Anthocyanin biosynthesis and accumulation in blood oranges during postharvest storage at different low temperatures

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Abstract

Blood oranges require low temperature for anthocyanin production. We have investigated the activation of anthocyanin biosynthesis and accumulation in the pulp of Moro blood and Pera blond oranges (*Citrus sinensis* L. Osbeck) stored at either 4 or 9 °C after harvesting. Both temperatures stimulated anthocyanin accumulation in blood but not in blond oranges. Nonetheless, blood orange fruits stored at 9 °C reached a darker purple coloration, higher anthocyanin contents and enhanced upregulation of genes from the flavonoid pathway in the pulp and juice than those kept at 4 °C. Our results indicated that dihydroflavonol channeling toward anthocyanin production was boosted during the storage at 9 °C compared to 4 °C, providing more leucoanthocyanidins to enzymes downstream in the pathway. Finally, despite both low temperatures stimulated the expression of key transcription factors likely regulating the pathway, their expression profiles could not explain the differences observed at 9 and 4 °C.

Keywords: Blood oranges; Anthocyanin biosynthesis; Cold temperature storage; Citrus

1 Introduction

Anthocyanins are polyphenolic compounds rendering a full spectrum of red to black colors to many fruits, flowers and vegetables. These pigments provide not only an aesthetic appeal, but they have also other physiological roles in plants, such as protecting them against pathogens and abiotic stress conditions (Zhang, Butelli, & Martin, 2014). Additionally, they confer phytonutritional traits to the fruits, including therapeutic properties beneficial to human health. For example, several studies report that anthocyanins intake prevents the onset and development of certain degenerative diseases, such as some types of cancer, type II diabetes or retinal degeneration (Martin, Butelli, Petroni, & Tonelli, 2011).

The anthocyanin biosynthesis pathway has been completely elucidated (Holton & Cornish, 1995). In recent years, research has been addressed to understand the molecular regulation of anthocyanin production in citrus fruits, and the genes encoding most enzymes of the pathway have been identified and their expression analyzed (Jaakola, 2013). Anthocyanins are biosynthesized via phenylalanine ammonia-lyase (PAL, which eliminates the ammonia group from the precursor phenylalanine) in the general phenylpropanoid pathway, which shares the same upstream regulation with flavonols synthesis until dihydroflavonols (DHF) formation (Fig. 1). Initial steps are controlled by the genes cinnamate 4-hydroxylase (*C4H*) and 4-hydroxy-cynnamoyl CoA ligase (*4CL*) involved in coumaric acid and coumaroyl formation, respectively, and chalcone synthase (*CHS*), chalcone isomerize (*CHI*) and flavonone 3-hydroxylase (*F3H*) catalyzing the initial steps of anthocyanins production to generate naringenin, which undergo different hydroxylations catalyzed by flavonoid 3′-hydroxylase (F3′H) and/or flavonoid 3′5′-hydroxylase (F3′5′H) to produce DHF. Then, the pathway branches to synthesis of flavonols and to DHF reduction to leucoanthocyanidins, reactions catalyzed by flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR), respectively. By catalysis of anthocyanin synthase (ANS),

the colorless leucoanthocyanidins are oxidized to anthocyanidins, which are glycosylated immediately by UDP-glucose-flavonoid 3-*O*-glucosyltransferase (UFGT) to form anthocyanins. Vacuolar anthocyanin import is facilitated by glutathione-S-transferases (GST) (Fig. 1).

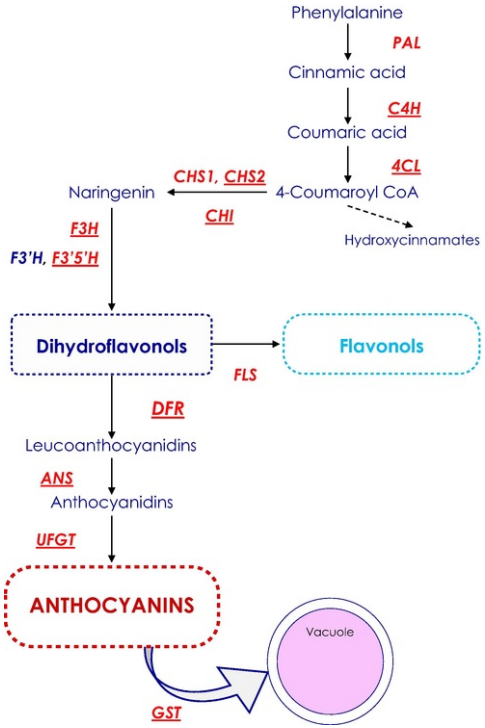

Fig. 1 A schematic representation of the flavonoid biosynthetic pathway leading to anthocyanins. Red named genes are those studied in this work and the underlined ones indicate genes higher upregulated during storage at 9 °C than at 4 °C. Gene names are abbreviated as follows: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-hydroxy-cynnamoyl CoA ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone 3-hydroxylase; *F3′H*, flavonoid 3′-hydroxylase; *F3′5′H*, flavonoid 3′5′-hydroxylase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *UFGT*, uridine diphosphate-glucose:flavonoid 3-*O*-glucosyltransferase and *GST*, glutathione-S-transferase.

Blood oranges (*Citrus sinensis* L. Osbeck) are the only commercial citrus fruits containing anthocyanins, responsible for their purple color. Different authors have described the beneficial effects of blood orange juice consumption for insulin sensitivity enhancement, reducing oxidative stress, protecting DNA against oxidative damage and decreasing cardiovascular risk factors (Bonina et al., 2002; Guarnieri, Riso, & Porrini, 2007). These effects have been associated with the antioxidant abilities of purple juices, which are related to their content in phenolic compounds, mainly anthocyanins (Rapisarda et al., 1999; Zou, Xi, Hu, Nie, & Zhou, 2016). Additionally, blood orange juice was more effective preventing fat accumulation than anthocyanins-enriched extracts, suggesting a synergistic effect of anthocyanins with other phytonutrients present in the orange juice (Titta et al., 2009).

Cyanidin 3-glucoside and cyanidin 3-(6′-malonylglucoside) are the two main anthocyanins identified in citrus though their content show seasonal oscillations among varieties (Fabroni, Ballistreri, Amenta, & Rapisarda, 2016). Moreover the content of anthocyanins in blood oranges differs greatly depending on multiple factors. For example, pigment levels of blood oranges grown under similar conditions vary highly depending on genotype, from the highest pigmentation of Moro orange fruits, to the lower pigmentation of Maltese orange fruits (Butelli et al., 2012; Lo Piero, 2015). Besides cultivar type, maturity and environmental growth conditions also influence anthocyanin accumulation profiles (Butelli et al., 2012; Lo Piero, 2015). In any case, anthocyanin content in blood oranges is low temperature-dependent (Butelli et al., 2012; Lo Piero, Puglisi, Rapisarda, & Petrone, 2005), being essential a wide day/night temperature range to activate its biosynthesis and get a deep purple color in mature fruits (Butelli et al., 2012). This limits reliable commercial production geographically to only a few citrus regions in the world, where anthocyanins accumulate consistently during winter (Butelli et al., 2012; Crifò, Petrone, Lo Cicero, & Lo Piero, 2012; Lo Piero et al., 2005). Cultivation of blood oranges under tropical/subtropical climates, as those of Brazil or Florida, yields blood orange fruit with very low or lack of purple coloration.

It has been widely shown that anthocyanin content in blood orange fruits may increase drastically through cold postharvest storage for a few weeks (Lo Piero et al., 2005; Rapisarda, Bellomo, & Intelisano, 2001). Refrigerated

storage is a commonly used practice to extend the commercial life of fresh citrus fruits, and it is required as a quarantine treatment for citrus export to different countries (El-Otmani, Ait-Oubahou, & Zacarías, 2011). However, blood orange storage at temperatures below 8 °C may cause chilling injuries and alteration in their organoleptic properties (Rapisarda et al., 2001). Nevertheless, storage periods of 15 to 77 days at 4 °C have been proposed as the best option to ensure purple color in Tarocco blood orange without compromising fruit quality (Crifò et al., 2012; Lo Piero et al., 2005). The effect of storage at low-moderate temperatures (8–10 °C) has been studied in several blood orange varieties for which a maximum anthocyanins content was obtained after 45 days of storage (Rapisarda et al., 2001).

Anthocyanin production is transcriptionally controlled by the MBD (MYB-bHLH-WD40) transcription factors complex. In sweet orange, Butelli et al. (2012) identified a R2R3-MYB protein that acted as a key positive regulator of anthocyanin biosynthesis. This transcription factor (*Ruby*) is overexpressed upon cold dependent activation of its LTR controlling retroelement thus triggering anthocyanin production in blood oranges. Anthocyanin accumulation is accomplished by upregulation of the main genes involved in their biosynthesis, such as *PAL*, *CHS*, *DFR*, *ANS*, *UFGT* and *GST* (Crifò et al., 2012; Lo Piero et al., 2005). Concurrent with the improvement of anthocyanin contents in Moro and Tarocco blood oranges stored at 4 °C, the transcript levels of *Ruby* and its LTR controlling retroelement increased (Butelli et al., 2012). Other transcription factors have been proposed to be involved in controlling anthocyanin biosynthesis in citrus, such as a NAC-like protein. *NAC* expression increased specifically in cold-exposed Tarocco blood oranges (Crifò et al., 2012; Lo Piero et al., 2005). Recently, RNAi suppression assays in citrus callus indicated that the R2R3-MYB transcription factor MYBF1 controls flavonol and hydroxycinnamic acid biosynthesis by activation of *CHS1* or *FLS* (Liu et al., 2016). Collectively, these results indicate that anthocyanin accumulation is induced during cold storage as result of increased expression of key biosynthetic genes controlled by relevant transcription factors.

Although the need of cold induction for anthocyanin accumulation in blood oranges is widely known, the influence of storage at different low temperatures on anthocyanin titers remains unclear. Enhancement of purple pigments content in blood oranges through proper cold postharvest storage and upregulation of responsible genes are explored here to assess whether it might be further exploited by citrus industries, more specifically in those countries where anthocyanin levels in blood orange varieties are lacking or limited due to constraining environmental conditions.

2 Materials and methods

2.1 Plant materials and storage conditions

Moro blood sweet orange (*C. sinensis* L. Osbeck) was chosen because the pulp of mature fruits displays a bright-purple color thus being suitable for anthocyanin accumulation studies. A blond sweet orange type which matures at the same time than Moro was used as control (*C. sinensis* L. Osbeck cv. Pera). Mature fruits were harvested at random from adult trees grown under standard conditions in three different commercial orchards in Sao Paulo State (Brazil): Paranapanema (Pa) (23°45′03.59″ S; 48°50′37.52″ O), Pardinho (Pr) (23°04′48.15″ S; 48°22′32.21″ O) and Maringá (Ma) (21°38′18.86″ S; 48°29′21.56″ O), in July of 2015 and 2016. Climatological conditions for every area were monitored (Fig. 1S). Fruits were uniform in size and color, and free of damage or external defects. Fruits were divided in two batches and stored for 45 days at either 9 °C or 4 °C, and 90–95% RH in constant darkness. At the time of harvest (1–5 h from collection time) and after 15, 30 and 45 days of storage, samples of pulp and juice were taken and stored at −80 °C until analysis. Pulp was separated with a scalpel, immediately frozen in liquid nitrogen and ground to a fine powder. Juice was extracted with a domestic squeezer and filtered through a metal sieve with a pore size of 0.8 mm. For all analyses, 3 replicate samples of 5 fruits each per temperature and storage time were used.

2.2 Determination of color and internal maturity index

Juice color was analyzed by spectrophotometric parameters as described by Kelebek, Canbas, and Selli (2008). Juice acidity was determined by titration with phenolphthalein and 0.1 N NaOH and was expressed as mg citric acid per 100 mL. The soluble solids content (°Brix) was estimated by refractometry, using an Atago® refractometer. The maturity index is expressed as the ratio of °Brix/acidity. The pH value was measured with a pH-meter Gehaka®.

2.3 Anthocyanins quantification

Juice samples were centrifuged at 5.000 rpm during 10 min and anthocyanins measured with a colorimetric kit (Flex-Reagent™, Initech Scientific, USA) following manufacturer instructions.

2.4 Total RNA isolation

Plant material used for total RNA isolation was the same as that used for anthocyanin analysis. Total RNA extraction was performed as described previously (Carmona, Zacarías, & Rodrigo, 2012).

2.5 Quantitative RT-PCR analysis

Total RNA was treated with DNase (Ambion®, Thermo Fisher Scientific, USA), accurately quantified by Nano Drop measurement (Thermo Fisher Scientific, USA) and used for cDNA synthesis (Invitrogen, Thermo Fisher Scientific, USA). Quantitative real-time was performed with a StepOne Plus Real Time PCR System (Applied Biosystem, USA) and data was analyzed using StepOne Software version 2.3. RT-PCR was carried out on 50 ng total cDNA adding 6 µL of SYBR Green PCR Master Mix (Applied Biosystems, USA) and 0.3 µM of gene specific primers in a total volume of 12 µL. The primers used for *MYBF1*, *C4H*, *4CL*, and *CHI* were those described by Liu et al. (2016). For *DFR*,

ANS, *UFGT* and *GST*, primers used were those described by Crifò et al. (2012). Primer pairs for *NAC*, *CHS1*, *CHS2*, *F3H*, *F3′5′H* and *FLS* were designed based on citrus coding sequences from *Citrus clementina* database available in the Plant Comparative Genomics portal (Phytozome v11.0). Primer sequences for each gene are detailed in Table 1S.

The RT-PCR procedure consisted of 95 °C 10 min followed by 40 cycles at 95 °C 15 s and 60 °C 40 s. Fluorescence intensity data were acquired during the 60 °C extension step and specificity of the reactions was checked by post-amplification dissociation curves. To evaluate the efficiency of each pair of primers and the dynamic range of all the genes analyzed, the variation of ΔCT was monitored by using a10-fold dilution series of a mix of cDNA samples from different tissues as a standard curve. The efficiency was found to be very similar for each pair of primers. To demonstrate the expression stability of the reference genes glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*) and ADP-Ribosylation factor A1F (*ADP*) under our experimental conditions, the algorithm geNorm was used (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al., 2002). The relative expression between cold-treated and control samples (zero time of Pera orange fruits) was determined by the method described by Livak and Schmittgen (2001). Values are presented as the mean of at least three independent analyses. Statistical analyses were performed using ANOVA.

3 Results

3.1 Blood orange pulp and juice appearance at different low temperature storages

To determine the effect of postharvest storage on fruit color at different low temperatures, Moro (blood type) and Pera (blond type) mature oranges, harvested from different areas to minimize the effect of natural environmental conditions, were kept at either 9 or 4 °C during 45 days. The visual aspect of pulp and juice of fruits stored at both temperatures was evaluated every 15 days (Figs. 2and 2S). Pulp and juice purple color of blood oranges was enhanced along cold storage, being this effect more noteworthy at 9 than at 4 °C, while in blond orange, as expected, no purple color was observed The maturity index remained constant from the harvest moment during the whole storage period and no differences were observed neither between fruits stored at 9 and 4 °C nor between cultivars (Fig. 3S). Similar results were obtained for pH values in Moro juices (Fig. 4S).

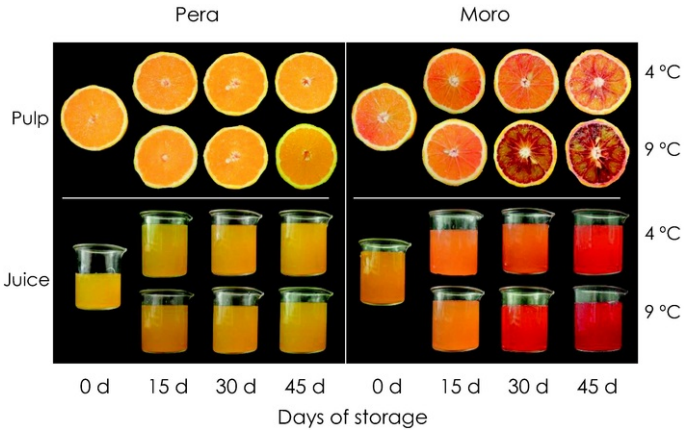

Fig. 2 Internal appearance of pulp (up) and juices (down) of Pera (left) and Moro (right) oranges from Paranapanema (Sao Paulo, Brazil) during storage at either 9 or 4 °C for 0, 15, 30 and 45 days.

3.2 Blood orange juice purple color development and anthocyanin accumulation at different low temperature storages

Progression of Moro juice purple color along storage was analyzed by monitoring color intensity (CI) and the yellow, red and blue proportions (Figs. 3 and 5S). In all the experiments, CI increased during all storage periods, being this effect notably higher in fruits kept at 9 °C. At the end of the storage period, CI augmented 2.6–4.2-fold in juice from fruits stored at 9 °C, while it only raised 1.7–2.2 times in those kept at the lower temperature. Red proportion progress displayed a similar profile to that of CI under both temperatures, being its increase also higher at 9 °C than at 4 °C (Fig. 3A and B). In contrast, yellow and blue color proportions did not show significant differences in any of the three replicates (Fig. 5S). The effect of storage temperature on juice anthocyanin content was also studied. At harvest, juices from Pa, Ma and Pr fruits had 72.4, 4.6 and 2.3 mg/L of anthocyanins, respectively. Accumulation of these pigments increased to 196.4, 60.0 and 33.5 mg/L in juice from fruits kept at 9 °C and to 124.5, 20.1 and 21.9 mg/L in juice from those fruits stored at 4 °C, in Pa, Ma and Pr, respectively (Fig. 3C). The final values of CI, red proportion and anthocyanin content were higher as deeper purple colored were the fruits at the harvest moment (Fig. 3), which was attributable to the environmental and growth conditions at the three locations investigated. Despite this, fruits from Pa, with the highest anthocyanin content and CI final values, displayed the lowest relative increment noticed in both parameters at both storage temperatures, 2.7–1.7-fold in anthocyanin contents and 2.6–1.6-

fold in CI values at 9 °C and 4 °C, respectively. On the contrary, the highest relative increase for both variables was observed in fruits from Pr, 14.6–9.5-fold in anthocyanin contents and 4.2–2.2-fold in CI at 9 °C and 4 °C, respectively, with the lowest absolute values reached at the end of the storage period (Fig. 3).

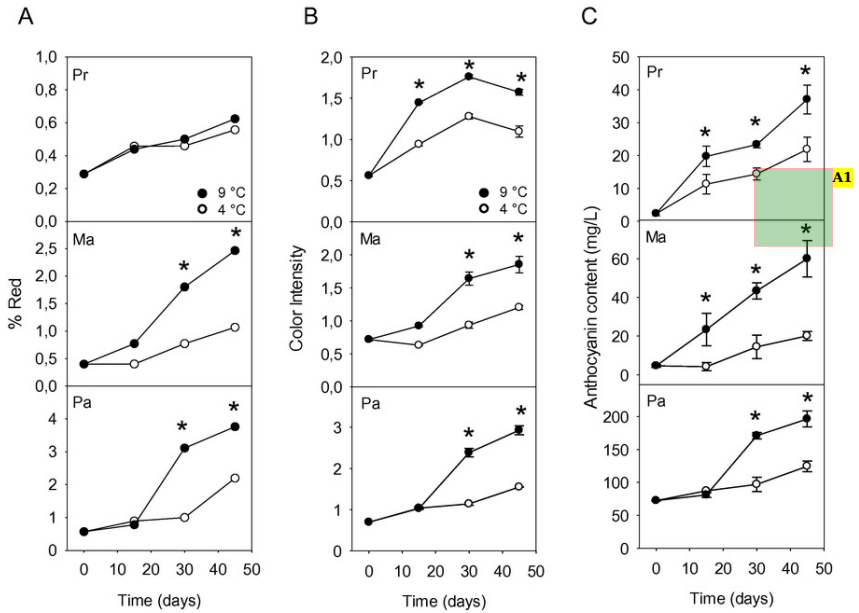

Fig. 3 Changes in % Red proportion (A), color intensity (B) and total anthocyanins content (C) in juice of Moro blood oranges from Pardinho (Pr), Maringá (Ma) and Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (●) or 4 °C (○) for 0, 15, 30 and 45 days. Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk indicates significantly different values ($P \leq 0.01$) for a given time.

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3.3 Gene expression profile differences between blond and blood oranges stored at different low temperatures

Expression profile of most genes involved in anthocyanin biosynthesis and three key transcription factors was analyzed along storage in pulp of both blood Moro and blond Pera oranges. Expression of biosynthetic genes (*PAL*, *C4H*, *4CL*, *CHS*s, *CHI*, *F3H*, *F3′5′H*, *FLS*, *DFR*, *ANS* and *UFGT*) increased in pulp of both varieties because of cold-temperature storage (Fig. 4; Figs. 7S and 8S). However, with the exceptions of *C4H* and *FLS*, upregulation induced by low temperature storage was considerably higher in blood than in blond orange. Conversely, the transcription factors *NAC* and *MYBF1*, which belong to two different families (Fig. 6S), showed similar or even higher expression levels in blond than in blood oranges, while that of *Ruby* was much higher in Moro because its expression was almost undetectable in Pera orange. It should be emphasized that several genes of the pathway such as *CHSs*, *DFR* and *GST*, displayed much boosted upregulation in blood than in blond oranges (Fig. 4; Figs. 7S and 8S).

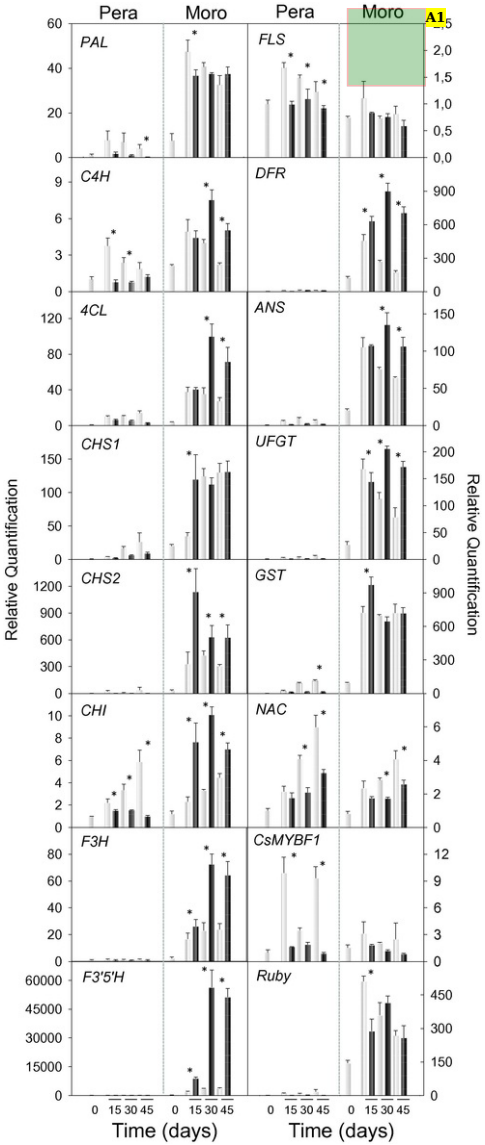

Fig. 4 Relative quantification of expression of *PAL, C4H, 4CL, CHSs, CHI, F3′H, F3′5′H, FLS, DFR, ANS, UFGT, GST* and the transcription factor genes *NAC, MYBF1* and *Ruby* in the pulp of Pera and Moro oranges from Maringá (Ma) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0, 15, 30 and 45 days. Data are presented as the mean relative expression ± SD of each individual sample as compared to the control sample (zero time of Pera oranges). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $P \leq 0.01$ (*).

Annotations:

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Comparison of transcript accumulation levels between postharvest storage at 9 and 4 °C revealed important differences for the pulp of Moro fruits. Although gene expression was enhanced at both temperatures, upregulation

of most genes of the pathway was much higher at 9 °C (Fig. 4; Figs. 7S and 8S). Among all genes analyzed, the initial gene *PAL* was the only one showing no consistent differences in expression between both temperatures (Fig. 4; Figs. 7S and 8S). In contrast, *C4H* and *4CL* transcripts as well as those of the initial genes *CHI*, *F3H* and *F3′5′H*, were in general higher at 9 °C from day 15 to 45, reaching at the end of the experiment values 1.5–2.6-fold (*CHI*), 1.6–2.7-fold (*F3H*) and 1.9–13.8-fold (*F3′5′H*) higher than those got at 4 °C (Fig. 4; Figs. 7S and 8S). In the case of *CHSs*, both genes were upregulated initially to a higher extent at 9 than at 4 °C although differences fade in the case of *CHS1* as storage time went by (Fig. 4; Figs. 7S and 8S). *FLS*, involved in the pathway bifurcation to flavonols, did not show important changes in expression along the experiments, while *DFR*, *ANS* and *UFGT*, involved in anthocyanin biosynthesis, were clearly boosted by low temperature storage. *DFR* upregulation was more noteworthy at 9 than at 4 °C at all postharvest storage times and at the three locations investigated, ranging between 1.4–2.5-fold higher at 15 days and 1.5–4.1-fold higher at 45 days of storage (Fig. 4; Figs. 7S and 8S). *ANS* and *UFGT* induction was generally higher at 9 than at 4 °C in Ma and Pr fruits, but after 30 days of storage their expression levels decreased, reaching values lower than those achieved at 4 °C in the case of Pa fruits (Fig. 4; Figs. 7S and 8S). Induction of *GST* was also higher initially at 9 than at 4 °C, but differences declined to levels becoming similar at both storage temperatures by day 45. Regarding master transcription factor genes *NAC, MYBF1* and *Ruby*, expression levels were comparable at most postharvest storage times and locations analyzed, with the only exception of Pr, where Moro fruits displayed a higher (1.3–1.9-fold) upregulation of *Ruby* at 9 than 4 °C (Fig. 4; Figs. 7S and 8S).

4 Discussion

In the last few years, the interest in blood oranges has increased because of their anthocyanin-related health-promoting properties. Being orange juices widely considered as healthy beverages due to their high content of vitamin C, flavonoids, carotenoids and micronutrients (Alharbi et al., 2016; Zou et al., 2016), anthocyanin accumulation of blood oranges may represent a surplus to further enhance orange juice attributes and broaden its organoleptic appeals. Nonetheless, there is not an ample global market for purple orange juices due to their unreliable blood color development associated to anthocyanin accumulation which depends largely on environmental conditions in the orchards. In fact, optimal purple coloration in blood oranges is only achieved when anthocyanin biosynthesis is induced by low temperatures (Butelli et al., 2012; Lo Piero et al., 2005), thus limiting commercial production to very few regions worldwide (Zarba & Pulvirenti, 2006). Despite the increasing demand of blood orange juices, the warm climates of the two world major regions for orange juice production (Florida, USA and Sao Paulo, Brazil) is preventing their accomplishment. It is known that anthocyanin accumulation can be promoted by keeping blood oranges at temperatures between 4- and 10 °C after harvesting (Butelli et al., 2012; Lo Piero et al., 2005; Rapisarda et al., 2001). Therefore, low temperature postharvest storage could be a good option to improve the purple coloration in their juices. However, there are no studies on the effect of storage at different low temperatures on anthocyanins accumulation. This is the first work in which the efficiency of fruit storage at two different low temperatures (below 10 °C) for enhancing purple color and anthocyanin accumulation was compared. Additionally, expression of relevant genes of the pathway was analyzed to gain insight into the cold-dependent regulation of anthocyanin biosynthesis in blood oranges.

Moro orange fruits stored at low temperature displayed an enhancement in the pulp and juice coloration, showing darker purple color at 9 than at 4 °C (Figs. 2 and 2S). These visual results were associated to a higher increase of both Red proportion and CI values at 9 °C (Fig. 3A and B). Purple coloration improvement was not due to changes in pH, which could affect anthocyanin colors (Zhang et al., 2014), because pH values did not vary along the storage period at any temperature (Fig. 4S). Instead, deeper purple color obtained at 9 °C could be explained by higher anthocyanin contents, which mostly paralleled CI increases (Fig. 3). The direct relationship between coloration and anthocyanin content has been described previously for different blood orange cultivars (Kelebek et al., 2008; Rapisarda et al., 2001).

In all cases our results showed that anthocyanin accumulation was increased progressively during the period of postharvest cold induction and that, at least in fruits from Pr and Ma, it was evident as soon as by day 15 of storage (Fig. 3C). At the end of the experiment, anthocyanin content in pulp was increased, in relation to the harvest time, for the three orchards investigated. Fruits from Pa showed the lowest relative increase in anthocyanin content, though final values were the highest, being the onset of cold-induced anthocyanin accumulation delayed in this case. Induction of purple pigment accumulation by cold storage was also noticed from 30- to 40 days of storage in other works (Lo Piero et al., 2005; Rapisarda et al., 2001). In one of these cases, initial pigment content was also high (about 240 mg/L) and increases reported were less than 1.2-fold at the end of the 45-day storage period (Rapisarda et al., 2001). In contrast, Moro fruits with less than 1 mg of anthocyanins per 100 g of fresh weight tissue increased their content by 5 times when stored at 4 °C during 45 days (Lo Piero et al., 2005). Collectively, these and our results suggest that postharvest cold-induced anthocyanin production depends on the initial anthocyanin content at the onset of storage, being the effect of cold storage relatively higher when natural anthocyanin accumulation in the blood orange fruits at the orchard were lower (or inexistent). Anthocyanins content and gene expression at harvest differed between the three locations, but these variations could not be attributed to global climatological conditions (Fig. 1S). Other environmental conditions may influence anthocyanin accumulation profiles such as nutrients, UV light, rootstock, soil pH, etc (Lo Piero, 2015). Further studies are required to unequivocally establish preharvest factors affecting anthocyanin content and the correlation between anthocyanin production at harvesting and accumulation after storage (Bernardi et al., 2010; Cotroneo, Russo, Ciuni, Recupero, & Lo Piero, 2006; Moriguchi, Kita, Tomono, Endo-Inagaki, & Omura, 2001), but our results suggest that higher initial anthocyanin content led to higher final anthocyanin concentration after storage and, consequently, to a deeper purple color development, being this effect much higher at 9 than at 4 °C. Moreover, it is remarkable that even poor colored blood oranges at the harvest moment could increase their anthocyanin content more than 10-fold by storing them at 9 °C, thus displaying a deep purple coloration in both pulp and juice. By storing fruits just for 45 days at 8–9 °C maturation index was not altered (Rapisarda et al., 2001) and undesired compounds leading to distasteful taste were not been produced yet (Rapisarda et al., 2001) so their juices would probably be highly appreciated by consumers.

To characterize the effect of storage at two different low temperatures on the anthocyanin biosynthesis pathway and to assess whether any changes were related to the effects observed on anthocyanin concentrations, the transcript levels of eight flavonoid biosynthesis initial genes (*PAL*, *C4H*, *4CL*, *CHS1*, *CHS2*, *CHI*, *F3H* and *F3′5′H*), the first gene involved in flavonols production (*FLS*), three anthocyanin biosynthesis structural genes (*DFR*, *ANS* and *UFGT*), a *GTS* gene and three transcription factors related with flavonoid biosynthesis regulation (*Ruby*, *MYBF1* and *NAC*) were analyzed in the pulp of Moro and Pera oranges stored at either 9 or 4 °C. Expression levels of most genes involved in the phenylpropanoid pathway (*PAL*, *C4H*, *4CL*, *CHS1*, *CHI* and *F3′5′H*) were induced by storage at low temperatures, and all but *C4H* to a higher level in the blood type than in the blond one. This may be likely related with the activation of the phenylpropanoid metabolism that occurs in citrus fruits as response to cold temperature stress (Lafuente, Sala, & Zacarias, 2004). Upregulation of these genes in Moro fruits could be related to a major demand of precursor for both flavonol and anthocyanin production, as it has been proposed before for *PAL* (Lo Piero et al., 2005). Expression of anthocyanin biosynthetic genes (*DFR*, *ANS* and *UFGT*), *GST* and *Ruby* was highly induced by cold temperatures in blood oranges. Instead, only a tiny or much lower upregulation was noticed in Pera fruits for these genes. Different transcription levels of *PAL*, *CHS1*, *ANS*, *UFGT*, *GST*, *NAC* and *Ruby* in blond and blood oranges have been reported before (Butelli et al., 2012; Cotroneo et al., 2006; Licciardello, Russo, Vale, & Recupero, 2008). Conversely, our results showed that expression of *NAC* and *MYB1* transcription factors was induced by cold-temperature storage to the same order of magnitude in both fruit types. This result somehow contradicts the cold-induced upregulation of the *NAC* transcription factor gene at low temperature (4 °C) in blood but not in blond Navel fruits reported by Crifò et al. (2012).

A common regulatory network comprising MYB-bHLH-WD40 (MBD) transcription factors has been described as regulator of the phenylpropanoid biosynthesis pathway (Azuma et al., 2008; Rahim, Busatto, & Trainotti, 2014). Accumulating evidences indicate that spatial and temporal expression of phenylpropanoid pathway genes are regulated by R2R3-MYB transcription factors (Holton & Cornish, 1995). Among them, the *MYBF1* transcription factor has been recently described as a controller of flavonol and hydroxycinnamic acid biosynthesis by promoting expression of the initial genes *PAL*, *C4H*, *4CL*, *CHS1* and *FLS* (Liu et al., 2016). All these genes and *CHI* presented a slight upregulation during cold storage in Pera fruits, which could be related with the transitory increase in *MYBF1* transcript levels (Fig. 4). In contrast, *MYBF1* expression was only detected at the beginning of cold storage in Moro fruits, indicating that there may be a different mechanism of flavonol regulation in blond and blood oranges (Cotroneo et al., 2006; Licciardello et al., 2008).

In Moro oranges, the increase of anthocyanin content induced by low temperature storage (which was higher at 9 than at 4 °C) correlated quite well with an overall upregulation of most genes of the pathway, which was also higher at 9 than at 4 °C. At the initial steps, the highest expression was attained by *CHSs* and *F3′5′H* (Fig. 4), related to flavonoid biosynthesis in citrus (Moriguchi, Kita, Tomono, Endo-Inagaki, & Omura, 1999; Moriguchi et al., 2001). *CHS2* has been also pointed out as responsible for anthocyanins accumulation during the maturation process (Cotroneo et al., 2006). Despite this, as far as we know, only *CHS1* expression has been reported in studies on cold-induced anthocyanin biosynthesis in citrus (Crifò et al., 2012; Lo Piero et al., 2005). In agreement with these works, we have found increased *CHS1* expression during cold storage of blood oranges, though no consistent differences were found comparing 9 and 4 °C. Otherwise, *CHS2* expression profile exhibited a higher upregulation at 9 °C, correlating better than *CHS1* with anthocyanin accumulation patterns. These data suggested that *CHS2* could have a more specific role in anthocyanin production in blood oranges, while *CHS1* may have a more general role in the phenylpropanoid metabolism, as proposed by Liu et al. (2016).

The branching point to anthocyanin or flavonol synthesis, initiated by *DFR* and *FLS*, respectively, constitutes an important key regulatory step in different plants (Tian et al., 2015; Wang et al., 2013). In general, *FLS* expression level correlates with flavonol concentration (Tian et al., 2015), while *DFR* concentration has a decisive role on anthocyanin content (Luo et al., 2016; Tian et al., 2015). In Moro oranges kept at low temperatures, *FLS* transcript accumulation remained stable while that of *DFR* increased hugely (Fig. 4). Upregulation of *DFR* expression by storage at 4 °C has been reported before (Crifò et al., 2012; Lo Piero et al., 2005), but it is interesting to stress that induction was much higher with postharvest storage at 9 °C, showing a positive correlation with purple pigmentation and anthocyanin accumulation. Different transcript accumulation for *FLS* and *DFR* in blood orange fruits stored at 9 and 4 °C indicated that precursor substrates were channeled to anthocyanins production, especially in those fruits kept at 9 °C. Due to the competitive relationship between FLS and DFR for DHF in determining the metabolic flux to flavonol and/or anthocyanin biosynthesis in fruits (Tian et al., 2015; Wang et al., 2013) and flowers (Lim et al., 2016; Luo et al., 2016), the *DFR*/*FLS* ratio revealed the relative balance between anthocyanin and flavonol production (Luo et al., 2016; Tian et al., 2015). In Moro oranges, values for the *DFR*/*FLS* ratio were higher than one at all but one of the postharvest times analyzed, and it was much higher in fruits stored at 9 °C than in those kept at 4 °C (Fig. 5), which was correlated with anthocyanin accumulation at both temperatures. Ratio values supported that the flux of DHF was favored to anthocyanins production particularly at 9 °C. Concurrent with this, downstream biosynthetic genes *ANS* and *UFGT*, and *GST*, related with the mechanism of anthocyanin vacuolar import (Lo Piero, Mercurio, Puglisi, & Petrone, 2009; Lo Piero, Puglisi, & Petrone, 2006), also exhibited higher upregulation in fruits kept at 9 °C. Taken together, not only there was a major flux toward leucoanthocyanidins production at 9 °C, but also their conversion to anthocyanins as well as their transport was favored at the low-moderate temperature.

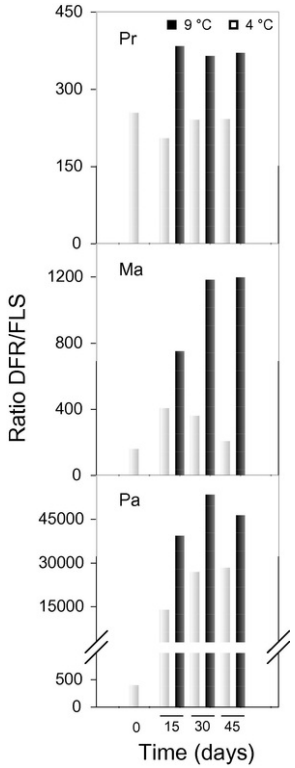

Fig. 5 Ratio of *DFR* versus *FLS* relative expression in the pulp of Moro blood oranges from Pardinho (Pr), Maringá (Ma) and Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0, 15, 30 and 45 days.

Anthocyanin content variations in different blood oranges, as well as the cold induction required for their synthesis, are governed by the R2R3-MYB transcription factor *Ruby* (Butelli et al., 2017, 2012). Accordingly, Moro oranges exhibited not only a higher basal *Ruby* expression at the onset of the experiment, but also a much higher induction during storage at low temperatures (Fig. 4). However, it is worth mentioning that the cold-regulation differences in expression exhibited by most of the initial and structural genes in blood oranges, with higher upregulation levels in fruits exposed to 9 than 4 °C, was not paralleled by *Ruby* expression profiles, which presented quite variable results in the replicates. Other transcription factor genes such as *NAC* and *MYBF1* showed higher upregulation when kept at 4 °C, indicating that higher accumulation of anthocyanin at 9 than at 4 °C was independent of the expression levels of the three master transcription factors investigated.

In conclusion, postharvest cold storage, a common practice to extend the shelf life and to keep the quality of citrus fruits (El-Otmani et al., 2011), could be a feasible practice in tropical countries to enhance anthocyanin accumulation of blood oranges, just by keeping the fruits at 9 °C for at least 15 or 30 days depending on their content at harvesting. The effect obtained will be higher the higher the anthocyanin content at the time of harvest. Gene expression analyses envisaged that cold-promoted induction of early genes from the phenylpropanoid pathway in blood oranges boosted DHF production making them available for additionally increasing the *DFR*/*FLS* ratio, which was much higher at 9 than at 4 °C of storage, directing the flux towards leucoanthocyanidins and then to anthocyanins, leading to a deeper purple fruit and juice pigmentation at the low-moderate temperature.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.05.076.

References

Alharbi M.H., Lamport D.J., Dodd G.F., Saunders C., Harkness L., Butler L.T. and Spencer J.P.E., Flavonoid-rich orange juice is associated with acute improvements in cognitive function in healthy middle-aged males, *European Journal of Nutrition* **55** (6), 2016, 2021–2029.

Azuma A., Kobayashi S., Mitani N., Shiraishi M., Yamada M., Ueno T., … Koshita Y., Genomic and genetic analysis of Myb-related genes that regulate anthocyanin biosynthesis in grape berry skin, *Theoretical and Applied Genetics* **117** (6), 2008, 1009–1019.

Bernardi J., Licciardello C., Patrizia Russo M., Luisa Chiusano M., Carletti G., Reforgiato-Recupero G. and Marocco A., Use of a custom array to study differentially expressed genes during blood orange (*Citrus sinensis* L. Osbeck) ripening, *Journal of Plant Physiology* **167** (4), 2010, 301–310.

Bonina F.P., Leotta C., Scalia G., Puglia C., Trombetta D., Tringali G., … Saija A., Evaluation of oxidative stress in diabetic patients after supplementation with a standardised red orange extract, *Diabetes, Nutrition & Metabolism* **15** (1), 2002, 14–19.

Butelli E., Garcia-Lor A., Licciardello C., Las Casas G., Hill L., Reforgiato-Recupero G., … Martin C., Changes in anthocyanin production during domestication of Citrus, *Plant Physiology* 2017, 01701.2016.

Butelli E., Licciardello C., Zhang Y., Liu J., Mackay S., Bailey P., … Martin C., Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges, *The Plant Cell* **24** (3), 2012, 1242–1255.

Carmona L., Zacarías L. and Rodrigo M.J., Stimulation of coloration and carotenoid biosynthesis during postharvest storage of “Navelina” orange fruit at 12 °C, *Postharvest Biology and Technology* **74**, 2012, 108–117.

Cotroneo P.S., Russo M.P., Ciuni M., Recupero G.R. and Lo Piero A.R., Quantitative real-time reverse transcriptase-PCR profiling of anthocyanin biosynthetic genes during orange fruit ripening, *Journal of the American Society for Horticultural Science* **131** (4), 2006, 537–543.

Crifò T., Petrone G., Lo Cicero L. and Lo Piero A.R., Short cold storage enhances the anthocyanin contents and level of transcripts related to their biosynthesis in blood oranges, *Journal of Agricultural and Food Chemistry* **60** (1), 2012, 476–481.

El-Otmani M., Ait-Oubahou A. and Zacarías L., Citrus spp.: Orange, mandarin, tangerine, clementine, grapefruit, pomelo, lemon and lime, In: *Postharvest Biology and Technology of Tropical and Subtropical Fruits,* 2011, 437–516e.

Fabroni S., Ballistreri G., Amenta M. and Rapisarda P., Anthocyanins in different Citrus species: An UHPLC–PDA-ESI/MS^n-assisted qualitative and quantitative investigation, *Journal of the Science of Food and Agriculture* **96** (14), 2016, 4797–4808.

Guarnieri S., Riso P. and Porrini M., Orange juice vs vitamin C: Effect on hydrogen peroxide-induced DNA damage in mononuclear blood cells, *The British Journal of Nutrition* **97** (4), 2007, 639–643.

Holton T.A. and Cornish E.C., Genetics and biochemistry of anthocyanin biosynthesis, *The Plant Cell* **7** (7), 1995, 1071–1083.

Jaakola L., New insights into the regulation of anthocyanin biosynthesis in fruits, *Trends in Plant Science* **18** (9), 2013, 477–483.

Kelebek H., Canbas A. and Selli S., Determination of phenolic composition and antioxidant capacity of blood orange juices obtained from cvs. Moro and Sanguinello (*Citrus sinensis* (L.) Osbeck) grown in Turkey, *Food Chemistry* **107**, 2008, 1710–1716.

Lafuente M.T., Sala J.M. and Zacarias L., Active oxygen detoxifying enzymes and phenylalanine ammonia-lyase in the ethylene-induced chilling tolerance in Citrus fruit, *Journal of Agricultural and Food Chemistry* **52** (11), 2004, 3606–3611.

Licciardello C., Russo M.P., Vale G. and Recupero R.G., Identification of differentially expressed genes in the flesh of blood and common oranges, *Tree Genetics & Genomes* **4** (2), 2008, 315–331.

Lim S.-H., You M.-K., Kim D.-H., Kim J.K., Lee J.-Y. and Ha S.-H., RNAi-mediated suppression of dihydroflavonol 4-reductase in tobacco allows fine-tuning of flower color and flux through the flavonoid biosynthetic pathway, *Plant Physiology and Biochemistry* **109**, 2016, 482–490.

Liu C., Long J., Zhu K., Liu L., Yang W., Zhang H., … Deng X., Characterization of a Citrus R2R3-MYB transcription factor that regulates the flavonol and hydroxycinnamic acid biosynthesis, *Scientific Reports* **6**, 2016, 25352.

Livak K.J. and Schmittgen T.D., Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method, *Methods* **25**, 2001, 402–408.

Lo Piero A.R., The state of the art in biosynthesis of anthocyanins and its regulation in pigmented sweet oranges (*Citrus sinensis* L. Osbeck), *Journal of Agricultural and Food Chemistry* **63** (16), 2015, 4031–4041.

Lo Piero A.R., Mercurio V., Puglisi I. and Petrone G., Gene isolation and expression analysis of two distinct sweet orange (*Citrus sinensis* L. Osbeck) tau-type glutathione transferases, *Gene* **443** (1–2), 2009, 143–150.

Lo Piero A.R., Puglisi I. and Petrone G., Gene isolation, analysis of expression, and in vitro synthesis of glutathione S-transferase from orange fruit (*Citrus sinensis* L. Osbeck), *Journal of Agricultural and Food Chemistry* **54**, 2006, 9227–9233.

Lo Piero A.R., Puglisi I., Rapisarda P. and Petrone G., Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage, *Journal of Agricultural and Food Chemistry* **53** (23), 2005, 9083–9088.

Luo P., Ning G., Wang Z., Shen Y., Jin H., Li P., ... Bao M., Disequilibrium of favonol synthase and dihydroflavonol-4-reductase expression associated tightly to white vs. red color flower formation in plants, *Frontiers Plant Science* **6**, 2016, 1257.

Martin C., Butelli E., Petroni K. and Tonelli C., How can research on plants contribute to promoting human health?, *The Plant Cell* **23** (5), 2011, 1685–1699.

Moriguchi T., Kita M., Tomono Y., Endo-Inagaki T. and Omura M., One type of chalcone synthase gene expressed during embryogenesis regulates the flavonoid accumulation in citrus cell cultures, *Plant Cell Physiology* **40** (6), 1999, 651–655.

Moriguchi T., Kita M., Tomono Y., Endo-Inagaki T. and Omura M., Gene expression in flavonoid biosynthesis: Correlation with flavonoid accumulation in developing citrus fruit, *Physiologia Plantarum* **111**, 2001, 66–74.

Rahim M.A., Busatto N. and Trainotti L., Regulation of anthocyanin biosynthesis in peach fruits, *Planta* **240** (5), 2014, 913–929.

Rapisarda P., Bellomo S.E. and Intelisano S., Storage temperature effects on blood orange fruit quality, *Journal of Agricultural and Food Chemistry* **49**, 2001, 3230–3235.

Rapisarda P., Tomaino A., Lo Cascio R., Bonina F., De Pasquale A. and Saija A., Antioxidant effectiveness as influenced by phenolic content of fresh orange juices, *Journal of Agricultural and Food Chemistry* **47**, 1999, 4718–4723

Tian J., Han Z.-Y., Zhang J., Hu Y., Song T. and Yao Y., The balance of expression of dihydroflavonol 4-reductase and flavonol synthase regulates flavonoid biosynthesis and red foliage coloration in Crabapples, *Nature Publishing Group* **5**, 2015, 12228.

Titta L., Trinei M., Stendardo M., Berniakovich I., Petroni K., Tonelli C., ... Giorgio M., Blood orange juice inhibits fat accumulation in mice, *International Journal of Obesity* **34**, 2009, 578–588.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. and Speleman F., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, *Genome Biology* **3** (7), 2002, 0034.

Wang H., Fan W., Li H., Yang J., Huang J. and Zhang P., Functional characterization of dihydroflavonol-4-reductase in anthocyanin biosynthesis of purple sweet potato underlies the direct evidence of anthocyanins function against abiotic stresses, *PLoS One* **8** (11), 2013, e78484.

Zarba A.S. and Pulvirenti G., The consumption of sicilian red oranges: Implications for firms involved in commercialization, *Journal of Business Chemistry* **3** (1), 2006, 22–41.

Zhang Y., Butelli E. and Martin C., Engineering anthocyanin biosynthesis in plants, *Current Opinion in Plant Biology* **19**, 2014, 81–90.

Zou Z., Xi W., Hu Y., Nie C. and Zhou Z., Antioxidant activity of Citrus fruits, *Food Chemistry* **196**, 2016, 885–896.

Appendix A. Supplementary data

Multimedia Component 1

Supplementary Table 1S Primer sequences used for the quantitative RT-PCR analyses.

Multimedia Component 2

Supplementary Fig. 1S Representative climatological condition parameters: Temperature (A), Relative humidity (B) and Precipitations (C) in different commercial orchards in season 2014–2015 (Paranapanema and Pardinho) and 2015–2016 (Maringá).

Multimedia Component 3

Supplementary Fig. 2S Internal appearance of pulp (up) and juices (down) of Pera (left) and Moro (right) oranges from Pardinho (A) and Maringá (B) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0, 15, 30 and 45 days.

Multimedia Component 4

Supplementary Fig. 3S Changes in maturation index (MI) of Pera (left) and Moro (right) oranges from Pardinho (Pr), Maringá (Ma) and Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (●) or 4 °C (○) for 0, 15, 30 and 45 days.

Multimedia Component 5

Supplementary Fig. 4S Changes in the pH values in juice of Moro blood oranges from Pardinho (Pr), Maringá (Ma) and Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (●) or 4 °C (○) for 0, 15, 30 and 45 days.

Multimedia Component 6

Supplementary Fig. 5S Changes in % Yellow (left) and % Blue (right) proportion in juice of Moro blood oranges from Pardinho (Pr), Maringá (Ma) and Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (●) or 4 °C (○) for 0, 15, 30 and 45 days.

Multimedia Component 7

Supplementary Fig. 6S Phylogenetic analysis of Valencia orange (*Citrus sinensis* L. Osb.) NAC and MYB transcription factors. Sequences were downloaded from PlantTFDB database (http://planttfdb.cbi.pku.edu.cn/index.php).

Multimedia Component 8

Supplementary Fig. 7S Relative quantification of expression of *PAL*, *C4H*, *4CL*, *CHSs*, *CHI*, *F3′H*, *F3′5′H*, *FLS*, *DFR*, *ANS*, *UFGT*, *GST* and the transcription factor genes *NAC*, *MYBF1* and *Ruby* in the pulp of Pera and Moro oranges from Paranapanema (Pa) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0, 15, 30 and 45 days. Data are presented as the mean relative expression ± SD of each individual sample as compared to the control sample (zero time of Pera oranges). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $P \leq 0.01$ (*).

Multimedia Component 9

Supplementary Fig. 8S Relative quantification of expression of *PAL*, *C4H*, *4CL*, *CHSs*, *CHI*, *F3′H*, *F3′5′H*, *FLS*, *DFR*, *ANS*, *UFGT*, *GST* and the transcription factor genes *NAC*, *MYBF1* and *Ruby* in the pulp of Pera and Moro oranges from Pardinho (Pr) (Sao Paulo, Brazil) during storage at either 9 (black bars) or 4 °C (grey bars) for 0, 15, 30 and 45 days. Data are presented as the mean relative expression ± SD of each individual sample as compared to the control sample (zero time of Pera oranges). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $P \leq 0.01$ (*).

Highlights

- Anthocyanin content in blood oranges was higher at 9 than at 4 °C of storage.
- This concurred with enhanced upregulation of genes from the pathway at 9 °C.
- Transcript ratio values showed favored flux to dihydroflavonols at 9 °C.
- Key transcription factors were not involved in regulating these differences.

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