Carmona-López, L.; Alquézar-García, B.; Tarraga Herrero, S.; Peña Garcia, L. (30-0). Protein analysis of moro blood orange pulp during storage at low temperatures. Food Chemistry. 277:75-83. https://doi.org/10.1016/j.foodchem.2018.10.108

PROTEIN ANALYSIS OF MORO BLOOD ORANGE PULP DURING STORAGE AT LOW TEMPERATURES

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Abstract

A protein analysis in the pulp of Moro blood oranges (*Citrus sinensis* L. Osbeck) at the onset and after 30 days of storage at either 4 or 9 °C was performed. All differential proteins belonged to different functional classes (sugar, amino acid and secondary metabolism, defense, stress response, oxidative process, transport and cellular component biogenesis), displaying a differential accumulation in those Moro oranges kept at 9 versus 4 °C, and in those stored at 4 ºC versus onset. Anthocyanin biosynthesis structural proteins chalcone synthases and flavonone 3-hydroxylase and different glutathione S-transferases related with their vacuolar transport were up-accumulated in fruits kept at 9 versus 4 °C and versus the onset. Proteins related with defense and oxidative stress displayed a similar pattern, concomitant with a higher anthocyanin content, denoting a possible role of defense and other stress response pathways in anthocyanin production/accumulation.

Keywords: Blood oranges, anthocyanins, cold storage, purple juice, proteome.

1. Introduction

Citrus fruits have been long considered healthy and nutritious, because they are rich in vitamin C, folic acid and minerals. They are also a source of phytochemicals such as carotenoids, terpenoids and flavonoids, with important health-related characteristics. Blood oranges are the only citrus fruits that also are rich in anthocyanins, water-soluble polyphenolic compounds which provide a distinctive purple coloration. Besides their aesthetic appeal, these pigments play decisive physiological roles in plants, such as protecting them against abiotic stress conditions and pathogen infections (Zhang, Butelli, & Martin, 2014). Anthocyanins present in blood oranges have been related with the antioxidant capacity of their purple juice. Due to this trait, the number of studies on the effects of blood orange juice consumption have raised in the last years, denoting its beneficial influence on several diseases such as oxidative insulin sensitivity or cardiovascular risk. Additionally, the synergistic effects of anthocyanins with others phytochemicals of blood orange juice on anti-inflammatory and anti-obesity properties have been described (Butelli et al. 2012).

The blood oranges pigmentation process is dependent on multiple factors such as genotype, fruit maturity and cultural/environmental factors, which also affect anthocyanin composition and concentration (Butelli et al., 2012; Hillebrand, Schwarz, & Winterhalter, 2004). Recent studies suggest that a given number of hours below 6 °C is essential to induce anthocyanin biosynthesis in blood orange fruits and to obtain a deep purple juice color (Continella et al., 2018), assuming that a wide day/night temperature range during fruit maturation is the main anthocyanin accumulation determinant (Butelli et al., 2012). This cold-dependency limits geographically a reliable quality in blood orange commercial production to only a few regions worldwide (Crifò, Petrone, Lo Cicero, & Lo Piero, 2012) and it is responsible for production of fruits with very low or lack of anthocyanins in blood oranges grown under tropical/subtropical climates (Butelli et al., 2012).

Research efforts have been conducted to understand the molecular regulation of anthocyanin biosynthesis and accumulation in citrus fruit. The genes encoding enzymes of most steps of the pathway have been identified and their expression analyzed (reviewed in Carmona et al. 2017). Anthocyanin biosynthesis occurs via the well-known flavonoid pathway, being the phenylalanine lyase (PAL) the first enzyme and phenylalanine the common precursor for multiple metabolites, such as 4-coumaroy CoA, which is catalyzed consecutively to generate naringenin by chalcone synthase (CHS) and chalcone isomerase (CHI). Subsequently this compound suffers different hydroxylations catalized by flavonone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H) and/or flavonoid 3'5'-hydroxylase (F3'5'H) to produce dihydroflavonols (DHF). Finally, dihydroflavonol 4-reductase (DFR) reduces DHF to leucoanthocyanidins, which are oxidized and glycosylated serially by anthocyanin synthase (ANS) and UDP-glucose-flavonoid 3-O-glucosyltransferase (UFGT) to form the colored anthocyanins. Glutathione S-transferase (GST) is involved in anthocyanins import to the vacuole (Licciardello et al., 2014). The relationship between anthocyanins accumulation and fruit storage at low-moderate temperature has been determined (Carmona, Alquézar, Marques, & Peña, 2017; Crifò et al., 2012; Rapisarda, Bellomo, & Intelisano, 2001), concluding that the maximum content of these pigments is obtained after 45 days of postharvest storage at 8-10 °C (Rapisarda, Bellomo, & Intelisano, 2001). Recently, a comparative study on the effect of low (4 °C) and moderate temperature (9 °C) on anthocyanin accumulation in Moro orange (*Citrus sinensis* L. Osbeck) during a storage period of 45 days showed that a feasible practice to enhance anthocyanin accumulation in blood oranges is to keep the fruits at 9 ºC for at least 15 or 30 days, depending on their pigment content at harvesting time (Carmona et al., 2017). Consequently, Moro orange fruits stored at 9 °C developed a deeper purple coloration due to a higher accumulation of anthocyanins than those kept at 4 °C. Likewise, a higher up-regulation of the structural genes *PAL*, *CHS*, *DFR*, *ANS*, *UFGT* and *GST* occurred, concomitant with the color enhancement observed at 9 °C. In this work, we

report a comparative protein profile analysis of Moro orange fruit pulp after 30 days of fruit storage at either 9 or 4 °C, in order to gain more insight into the biochemical features associated with the differential increases in anthocyanin contents at both postharvest storage temperatures.

2. Materials and methods

2.1. Plant material and storage conditions

Moro blood sweet orange fruit (*C. sinensis* L. Osbeck) was selected due to the bright-purple color of its pulp and peel. Mature fruits were harvested at random from adult trees grown under standard conditions in two different commercial orchards in Sao Paulo State (Brazil): Paranapanema (Pa) (23°45'03.59'' S; 48°50'37.52'' O) and Maringá (Ma) (21°38'18.86'' S; 48°29'21.56'' O), where Moro fruit accumulates little anthocyanin content due to the environmental conditions, with minimum temperatures in winter of 8-10 °C in Pa vs 14-16 °C in Ma. Fruits were uniform in size and color, and free of damage and external defects. Fruits were divided in two lots and stored for 30 days at 9 and 4 °C and 90-95 % RH in constant darkness. At the time of the harvest and after 30 days of storage, pulp was separated with a scalpel, immediately frozen in liquid nitrogen and stored at - 80 °C until analysis. Three replicate samples of 10 fruits each per temperature and storage time were used for analysis.

2.2. Determination of color, internal maturity index, pH and anthocyanin quantification

Pulp color, internal maturity, pH and anthocyanin quantification were determined as described by Carmona et al. (2017).

2.3. Protein extraction

Frozen pulp (1 g) of each sample was separately homogenized in liquid nitrogen with 0.05% PVP. Then samples were homogenized in 1.5 mL extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 0.2 % β-mercaptoethanol) and centrifuged at 4 °C and 13.200 rpm during 20 min. Supernatant was supplemented with the pellet (containing insoluble proteins) resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS), and centrifuged at 13.200 rpm for 15 min at 4 °C. Supernatant was mixed, with an equal amount of cold 20 % TCA (trifluoroacetic acid, Sigma) and incubated for 2 h at 4 °C. Finally, samples were centrifuged at 13.200 rpm for 15 min at 4 °C. The protein aliquots were stripped of non-protein contaminants using a 2-D Clean-Up Kit following the manufacturer's instructions (GE-Healthcare, USA). Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, USA) using bovine serum albumin (BSA) as a standard.

2.4. Fluorescent labelling

Equal amounts (50 µg) of each protein sample were labelled using CyDyes DIGE fluors (Cy2, Cy3 and Cy5) according to the manufacturer's instructions (GE-Healthcare, USA). Samples corresponding to freshly harvest (zero days) were labelled with Cy2, samples from fruit stored for 30 days at 4 °C were labelled with Cy3 and those from fruit kept at 9 °C were labelled with Cy5. After labeling, samples were pooled. Lysis buffer was added to make up the volume to 40 ml. Then, the pool was mixed with 40 µl of isoelectrofocusing (IEF) rehydration buffer (8 M urea, 4% CHAPS, 0.005% bromophenol blue) containing 65 mM DTT and 1% IPG buffer pH 3-11, and it was loaded in the gel.

2.5. 2D-electrophoresis

For the 2D analysis, 24-cm long strip with an immobilized pH gradient of 3-11 were hydrated overnight at room temperature with 450 µl of IEF rehydration buffer, containing the reagents Destreak and Pharmalyte, pH 3-10, according to the

manufacturer's instructions (GE Healthcare, USA). The CyDyes-labelled pool sample (150 µg of protein) was loaded on the hydrated strip. IEF was performed in an IPGphor unit (GE Healthcare, USA) at 20 °C and at a maximum current of 50 mA per strip at the following settings: 300 V for 1 h, an increasing voltage gradient to 1000 V for 6 h, an increasing voltage gradient to 8000 V for 3 h, before finally holding at 8000 V for a total of 32,000 Vh. After IEF, the strip was equilibrated separately for 15 min in 10 mL equilibration solution I (0.05 M Tris-HCl buffer, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 2% DTT), followed by equilibration solution II (0.05 M Tris-HCl buffer pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, 0.01% bromophenol blue) before being applied directly to the second dimension 12.5% SDS-PAGE gels. Gels were run at 20 °C by applying 2W/gel for 30 min and 20 W/gel for the remaining 5-6 h in an Ettan DALTsix unit (GE-Healthcare, USA). A running buffer of 25 mM Tris, pH 8.3, 192 mM glycine and 0.2% SDS was used.

2.6. Gel imaging and data analysis

Proteins were visualized by scanning using a Typhoon Trio scanner (GE Healthcare) with the relevant wavelengths for each CyDye. The three images of each gel were processed with DIA (Differential in-gel analysis) to investigate differential protein accumulation between control sample (zero days) and those stored at 4 and 9 °C. DIA module was used for the spot detection, spot volume quantification and volume ratio normalization of different samples in the same gel.

Spots of interest (showing differential accumulation between analyzed samples) were excised from silver-stained gel (Staining Kit protein, GE Healthcare), distained by 2 5-min washes with acetonitrile (ACN):water (1:1) and rehydrated with 50 mM ammonium bicarbonate for 5 min and 25 mM ammonium bicarbonate in 50% (v/v) ACN for 15 min.

2.7. Protein identification by mass spectrometry analysis

161 Samples were digested with 10 ng of trypsin (o/n at 37 °C). The digestion was stopped
162 with 7 mL of 0.1% TFA, and 1 µL was spotted onto the MALDI target plate. After air-
163 drying the droplets at room temperature, 0.5 µL of matrix (5 mg/mL CHCA) (α-cyano-4-
164 hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/H_2O (1:1, v/v) was added and allowed
165 to air-dry at room temperature. A 4700 Proteomics Analyzer (Applied Biosystems,
166 Foster City, USA) was used for analyzing the resulting mixtures. Five of the most
167 intense precursors (according to the threshold criteria: minimum signal-to-noise: 10,
168 minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap:
169 4) were selected for every position for the MS/MS analysis. The MS/MS data were
170 acquired using the default 1 kV MS/MS method and the MS and MS/MS information
171 was sent to MASCOT via the Protein Pilot software (Applied Biosystems). Database
172 searches was performed on NCBI EST_citrus.

173 The samples without a positive identification were analyzed by LC/MS/MS. Peptide
174 separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC
175 Packings) and a QSTAR XL Q-TOF hybrid mass spectrometer (AB Sciex). Samples (5
176 µL) were delivered to the system using a FAMOS autosampler (LC Packings) at 30 µL/
177 min, and peptides were trapped onto a PepMap C18 pre-column (5 mm x 300 mm i.d.;
178 LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15
179 cm x 75 mm i.d.; LC Packings) at 300 nL/min and were separated using a 30 min
180 gradient of 5-45% ACN. QSTAR XL was operated in the information dependent
181 acquisition mode, in which a 1-s TOF MS scan from 400 to 2000 m/z was performed,
182 followed by 3-s product ion scans from 65 to 2000 m/z on the three most intense
183 doubly or triply charged ions. The MS/MS information was sent to MASCOT via the
184 MASCOT DAEMON software (MATRIX SCIENCE). The search parameters were
185 defined as for the MS-MS/MS analysis.

186 2.8. Quantitative RT-PCR analysis

187 Total RNA, extracted as described previously in Carmona et al. (2017), was treated
188 with DNase (Ambion®, Thermo Fisher Scientific, USA), accurately quantified by Nano
189 Drop measurement (Thermo Fisher Scientific, USA) and used for cDNA synthesis
190 (Invitrogen, Thermo Fisher Scientific, USA). Quantitative real-time PCR was performed
191 with a StepOne Plus Real Time PCR System (Applied Biosystem, USA) and data was
192 analyzed using StepOne Software version 2.3. The RT-PCR procedure and primers
193 used for analyzed genes were those described by Carmona et al. (2017). The relative
194 expression between cold-treated and control samples (zero time of Moro orange fruits)
195 was determined by the method described by Carmona et al. (2017). Values are
196 presented as the mean of at least three independent analyses. Statistical analyses
197 were performed using ANOVA.

198 **3. Results**

199 3.1. Blood orange pulp appearance and quality parameters at different low
200 temperature storages

201 Visual aspect, maturity index (MI), pH values, color index (CI) and anthocyanin content
202 of the pulp before and after postharvest storage at 4 or 9 °C were analyzed in Moro
203 pulp oranges harvested from two different locations and showing different pigmentation
204 intensities (Table 1). After 30 days of storage, changes in pulp coloration were evident
205 at both temperatures, although color reached higher intensity in those fruits kept at
206 9 °C. Consistently, CI and anthocyanin content were higher in those fruits stored at
207 9 °C independently of their initial anthocyanin content at harvest time, reaching CI
208 values of 1.6 and 2.4 and a anthocyanin content of 43.4 and 171.0 mg/L in Maringa
209 (Ma) and Paranapanema (Pa) fruits, respectively, while fruits kept at 4 °C presented
210 much lower values for both parameters (Table 1). At harvest time, pH and MI were
211 similar in fruits for both locations and did not change significantly along the storage
212 period at any temperature studied (Table 1).

3.2. Blood oranges proteome profile at different low temperatures

To elucidate changes on protein accumulation patterns, a 2D-electrophoresis of proteins from Moro pulp at harvest time and at day 30 after storage at either 9 or 4 °C was performed (Fig. 1 and Fig. Supp. 1). Subsequent analyses to select proteins accumulated differentially were performed. Initially, a screening to select those proteins with a minimum of 1.2-fold differential accumulation between the onset of the experiment and fruit samples stored at either 9 or 4°C was performed. A total of 90 and 121 spots with differential accumulation were selected in fruits from Pa and Ma, respectively. Afterward, spots with at least 1.2-fold differential accumulation between samples stored at either 9 or 4 °C were selected for their identification. Based on this criterion, a total of 54 (43 up-accumulated and 9 down-accumulated in 9 vs 4 ºC and vs harvest time) and 32 (23 up-accumulated and 9 down-accumulated in 9 vs 4 ºC and vs harvest time) differentially accumulated spots were chosen from the pulp of Moro oranges from Pa and Ma, respectively.

Spots showing differential intensities under storage at either 9 or 4°C were excised from 2D-PAGE gels and subjected to identification. The data obtained were searched via MASCOT against the non-redundant protein database NCBI EST_citrus (Table 2 and 1S). Some proteins were identified more than once in different spots, reflecting different isoforms or posttranslational modifications. For instance, among the spots of Moro pulp proteome from Pa, four spots were identified as fructose bisphosphate aldolase (spots 540, 566, 593 and 596), two spots as enolase (spots 379 and 567) and two spots as germin-like protein subfamily 1 member 17 (spots 1120 and 1081) (Table 2). Similarly, in the proteome analysis of Moro pulp from Ma (Table 1S) two spots were identified as V-type H^+ ATPase catalytic subunit A (spots 368 and 406) and other two as glutathione-S transferase F6 (spots 1178 and 1191), among others.

238 Identified protein functions were inferred using the UniProt database
239 (http://www.uniprot.org). According to their biological properties, differentially
240 accumulated proteins from Pa and Ma were classified into 9 and 8 functional
241 categories, respectively (Fig. 2). The main functional group represented was that of
242 stress response (40.7% in Pa and 37.5% in Ma), being the main proteins in this group
243 (35%) glutathione S-transferases (GSTs) (Table 2 and 1S). The second most abundant
244 class corresponded to proteins involved in the sugar metabolism (16.7% and 21.9% in
245 Pa and Ma, respectively) (Fig. 2). Secondary metabolism was the third group with a
246 representation of 11.1% in Pa and 9.4% in Ma, respectively (Fig. 2). This category
247 included malate dehydrogenases (MDH), isoflavone reductases (IFR) and cinnamoyl-
248 CoA reductases (CCR). Additionally, in the case of proteome from Pa fruits, two
249 chalcone synthases (CHS) with a ratio of 1.78 (spots 563) and 1.31 (spots 555) and
250 one flavonone 3-hydroxylase (F3H) with a ratio of 1.54 (spot 554) were identified
251 (Table 2).

252 Other represented functional classes were oxidative process, defense and amino acid
253 metabolism (Fig. 2). Interestingly, all the proteins belonging to defense, sugar, amino
254 acid and secondary metabolism functional classes displayed a higher accumulation in
255 those Moro oranges kept at 9 °C than in those stored at 4 °C, and in those stored at
256 4 ºC vs harvest time. On the other hand, those proteins down-accumulated at 9 °C vs
257 4 °C (and vs harvest time) belonged to the stress response, oxidative process and
258 transport categories (Table 2 and 1S).

259 3.3. Quantitative qRT-PCR validation

Four genes, encoding significantly differential accumulated proteins between samples kept 30 days at either 4 or 9 ºC, were selected for qRT-PCR validation (Fig. 3). *Chalcone synthases* genes (*CHS1* and *CHS2*) were found to be up-regulated by low-temperature storage, to a larger extent at 9 than at 4 ºC. The expression of *flavonone 3-hydroxylase* gene (*F3H*) also augmented in cold-stocked samples and again more at 9 than at 4 ºC. Similarly, *glutathione S-transferase* (*GST*) presented higher expression level in those fruits stored at 9 °C in comparison with those kept at 4 °C, being both superior to the level found at harvest time.

4. Discussion

Anthocyanin content in blood oranges is low-temperature dependent (Butelli et al., 2012), being the number of hours exposed below 6 °C a critical factor to get a deep purple coloration (Continella et al., 2018). This condition limits consistent commercial production of these varieties to a few areas in the world (Butelli et al., 2012; Carmona et al., 2017; Crifò et al., 2012). Recently, a comparative study of fruit postharvest storage at two low-temperatures demonstrated that purple coloration in Moro orange pulp improves when fruits are stored at 9 instead of at 4 °C, proposing that keeping the fruits at 9 °C for at least 15 days, could be a feasible practice in tropical countries to enhance anthocyanin accumulation in blood oranges (Carmona et al., 2017). Here, to expand the knowledge on the biochemical mechanisms that regulate the cold-induced accumulation of anthocyanins in blood orange fruits, a proteomic analysis was carried out.

Moro pulp showed a higher enhancement of purple coloration in fruits stored at 9 than 4 °C, which was correlated with an increase in both, CI and anthocyanin content, (Table 1). The relationship between coloration and anthocyanin content has been described previously (Carmona et al., 2017; Rapisarda et al., 2001). Cold-induced anthocyanin production depends on their concentration at the onset of postharvest

286 storage, being lower the initial anthocyanin content, greater the effect of cold storage
287 (Carmona et al., 2017). Accordingly, the higher increase on anthocyanin content was
288 detected in Ma fruits (9.4 fold). However, the final highest concentration of pigments
289 was found in Pa fruits, which also had higher anthocyanin content at the onset of the
290 experiment (Table 1). This is due to the better environmental conditions of Pa to grow
291 blood orange trees, with minimum temperatures in winter of 8-10 °C vs 14-16 °C in Ma.

292 Moro orange pulps from fruits stored at either 9 or 4 °C for 30 days presented
293 differentially accumulated proteins (Fig. 1 and Supp. 1). Most of these proteins were
294 related to stress response, sugar metabolism, secondary metabolism and oxidative
295 processes (Fig. 2). Amino acid metabolism, transport and defense functional classes
296 were also represented (Fig. 2). Additionally, these functional classes appeared over-
297 represented when the Moro pulp proteome was compared with that of a blond cultivar
298 (Muccilli et al., 2009).

299 Within the sugar metabolism class, a glyceraldehyde 3-phosphate dehydrogenase
300 (GADPH) (spot 523, Table 2) and different isoforms of enolases (spots 379 and 567,
301 Table 2; spots 798 and 829, Table 1S), fructose bi-phosphate aldolases (spots 566,
302 596, 540 and 593, Table 2; spots 790 and 815, Table 1S), triose phosphate
303 isomerases (spots 851 and 893, Table 2) and pyruvate decarboxylases (spots 405 and
304 407, Table 1S) were identified (Table 2 and 1S). All these proteins are involved in the
305 production of pyruvate and metabolites for the tricarboxylic acid (TCA) cycle in the
306 mitochondrion, providing NADH for the electron transport pathway. The relationship
307 between sugar and anthocyanins accumulation has been established previously. In
308 blood-blond oranges comparative analysis, higher accumulation of enzymes involved in
309 sugar metabolism was found in the former (Muccilli et al., 2009;Wang et al., 2017), and
310 this has been related with higher sugar metabolism requirement in the blood cultivars
311 to satisfy the need of carbon skeletons necessary for anthocyanin biosynthesis
312 (Muccilli et al., 2009). Supporting this, all proteins belonging to sugar metabolism class

were up-accumulated in those blood oranges kept at 9 °C vs 4 ºC (Table 2 and 1S), concomitant with a higher anthocyanin accumulation at 9 °C (Table 1, Carmona et al., 2017). Other proteins such as alanine (spots 334, Table 2; spot 562, Table 1S) and aspartate aminotransferases (spot 568, Table 1S) and malate dehydrogenases (MDHs) (spot 550, Table 2; spot 818, Table 1S) showed also an increase in their accumulation at 9 °C vs 4 ºC, leading towards the production of TCA cycle metabolites and contributing to the input of energy (Hillebrand et al., 2004). Under low temperature (9 days at 4 °C), sugar and citrate from TCA cycle might be used as energy source in the flavonoids biosynthesis (Lo Piero, Lo Cicero, & Puglisi, 2014) and higher transcription of TCA cycle genes in blood vs blond orange cultivars has been also previously reported (Wang et al., 2017). Taken together, the higher accumulation of all these proteins in fruits stored at 9 °C indicates that the input of energy was higher in these fruits than in those kept at 4 °C, and in the later higher than at harvest time (Fig. 4).

In the secondary metabolism category, fruits kept at 9 ºC showed higher accumulation of defense-related proteins, such as cinnamoyl-CoA reductases (CCRs) (spot 602, Table 2; spot 803, Table 1S) involved in lignin biosynthesis and acting as effector signals in plant defense responses (Kawasaki et al., 2006), or isoflavone reductases (IFR) (spot 570, Table 2; spot 853, Table 1S) involved in the biosynthesis of isoflavonoid phytoalexins essential to reduce the oxidative damage caused by abiotic stresses (Cheng et al., 2015). IFRs up-accumulation has been also reported in pummelo fruits stored at 8-10 °C (Yun et al., 2012) and in blood orange compared with blond one (Muccilli et al., 2009). Moreover, the secondary metabolism class was also represented by two chalcone synthases (CHSs) (Spots 563 and 555) and a flavanone 3-hydroxylse (F3H) (Spot 554) (Table 2) involved in the initial steps of anthocyanin biosynthesis. In citrus, *CHSs* and *F3H* expression levels parallel anthocyanin accumulation (Licciardello, Russo, Vale, & Recupero, 2008; Wang et al., 2017). Additionally, CHS and F3H proteins were found in mature blood varieties, while none of

them were identified in the blond counterparts (Muccilli et al., 2009;Wang et al., 2017). Similarly, during postharvest storage CHSs and F3H were accumulated at higher levels in fruits from Pa stored at 9 °C (Table 2), correlated with the higher expression level of the corresponding genes (Fig. 3A) and with the increase of the anthocyanin content found in these fruits (Table 1). However, a poor correlation between transcripts and proteins was found in Ma fruits. Despite the differences on *CHSs* and *F3H* expression and anthocyanin accumulation observed in Ma fruits stored at 4 and 9 ºC (Fig. 3B and Table 1), no significant differences were found for anthocyanin-related proteins (Table 1S). This lack of correlation between gene expression and protein accumulation in the case of Ma fruit showing very low accumulation of anthocyanins at the beginning of the experiment due to improper environmental conditions for cultivation of blood oranges (with relatively high temperatures at night) may be explained by the temporal lag between transcription and translation, the regulation of mRNA translation and/or posttranslational modifications (Wang et al., 2017).

Proteins belonging to the stress response category constituted the largest functional class (Fig. 2), mainly represented by heat shock proteins (HSPs) and glutathione-S transferases (GSTs) (Table 2 and 1S). Among HSPs, a total of 3 isoforms of HSP 70 KDa (spots 326, 365 and 372; Table 1S) and different small HSPs were over-accumulated at 9 °C vs 4ºC and vs harvest time (Table 2 and 1S). Both kinds of proteins cooperate to avoid protein denaturation in stress conditions. The HSP 70 KDa cold-induction has been described in peach and citrus (Renaut et al., 2008; Yun et al., 2012). Among the small HSPs, a HSP 17.4 KDa (Spot 1001, Table 2; spot 1489, Table 1S) presented the most down-accumulated profile with a ratio of -2.11 in Pa and -1.73 in Ma fruits. These small HSPs are induced by low temperatures in carrot, tomato and wheat (Sabehat, Lurie, & Weiss, 1998), but they are also related with heat-stress in blueberry (Shi et al., 2017), envisaging their versatile role in response to temperature stresses. Belonging to this class, a cystathionine β-synthase (CBS) domain protein

(CBSX3) was down-accumulated in fruits stored at 9 °C with a -1.43 (Spot 1101, Table 2) and -1.33 (Spot 1364, Table 1S) ratio in Pa and Ma fruits, respectively. CBSX proteins stabilize cellular redox homeostasis and modulate plant development via regulation of NADP-Thioredoxin (TRX) systems in the cytosol and mitochondrion under threatening conditions (Yoo et al., 2011). Two TRX-related proteins (Spots 1334, Table 2; spot 925, Table 1S) and other proteins involved in the redox homeostasis such as Mn and Cu-Zn superoxide dismutase (Spots 1257 and 1414, Table 1S) were found down-accumulated in our study.

A total of 8 and 4 GSTs were identified as differentially accumulated in the pulp of Pa and Ma fruits, respectively (Table 2 and 1S). GSTs are classified into 6 different classes, being Phi and Tau the most representative ones in citrus (Licciardello et al., 2014). In Tarocco blood orange fruits, a *GST-F12* gene involved in glutathione tagging of anthocyanins was identified and characterized (Lo Piero, Puglisi, & Petrone, 2006), showing an increase of its expression in mature fruits (Licciardello et al., 2014; J. Wang et al., 2017). Interestingly, a GST protein identified as GST-F12 (spot 1176, Table 1S) presented an over-accumulation in those fruits stored at 9 °C, together with other Phi GSTs such as GST-F9 (spot 900, Table 2) and GST-F6 (spots 1178 and 1191, Table 1S). In Tarocco and Moro fruits, *GSTU1* gene is induced by low temperature storage (Carmona et al., 2017; Crifò et al., 2012) and this effect is higher at 9 than at 4 ºC, being correlated with anthocyanin content (Table 1, Fig 3A, Carmona, 2017). Accordingly, higher accumulation (ratio 1.32) of the corresponding protein (spot 870, Table 2) was found in fruits kept at 9 ºC. Collectively, the higher accumulation of different GSTs in fruits stored at 9 °C in comparison with those kept at 4 °C (Table 2), correlated with increased anthocyanin accumulation in the former (Table 1), supporting the involvement of GSTs in the vacuolar transport of anthocyanins in blood orange fruits (Lo Piero et al., 2006; Carmona et al., 2017; Licciardello et al., 2014) (Fig. 4). GST enzymes are required for vacuolar anthocyanin transport because retention of

anthocyanins in the cytoplasm is toxic to the cell and prevents anthocyanin biosynthesis (Sun, Li, & Huang, 2012). Abnormal vacuolar anthocyanin accumulation due to deficient GST activity was reported in other plants, such as petunia, carnation, grape, and Arabidopsis (reviewed in Shitan & Yazaki (2013). In grapevine, a VvABCC1 transporter involved in vacuolar accumulation of glycosylated anthocyanins has been described. It has been proposed that a C-type ABC transporter with substrate preference for glutathione conjugates may be involved in anthocyanin transport/accumulation (Francisco et al., 2013). However, anthocyanin–glutathione conjugates have not been found in plants (Zhao & Dixon, 2010). Moreover, under low-temperature storage, ABC-transporters (spots 1237 and spot 788, Table 1S) displayed a strong down-accumulation (ratios -1.42 and -1.73) in purple-colored Moro fruits kept at 9 °C.

Together with ABC-transporters, a probable polyamine transporter (spot 979, Table 2) and two isomers of V-type H^+ ATPase catalytic subunit A (V-ATPase) (spot 368 and 406, Table 1S) constitute the differentially accumulated representatives of the transport functional class. A relationship between vacuolar acidification and anthocyanin accumulation has been proposed (Faraco et al., 2014), though the mechanism of interaction has not been uncovered yet. Different anthocyanin vacuolar transporters have been identified in fruits (de Brito Francisco & Martinoia, 2018), such as a V-ATPase in apple, which modulates the pH and the vacuolar anthocyanin accumulation (Hu et al., 2016). Although in citrus fruits no specific transporter involved directly in anthocyanins accumulation has been identified, two V-ATPases presented an up-accumulation (ratio 1.57 and 1.63) in those fruits stored at 9 °C (Fig. 4 and Table 2) . However, no differential accumulation was found between mature blond and blood oranges in relation to V-ATPase subunit A, while the later presented over-accumulation of F1-ATP synthase subunit B (Muccilli et al., 2009).

420 Within the defense functional class, polygalacturonase-inhibiting proteins (PGIPs) and
421 germin proteins presented an over-accumulation in fruits stored at 9 °C vs 4 ºC
422 (Table 2 and 1S). PGIPs inhibit the pectin-depolymerizing activity of
423 polygalacturonases secreted by microbial pathogens (Kalunke et al., 2015), originating
424 the accumulation of oligogalacturonides (OGs). OGs could work as elicitors of defense
425 responses, as the induction of anthocyanin biosynthesis via increasing *CHS* expression
426 (Muccilli et al., 2009). On the other hand, germins are versatile proteins implicated in
427 different processes including response to stress (Barman & Banerjee, 2015; Cheng et
428 al., 2014), for example they are accumulated by cold in the peel of heat-treated
429 Valencia oranges during postharvest cold storage (Perotti et al., 2015). In addition, high
430 levels of germin protein are accumulated together with an ascorbate peroxidase (APX)
431 in wheat under oxidant conditions (Barman & Banerjee, 2015). APX proteins are
432 involved in the response to cold by reducing H_2O_2 via ascorbic acid (Caverzan et al.,
433 2012). Interestingly, the protein with the highest ratio at 9 vs 4 °C in the proteome of Pa
434 fruits was an APX (ratio 1.96) (spot 888, Table 2). As anthocyanins together with
435 ascorbic acid confer to blood oranges characteristic antioxidant properties (Arena,
436 Fallico, & Maccarone, 2001), it has been proposed that anthocyanins can act as
437 substrates to reduce the H_2O_2 that escapes from organelles (Niu et al., 2017). This
438 hypothesis could explain not only the higher APX accumulation in mature Moro pulp,
439 but also its over-accumulation when the fruit is stored at low-temperatures (Table 2,
440 Muccilli et al., 2009). Furthermore, the increase of anthocyanin content together with
441 the APX over-accumulation observed at 9 °C (Table 1 and 2) may suggest that, under
442 these conditions, a better oxidative damage control is required.

443 A thermostable pectinesterase (PME) (spot 901, Table 2) was the only differentially
444 accumulated protein identified belonging to cell component biogenesis functional class.
445 PME plays an important role in cell wall metabolism during fruit ripening and it was
446 over-accumulated in fruits kept at 9 °C vs 4 ºC. A high *PME* expression was found in

advanced maturing stages of Moro blood oranges (Licciardello et al., 2008), while its expression was undetectable in a blond cultivar. In agreement with this, the comparison of Moro and Cadenera (blond) pulp proteome showed an over-accumulation of a PME in the blood cultivar (Muccilli et al., 2009).

In conclusion, cold storage promotes the differential accumulation of proteins belonging to secondary, sugar and amino acid metabolism, defense, oxidative processes, transport, and stress response between the onset of the storage period and after 30 days at either 4 °C or 9 ºC. This response was more marked when storage at 9 was compared with that at 4 °C, which was correlated with higher anthocyanin accumulation at the former temperature. Protein analysis showed that the storage at low-moderate temperature (9 °C) induced a higher accumulation of anthocyanins biosynthesis structural proteins and other proteins associated with the input of energy required for anthocyanin biosynthesis such as those from sugar and amino acid metabolism categories (Fig. 4). Moreover, these data support the notion that keeping blood orange fruits at 9 °C for 30 days could be a feasible practice in tropical areas to improve anthocyanin accumulation. Furthermore, the over-accumulation of proteins related with defense and oxidative stress after storage at 9 ºC suggests a correlative association between these categories and anthocyanin accumulation/production and raises the question on whether triggering of defense and oxidative stress responses through specific treatments of the fruit during postharvest cold storage may enhance further the accumulation of anthocyanins in blood oranges.

Acknowledgments

This work was supported by the São Paulo Research Foundation (FAPESP, Brazil) project (FAPESP 2014/12616-9) and Fundecitrus. LC was funded by a grant from FAPESP (2014/23447-3). We appreciate the help of Citrosuco for providing Moro fruits.

Conflict of Interest Statement: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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601

Figures and tables

Table 1. Visual aspect of the pulp, maturity index, pH, color index and anthocyanin content of Moro oranges from Maringá and Paranapanema (São Paulo, Brazil) during storage at either 9 or 4 °C for 0 and 30 days. Statistical analyses were performed using analysis of variance (ANOVA) and different letters indicate significantly different values ($P \leq 0.01$) for a given time.

Table 2. List of differentially accumulated proteins identified in pulp of Moro blood oranges from Paranapanema (São Paulo, Brazil) stored at either 9 or 4 °C for 30 days.

Figure 1. (A) Two-dimensional electrophoresis maps of the total labeled proteins from Moro orange pulp at 0 days and 30 days stored at either 4 or 9 °C (Paranapanema). (B) Mapped images of labelled proteins gel. (C) Volumetric quantification of spots of interest identified. Abundance values are expressed as the ratio obtained from volumetric image comparisons.

Figure 2. Functional classification and the correspondence percentage of each class from the analysis of differentially accumulated proteins in pulp of Moro oranges from Paranapanema (A) and Maringá (B) (São Paulo, Brazil) stored at either 4 or 9 °C for 30 days.

Figure 3. Relative quantification of expression of *CHSs*, *F3H* and *GST* in the pulp of Moro oranges from Paranapanema (A) and Maringá (B) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0 and 30 days. Data are presented as the mean relative expression ± SD of each individual sample as compared to the control sample (zero time). Statistical analyses were performed using analysis of variance (ANOVA) and different letters indicate significantly different values ($P \leq 0.01$) for a given time.

Figure 4. Representative scheme of differentially accumulated proteins from pulp of Moro oranges stored at either 4 or 9 °C for 30 days. Proteins over- (red and with red arrows) and down- (green) accumulated at 9 °C vs 4 and 0 days are represented. Each functional class is represented by a different color (colored backgrounds): amino acid (pink), sugar (yellow), secondary metabolism (purple), defense (blue), oxidative processes (green), transport (dark blue), stress response (orange) and cellular component biogenesis (fluor green). Protein abbreviations: ABCT, ABC transporter; AGL, α-glucosidase; ALR, alanine transferase; APX, ascorbate peroxidase; AST, aspartate transferase; CBS, CBS domain contain protein; CHS, chalcone synthase; CCR, cinnamoyl-CoA reductase; ENO, enolase; F3H, flavanone 3-hydroxulase; FBA, fructose bisphosphate aldolase; GMP, germin protein; GPD, glucose 3-phosphate dehydrogenase; GST; glutathione S-transferase; sHSP, small heat shock proteins; IFR, isoflavone reductase; MDH, malate dehydrogenase; MDR, monodehydroascorbate reductase; NDH, NAD(P)H dehydrogenase; PAT, polyamine transporter; PDC, pyruvate decarboxylase; PIGP, polygalacturonase-inhibiting protein; PME, thermostable pectinesterase; PNDH, photosiynthetic NDH subunit; SDM, superoxide dismutase; TRI, triosephosphate isomerase; TRX, thioredoxin; V-ATPase, V-H^+ ATPase catalytic unit. Metabolite abbreviation: F6P, fructose 6-phosphate; DAP, dihydroxyacetone; G3P, glyceraldehyde 3-phosphate, 3PGA, 1,3-biphosphoglicerate; 2PGA, 1,2-biphosphoglicerate; PEP, phosphoenolpyruvate.

Supplemental data

Supplemental Table 1. List of differentially accumulated proteins identified in pulp of Moro blood oranges from Maringá (São Paulo, Brazil) stored at either 9 or 4 °C for 30 days.

Figure 1 Supp. (A) Two-dimensional electrophoresis maps of the total labelled proteins from Moro orange pulp at 0 days and 30 days stored at either 4 or 9 °C (Maringá). (B) Mapped images of labelled proteins gel. (C) Volumetric quantification of spots of interest identified. Abundance values are expressed as the ratio obtained from volumetric image comparisons.

Table

Table 1. Visual aspect of the pulp, maturity index, pH, color index and anthocyanin content of Moro oranges from Maringá and Paranapanema (São Paulo, Brazil) during storage at either 9 or 4 °C for 0 and 30 days. Statistical analyses were performed using analysis of variance (ANOVA) and different letters indicate significantly different values ($P \leq 0.01$) for a given time.

	Maringá			Paranapanema		
Visual aspect and treatments	 0 days	 30 days at 4 °C	 30 days at 9 °C	 0 days	 30 days at 4 °C	 30 days at 9 °C
Maturity index (MI)	9.5 ± 0.7[a]	8.9 ± 0.6[a]	9.2 ± 0.5[a]	7.6 ± 0.0[a]	8.2 ± 0.5[a]	8.3 ± 0.4[a]
pH	3.6 ± 0.2[a]	3.7 ± 0.1[a]	3.8 ± 0.1[a]	3.3 ± 0.2[a]	3.6 ± 0.2[a]	3.5 ± 0.1[a]
Color index (CI)	0.7 ± 0.0[a]	0.9 ± 0.0[a]	1.6 ± 0.1[b]	0.7 ± 0.0[a]	1.2 ± 0.0[b]	2.4 ± 0.1[c]
Anthocyanin content (mg/L)	4.6 ± 0.7[a]	14.5 ± 6.1[a]	43.4 ± 14.2[b]	72.4 ± 0.6[a]	97.1 ± 0.1[b]	171.0 ± 4.7[c]

Table 2. List of differentially accumulated proteins identified in pulp of Moro blood oranges from Paranapanema (São Paulo, Brazil) stored at either 9 or 4 °C for 30 days.

Spot No.	Ratio 9 °C vs 4 °C	Acc. Num.	MASCOT Score[a]	Prot. Mass	Protein annotation (Organism)[b]	Functional classification
334	1.30	CV998078.1	440	36700	Alanine aminotransferase 2 (*Citrus sinensis*)	Aminoacid metabolism
379	1.38	CX663372.1	5395	32214	Enolase (*Citrus sinensis*)	Sugar metabolism
523	1.26	DC899161.1	552	52809	Glyceraldehyde-3-phosphate dehydrogenase (GAPC1) (*Citrus sinensis*)	Sugar metabolism
540	1.31	DN794998.1	5870	32212	Fructose-bisphosphate aldolase cytoplasmic isozyme (*Citrus sinensis*)	Sugar metabolism
550	1.37	DY298078.1	1475	37024	Malate dehydrogenase, mitochondrial (*Citrus sinensis*)	Secondary metabolism
554	1.54	CB291637.1	276	33145	Flavanone 3-hydroxylase (*Citrus maxima*)	Secondary metabolism
555	1.31	DY283115.1	111	35564	Chalcone synthase-like (*Citrus sinensis*)	Secondary metabolism
562	1.26	DY284689.1	442	37935	Aspartate aminotransferase (*Citrus sinensis*)	Aminoacid metabolism
563	1.78	CX045954.1	102	30134	Chalcone synthase-like (*Citrus sinensis*)	Secondary metabolism
566	1.22	DY295805.1	412	43923	Fructose-bisphosphate aldolase (*Citrus sinensis*)	Sugar metabolism
567	1.32	DC887176.1	475	60533	Enolase (*Citrus sinensis*)	Sugar metabolism
570	1.26	CV885237.1	110	32596	Isoflavone reductase (*Citrus sinensis*)	Secondary metabolism
582	1.27	CN186533.1	2231	25978	Polygalacturonase-inhibiting protein (PIGP) (*Citrus hystrix*)	Defense
593	1.34	DY267276.1	1150	39054	Fructose-bisphosphate aldolase (*Citrus sinensis*)	Sugar metabolism
596	1.28	CN191172.1	717	29094	Fructose-bisphosphate aldolase (*Citrus sinensis*)	Sugar metabolism
602	1.22	CX670135.1	263	33259	Cinnamoyl-CoA reductase 1-like (*Citrus sinensis*)	Secondary metabolism

Table 2. Continued.

Spot No.	Ratio 9 °C vs 4 °C	Acc. Num.	MASCOT Scorea	Prot. Mass	Protein annotation (Organism)b	Functional classification
849	1.43	CF830958.1	131	30941	Glutathione S-transferase DHAR2-like (*Citrus sinensis*)	Stress response
851	1.22	CB291742.1	627	30576	Triosephosphate isomerase, cytosolic(*Citrus clementina*)	Sugar metabolism
853	1.22	CK934815.1	125	32708	20 kDa Chaperonin (*Citrus sinensis*)	Stress response
859	1.27	CB290506.1	355	30614	Small heat shock protein (*Citrus sinensis*)	Stress response
868	1.38	CB290506.1	500	30614	Small heat shock protein (*Citrus sinensis*)	Stress response
870	1.32	FC924648.1	112	27669	Glutathione S-transferase (GSTU1) (*Citrus sinensis*)	Stress response
886	1.82	CX672355.1	183	35015	Glutathione S-transferase DHAR3 (*Citrus sinensis*)	Stress response
888	1.96	CV884630.1	845	31663	L-ascorbate peroxidase (*Citrus sinensis*)	Oxidative process
893	1.37	EY745912.1	629	28133	Triosephosphate isomerase (*Citrus sinensis*)	Sugar metabolism
896	1.29	CB293009.1	159	29091	Glutathione S-transferase L3-like (*Citrus sinensis*)	Stress response
899	1.28	CF508590.1	322	21873	Probable NAD(P)H dehydrogenase (quinone, FQR1) (*Citrus clementina*)	Oxidative process
900	1.57	CK933143.1	223	31944	Glutathione S-transferase F9 (*Citrus sinensis*)	Stress response
901	1.28	NC_023049	213	31949	Putative thermostable pectinesterase (PME4) (*Citrus sinensis*)	Cellular component biogenesis
903	1.33	CN185579.1	755	27357	Small heat shock protein HSP20 (*Citrus sinensis*)	Stress response
904	1.33	DY280073.1	299	34636	Glutathione S-transferase DHAR2-like (*Citrus sinensis*)	Stress response
911	1.41	CN185579.1	506	27357	Small heat shock protein (*Citrus sinensis*)	Stress response
912	1.25	CN185690.1	219	32663	26.5 kDa Heat shock protein (*Citrus sinensis*)	Stress response
915	1.70	CB293436.1	147	32217	Glutathione S-transferase L3-like (*Citrus sinensis*)	Stress response

Table 2. Continued.

Spot No.	Ratio 9 °C vs 4 °C	Acc. Num.	MASCOT Score[a]	Prot. Mass	Protein annotation (Organism)[b]	Functional classification
928	1.25	CN183970.1	191	29916	Glutathione S-transferase L3-like (*Citrus sinensis*)	Stress response
931	1.20	CB292454	163	31344	Hypothetical protein (*Citrus sinensis*)	Unknown
941	1.32	CN186533.1	265	25978	Polygalacturonase-inhibiting protein (PIGP) (*Citrus hystrix*)	Defense
961	1.35	CN187365.1	3726	28128	Small heat shock protein (*Citrus sinensis*)	Stress response
979	1.25	EY884305.1	229	34327	Probable polyamine transporter At3g19553 (*Citrus sinensis*)	Transport
1027	1.23	AU300816.1	176	27426	Hypothetical protein (*Citrus sinensis*)	Unknown
1043	1.24	CB290506.1	248	30614	Small heat shock protein (*Citrus sinensis*)	Stress response
1072	1.45	EY703395.1	2374	29966	18.2 kDa Heat shock protein class I (*Citrus sinensis*)	Stress response
1081	1.34	CX297938.1	111	11731	Germin-like protein subfamily 1 member 17 (*Citrus clementina*)	Defense
1120	1.29	CN186507.1	255	26869	Germin-like protein subfamily 1 member 17 (*Citrus clementina*)	Defense
1125	1.23	CX638325.1	307	29605	Photosynthetic NDH subunit of lumenal *Citrus sinensis*)	Oxidative process
1001	-2.11	BQ623024.1	94	22614	17.4 kDa class I heat shock protein-like (*Citrus sinensis*)	Stress response
1058	-1.29	BQ625000.1	497	22420	17.4 kDa Heat shock class I (*Citrus sinensis*)	Stress response
1101	-1.43	CF835566.1	425	26195	CBS domain-containing protein (CBSX3) (*Citrus sinensis*)	Stress response
1183	-1.66	BQ623121.1	104	20136	Heat shock protein-like class I (*Citrus sinensis*)	Stress response
1237	-1.42	EY725174.1	59	31783	ABC transporter I family member 17 (*Citrus clementina*)	Transport

Table 2. Continued.

Spot No.	Ratio 9 °C vs 4 °C	Acc. Num.	MASCOT Score[a]	Prot. Mass	Protein annotation (Organism)[b]	Functional classification
1257	-1.33	CB292466.1	156	28519	Manganese superoxide dismutase (*Citrus sinensis*)	Oxidative process
1334	-1.28	BQ625161.1	214	24285	Thioredoxin-1 (*Citrus sinensis*)	Oxidative process
1396	-1.23	CK938352.1	99	11461	Uncharacterized (*Citrus sinensis*)	Unknown
1398	-1.26	BQ623024	56	22614	17.4 kDa class I heat shock protein-like (*Citrus sinensis*)	Stress response

[a] Score ≥ 46 indicates identity ($P \leq 0.05$)

[b] Identification performed by searching MSMS data by MASCOT software against NCBInr proteins database.

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Figure 1 (Carmona *et al.*, 2018)

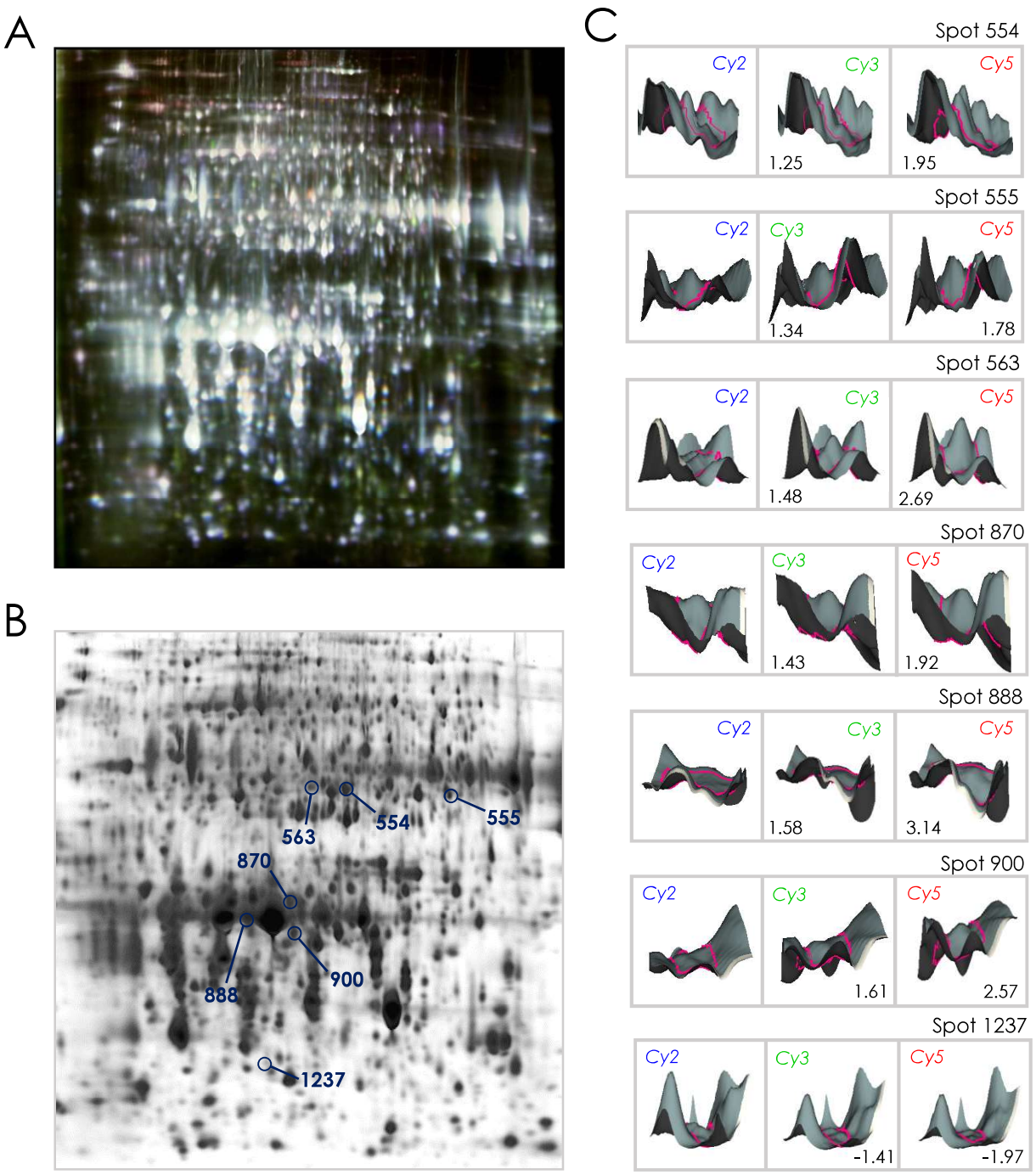

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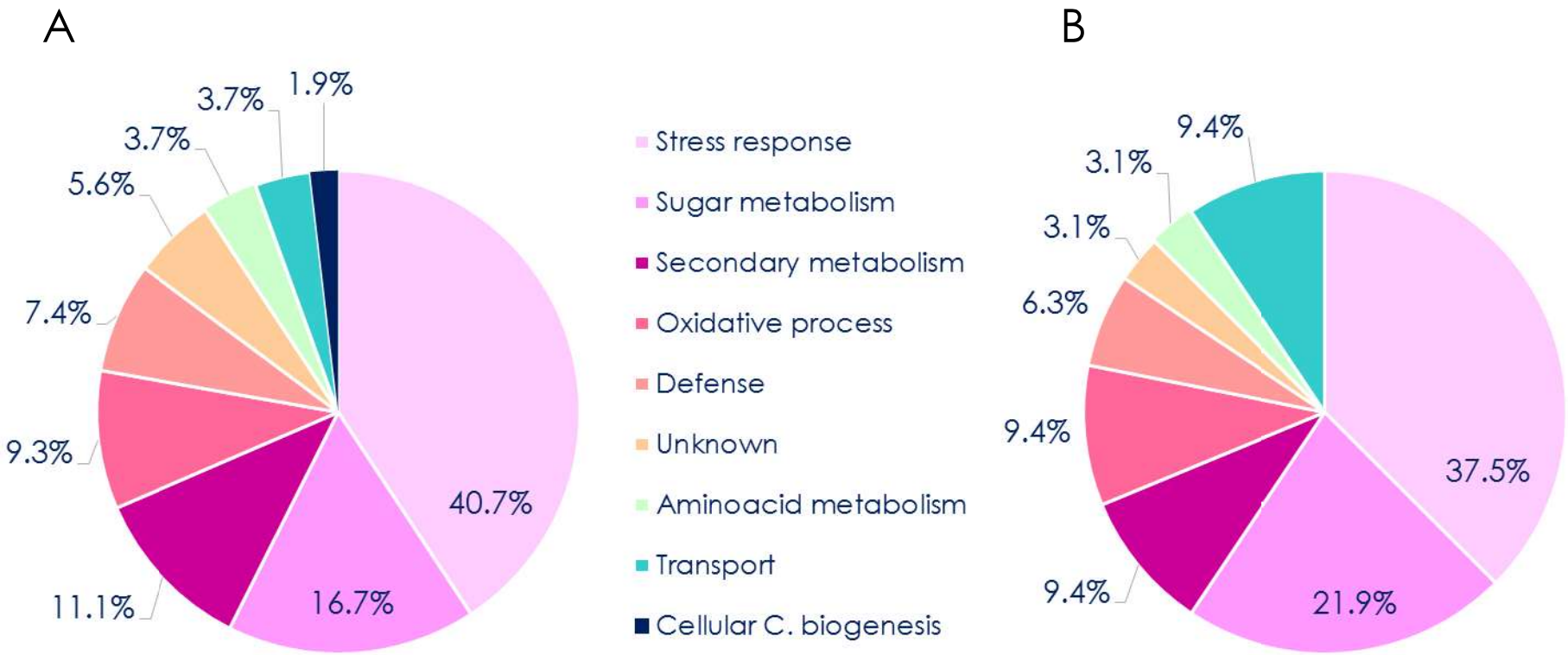
Figure 2 (Carmona *et al.*, 2018)



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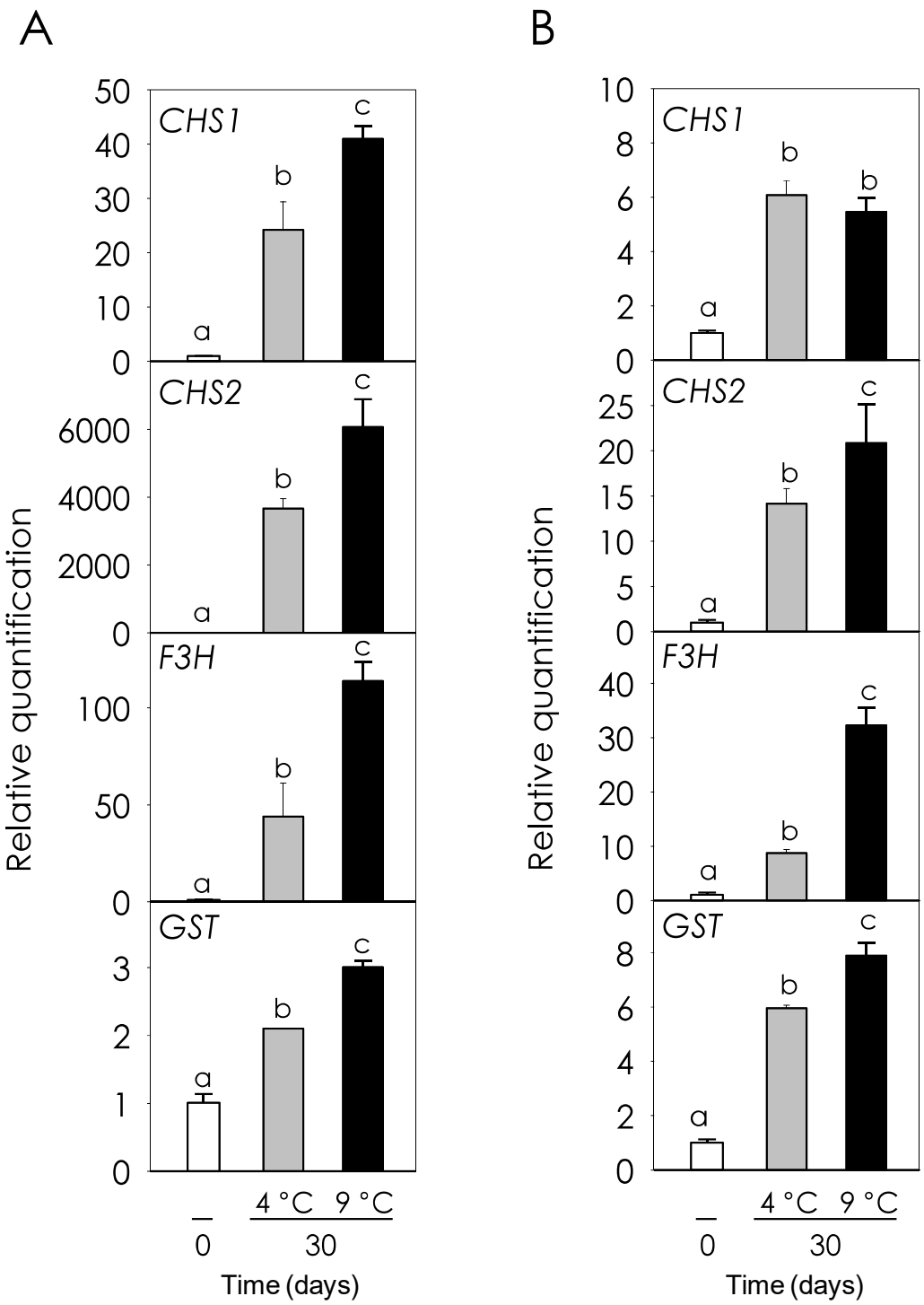

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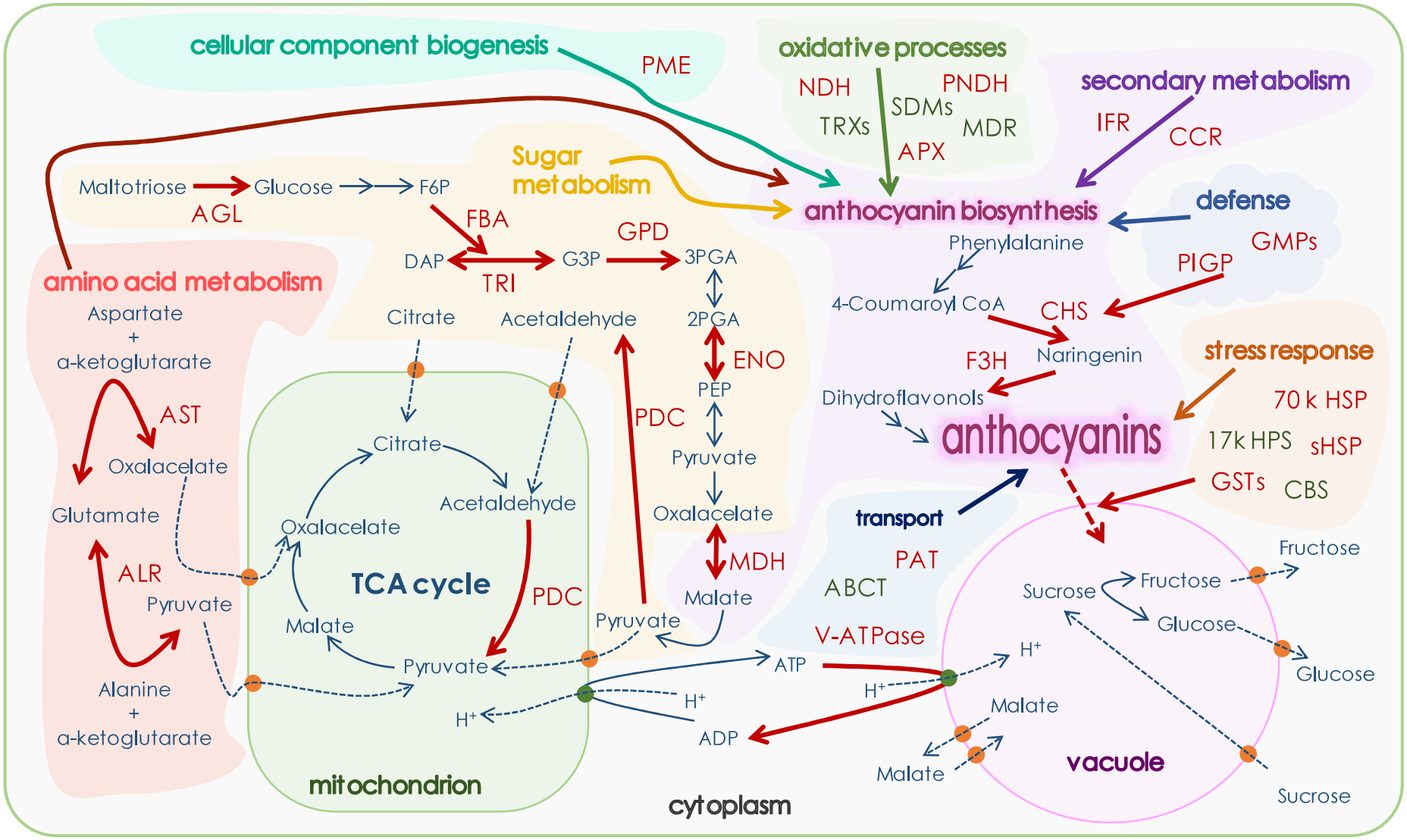

Supplementary Material

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Supplementary Material

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