.
 - 17 Amemiya T, Kanayama Y, Yamaki S, Yamada K & Shiratake K, Fruit-specific V-ATPase suppression in antisense-transgenic tomato reduces fruit growth and seed formation, *Planta*, 223 (2006) 1272-1280.
 - 18 Shiratake K & Martinoia E, Transporters in fruit vacuoles, *Plant Biotechnol*, 24 (2007) 127-133.
 - 19 Sze H, Schumacher K, Müller M L, Padmanaban S & Taiz L, A simple nomenclature for a complex proton pump: *VHA* genes encode the vacuolar H⁽⁺⁾-ATPase, *Trends Plant Sci*, 7 (2002) 157-161.
 - 20 Ma B, Xiang Y & An L, Structural bases of physiological functions and roles of the vacuolar H⁺-ATPase, *Cell Signal*, 23 (2011) 1244-1256.
 - 21 Shiratake K, Kanayama Y, Maeshima M & Yamaki S, Changes in H⁺-pumps and a tonoplast intrinsic protein of vacuolar membranes during the development of pear fruit, *Plant Cell Physiol*, 38 (1997) 1039-1045.
 - 22 Terrier N, Sauvage F X, Ageorges A & Romieu C, Changes in acidity and in proton transport at the tonoplast of grape berries during development, *Planta*, 213 (2001) 20-28.
 - 23 Coker J S, Jones D & Davies E, Identification, conservation, and relative expression of V-ATPase cDNAs in tomato plants, *Plant Mol Biol Rep*, 21 (2003) 145-158.
 - 24 Altschul S F, Madden T L, Schäffer A A, Zhang J, Zhang Z, *et al*, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res*, 25 (1997) 3389-3402.
 - 25 Altschul S F, Wootton J C, Gertz E M, Agarwala R, Morgulis A *et al*, Protein database searches using compositionally adjusted substitution matrices, *FEBS J*, 272 (2005) 5101-5109.
 - 26 Larkin M A, Blackshields G, Brown N P, Chenna R, McGettigan P A *et al*, Clustal W and Clustal X version 2.0. *Bioinformatics*, 23 (2007) 2947-2948.
 - 27 Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins M R *et al*, Protein identification and analysis tools on the ExPASy server, in *The Proteomics Protocols Handbook*, edited by J M Walker (Totowa, Humana Press) 2005, 571-607.
 - 28 Rost B, Yachdav G & Liu J, The Predict Protein Server, *Nucleic Acids Res*, 32 (Web Server issue) (2004) W321-W326.
 - 29 Hofmann K & Stoffel W, TMbase - A database of membrane spanning proteins segments, *Biol Chem Hoppe-Seyler*, 374 (1993) 166.
 - 30 Laplace L, Ribeiro A, Franche C, Duhoux E, Auguy F *et al*, Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homolog of *Alnus glutinosa ag12*, *Mol Plant-Microbe Interact*, 3 (2000) 113-117.
 - 31 AOAC, Official method of analysis 934.01. Maryland: Association of Official Analytical Chemists, 2000.
 - 32 Loke J C, Stahlberg E A, Strenski D G, Haas B J, Wood P C *et al*, Compilation of mRNA polyadenylation signals in *Arabidopsis* revealed a new signal element and potential secondary structures, *Plant Physiol*, 138 (2005) 1457-1468.
 - 33 Ocheretina O & Scheibe R, Cloning and sequence analysis of cDNAs encoding plant cytosolic MDH, *Gene*, 199 (1997) 145-148.
 - 34 Yao Y, Hao Y, Li M, Pang M, Liu Z & Zhai H, Gene clone, expression and enzyme activity assay of a cytosolic malate dehydrogenase from apple fruits, *Front Agric China*, 2 (2008) 307-313.
 - 35 Miller S S, Driscoll B T, Gregerson R G, Gantt J S & Vance C P, Alfalfa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH, *Plant J*, 15 (1998) 173-184.
 - 36 Hasenfratz M P, Tsou C L & Wilkins T A, Expression of two related vacuolar H⁺-ATPase 16-kilodalton proteolipid genes is differentially regulated in a tissue-specific manner, *Plant Physiol*, 108 (1995) 1395-1404.
 - 37 Yao, Y X, Li M, Zhai H, You C X & Hao Y J, Isolation and characterization of an apple cytosolic malate dehydrogenase gene reveal its function in malate synthesis, *J Plant Physiol*, 168 (2011) 474-480.
 - 38 Lu X P, Liu Y Z, Zhou G F, Wei Q J, Hu H J *et al*, Identification of organic acid-related genes and their expression profiles in two pear (*Pyrus pyrifolia*) cultivars with difference in predominant acid type at fruit ripening stage, *Sci*, 129 (2011) 680-687.
 - 39 Müller M L, Irkens-Kiesecker U, Rubinstein B & Taiz L, On the mechanism of hyperacidification in lemon. Comparison of the vacuolar H⁽⁺⁾-ATPase activities of fruits and epicotyls, *J Biol Chem*, 271 (1996) 1916-1924.