Postharvest Biology and Technology 99 (2015) 80–87

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Biochemical characterization of the flavedo of heat-treated Valencia orange during postharvest cold storage

Valeria E. Perotti[a,1], Alejandra S. Moreno[a,1], Karina Trípodi[a], Hernán A. Del Vecchio[a], Guillermo Meier[b], Fernando Bello[b], Mariángeles Cocco[b], Daniel Vázquez[b], Florencio E. Podestá[a,*]

[a] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina
[b] Estación Experimental Concordia, Instituto Nacional de Tecnología Agropecuaria (INTA), Colonia Yeruá, Argentina

ARTICLE INFO

Article history:
Received 6 June 2014
Accepted 8 August 2014

Keywords:
Citrus fruit
Glycolysis
Fruit biochemistry
Orange
Postharvest
Proteomics

ABSTRACT

Heat treatment is a powerful and eco-friendly method to prevent *Penicillium* infection in citrus fruit during the postharvest. Several studies have been dedicated to investigate the general chemical changes that justify the immediate reaction responsible for the induced tolerance; but just how primary metabolism and enzymology are affected by heat treatment and along a prolonged cold storage is still unclear. In this work, the main enzymes of carbon metabolism of Valencia orange flavedo were analyzed during the postharvest period after a heat treatment (HT) of 48 h at 37 °C. Enzymatic activity measurements indicated that the NADPH producing enzymes glucose 6-phosphate dehydrogenase and non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase have lower levels in HT fruit. In parallel, a higher synthesis of sucrose from organic acids was observed in HT epicarp. Sucrose-phosphate synthase would have an important role in sucrose accumulation. The pathway of carbon through glycolysis was affected by cold storage, independently of HT, in a way that it favors the ATP-dependent phosphofructokinase over the PPi-dependent homologous enzyme and the use of phosphoenolpyruvate (PEP) by PEP carboxylase instead of pyruvate kinase. Similarly, phenylpropanoid compounds did not show major changes in response to HT, although some of them showed a marked descent along the cold storage. Proteomic studies revealed alterations in the abundance of ascorbate peroxidase, two germin-like proteins and small HSPs, completing the description of the main metabolic changes in this tissue.

1. Introduction

Heat treatment (HT) is a frequently used conditioning method that help fruit and many other crop products withstand the postharvest period with low incidence of decay, chilling injury, insect attack and microbial infections (Ghasernnezhad et al., 2008; Lafuente et al., 2011; Lurie, 1998; Palou, 2013; Paull, 1990; Perotti et al., 2011; Sapitnitskaya et al., 2006; Schirra et al., 2011). It is a very low environmental impact method that can be applied alone or in combination with chemical treatments, reducing the amount to be applied and increasing its effectiveness (Palou, 2013). Ideally, HT must protect the fruit against decay while maintaining both internal and external quality unaltered (Palou, 2013). Different types of fruit respond differently to HT and, even within the same genus, considerable different responses may exist that rule out the possibility of using a single protocol in all cases (Lurie, 1998; Schirra et al., 2011).

The effects of HT on the general biochemistry of citrus fruit, which has been explored in the past, has received a boost in recent years by the use of modern techniques, mainly the application of the "omics" to unravel the molecular mechanisms subjacent to the response that HT elicits in the fruit tissues (Katz et al., 2007; Lara et al., 2009; Muccilli et al., 2009; Pan et al., 2009; Yan et al., 2006; Yun et al., 2013; Zhang et al., 2010).

In a recent paper, evidence was presented that HT bring out a number of responses in Valencia orange that are consistent with the improved resilience of the fruit during the postharvest period (Perotti et al., 2011). Most of the results and all the proteomic analysis were performed on the endocarp. This work completes the former study with a proteomic and biochemical examination of the flavedo in the same fruit. The flavedo of citrus fruit is a metabolically

* Corresponding author. Tel.: +54 341 4371955; fax: +54 341 4371955.
E-mail address: podesta@cefobi-conicet.gov.ar (F.E. Podestá).
[1] These two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.postharvbio.2014.08.007
0925-5214/

active tissue (much more than the endocarp at maturity) (Falcone Ferreyra et al., 2006) that, although does not constitute the edible part of the fruit, is essential to judge the quality of the fruit from a consumer's point of view. In citrus fruit, great differences exist in the response of this tissue to postharvest treatments and conditions. The study described here looks into the several metabolic changes introduced by postharvest condition in Valencia orange, probably the most consumed citrus fruit and one of the more resistant to the abiotic stress posed by postharvest management.

2. Materials and methods

2.1. Plant material and treatments

Assays were conducted with orange fruit [*C. sinensis* (L.) Osbeck] cv 'Valencia late' grown in the Estación Experimental Agropecuaria INTA, Concordia, Entre Ríos, Argentina (EEAC), harvested at full maturity in October/November 2007; and repeated with fruit grown during 2008. Immediately after harvest, fruit were manually selected for uniformity of color and size and divided into two groups of 50 each. The first group was used as control, remaining for 72 h at 20 °C. The second group received a heat treatment, which consisted of 48 h at 37 °C and 90% relative humidity followed by 24 h at 20 °C. This treatment usually is applied to oranges from the EEAC because its effectiveness in controlling *Penicillum digitatum* has been demonstrated previously (Cocco et al., 2008). Groups were labeled control (C) and heat treated (HT), respectively, and this couple of samples are called "fist pair". In turn, one subgroup of each category was stored at 4 °C for 60 d, simulating commercial conditions (C + 60 d and HT + 60 d, called "second pair"). Immediately after each treatment, the flavedo (epicarp) were frozen in liquid nitrogen and stored at −80 °C for further experiments.

2.2. Total protein extraction

Fruit tissue (approximately 0.5 g of epicarp) was powdered with liquid nitrogen in a mortar and then homogenized with ten volumes of extraction buffer (100 mM KPi pH 7.0, 1 mM PMSF). The final pH of the crude extracts thus obtained was near 7.0. The homogenates were centrifuged for 15 min at 4 °C in an Eppendorf microcentrifuge at maximum speed. The resulting supernatant was desalted in a Sephadex G-50 column previously equilibrated with five volumes of extraction buffer (Penefsky, 1977). The eluate was used as the source for enzyme activity measurements and inmunoblotting.

2.3. Protein quantification

Protein concentration was determined in crude extracts using a detergent-compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection of total protein and bovine serum albumin as standard.

2.4. Activity assay

All enzymes were assayed at 30 °C in a Hitachi 150-20 (Hitachi Corp., Tokyo, Japan) spectrophotometer following the oxidation of NAD(P)H al 340 nm in a final volumen of 1 mL, except for fumarase, sucrose-phosphate synthase and succinate dehydrogenase (see below). The assays were optimized as described by Falcone Ferreyra et al. (2006). One basic experiment in which enzymatic activities were measured represents the mean of at least 3 determinations per fruit made in 3 fruit from each group. Each experiment was repeated at least twice. The reaction mixtures for each assay were as follows.

Glyceraldehyde-3-phosphate dehydrogenase (Ga3PDH, EC 1.2.1.12): 50 mM Tricine-NaOH, pH 8.5, 4 mM NAD, 2 mM fructose-1,6-bisphosphate (Fru-1,6-P_2), 10 mM arsenate and 1 U aldolase. Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (npGa3PDH, EC 1.2.1.9): 50 mM Tricine-NaOH, pH 8.5, 0.4 mM NADP, 2 mM Fru-1,6-P_2 and 1 U aldolase. Malate dehydrogenase (MDH, EC 1.1.1.37): 50 mM imidazole, pH 6.9, 1 mM oxaloacetate and 0.15 mM NADH. NAD-malic enzyme (NAD-ME, EC 1.1.1.39): 50 mM HEPES-NaOH, pH 7.3, 2 mM NAD, 2 mM L-malate, 5 mM dithiothreitol (DTT), 75 μM CoA, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 U MDH. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49): 100 mM Tris–HCl, pH 8, 0.2 mM NADP and 2 mM glucose-6-phosphate. Hexokinase (HK, EC 2.7.1.1): 30 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM NAD, 2 mM glucose, 1 mM ATP, 1 U NAD-dependent glucose-6-phosphate dehydrogenase. ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11): 50 mM Tris–HCl, pH 7.5, 0.15 mM NADH 4 mM fructose-6-phosphate (Fru-6-P), 0.5 mM ATP, 5 mM $MgCl_2$, 5% (w/v) polyethylene glycol (PEG), 1 mM dithiothreitol (DTT), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase triose phosphate isomerase. Pyrophosphate-dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90): 50 mM Tris–HCl, pH 7.5, 0.15 mM NADH, 4 mM Fru-6-P, 0.5 mM PPi, 5 mM $MgCl_2$, 5%(w/v) PEG, 1 mM DTT, 1 μM fructose-2,6-bisphosphate (Fru-2,6-P_2), 0.2 U aldolase and 0.1 U glycerophosphate dehydrogenase/triose phosphate isomerase. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11): 50 mM HEPES-NaOH, pH 7.5, 0.1 mM (Fru-1,6-P_2), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.5 mM $NADP^+$, 2 U glucose-6-phosphate dehydrogenase and 1 U hexose phosphate isomerase. Phosphoenolpyruvate carboxykinase (PCK, EC 4.1.1.49): 50 mM HEPES-NaOH, pH 7.3, 2.5 mM $MgCl_2$, 2.5 $MnCl_2$, 10 mM $NaHCO_3$, 4 mM phosphoenolpyruvate (PEP), 0.15 mM NADH, 3 mM ADP and 2 U MDH. Activity was corrected for interference by PEPC activity by omitting ADP from the reaction mixture. Phosphoglucose isomerase (PGI, EC 5.3.1.9): 50 mM Tris–HCl, pH 8.1, 1 mM fructose-6-phosphate, 0.24 NADP and 2 U G6PDH. Phosphoglucomutase (PGM, EC 2.7.5.1): 20 mM imidazole, pH 7.85, 10 mM $MgCl_2$, 3 mM EDTA, 0.1 mM glucose-1,6-bisphosphate, 0.5 mM NADP and 0.8 U glucose-6-phosphate dehydrogenase. PEP carboxylase (PEPC, EC 4.1.1.31): 100 mM HEPES-NaOH, pH 8, 10% (v/v) glycerol, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 4 mM PEP, 0.15 mM NADH and 0.6 U MDH. NADP-malic enzyme (NADP-ME, EC 1.1.1.40): 50 mM Tris–HCl, pH 7.5, 0.5 mM NADP, 10 mM $MgCl_2$ and 10 mM L-malate. Pyruvate kinase (PK, EC 2.7.1.40): 25 mM HEPES-NaOH, pH 7.2, 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.15 mM NADH, 5% (w/v) PEG, 1 mM ADP and 0.4 U LDH. This enzymatic activity was corrected for interference by PEP-phosphatase activity by omitting ADP from the reaction mixture. Alanine aminotransferase (Ala-AT, EC 2.6.1.2): 100 mM Tris–HCl, pH 8.0, 25 mM alanine, 10 mM 2-ketoglutarate, 0.15 mM NADH and 0.2 U LDH. Glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1): 80 mM Tris-Cl, pH 7.8, 20 mM L-aspartate, 15 mM 2-ketoglutarate, 0.15 mM NADH, 0.8 U LDH and 0.6 U MDH. Fumarase (EC 4.2.1.2): 100 mM KPi, pH 7.4 and 50 mM L-malate. This activity was measured following the production of fumarate at 240 nm. Neutral invertase (NI, EC 3.2.1.26): 200 mM HEPES-NaOH, pH 7.5, 200 mM sucrose. The mixture was incubated at 30 °C for different times and the progress of the reaction was followed detecting the amount of glucose produced by using glucose oxidase/peroxidase commercial kit (Wiener Lab, Rosario, Argentina). Acid invertase (AI, EC 3.2.1.26) was assayed under the conditions described above, although the reaction mixture contained 100 mM acetic acid/sodium acetate buffer, pH 5.0. In the case of AI, the aliquot was neutralized prior to glucose determination. Sucrose Synthase (SS, EC 2.4.1.13): 100 mM MES, pH 6.5, 3 mM $MgCl_2$, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 50 mM sucrose, 0.02 mM glucose-1.6-bisphosphate, 0.5 mM NAD, 1 mM UDP, 1 mM PPi, 1 U PGM, 1 U G6PDH and 1 U UDP-glucose pyrophosphorylase. Sucrose-phosphate synthase (SPS, EC 2.4.1.14): the reaction mixture of 200 μl contained 100 mM

Hepes–NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA, 5 mM fructose 6-phosphate, 15 mM glucose 6-P, 10 mM UDPG. The reaction was initiated by addition of 100 μl of extract and incubated at 30 °C at different times from 0 to 20 min. Termination was accomplished by addition of 30% KOH. Tubes were placed in boiling water for 10 min to destroy any remaining fructose or fructose-6-phosphate. After cooling, 3.5 mL of a mixture of 0.15% anthrone in 80% H_2SO_4 was added to the tube, which was then incubated in a 40 °C water bath for 20 min. After cooling to room temperature, color development was measured at 620 nm. Succinate dehydrogenase (Succinate DH, EC 1.3.5.1): 500 mM Na_3PO_4, pH 7.4, 5% (v/v) Triton X-100, 500 sodium succinate, 15 mM nitro blue tetrazolium. The mixture was incubated at 37 °C for 30 min, the reaction was stopped by adding 2 mL 2% SDS and activity was measured following the reduction of NBT, an artificial electron acceptor, at 630 nm.

2.5. Electrophoresis and immunoblotting

SDS-PAGE was carried out according to the method of Laemmli (1970) using a Bio-Rad mini-gel apparatus (Bio-Rad, Hercules, CA). The final acrylamide concentration was 10% for HSP 70 and 15% for HSP 21 detection. The following standard proteins were used: 200 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 35 kDa, and 20 kDa from a commercial kit (Promega). Immunoblotting was performed according to the method of Bollag and Edelstein (1991). Samples were run on denaturing PAGE and transferred to nitrocellulose membrane. The antibodies used for detection were raise in rabbit against *Solanum lycopersicum* purified HSP21(Polenta et al., 2007) and against *Nicotiana tabacum* recombinant HSP70 (Lara et al., 2005).

2.6. Bidimensional electrophoresis

Protein extraction, labeling with dyes, gel image analysis and protein identification were carried out as previously described (Perotti et al., 2011).

2.7. Crude extracts preparation for LC–MS metabolite analysis

Phenolic compounds from flavedo of citrus fruits were analyzed as described (Ballester et al., 2013) with slight modifications. HPLC–MS analysis was performed using an Agilent 1200 HPLC system, coupled to a G1314C VWD UV detector and a Bruker micrOTOF-Q II spectrometer (Bruker-Daltonics). Fruit tissue (approximately 0.5 g of epicarp) was powdered with liquid nitrogen in a mortar and then homogenized with 1 mL of methanol 80% v/v. The homogenates were centrifuged for 15 min at 4 °C in an Eppendorf microcentrifuge at maximum speed. The resulting supernatant was filtered through 0.22 μm GE nylon filter. Samples were separated by reverse phase HPLC in a Zorbax XDB 1.8 μ C18 column (50 mm × 3.0 mm, Agilent), using a binary gradient elution of acetonitrile and water (pH 2.5). The flow rate was 3.33×10^{-3} mL s^{-1} and the injection volume, 5 μl. Elution was monitored at 280 nm. MS settings whereas follows: source type, ESI; ion polarity, positive; nebulizer, 1.0 bar; dry heater, 200 °C; dry gas, 66.7 mL s^{-1}; capillary, 4500 V; end plate offset, 500 V; collision cell RF, 150.0 Vpp. Peak area was normalized using the amount of the sample fresh weight and the relative compounds levels were expressed as the ratio of the area in the UV chromatogram respect to the control.

2.8. Statistical analysis

Data from experiments were tested using two-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Holm-Sidak test ($\alpha = 0.05$) using the Sigma Stat Package.

3. Results and discussion

3.1. Carbohydrate metabolism in citrus epicarp is affected by HT and cold storage

In order to complete and extend the biochemical characterization of the changes caused by heat treatment in orange fruits during postharvest storage (Perotti et al., 2011) activity measurements of the main enzymes involved in carbohydrate metabolism in the flavedo were performed (Figs. 1 and 2). Alterations from each pathway are grouped for a better analysis of the results.

3.1.1. Sucrose metabolism

Sucrose synthase and acid invertase activities did not show significant variations between treatments. Conversely, both of them were affected by storage time: while showed a decrease after 60 d, acid invertase increased significantly. In all, there was a net important reduction of the sucrose degradation rate along the storage, because the increase of acid invertase was not enough to compensate the sucrose synthase activity decrease. Instead, neutral invertase activity only decreased in HT + 60 d samples. However, the rise in sucrose concentration observed previously in HT epicarp with respect to the control (Perotti et al., 2011) cannot be explained by a lower sucrose degradation rate, since there are not significant differences between the degradative activities in the first pair. In this case, a higher synthesis by sucrose-phosphate synthase would explain that result (Fig. 1), although a simultaneous induced import of this metabolite from the albedo could not be discarded. Organic acids could be the carbon source for sucrose synthesis, since the main acids decreased in HT samples (Perotti et al., 2011). Similarly, there was a sudden and transient transformation of organic, amino and fatty acids into sugars driven by HT in Satsuma mandarin, which was stopped during the later stages of storage (Yun et al., 2013).

3.1.2. Primary metabolism: glycolysis and gluconeogenesis

Hexokinase showed a behavior similar to acid invertase while phosphoglucoisomerase was detected only in the second pair. Phosphoglucomutase did not show significant changes in any case. PPi-PFK activity was not detected after 60 d whereas ATP-PFK increased notoriously at the same point of storage. This behavior means a higher dependence on ATP rather than PPi to phosphorylate Fru6P and is certainly intriguing that it appears at a time when energy demands may be critical (Plaxton and Podestá, 2006). PPi-PFK activity tends to decline along fruit development in pepper (Nielsen et al., 1991), although it remains at a low but detectable level in the mature fruit. In Valencia orange fruit it also decreases during development (Sansevich and Podestá, unpublished results) and probably this tendency remains during the postharvest storage. Interestingly, this trend also was observed at the protein level by specific inmunodetection of both subunits of this enzyme (Fig. 3). Even more, an examination of the subunit composition of PPi-PFK revealed a decrease in the relative amount of the α subunit along the storage. This subunit has been proposed to confer sensitivity to the activator, while the β subunit is catalytic (Podestá and Plaxton, 1994). In this way, PPi-PFK activity would be lower due to a lower catalytic capacity and to a lower response to the activator Fru-2,6-P_2.

Regarding the enzymatic activities related to PEP metabolism, the most notable change was the decrease in PK activity immediately after heat treatment to levels which continued low during the postharvest period. PEPC, MDH (Fig. 1) activities increased along the storage both in control and HT fruit, while NAD-ME, which was undetectable at the start of the experiment, displayed a low but measurable activity after 60 d. All these variations can be summarized as a higher flux of carbon via PEPC, MDH and NAD-ME rather

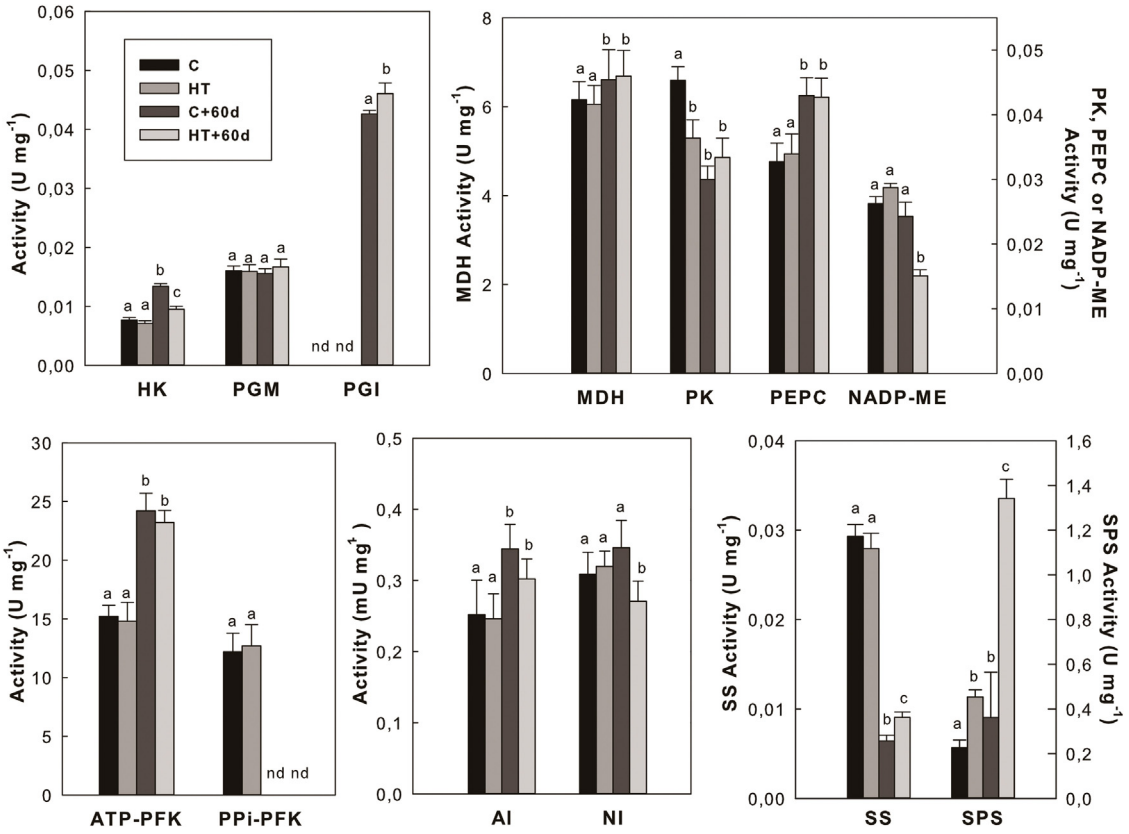

Fig. 1. Activity of enzymes in Valencia orange flavedo of control and cured samples. Enzymatic activities are expressed in U mg^{-1} protein, with the exception of invertases activities, which are expressed in mU mg^{-1} protein. For each enzyme, bars with the same letters are not significantly different ($^{*}P < 0.05$). HK, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; SS, sucrose synthase; SPS, sucrose-phosphate synthase; AI, acid invertase; NI, neutral invertase; MDH, NADmalate dehydrogenase; PK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; NADP-ME, NADP-malic enzyme; ATP-PFK, ATP-dependent phosphofructokinase; PPi-PFK, pyrophosphate-dependent phosphofructokinase.

than through cytosolic PK, as occurs upon specific conditions such as oilseed embryogenesis or prolonged Pi starvation (Nagano et al., 1994; Schwender et al., 2004). It could also be said that cold storage during the postharvest period stimulates intramitochondrial pyruvate production at the expense of cytosolic ATP production. It must be noted that the respiration rate remained unchanged during the 60 d, regardless of the treatment (Perotti et al., 2011), so these findings indicate that the amount of ATP produced by mitochondrial respiration is sufficient to meet energy needs in the stored fruit. Since the amount of the variation in PK and PEPC activities, albeit significant, is small, it might well be than rather than reflecting a respiratory variation these changes are adjustments to a new metabolic situation that calls for an increased anapleurotic metabolism (i.e. lignin and/or aminoacid synthesis). This makes sense if it is considered that upon harvest the fruit does no longer receive photosynthate from the plant. NADP-ME role in plants is the provision of NADPH for several biosynthetic processes in the cytosol or lipids in the plastid. The location of this enzyme in citrus flavedo is not known. This enzyme's activity falls sharply in HT fruit after 60 d. It could be hypothesized that HT fruit is less requiring of reductive power, as will be discussed in Section 3.2.

From an analysis of the opposite variations along the postharvest of the ATP-PFK/PPi-PFK and PK/PEPC couples, the key plant glycolysis enzymes, it could be concluded that the net glycolytic flux remains constant along cold storage, independently of the HT application. Parallel increases in PPi-PFK and PEPC usually appear as a result of a stress situation that might decrease adenylates levels (Plaxton and Podestá, 2006). The changes in ATP-PFK and PPi-PFK suggest that this is not the situation that develops on the stored flavedo. The reason why PPi-PFK drops is not clear, but it may be reasoned that as the natural senescence process progresses, biosynthetic pathways that produce PPi will progressively be shut down and with it the availability of PPi, making the role of the PPi-PFK unnecessary.

Two of the most prominent enzymes involved in gluconeogenesis, PCK and FPBase, were measured (Fig. 2). PCK activity could only be detected in HT + 60 d samples, suggesting a potential surge of gluconeogenic activity at this point. However, since at the same time there is an about constant level of glycolytic enzymes, and given that the sum of PK and PEPC activities is nearly 8 times greater than PCK activity after 60 d, the glycolytic flux clearly predominates at all times.

Even though several reports describe the effect of pathogen infection on carbohydrate metabolism, there is still a considerable lack of knowledge regarding how these changes influence the outcome of plant–pathogen interactions (Berger et al., 2007). Moreover, how an increase in sugar levels previous to the pathogen infection could help the fruit to prevent fungal proliferation is currently not known. Several factors contribute to the complexity of the mutual relation between carbohydrate status and development of disease/resistance. Sugars are not only nutrients; but also behave as signals that regulate gene expression in plants promoting the defense response (Berger et al., 2007; Koch, 1996; Pego et al., 2000) and probably regulate the pathogen's gene expression as well. Functional approaches are necessary to elucidate how the alterations in flavedo carbohydrate metabolism observed in this study could affect the plant–pathogen interaction.

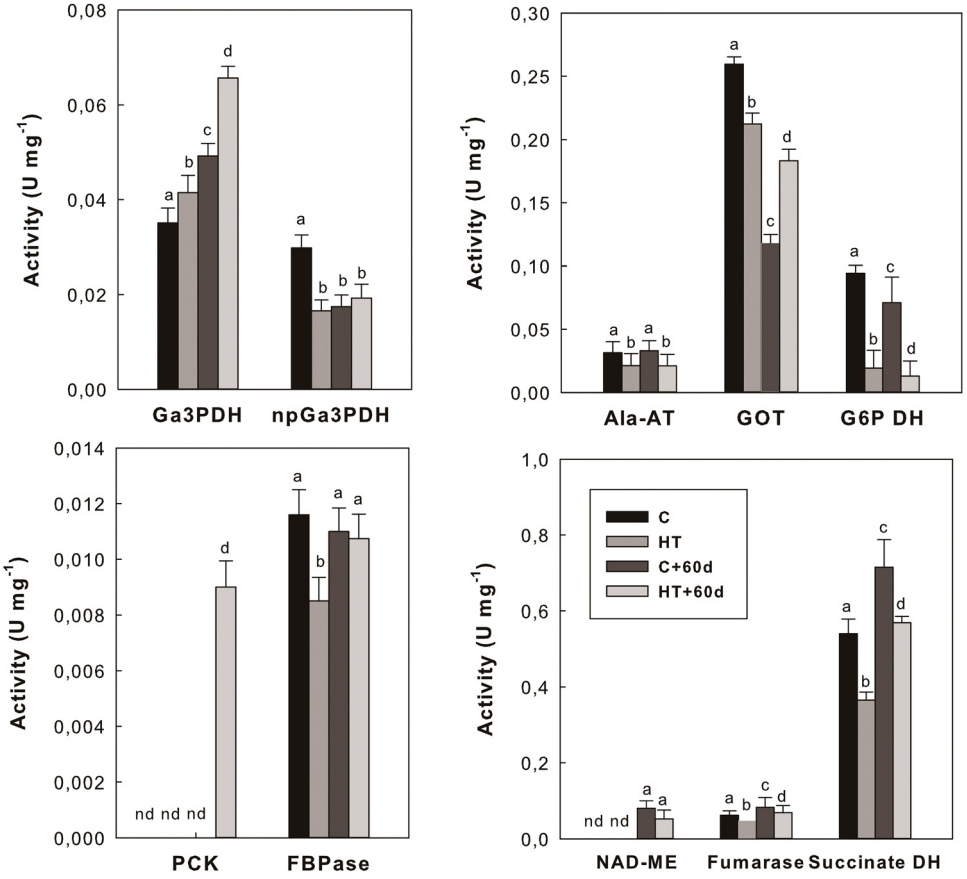

Fig. 2. Activity of enzymes in Valencia orange flavedo of control and cured samples. Enzymatic activities are expressed in U mg^{-1} protein. For each enzyme, bars with the same letters are not significantly different (*$P < 0.05$). GAPC, glyceraldehyde-3-phosphate dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Ala-AT, alanine aminotransferase; GOT, glutamate oxaloacetate transaminase; G-6-P DH, glucose-6-phosphate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase; NAD-ME, NAD-malic enzyme.

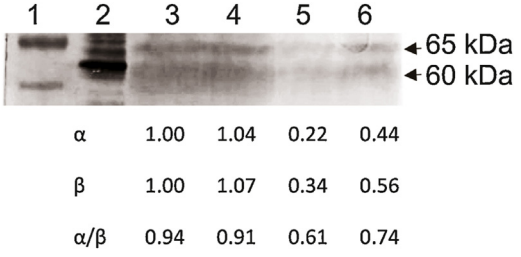

Fig. 3. Immunodetection of PPi-PFK from Valencia orange epicarp extracts. Membranes were probed with rabbit immune serum raised against potato tuber PPi-PFK. Lane 1: standard molecular mass markers; lane 2: crude extract from potato tuber; lane 3: crude extract from control Valencia orange fruit (C); lane 4: crude extracts from HT Valencia orange fruit (HT); lane 5: crude extracts from C samples after 60 d of storage at 4 °C (C + 60 d); lane 5: crude extracts from HT samples after 60 d of storage at 4 °C (HT + 60 d). Twenty μg of total protein was loaded per lane. The numbers below the figure indicate the relative intensities of reactive bands from α and β subunits as determined by densitometry with respect to control samples (C) and the ratio α/β for each extract. The numbers on the side of each gel indicate the mass of each subunit.

3.2. Redox status

npGa3PDH, a source of NADPH in the plant cell cytosol (Bustos et al., 2008), diminishes by almost a half upon heat treatment and by storage time only in control fruit, whereas in HT fruit it remains at the same levels (Fig. 2). Although the function of npGa3PDH in non-photosynthetic tissue is not clearly resolved, there are clues to its protecting role against oxidative stress (Bustos et al., 2008; Piattoni et al., 2013). Moreover, it has been proposed (Piattoni et al., 2013) that elevated H_2O_2 levels would lower Ga3PDH but increase npGa3PDH. According to this hypothesis, the observations reported here do not suggest that there is an increase in H_2O_2 levels. Previous work (Perotti et al., 2011) suggested that the potential increase in H_2O_2 would not be high enough to induce a full antioxidative response and/or that the basal activities of ascorbate peroxidase and glutathione reductase are high enough to cope with existing H_2O_2.

On the other hand, G6PDH presented the same pattern in both pairs: an evident drop by HT. These results suggest that HT modifies the oxidative glucose catabolism toward predominance of glycolysis over the pentose phosphate pathway. Taking together this result with the low npGa3PDH and NADP-ME levels observed suggests that NADPH production during the postharvest period is not critical.

3.3. Mitochondrial function

The lower activities of succinate DH and fumarase in both HT samples would indicate an alteration of mitochondrial function brought about by HT, as had been suggested by previous work (Perotti et al., 2011).

Alanine serves as a storage form of pyruvate in the vacuole, controlling the supply of the substrate to LDH and PDC (and hence the flux to lactate and ethanol) and also to the pyruvate dehydrogenase complex (Muench et al., 1998). Miyashita et al. (2007) suggested that Ala-AT is mainly involved in Ala degradation. Therefore, a lower demand of pyruvate by the mitochondrion could justify the lower Ala-AT level observed upon HT in both pairs.

Table 1
List of differentially expressed proteins in control and cured Valencia orange epicarp. The data are grouped according to the storage period (0 and 60 days). The second column shows changes of spot abundance ratios: negative values, if protein levels decrease in HT fruit with respect to control, or positive values, in the opposite case. For each spot, the MASCOT score, the accession number, the protein annotation, the sequence coverage, the number of matched and fragmented peptides and finally the theoretical and experimental relative molecular mass (Mr) and isolectric point (pI) are indicated. When the search was carried out using the Viridiplantae ESTs database, the EST accession number and the E-value associated to the best matching protein obtained via BLASTx are also indicated.

Spot no	Ratio	MASCOT score	EST Acc. no translated ORF	Acc. no of homologous found in NCBInr	Protein annotation of homologous found in NCBInr [organism]	*E*-value	Coverage %	Matched peptides	Fragmented peptides	Theoretical		Observed	
										Mr	pI	Mr	pI
Epicarp comparisons													
C vs HT													
1	+10,053				No significant hit							50	6.5
2	+1145				No significant hit							25	6.0
3	+2098				No significant hit							60	6.0
4	−1112	85		ACM17464	Ascorbate peroxidase 2 [*Citrus maxima*]		24%	7	2	27.7	5.55	35	6.0
C + 60 d vs HT + 60 d													
5	+1053				No significant hit							27	6.2
6	+1615	102	EY694807	AFN02126	Germin-like protein [*Citrus limon*]	6E−108	38%	9	1	26.4	8.00	30	6.4
7	1671	96	EY714064	AFN02126	Germin-like protein [*Citrus limon*]	7E−119	37%	10	1	29.5	9.62	65	5.7

Postharvest storage, on the other hand, affected the values of the mentioned mitochondrial enzymes plus NAD-ME, which results in an increase in the total activity of the three enzymes, most notably NAD-ME, which could not be detected at the start of the experiment. This suggests that there is an increase in mitochondrial capacity, either in the number of these organelles or the enzymatic content, along the storage period. HT does not prevent this increase, although the cured flavedo cannot recover to control levels. Although these variations that probably reflect an alteration in mitochondrial function are not paralleled by a modification in respiration in the HT and/or stored fruit (Perotti et al., 2011), it must be noted that whole fruit and not the flavedo alone was used in that study.

3.4. Changes in the fruit epicarp proteome after exposure to HT

The differentially expressed proteins were identified by MS analysis and are listed in Table 1. Two approaches were employed to identify the selected spots: searching MS–MS derived peptide sequence against NCBInr protein databases on all available higher plant proteins (Viridiplantae), and searching against ESTviridiplantae database. The complete list of peptide sequences from identified proteins is shown in Supplementary Table 1

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2014.08.007.

Among the seven differential proteins detected using the software Image Master 2D-Platinum (GE Healthcare Life Sciences), only three could be identified successfully. Two of them matched germin-like proteins, and showed contrasting variations at 60 d after HT. Many proteins of this highly diverse family are glycoproteins associated with the extracellular matrix and involved in the response to various stress conditions. The third one was ascorbate peroxidase (APX), which showed a decrease immediately after HT. This reduced protein abundance is coherent with the lower APX activity in cured epicarp reported before (Perotti et al., 2011). APX and GR enzymes act coordinately in the system called APX/GR to eliminate H_2O_2 excess at the expense of reduced ascorbate. This system would not seem to be up-regulated in oranges affected by the heat treatment, suggesting once more that there is not an increase of H_2O_2 levels. In the same direction, it was demonstrated that HT down-regulates H_2O_2 content in pericarp of Satsuma mandarin (Yun et al., 2013).

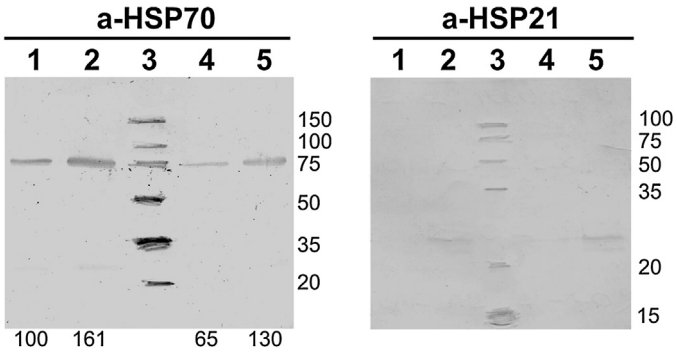

Fig. 4. Immunodetection of HSP70 and small HSPs enzymes from Valencia orange epicarp extracts. Immune sera raised against *Nicotiana tabacum* recombinant HSP70 and *Solanum lycopersicum* purified HSP21, respectively, were used. Lane 1: crude extracts from control fruit (C); lane 2: crude extracts from HT fruit (HT); lane 3: standard molecular mass markers; lane 4: crude extracts from C samples after 60 d of storage at 4 °C; lane 5: crude extracts from HT samples after 60 d of storage at 4 °C. Twenty μg of total protein was loaded in each lane. The numbers below the figure indicate the relative intensities of reactive bands determined by densitometry respect to control samples (100%). The numbers on the side of each gel indicate the mass of the standard molecular weight markers.

3.5. Induction of HSPs

The heat stress response is characterized by the induction of proteins that constitute a complex mixture of polypeptides with high and low molecular weight, known as heat shock proteins (HSPs). The abundance of these proteins in flavedo samples was evaluated by immunoblot analyses. This study confirmed the induction of both small HSPs and HSP70 family proteins by HT in epicarp (Fig. 4). Interestingly, endocarp samples did not show any significant variation of these immunoreactive proteins (Perotti et al., 2011), pointing again that the epicarp is the tissue most affected by the curing treatment.

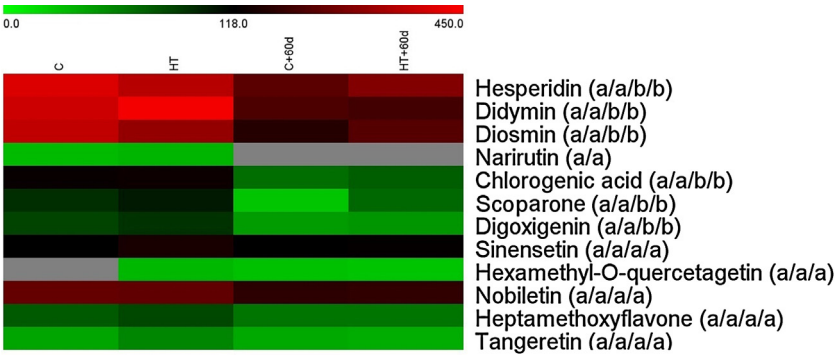

Fig. 5. Phenylpropanoid and flavonoid relative quantification in Valencia orange epicarp extracts. The relative compounds levels were expressed as the ratio of the area (mAUs) of the peak in the UV chromatogram respect to the control (C), with the exception of hexamethyl-O-quercetagetin, which was expressed relative to the control of the second pair (C + 60 d). Data visualization was performed using TM4 MultiExperiment Viewer Software. A gray box indicates 'non detected'. For each metabolite, along the four different points, the same letters indicate that differences are non significant ($^*P < 0.05$).

3.6. *Effect of HT and cold storage on phenylpropanoids metabolic profiles*

Phenylpropanoid compounds play a vital role in fruit resistance because they are involved in local and systemic signaling for defense-gene induction (Dixon et al., 2002). The present study revealed that most of the phenolic compounds identified did not show major changes in response to HT. Conversely, many of them showed a marked descent along cold storage (Fig. 5). Similarly, Ballester et al. (2010) observed only small changes induced by HT in the most abundant citrus flavonoids in Navelate oranges. Even more, HT decreased the level of some phenylpropanoids in Satsuma mandarin (Yun et al., 2013). Little information is available related to which particular class of phenylpropanoid compounds plays a core role in defensive functions, and their detection seems to be difficult probably because of the transient nature of their appearance.

The coumarin scoparone presented an important induction in fungus-inoculated HT Navelate oranges (Ballester et al., 2010). However, this study, performed in Valencia orange, failed to detect any significant changes. Whether the effect is dependent on fungal infection but not HT or there is a variety-dependent response cannot be ascertained at this time. Further research or refinement of detection techniques may be necessary to establish whether there is any alteration in this family of compounds upon HT and assess its importance in the flavedo biochemistry. The results presented herein show a decrease after 60 d in 7 of the 12 compounds assessed, which suggests that the defensive capacity associated to phenylpropanoids is reduced during the postharvest time.

3.7. *Concluding remarks*

In this study, several biochemical parameters of Valencia orange flavedo were analyzed during the postharvest period after HT. The major modifications in carbohydrate metabolism and phenylpropanoids content seem to be caused by cold storage rather than by HT. Cold storage favored the intramitochondrial pyruvate production while it bolstered the use of PEP by PEPCase instead of PK. Simultaneously, many phenylpropanoids showed a marked descent along the cold storage, in concordance with previous observations. In parallel, a higher synthesis of sucrose from organic acids takes place in HT epicarp, which highlights the role of sucrose–phosphate synthase in sugar metabolism upon HT condition.

Acknowledgements

This work was made possible by grants PICT 2011-01122 from ANPCyT and PIP 2159 from CONICET. ASM and VEP are Fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, and FEP is a member of the Investigator Career of the same Institution and Professor at the Univeridad Nacional de Rosario.

References

Ballester, A.R., Izquierdo, A., Lafuente, M.T., González-Candelas, L., 2010. Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in citrus fruit. Postharvest Biol. Technol. 56, 31–38.

Ballester, A.R., Lafuente, M.T., de Vos, R.C.H., Bovy, A.G., González-Candelas, L., 2013. Citrus phenylpropanoids and defence against pathogens. Part I: metabolic profiling in elicited fruits. Food Chem. 136, 178–185.

Berger, S., Sinha, A.K., Roitsch, T., 2007. Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. J. Exp. Bot. 58, 4019–4026.

Bollag, D.M., Edelstein, S.J., 1991. Protein Methods. Wiley-Liss Inc., New York.

Bustos, D.M., Bustamante, C.A., Iglesias, A.A., 2008. Involvement of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in response to oxidative stress. J. Plant Physiol. 165, 456–461.

Cocco, M., Vázquez, D.E., Albors, A., Cháfer, M., Meier, G.E., Bello, F., 2008. Combinación de tratamientos térmicos y bicarbonato de sodio para el control de Penicillium digitatum en frutos cítricos. Rev. Iberoam. Tecnol. Postcos. 9, 55–62.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S.S., Wang, L.J., 2002. The phenylpropanoid pathway and plant defence – a genomics perspective. Mol. Plant Pathol. 3, 371–390.

Falcone Ferreyra, M.L., Perotti, V., Figueroa, C.M., Garrán, S., Anderson, P.C., Vázquez, D., Iglesias, A.A., Podestá, F.E., 2006. Carbohydrate metabolism and fruit quality are affected in frost-exposed Valencia orange fruit. Physiol. Plant. 128, 224–236.

Ghasernnezhad, M., Marsh, K., Shilton, R., Babalar, M., Woolf, A., 2008. Effect of hot water treatments on chilling injury and heat damage in 'satsuma' mandarins: antioxidant enzymes and vacuolar ATPase, and pyrophosphatase. Postharvest Biol. Technol. 48, 364–371.

Katz, E., Fon, M., Lee, Y., Phinney, B., Sadka, A., Blumwald, E., 2007. The citrus fruit proteome: insights into citrus fruit metabolism. Planta 226, 989–1005.

Koch, K.E., 1996. Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 509–540.

Lafuente, M.T., Ballester, A.R., Calejero, J., Zacarías, L., González-Candelas, L., 2011. Effect of heat-conditioning treatments on quality and phenolic composition of cold stored 'Fortune' mandarins. Food Chem. 128, 1080–1086.

Lara, M.V., Borsani, J., Budde, C.O., Lauxmann, M.A., Lombardo, V.A., Murray, R., Andreo, C.S., Drincovich, M.F., 2009. Biochemical and proteomic analysis of 'Dixiland' peach fruit (*Prunus persica*) upon heat treatment. J. Exp. Bot. 60, 4315–4333.

Lara, M.V., Drincovich, M.F., Müller, G.L., Maurino, V.G., Andreo, C.S., 2005. NADP-malic enzyme and Hsp70: co-purification of both proteins and modification of NADP-malic enzyme properties by association with Hsp70. Plant Cell Physiol. 46, 997–1006.

Lurie, S., 1998. Postharvest heat treatments. Postharvest Biol. Technol. 14, 257–269.

Miyashita, Y., Dolferus, R., Ismond, K.P., Good, A.G., 2007. Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. Plant J. 49, 1108–1121.

Muccilli, V., Licciardello, C., Fontanini, D., Russo, M.P., Cunsolo, V., Saletti, R., Reforgiato Recupero, G., Foti, S., 2009. Proteome analysis of *Citrus sinensis* L. (Osbeck) flesh at ripening time. J. Proteomics 73, 134–152.

Muench, D.G., Christopher, M.E., Good, A.G., 1998. Cloning and expression of a hypoxic and nitrogen inducible maize alanine aminotransferase gene. Physiol. Plant. 103, 503–512.

Nagano, M., Hachiya, A., Ashihara, H., 1994. Phosphate starvation and a glycolytic bypass catalyzed by phosphoenolpyruvate carboxylase in suspension cultured *Catharanthus roseus* cells. Z. Naturforsch. 49, 742–750.

Nielsen, T.H., Skjærbæ, H.C., Karlsen, P., 1991. Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum*) plants. Physiol. Plant. 82, 311–319.

Palou, L., 2013. Mini-review: heat treatments for the control of citrus postharvest green mold caused by *Penicillium digitatum*. In: Méndez-Vilas, A. (Ed.), Microbial Pathogens and Strategies for Combating them: Science, Technology and Education. Formatex, Badajoz, Spain, pp. 508–514.

Pan, Z., Guan, R., Zhu, S., Deng, X., 2009. Proteomic analysis of somatic embryogenesis in Valencia sweet orange (*Citrus sinensis* Osbeck). Plant Cell Rep. 28, 281–289.

Paull, R.E., 1990. Postharvest heat treatments and fruit ripening. Postharvest News Inf. 1, 355–363.

Pego, J.V., Kortstee, A.J., Huijser, C., Smeekens, S.C.M., 2000. Photosynthesis, sugars and the regulation of gene expression. J. Exp. Bot. 51, 407–416.

Penefsky, H.S., 1977. Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. J. Biol. Chem. 252, 2891–2899.

Perotti, V.E., Del Vecchio, H.A., Sansevich, A., Meier, G., Bello, F., Cocco, M., Garrán, S.M., Anderson, C., Vázquez, D., Podestá, F.E., 2011. Proteomic, metabalomic, and biochemical analysis of heat treated Valencia oranges during storage. Postharvest Biol. Technol. 62, 97–114.

Piattoni, C., Guerrero, S., Iglesias, A., 2013. A differential redox regulation of the pathways metabolizing glyceraldehyde-3-phosphate tunes the production of reducing power in the cytosol of plant cells. Int. J. Mol. Sci. 14, 8073–8092.

Plaxton, W.C., Podestá, F.E., 2006. The functional organization and control of plant respiration. Crit. Rev. Plant Sci. 25, 159–198.

Podestá, F.E., Plaxton, W.C., 1994. Regulation of carbon metabolism in germinating *Ricinus communis* cotyledons, I. Developmental profiles for the activity, concentration, and molecular structure of pyrophosphate- and ATP-dependent phopshofructokinases, phosphoenolpyruvate carboxylase, and pyruvate kinase. Planta 194, 374–380.

Polenta, G.A., Calvete, J.J., González, C., 2007. Isolation and characterization of the main small heat shock proteins induced in tomato pericarp by thermal treatment. FEBS J. 274, 6447–6455.

Sapitnitskaya, M., Maul, P., McCollum, G.T., Guy, C.L., Weiss, B., Samach, A., Porat, R., 2006. Postharvest heat and conditioning treatments activate different molecular responses and reduce chilling injuries in grapefruit. J. Exp. Bot. 57, 2943–2953.

Schirra, M., D'Aquino, S., Cabras, P., Angioni, A., 2011. Control of postharvest diseases of fruit by heat and fungicides: efficacy, residue levels, and residue persistence. J. Agric. Food Chem. 59, 8531–8542.

Schwender, J., Ohlrogge, J., Shachar-Hill, Y., 2004. Understanding flux in plant metabolic networks. Curr. Opin. Plant Biol. 7, 309–317.

Yan, S.-P., Zhang, Q.-Y., Tang, Z.-C., Su, W.-A., Sun, W.-N., 2006. Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol. Cell. Proteomics 5, 484–496.

Yun, Z., Gao, H., Liu, P., Liu, S., Luo, T., Jin, S., Xu, Q., Xu, J., Cheng, Y., Deng, X., 2013. Comparative proteomic and metabolomic profiling of citrus fruit with enhancement of disease resistance by postharvest heat treatment. BMC Plant Biol. 13, 44.

Zhang, J.J., Wang, X., Yu, O., Tang, J.J., Gu, X.G., Wan, X.C., Fang, C.B., 2010. Metabolic profiling of strawberry (Fragariaxananassa Duch.) during fruit development and maturation. J. Exp. Bot. 62, 1103–1118.