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Molecular Insights into the Effects of Rootstocks on Maturation of Blood Oranges

Giuseppe Lana [1,†], Giulia Modica [1,†], Giuseppina Las Casas [2], Laura Siracusa [3], Stefano La Malfa [1], Alessandra Gentile [1], Angelo Sicilia [1], Gaetano Distefano [1,*] and Alberto Continella [1]

[1] Department of Agriculture, Food and Environment (Di3A), University of Catania, Via Valdisavoia 5, 95123 Catania, Italy; giuseppe.lana51@gmail.com (G.L.); giulia.modica@unict.it (G.M.); slamalfa@unict.it (S.L.M.); gentilea@unict.it (A.G.); angelo.sicilia@unict.it (A.S.); acontine@unict.it (A.C.)
[2] CREA, Research Centre for Olive, Fruit and Citrus Crop, Corso Savoia, 95024 Acireale, Italy; giuseppina.lascasas@crea.gov.it
[3] Istituto di Chimica Biomolecolare del CNR (ICB-CNR), 95100 Catania, Italy; laura.siracusa@icb.cnr.it
* Correspondence: distefag@unict.it; Tel.: +39-095-4783403
† These authors contributed equally.

Abstract: Rootstock choice has important effects on the horticultural and pathological traits of the citrus cultivars. Thus, the scion/rootstock combination can affect tree vigour, nutrition, and stress resistance; it can also have positive influences on the fruit quality traits. Although the study of rootstock effects has been a relevant research topic in citrus for many years, the main body of such study has been conducted at the biochemical level, while little effort has been directed to the determination of the rootstock influences at the molecular level. A comparative study of three combinations of scion and rootstock shows a positive correlation between the regulation of the fruit quality-related genes and the accumulations of bioactive compounds, as well as with acid degradation. Monitoring the anthocyanin accumulation during ripening shows the scion/rootstock combination can increase anthocyanin synthesis in the fruit, as well as vitamin C accumulation and acid degradation. Our results show that the rootstock genotype can exert important influences on citrus fruit quality by affecting gene expression in the scion. New insights into the molecular interactions between scion and rootstock may help unravel the systems through which rootstocks exert their influences on the regulatory networks in the scion, so as to influence relevant agronomic traits. This information should result in an improved rootstock breeding selection and definition of scion/rootstock combinations to enhance fruit quality traits.

Keywords: scion/rootstock combination; tarocco sciré; citrus fruit quality; anthocyanin; vitamin C; gene expression

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1. Introduction

Although a very ancient practise, grafting remains a widespread and almost indispensable technique in modern fruit culture. The use of rootstocks offers several advantages, such as better adaptation to limiting soil conditions, improved tolerance to temperature and water stress, and, especially, improved resistance/tolerance to pests and diseases. The relationship between rootstock and the scion can strongly modify the performance of the whole plant combination [1]. The main fruit quality traits are highly dependent on the scion genotype, even though some of them can also be strongly influenced by rootstock genotype. Thus, a particular scion, in combination with different rootstocks, can lead to very different results, in terms of fruit size, shape, pigmentation, juice content, and soluble solid concentration [2]. For this reason, the rootstock choice is a key first step in the development of a productive and profitable orchard [3]. Although, it is well known that some rootstocks can provide fruits of better internal quality than others, the current

understanding suggests the rootstock influences on fruit flavour depend on specific interactions established between a particular scion cultivar and the rootstock itself [4]. Evidence for the influence of rootstock on fruit qualitative traits in the *Citrus* species have been investigated extensively [5,6]. Thus, the trifoliate orange rootstocks (*Poncirus trifoliata* L. Raf.) enhances fruit quality and anthocyanin content, while the Carrizo citrange rootstock [*C. sinensis* (L.) Osb. cv. Washington navel × *P. trifoliata* (L.) Raf.] (widespread in the USA and Europe) confer good internal qualitative traits. The *C. volkameriana* rootstock produces very vigorous and productive trees, but the fruit quality is poor. Meanwhile, Swingle citrumelo [*C. paradisi* (Macfadyen) × *P. trifoliata* (L.) Raf.] is highly productive but induces low fruit acidity and performs poorly in heavy and calcareous soils [7].

Fruit colour is the first characteristic perceived by consumers and, therefore, is one of the key factors influencing a fruit's aesthetic value and market appreciation. In the case of blood oranges, the red pigments are also associated with perceived health benefits. The changes in colour that take place during ripening are related to the synthesis and degradation of three main classes of compounds: chlorophylls, carotenoids, and anthocyanins [8]. Chlorophylls are the predominant pigments in the green skin of an unripe citrus fruit, while the yellow–orange colour, typical of a mature fruit, is due to the carotenoids. The synthesis of anthocyanins is limited to the blood orange group and its hybrids, which are responsible for the purple–red colour of both their flesh and skin [9,10].

The pigmentation is regulated by a number of factors, including environmental conditions, cultivation practices, water, and nutrient availability; it also depends on both the maturity of fruit [11] and can be affected by the postharvest storage temperature [12]. As in other foods, due to their antioxidant properties, anthocyanins protect from oxidative stress and help prevent cardiovascular disease and cancer [13,14].

The use of different citrus rootstocks can significantly influence the timing of fruit development, ripening, and harvesting of pigmented fruits, as well as modifying their final chemical composition [15]. Ordóñez-Díaz et al. [16] drew attention to the strong influence of rootstock on the composition of some bioactive compounds developed during fruit maturation in the cvs. Salustiana and Tarocco. The maturation process influences total phenol content and increases the concentrations of anthocyanins at harvest. Indeed, the increase in bioactive compounds during maturation has also been observed in blood oranges [11,17].

Most of the genes encoding the enzymes that are involved in anthocyanin synthesis have been identified and characterised in a range of species, including in citrus species. Phenylalanine ammonia-lyase (PAL) is considered a key gene in anthocyanin metabolism because phenylalanine is the main precursor of these pigments. The first enzyme of the anthocyanin synthetic pathway is chalcone synthase (CHS), which is also involved in the production of other phenols. In the next steps, dihydroflavonol 4-reductase (DFR) reduces the dihydroflavonols to leucoanthocyanidins; these colourless compounds are then transformed into coloured anthocyanins by anthocyanidin synthase (ANS). The last step of synthesis is driven by UDPglucose-flavonoid glucosyl transferase (UFGT), which increases the stability and water solubility of the recently produced anthocyanins by adding a glucose molecule in the 3-OH position. The anthocyanin synthesis genes are regulated by a complex system of transcription factors, referred to as the MBW complex, of which *Ruby* is the R2R3 MYB activator in citrus fruits [10,18].

The taste of citrus flesh is highly dependent on its sugar and acid content and, in particular, on the ratio between total soluble solids (TSS) and titratable acidity (TA), which has a strong effect on the perception of sweetness and sourness [19]. This is the reason the ratio TSS/TA is considered one of the most important fruit quality traits and the most common parameter used to define the ripening index in some citrus fruit, such as oranges or mandarins. In *citrus* spp., sugars accumulate in juice sacs during maturation, while organic acids are gradually degraded. Blood oranges are generally more acidic than blonde ones at harvest. This can mean that they do not reach an adequate sugar/acid ratio when

fully ripe, with a consequent loss of consumer acceptance. For this reason, the control of fruit acidity is of considerable economic relevance [20–22].

Recent studies have identified and characterised a number of structural genes involved in the synthesis and catabolism of organic acids in citrus flesh. Among these, citrate synthase (*CS*) participates in the synthesis of citrate, while isocitrate dehydrogenase (*IDH*) and ATP citrate lyase (*ACL*) encode the enzymes involved in citrate utilization [23]. The transcription of the sucrose phosphate synthase (*SPS*) gene has been pointed out as one of the major factors regulating sucrose synthesis and storage in mature fruit. The enzyme produced by this gene catalyses the conversion of fructose-6-phosphate and UDP glucose to sucrose-6-phosphate and is responsible for sucrose synthesis in plants [24,25].

Citrus fruits are a great source of vitamin C for humans, who are not able to synthesise it themselves and, thus, are obliged to meet their requirement through a daily dietary intake. L-ascorbic acid (AsA) is produced in large amounts in plants, where it protects tissues from oxidative damage, related to numerous biotic and abiotic stresses [26]. Due to its free-radical scavenging activity and role as a cofactor in many chemical reactions, AsA is also an essential component of the human diet. The reaction catalysed by the enzyme encoded by GDP-L-galactose phosphorylase (GGP) is considered the first committed step of the main pathway, as well as the most important of AsA synthesis. A second key gene in AsA synthesis is D-galacturonate reductase (GalUR), since it regulates one of the alternative routes for AsA synthesis [27]. Blood orange is a rich source of vitamin C; indeed, the Tarocco clones have higher concentrations than many blonde orange varieties, ranging from 500 to 800 mg/L of juice [28,29], similar to those observed in the Moro and Sanguinello cultivars [30].

Nevertheless, it is still unclear how rootstocks can exert their influences on fruit quality parameters, such as anthocyanins, sugars, acids, and vitamin C. In this study we attempt to determine if any rootstock effects are related to changes in the expressions of the key quality trait genes in the scion. With this aim, we carried out metabolic studies and gene expression analyses on fruits of the blood orange selection (*C. sinensis* (L.) Osb. cv Tarocco Sciré), grafted on to one of three different rootstocks.

2. Materials and Methods

2.1. Plant Material

Fruits of Tarocco Sciré sweet orange were harvested from nine-year-old trees, grafted on one of three different rootstocks: (1) Carrizo citrange (CAR) [*C. sinensis* (L.) Osb. cv. Washington navel × *P. trifoliata* (L.) Raf.], (2) Bitters (BIT), or (3) Furr (FUR) [*C. sunki* Hort. ex Tan. × *P. trifoliata* (L.) Raf.]. Plants were cultivated in an experimental field, located in Lentini (37°17′04″ N, 14°53′16″ E, Syracuse, Italy), and subjected to standard cultural practice. Three biological replicates for each of the scion/rootstock combinations were selected in a randomised block design. Samples were taken every 30 d at mid-month, from November 2018 to March 2019, at five maturity stages: 184 days after full bloom (DAFB), 214, 245, 276, and 304 DAFB. These timings ranged from the onset of ripening to the fully mature stage (Figure 1A). For each block, 28 randomised fruits were collected from 9 trees and quickly transferred to the laboratory. Fruit juice was extracted with a commercial juice extractor (Kenwood Citrus Juicer JE290, Havant, UK), filtered, quick-frozen in liquid nitrogen, and stored at −80 °C until processing.

2.2. Biochemical Analysis

HPLC/DAD and HPLC/ESI/MS analyses were used to quantify total anthocyanin content (TAC) for each scion/rootstock combination. All solvents and reagents used were high purity laboratory solvents from VWR (Milan, Italy); HPLC grade water and acetonitrile were also obtained from VWR. Cyanidin 3-O- glucoside was purchased from Sigma (Sigma-Aldrich, Milan, Italy). Small samples (2 mL) of the juices were placed in 15 mL plastic sample tubes, and 100 µL of formic acid (98%) was added. Samples were sonicated for 5 min, then centrifuged (5417R Eppendorf, Osterode, Germany) at 800 g force

for 15 min to separate the solid portion of the juice. Next, 1 mL of the clear supernatant was transferred to a 2 mL HPLC amber vial and immediately analyzed. Chromatographic analyses were carried out on an Ultimate3000 UHPLC focused instrument (equipped with a binary high-pressure pump), photodiode array detector, thermostatted column compartment, and automated sample injector (Thermo Fisher Scientific, Inc., Milan, Italy). The chromatographic column, elution program, and DAD acquisition parameters used were the same as previously reported [31]. Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. The results are expressed as mg of cyanidin-3-glucoside equivalents per litre. The TSS content was measured using a digital refractometer (Atago Co., Ltd., model PR-32 α, Tokyo, Japan) and expressed as °Brix. According to the AOAC method (AOAC, 1995), the TA was determined by potentiometric titration (Hach, TitraLab AT1000 Series) of the juice using 0.1 N NaOH at pH over 8.1, results are expressed as g L^{-1} of citric acid equivalent. Vitamin C (L-ascorbic acid, AsA) was determined using an automatic titration apparatus (702 SM Titrino, Metrohm, Herisau, Switzerland) with 0.001 M I_2, and the results are expressed as mg L^{-1}. Ripening index (RI) represents the ratio between TSS and TA. Juice colour was recorded with a Minolta CR-400 chroma meter (Minolta Corp., Osaka, Japan), as described by Caruso et al. [15]. The results were expressed as citrus colour index (CCI = a*1000/L*b), a maturation index widely used in the citrus industry [32].

2.3. Gene Expression Analysis

The plant material used for total RNA extraction was the same as that used for metabolic study. Total RNA was isolated from 3 mL of juice, as described by Butelli and colleagues [33], and subsequently treated with RNAse-free DNAase (DNase treatment and removal, Ambion, Madrid, Spain). Total RNA concentration and purity were assessed using a spectrophotometer (NanoDrop-2000, Thermo Scientific, Wilmington, NC, USA). The absence of DNA was checked by gel electrophoresis; cDNA was synthesised from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Inchinnan, UK), following the procedure indicated by the manufacturer.

Quantitative real-time polymerase chain reaction (qPCR) was carried out on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) in 10 µL total reaction volume containing 1 × PCR buffer II, 2 mM MgCl2, 0.2 mM dNTPs, 0.3 µM of forward and reverse primers (Table 1) (Life Technologies, Inchinnan, UK), 1.5 µM SYTO9 (Life Technologies, Inchinnan, UK), 20 ng of cDNA, and 1U of MyTaq DNA polymerase (Bioline, London, UK). Thermal cycling conditions included a pre-incubation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 10 s for denaturation and 60 °C for 60 s for a single annealing-elongation step. The ΔΔCt method was used to normalise the raw Ct data, the elongation factor 1-alpha (EF-1α) was chosen as reference gene, while CAR gene expression of November was taken as calibrator. The results are the average of three independent biological sample replicates.

2.4. The qPCR Data and Statistical Analysis

The outputs of the different analyses were processed and visualised using R software (R core team, Foundation for Statistical Computing 2016, Vienna, Austria). The bar plots were produced through the base package. The qPCR data were normalised using the 'HTqPCR' package [36] and the outputs were visualised using the 'heatmap3' package [37]. The ANOVA tests were conducted with the 'aov' function of the base package, and a Tukey test was applied to the results. Samples had previously been submitted to the Shapiro–Wilk test to check the normality of their distribution. Significant differences in metabolic data were represented on plots by the letters a, b, and c (p value < 0.01).

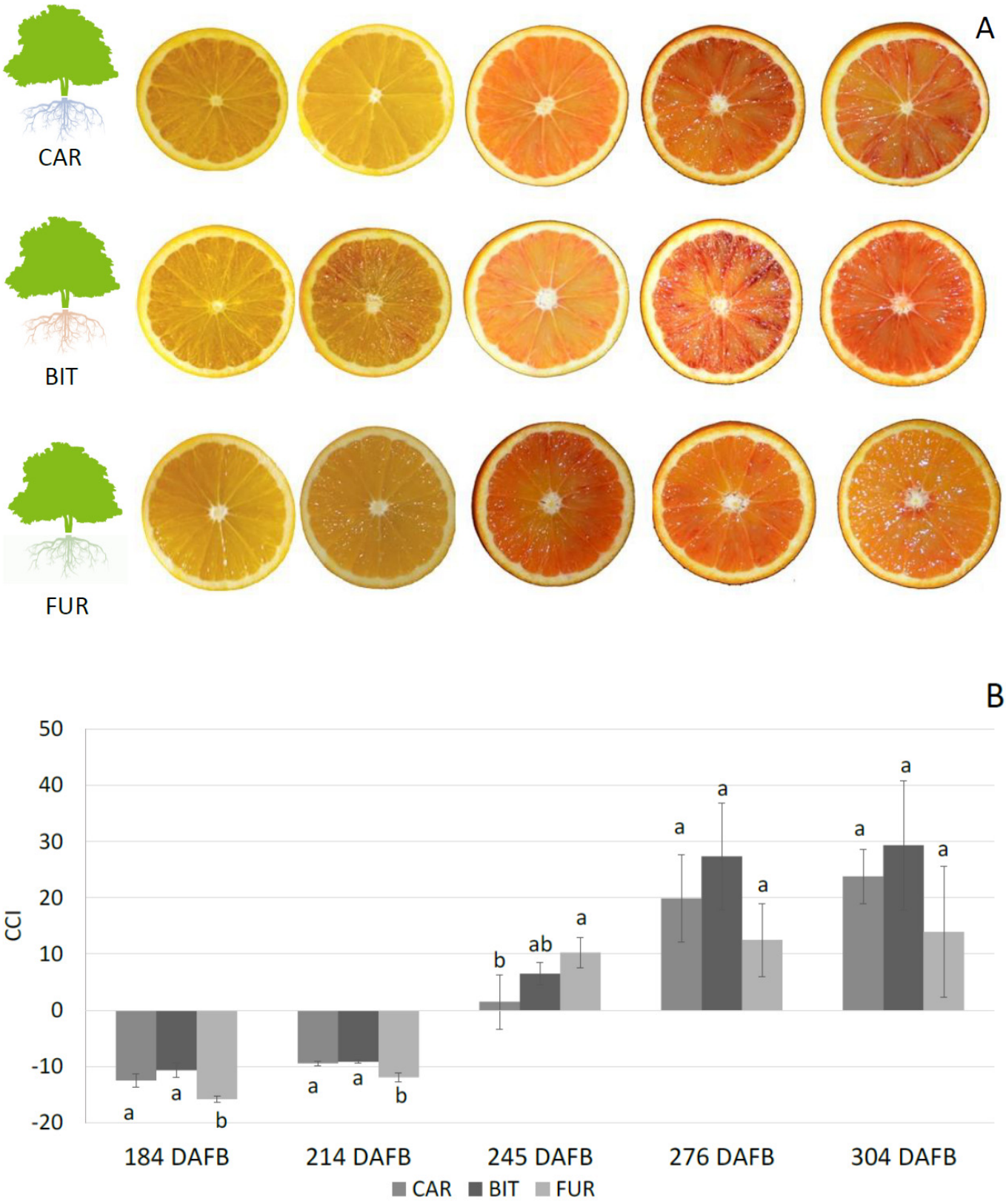

Figure 1. (**A**) Flesh colour of the sweet orange Tarocco Scirè, grafted on the rootstocks Carrizo (CAR), Bitters (BIT), and Furr (FUR) during the different harvest dates (DAFB: days after full bloom). (**B**) Citrus Colour index (CCI) of Tarocco Scirè juice, measured at the different harvest dates (n = 84: mean values of 28 fruits × 3 replicates). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (p value < 0.01) are represented by different letters.

Table 1. List of the primers used for qPCR analysis.

Accession Number	Gene Name	Forward 5′-3′	Reverse 5′-3′	Reference
XM_006481431.3	PAL	GATTTGAGACATTTGGAGGA	ATGGATGAAGCTCTCCACTA	
XM_006420545.2	CHS	TCTATCGACGGGCATCTTC	TGCCTCGGTTAGGCTTTT	[34]
NM_001288931.1	DFR	GCTGTTCGTGCTACTGTTC	GGCTAAATCGGCTTTCCATA	
XM_025097974.1	ANS	GGGTGACTGCTAAATGTGTT	CAAGTCCCCTGTGAAGAATA	
NM_001320060.1	UFGT	TCTTCAGCACTCCGCAATC	TCCATCGGATACGTCGTAAG	
NM_001288889.1	R2R3Myb	ACAATCCACCCCGTCTGATC	CTGGCCTGCTTCAATGACTC	
XM_006483335.2	SPS	TTGTAACTAGCACCCGACAGG	CAACCATACGAGGCATAAACC	[4]
XM_006480234.2	ACL	GATACTGTTGGAGACTTGGG	GCTCTCTTACGACCATCAGG	[23]
XM_006494513.2	NADP-ID184 DAFB	GAAAATTGGGGATTGGGATT	CAACAGAGGTGCAGCTCAAA	[23]
XM_006482744.2	CS	GGTGCCCCCAATATTAACAA	AGAGCTCGGTCCCATATCAA	[23]
XM_006474957.2	GGP	TACCAAAGTGGGGCAAGAAG	TGGCAACAACACTTGGAGAA	[35]
XM_006492225.2	GalUR-12	CCCAGGTTTCTTTGAGGTGGGTTTATC	TACTGTGGAATTTGTTCGATCTTTTGCAGC	[35]
AY498567	EF	CACCACCCCCAAGTACTC	GTTGTCACCCTCGAAACC	

3. Results

3.1. Biochemical Analysis

The citrus colour index (CCI) of the juice is dependent on the stage of ripening. At 184 and 214 DAFB, the value of CCI was negative because of the green appearance (Figure 1B). The lowest values were in FUR, −15.81 (184 DAFB) and −11.94 (214 DAFB), while BIT and CAR were almost of the same colour level (Figure 1A). Juice colour changed progressively from yellow/orange to deep orange/reddish at 245 DAFB for FUR, which showed the highest value (10.25). Nevertheless, at full ripening (304 DAFB), FUR (13.95) had a lower value than for either BIT (29.37) or CAR (23.80).

The TSS values increased progressively until January (245 DAFB) in BIT and FUR, subsequently they maintained quite constant values; CAR was the only one in which the sugar content increased from January (245 DAFB) to March (304 DAFB) (Figure 2A). No differences among the three rootstocks were recorded for TSS at 304 DAFB, even though statistical differences indicated a lower content of TSS in the fruits of FUR during November (184 DAFB) (9.7) and December (214 DAFB) (10.53). At the end of maturation, the pH values in the blood orange samples were between 3.57 (BIT) and 3.88 (CAR). Marked differences were found in TA, which was consistently higher in FUR than in the other two rootstocks (Figure 2B). Although TA values were similar in CAR and BIT, the TA of CAR decreased from 245 to 304 DAFB, while in BIT, the TA was relatively constant. Even in this case, the largest changes in metabolite reduction were observed at 245 DAFB, when TA decreased substantially in all samples. In CAR, there was a simultaneous decrease of TA and an increase of TSS during the last three stages of ripening, resulting in the higher TSS/TA values, while MI showed slight changes in FUR and BIT during 276 and 304 DAFB (Figure 2C).

Total anthocyanin content appeared at the second month of sampling (214 DAFB) in all three scion/rootstock combinations, even though TAC was not clearly detectable colourimetrically (Figure 1A). The TAC content was significantly higher in BIT at 214 DAFB, but it increased in January (245 DAFB) and again in February (276 DAFB), then maintaining these values until March (304 DAFB). The rootstock reaching the highest concentration during the last three months was BIT, which accumulated significantly higher amounts of anthocyanins after 245 DAFB (Figure 3A). Meanwhile, FUR showed the lowest values, compared to the other two rootstocks, except in January (245 DAFB), when the lowest anthocyanins content was in CAR. The only rootstock showing a significant increment between 276 and 304 DAFB was CAR.

The vitamin C content decreased slightly during maturation, reaching a lowest value in 304 DAFB (Figure 3B). Among the samples, the lowest AsA content was recorded in CAR, where levels were consistently lower than in BIT and FUR. The variations between these last two rootstocks were not statistically significant, except in 276 DAFB, when BIT was significantly higher than FUR.

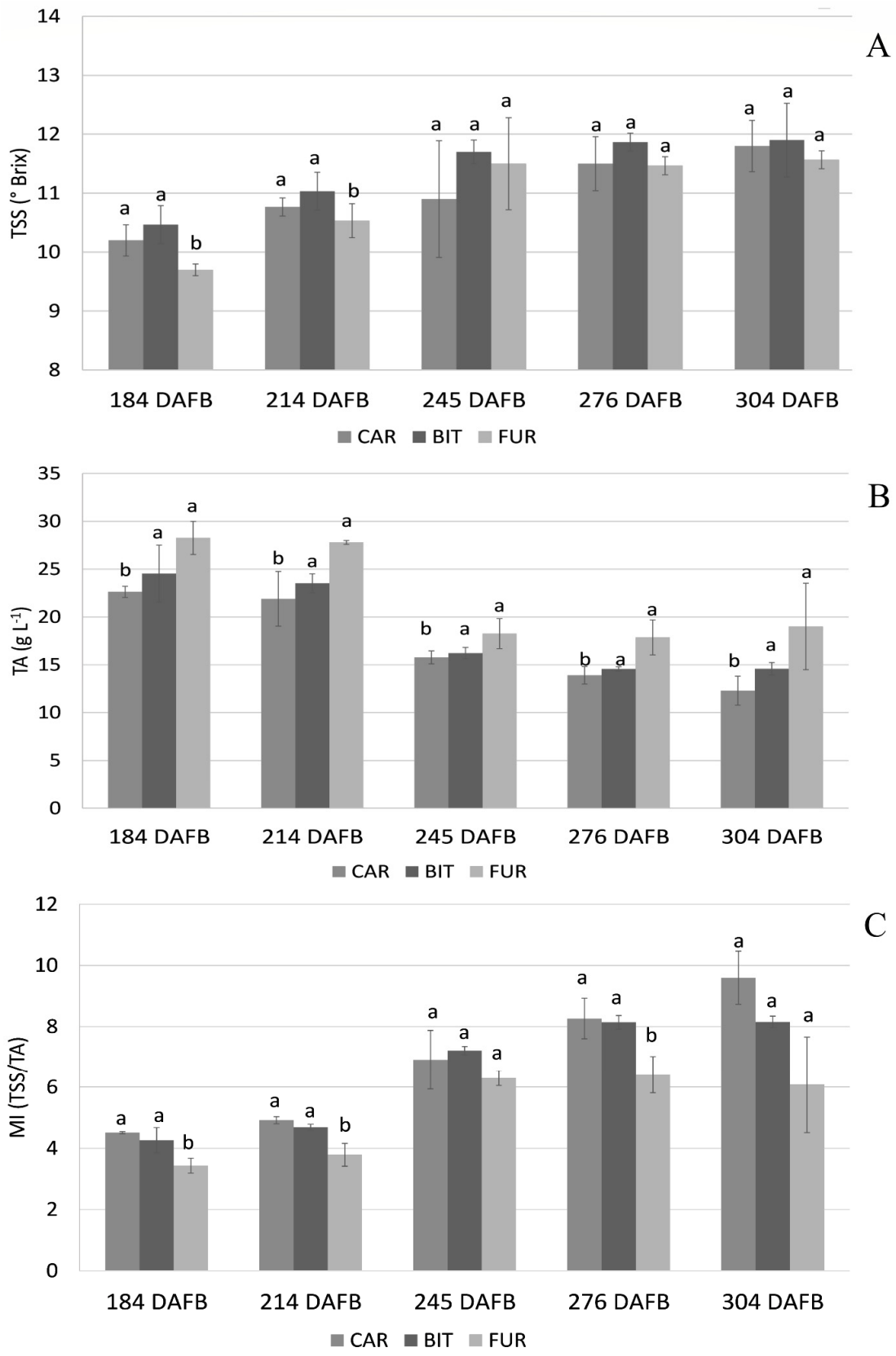

Figure 2. Evolution of biochemical data recorded on the juice of the sweet orange Tarocco Sciré, grafted onto Carrizo (CAR), Bitters (BIT), or Furr (FUR) rootstocks during the different harvest dates (DAFB: days after full bloom). (**A**) TSS, total soluble solids (°Brix). (**B**) TA, titratable acidity (g L^{-1}). (**C**) Maturity index expressed as total soluble solids/titratable acidity (TSS/TA). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (p value < 0.01) are represented by different letters.

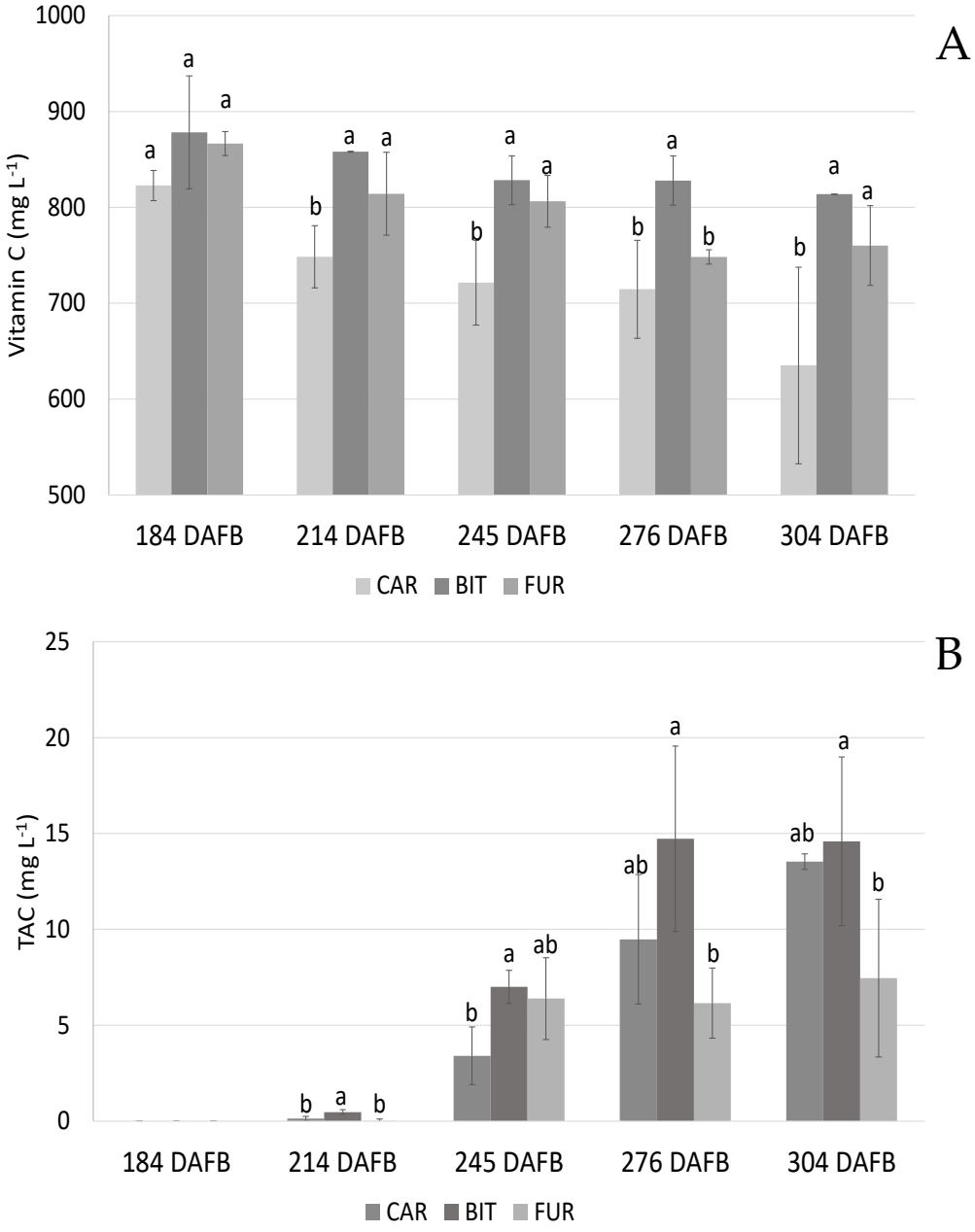

Figure 3. Pattern of biochemical data recorded on juice of sweet orange Tarocco Sciré, grafted onto Carrizo (CAR), Bitters (BIT), or Furr (FUR) rootstocks during the different harvest dates (DAFB: days after full bloom). (**A**) TAC, total anthocyanin content (mg L^{-1}). (**B**) Vitamin C (mg L^{-1}). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (p value < 0.01) are represented by different letters.

3.2. Gene Expression Analysis

Transcription of *PAL* was clearly higher in BIT than in the other two rootstocks, at all inspection times, except for 245 and 276 DAFB, when the values for FUR and CAR were very similar to the one for BIT (Figure 4). Similarly, *CHS* expression reached a high for BIT at 214 DAFB and in FUR at 245 DAFB. At 214 and 276 DAFB, CAR values were significantly lower than those for BIT. The highest transcription levels of *DFR* were recorded at 214 DAFB for BIT and at 276 DAFB for FUR. The expression of this gene was not significantly different in the two rootstocks at 184 and 304 DAFB but was more elevated at 214 and 276 DAFB for BIT and at 245 DAFB for FUR. In comparison to CAR, *DFR* showed increased transcription for BIT at 214 DAFB, but the differences (245, 276, and 304 DAFB)

were not significant. Anthocyanidin synthase was highly expressed during 214 and 245 DAFB for both BIT and FUR; for BIT, the peak of expression was anticipated at 214 DAFB, whereas in FUR the highest FC value was reached at 245 DAFB. It is worth noting that the expressions of *ANS* were generally lower for CAR than for BIT and FUR, and the highest FC value was reached only at 276 DAFB. Transcription of *UFGT* was relatively constant in all three rootstocks, slight differences were found exclusively at 184 DAFB, when the accumulations of gene transcripts for CAR and FUR were higher than for BIT, and at 276 DAFB, when they were higher than for either BIT or FUR. The only differences in *Ruby* expression were recorded at 276 DAFB, when CAR showed a higher accumulation of mRNA than for BIT at 276 DAFB.

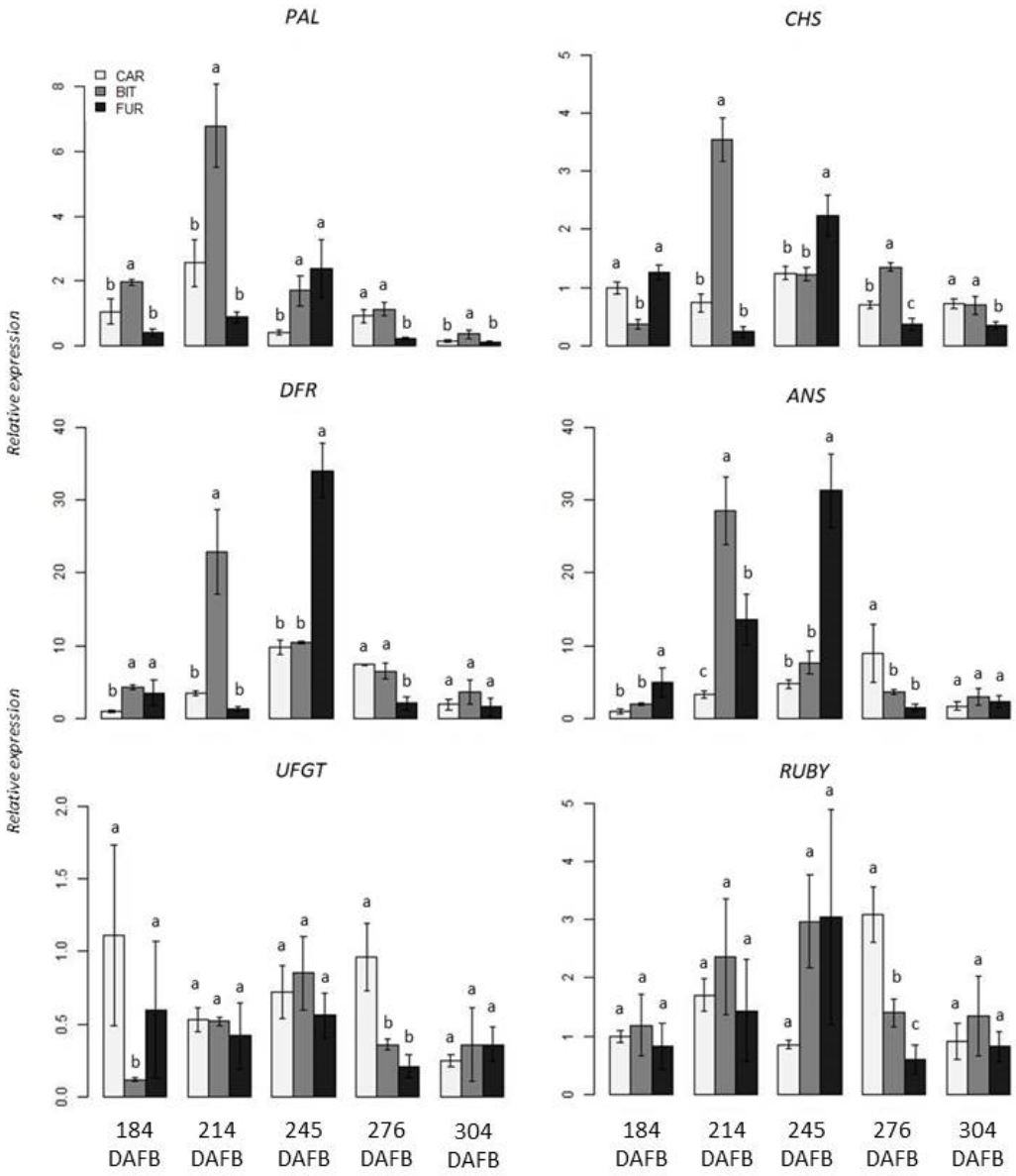

Figure 4. Expression of genes involved in anthocyanin synthesis. Histograms of qPCR data (FC, fold change) detected in juice of 'Tarocco Sciré' grafted on Carrizo (CAR), Bitters (BIT), or Furr (FUR) rootstocks at five sampling stages (DAFB: days after full bloom). PAL, Phenylalanine ammonia-lyase. *CHS*, chalcone synthase. *DFR*, dihydroflavonol 4-reductase. *ANS*, anthocyanidin synthase. *UFGT*, UDPglucose-flavonoid glucosyl transferase. *Ruby*, R2R3Myb. Vertical bars indicate standard deviations. The ANOVA tests were conducted with the 'aov' function of the base package, and a Tukey test was applied to the results. Statistically significant differences are represented by letters a, b, c (p value < 0.01).

The expression of *SPS* showed no significant differences among the rootstocks during the whole process of ripening, except at 214 DAFB, when the gene was more strongly expressed for CAR than for the other two rootstocks (Figure 5).

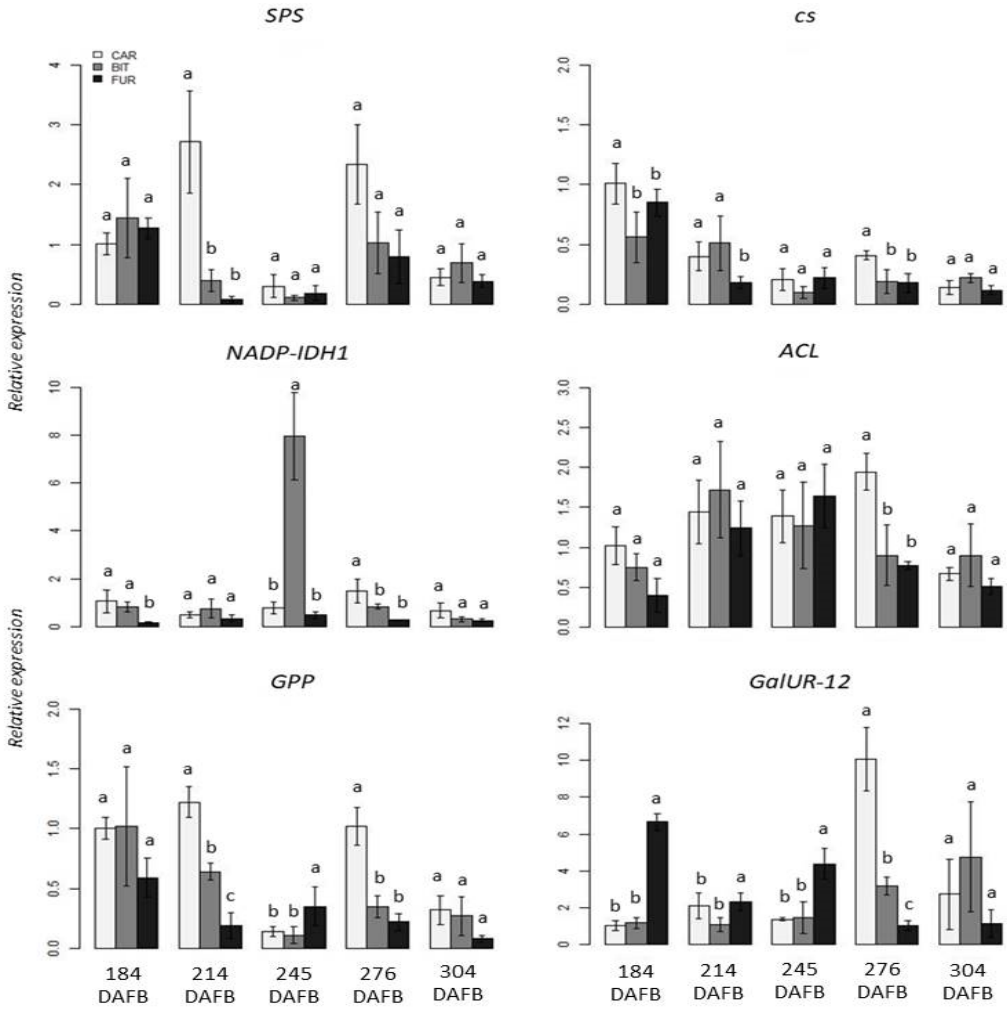

Figure 5. Histograms of qPCR data (FC, fold change) detected in juice of 'Tarocco Sciré', grafted onto Carrizo (CAR), Bitters (BIT), or Furr (FUR) rootstocks at the five sampling stages (DAFB: days after full bloom). *SPS*, sucrose phosphate synthase. *CS*, citrate synthase. *NADP-IDH1*, isocitrate dehydrogenase. *ACL*, ATP citrate lyase. *GGP*, GDP-L-galactose phosphorylase. *GalUR-12*, D-galacturonate reductase. Vertical bars indicate standard deviation. The ANOVA tests were conducted with the 'aov' function of the base package, and a Tukey test was applied to the results. Statistically significant differences are represented by letters a, b, c (p value < 0.01).

At 184 and 276 DAFB, *CS* was downregulated for BIT and FUR, in comparison with CAR; at 214 DAFB, *CS* was more expressed for CAR and BIT than for FUR (Figure 5). The gene was also upregulated for CAR at 276 DAFB. During the other months, no significant differences were found. A slight downregulation of *NADP-ID184 DAFB* was noted for FUR at 184, 245, and 276 DAFB, compared to for BIT and CAR. Any significant divergence was highlighted by statistical analysis of the accumulation of *ACL* transcript levels, except for 276 DAFB, when CAR accumulated higher amounts of mRNA, in comparison with BIT or FUR.

The expression of *GPP* was relatively variable across the five sampling stages, but CAR was associated with the highest expressions at 214 and 276 DAFB; conversely, FUR was associated with the lowest ones (Figure 5). For FUR, *GPP* was upregulated at 245 DAFB, compared with either CAR or BIT. At 214 and 276 DAFB, the highest FC values were for CAR, and the lowest were for FUR. Early upregulation of *GalUR-12* was evident for FUR

from the first stages of development. Although *GalUR-12* transcription was lower for BIT and CAR than for FUR at 184, 214, and 245 DAFB, but it increased significantly at 276 and 304 DAFB. The highest peak of expression was for FUR at 184 DAFB, before the FC value dropped at 214 DAFB, but was still higher than for BIT. At 245 DAFB, *GalUR-12* was upregulated for FUR, compared with for BIT and CAR; at 276 DAFB, it was the CAR rootstock that showed the highest regulation of the gene, while FUR showed the lowest values of the three rootstocks.

4. Discussion

Several studies have documented the increase in anthocyanin content of pigmented oranges during ripening [17,38] but few in blood orange, as a function of rootstock [16]. A recent study demonstrates that some recently released rootstocks are better than others, with respect to the production of fruit with high levels of pigmentation. These enhance the synthesis and accumulation of anthocyanins in the flesh of citrus fruit [39]. By monitoring anthocyanin accumulation during fruit ripening, our results seem to support these findings, since the metabolic data indicates clear differences in fruit anthocyanin content among the three rootstocks tested.

Total anthocyanin content was at almost high levels by the third sampling (245 DAFB) for all three scion/rootstock combinations; though, for the rootstock BIT, a higher content was already significantly evident at the second sampling (214 DAFB). Positive CCI values were recorded only in January (245 DAFB), when TAC settled to about 5 mg L^{-1} in all three samplings. The anthocyanin accumulation is especially relevant inside the juice sacs adherent to the segments, and particularly so in the ones nearest the albedo (Figure 1); the influence of rootstock was first clearly evident colourimetrically in the early stages of synthesis (Figure 1). The rootstock CAR was the only one that increased the increment of TAC from 245 to 304 DAFB, while the two citrandarins (BIT and FUR) seemed to be more precocious, already achieving their maximum anthocyanin levels at 276 DAFB. The fruits of CAR seemed to synthesise anthocyanins later on, reaching the highest values at 304 DAFB. Meanwhile, BIT showed intense pigmentation from January (245 DAFB), the month in which anthocyanin was most markedly accumulated.

According to our earlier results [16], the concentration of anthocyanins increased progressively during the sampling period. These authors detected the influence of rootstock on the accumulation of anthocyanins in the juice of the blood orange cultivar Sanguinelli. They noted that FA5 and Cleopatra rootstocks were the ones with the highest total content, while Carrizo citrange slightly enhanced the pigmentation, and Volkameriana scarcely had any effect. Also, Morales et al. [11] observed similar anthocyanin contents in the fruits of cv. Tarocco Rosso, assessing FA13, FA5, and Cleopatra mandarin as the rootstocks that most determined increases in pigmentation, while Carrizo just affected it and *C. macrophylla*; Swingle citrumelo showed the least effects.

The higher TAC recorded in BIT (Figure 3A) matched an early up-regulation of anthocyanin synthetic gene (Figure 4). qPCR data showed that *PAL*, CHS, *DFR* and *ANS* showed their highest peaks of expression in BIT at 214 DAFB, while FUR, *DFR* and *ANS* showed highest expressions at 245 DAFB, a month later than for BIT (Figure 4). In pistachio, it has been found that rootstock can influence the activity of PAL, which plays a pivotal role in the production of phenolic compounds, flavonoid, and anthocyanin [40]. While, in *Arabidopsis* a mutation of *CHS* led to a phenotype with flavonoid synthesis deficiency [41].

A key gene for synthesis of anthocyanin is *DFR* because its transcription is generally not detected in blonde oranges [42] and it is highly up-regulated in the flesh of pigmented citrus fruits [43]. Both *DFR* and *ANS* were generally downregulated for CAR compared to for BIT or FUR and seemed to reach highest expression only at 276 DAFB, two months later than for BIT and one month later than for FUR (Figure 4). This delayed activation of gene transcription may explain why for CAR, fruit anthocyanin accumulation began later than for BIT or FUR. Indeed, both *DFR* and *ANS* are part of the downstream gene of the pathway and, for this reason, are called late synthetic genes (LBGs). These are the opposite

of the early synthetic genes (EBGs) such as *PAL* and *CHS*, which encode for precursors in common with several other pathways. The LBGs encode specifically for enzymes involved in the synthesis of anthocyanins [44].

Although fruits for FUR reached a higher TAC earlier than for CAR, by the end of ripening FUR had the lowest TAC of the three rootstocks (Figure 2A). Unlike for BIT, where the expression of anthocyanin synthetic genes decreased after the peak at 214 DAFB, for FUR, the transcription of these genes dropped suddenly at 276 DAFB (Figure 4). The amount of pigment accumulated in the citrus pulp tissues was positively correlated with the expressions of the anthocyanin synthetic genes [45]. The lower TAC in the ripe fruit for FUR may, therefore, be connected with the early downregulation of these genes.

The accumulation of anthocyanins in citrus fruit pulp is regulated not only by the structural genes just discussed, but also by *Ruby*, a transcriptional factor belonging to the MYB family [18]. The transcription of this gene is activated in blood orange by the insertion of a retrotransposon called Tcs1, which is cold-dependent and for this reason is also responsible for temperature-dependent anthocyanin synthesis [18,34,46]. During our study, no remarkable differences in *Ruby* expression were detected among the three rootstocks (Figure 4).

During maturation, juice pH showed an increase in all three rootstocks, as also reported in other studies with citrus fruits [47]. Our results are similar to those reported for blood orange cultivars, such as Tarocco Rosso and Tarocco Ippolito [17].

Sugars are important components of the chemical composition of blood oranges and their profiles depend on cultivar, harvest time and environmental conditions. Several authors have studied the influence of rootstock on the sugar profiles of citrus fruits [5]. Morales et al. [11] investigated rootstock effects on fruit quality of the blood oranges Moro and Tarocco Rosso: the FA5 rootstock showed the highest levels of TSS in fruits of both cultivars during ripening. This contrasts with those grafted on Carrizo citrange where TSS scarcely increased. In our study, CAR showed a late increase in TSS and a decrease in TA, compared with the other two citrandarin rootstocks. This confirms a delay in the whole ripening process, with a lag of about a month. Also, Domingues et al. [48] reported both higher and anticipated TSS and maturation index (MI) of fruits of Valencia grafted onto some citrandarins (US–852 and IPEACS–256), like BIT and FUR, with respect to some other rootstocks (such as some citrumelo clones). Values obtained during the evolution of TSS and TA at full maturation stage agreed with those reported by Abdelaali et al. [49], Continella et al. [39], and Cebadera-Miranda et al. [17]. Since no large differences were recorded in the TSS content in the final stages of maturation (276 and 304 DAFB), the TSS/TA ratio (MI) was more influenced by TA (Figure 2C).

A slightly lower content of TSS was found for FUR at 184 and 214 DAFB (Figure 2B), but it was not possible to correlate this with changes in SPS transcription (Figure 5). The regulatory network that controls sugar accumulation is strictly related to sink strength, the competitive ability of fruits to attract assimilates, compared with other plant organs [24]. Thus, the lower values assumed for FUR during 184 and 214 DAFB may be related to other factors involved in sugar accumulation, not to *SPS* expression.

Although TA was consistently higher for FUR than for BIT or CAR (Figure 2C), *CS* was generally downregulated for FUR (Figure 5). The acidity of citrus fruits largely depends on citrate accumulation, since 90% of the organic acids contained in the juice vesicles is represented by citrates. However, the reduction of acidity taking place during fruit ripening seems to be due to increased catabolism of citrate rather than to its reduced synthesis [50]. As suggested in several studies, the main regulator of citrate content is a group of genes related to the citrate degradation pathways and not *CS*, which is involved in the synthesis of these compounds [4,23,51,52]. One of the key genes involved in citric acid catabolism is *NADP-ID184 DAFB* and its upregulation has been related to reduced accumulation of citrate [23]. Clear downregulation of this gene was noted for FUR, in comparison to BIT or CAR (Figure 5). It is possible to speculate that the higher TA values recorded for FUR (Figure 2C) are related to a lower rate of citrate degradation. A second important

gene participating in acidity reduction is *ACL*, which catalyses the conversion of citrate to oxaloacetate. Although this gene plays a fundamental role in citric acid utilization [53], no noteworthy changes of its transcription were observed during our study.

Citrus fruits are beneficial in human nutrition because of their antioxidant activity and high concentration of ascorbic acid that is influenced both by the stage of ripening and by rootstock [28,54]. Previous studies with blood orange have reported concentrations of ascorbic acid in Moro and Sanguinello above 500 mg/L, with higher contents than in blonde orange cultivars [28,55,56].

Influences of rootstock on vitamin C content have been reported by Morales et al. [54], where mandarins have the highest levels of ascorbic acid in January, but this decreases by the end of February. Rootstock strongly influenced the total amount of vitamin C, with FA5 showing higher values than for Carrizo citrange. Accordingly, the same behaviour has also been observed on Tarocco Sciré: vitamin C decreases during maturation, with BIT showing the highest vitamin C content at full maturation in March (304 DAFB).

In recent years, the regulation of AsA has been subjected to in-depth study because of its known positive effects on both plant and human health. Accumulation of AsA depends on the balance between its synthesis and oxidation rates, which are characteristics of each genotype and tissue. The regulation of *GPP* has been strongly related to AsA concentration in the tissues of several plants [27]. However, the steadily rising content of AsA for FUR (Figure 3B) may not be related to the transcription of this gene, since it was generally downregulated for FUR compared with for CAR or BIT (Figure 5). More likely, the higher level of AsA for FUR was caused by an early upregulation of *GalUR-12*. The expression of the gene for CAR and BIT increased from 276 to 304 DAFB, while for FUR the transcription of *GalUR-12* was quite high from 184 to 245 DAFB, before a decreasing trend was initiated (Figure 5). *GalUR-12* is highly expressed in citrus fruits and represents the rate-limiting step of the galacturonate pathway, an alternative synthetic route for AsA accumulation to the main L-Galactose pathway. However, recent evidences suggest the GalUR genes may be the main ones responsible for the high accumulation of vitamin C in citrus fruit [57].

Despite it being well-documented that grafting can affect fruit organoleptic and nutritive qualities such as the content of bioactive compounds [58–62], it is still unclear how rootstocks exert their influence on these characteristics. The results discussed here suggest that the performances of grafted trees are related to more specific interactions between scion and rootstock and not simply to factors such as plant water and nutrient status or crop yield [3]. The higher accumulation of anthocyanins for BIT and of AsA for FUR was connected to the upregulation of genes encoding for the key enzymes involved in the synthesis of these metabolites. While the higher level of acidity recorded for FUR was correlated with the downregulation of a gene that activates the degradation of citrates. Not much is known about the effects of rootstock at the molecular level; however, recent advances have shown that the use of a rootstock can affect scion gene regulation [63,64]. Moreover, it seems that scion gene expression may be induced by the movement of proteins and small RNA across the graft junction [65,66]. In conclusion, on the basis of our results, it is reasonable to assert that a rootstock plays a fundamental role in the control of the gene regulatory networks of the citrus scion that are involved in the fruit quality traits.

5. Conclusions

This comparative study compared three combinations of scion/rootstock and indicates a positive correlation between rootstock and the regulation of fruit-quality related genes in the scion and the accumulation of bioactive compounds in the fruit. Monitoring the anthocyanin accumulation during fruit development and ripening shows certain combination of scion and rootstock can enhance the synthesis of these compounds, as well as of vitamin C accumulation and acid degradation. Ripening index and CCI are consistent indicators of fruit maturation in blood oranges. Among the rootstocks evaluated citrandarins, Bitters and Furr were effective in causing earlier maturation of fruits. It is

possible to conclude that rootstock genotype can exert important influences on citrus fruit quality by affecting gene expression in the scion.

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