Citrus R&T (e-ISSN 2236-3122)
Citrus Research & Technology, v. 38, n. 1, 2017
http://dx.doi.org/10.4322/crt.ICC068

Artigo/Article

Study of the relationship between flavonoids content and changes in the enzymes implicated in their biosynthetic pathway, during development of *Citrus sinensis* cv. Sanguinelli fruits

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SUMMARY

Citrus sinensis (L.) Osbeck cv. Sanguinelli is the most common blood orange cultivated in Spain. Flavonoids as hesperidin and naringin are the most important plant secondary metabolites present in *Citrus* fruits. These are the phenolic compound most predominant in *Citrus* fruits and juices. Some *Citrus* fruits, as the blood orange, contain also anthocyanins as cyanidin 3-glucoside, cyanidin 3.5-diglucoside, delphinidin 3-glucoside, peonidin 3-(6"-malonylglucoside), and cyanidin 3-(6"-dioxalylglucoside). These flavonoids have been shown to be antioxidants, free radical scavengers, quenching capacity of singlet oxygen, superoxide anion scavenging activity, and antilipoperoxidant activity. The aim of this study is to deepen in knowledge about changes of main flavonoids and anthocyanins accumulation levels and to study the enzymes that regulate the anthocyanins biosynthesis during the ripening process. The levels of main flavanones and anthocyanins presents in fruits during fruits development have been identified and quantified by HPLC-MS/MS and HPLC-DAD-TOF. Also, transcriptions levels of 6 genes directly implicated in anthocyanins biosynthesis have been study by RT-PCR during this period. The selected genes were (i) chalcone synthase (CHS); (ii) flavonoid-3-hydroxilase (F3H); (iii) dihydroflavonol 4-reductase (DFR); (iv) anthocyanidin synthase (ANS); (v) UDP-glucose-flavonoid-glucosyltransferase UFGT; (vi) glutation-S-transferase (GST). The results show that accumulation of anthocyanins is produced in pulp, starting in October-November (when temperature begins to decrease). During this period, the expression of related genes with the biosynthesis pathway, as ACS, DFR and GST, increase in a significantly way (amounts of 1400, 1800 and 12000 fold expression respectively in contrast to the basal levels). The relationship among flavonoids content and the degree of expression of enzymes implicated in their biosynthetic pathway permits to determine which are the enzymes responsible for control the biosynthesis of these secondary metabolites in *Citrus*.

Index terms: cyanidin 3-glycoside, cianidin 3-(6" malonyl glucoside), peonidin 3-(6" malonyl glucoside), hesperidin, chalcone synthase, flavonoid-3-hydroxilase, dihydroflavonol 4-reductase, anthocyanidin synthase, UDP-glucose-flavonoid-glucosyltransferase, glutation-S-transferase.

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Estudo da relação entre o conteúdo de flavonoides e as alterações das enzimas implicadas no processo biosintético, durante o desenvolvimento de frutos de *Citrus sinensis* cv. Sanguinelli

RESUMO

Citrus sinensis (L.) Osbeck cv. Sanguinelli é a laranja sanguínea mais cultivada na Espanha. Os flavonoides hesperidina e a naringina são os metabólitos secundários mais importantes, presentes em frutas cítricas. Estes são também os compostos fenólicos predominantes em frutas cítricas e sucos. Alguns citros, como as laranjas sanguíneas, contêm também antocianinas como cianidina 3-glucosidio, cianidina 3,5-diglucosidio, delfinidina 3-glucosidio, peonidina 3-(6"-malonilglucosidio) e cianidina 3-(6"-dioxalilglucosidio). Estes flavonoides demonstraram ter atuação como antioxidantes, eliminadores de radicais livres, com capacidade de extinguir o oxigênio *singlete*, ativadores de eliminação de ânions superóxido, além da atividade antilipoperoxidante. O objetivo deste estudo foi aprofundar o conhecimento sobre mudanças nos principais níveis de acumulação de flavonoides e antocianinas, além de estudar as enzimas que regulam a biossíntese de antocianinas durante o processo de amadurecimento. Os níveis das principais flavanonas e antocianinas presentes em frutas durante o desenvolvimento dos mesmos, foram identificados e quantificados por HPLC-MS/MS e HPLC-DAD-TOF. Além disso, os níveis de transcrição de seis genes diretamente implicados na biossíntese de antocianinas foram estudados por RT-PCR durante esse período. Os genes selecionados foram (i) chalcone sintase (CHS); (ii) flavonoide-3-hidroxilase (F3H); (iii) di-hidroflavonola 4-redutase (DFR); (iv) antocianidina sintase (ANS); (v) UDP-glicose-flavonoide-glucosiltransferase UFGT; (vi) glutatione-S-transferase (GST). Os resultados mostram que o acúmulo de antocianinas ocorre na polpa, começando em outubro-novembro (quando a temperatura começa a diminuir). Durante este período, a expressão de genes relacionados com a via de biossíntese, como ACS, DFR e GST, aumenta de forma significativa (quantidades de 1400, 1800 e 12000 vezes de expressão, respectivamente, em contraste com os níveis basais). A relação entre o conteúdo de flavonoides e o grau de expressão de enzimas implicadas na via biossintética permite determinar quais são as enzimas responsáveis pelo controle da biossíntese desses metabólitos secundários em *Citrus*.

Termos de indexação: cianidina 3-glicosidio, cianidina 3-(6" malonil glucosidio), 3- peonidina 3-(6" malonil glucosidio), hesperidina, chalcone sintase, flavonoide-3-hidroxilase, di-hidroflavonola 4-redutase, antocianidina sintase, UDP-glucose-flavonoide-glucosiltransferase, glutatione-S-transferase.

INTRODUCTION

The genus *Citrus* is one of the most relevant crops in the world, with a production upper than 100 million tonnes per year . This fact is due in part to the large number of bioactive compounds beneficial to human health that present the fruits of this genus (Bernardi et al., 2010).

Sweet orange [*Citrus sinensis* (L.) Osbeck] fruits are very rich in secondary metabolites, such as carotenoids, vitamin C or flavonoids. Among the flavonoids, there are different classes such as flavanones, flavones or polimexiflavones among others, being probably the most important secondary metabolites present in *Citrus* genera, because they have a great physiological relevance and a great pharmacological potential. No other class of secondary metabolites has had so many key functions for the growth of the plant and its development, often becoming indispensable for the plant's own survival. Some examples of this are their involvement in the attraction of pollinating insects to aid in seed dispersal, they participate in the prevention of tissue damage by photooxidation (Feild et al., 2001) or stimulation of new shoots. In addition, flavonoids have also been described as potent phytoalexins (Arcas et al., 2000; Harbone & Grayer, 1993; Hedin & Jenkins, 1977).

It is worth mentioning its enormous potential use as pharmaceutical compounds, since its antioxidant activity has been recognized in many studies, as well as free radical scavengers, superoxide anion scavenging and antilipoperoxidant activities (Gould & Lister, 2006). They may also act as modulator substances in the carcinogenesis process, presenting antimutagenic and anticarcinogenic effects (Liao et al., 2013; Park et al., 2012; Stavric, 1994).

Anthocyanins belong to the family of flavonoids and are a class of water-soluble metabolites with a reddish pigmentation, which are in the fruits and leaves of blood oranges (a special type of sweet oranges). There are a lot of varieties, but *C. sinensis* cv Sanguinelli is the

most cultivated in Spain, having a great nutritional value due to an accumulation a great concentrations of anthocyanins in pulp. As with the rest of the flavonoids, anthocyanins and their glycosides, anthocyanidins have important nutritional and pharmaceutical properties, very relevant to human health (Barreca et al., 2011a, 2011b, 2013; Chinapongtitiwat et al., 2013; Zhang et al., 2011; Nogata et al., 2006; Nielsen et al., 2006; Del Río et al., 2004; Kefford & Chandler, 1970; Horowitz, 1964).

The anthocyanin biosynthesis pathway is well known and the genes involved have been identified (Holton & Cornish, 1995). The general scheme of biosynthesis of anthocyanins is shown in Figure 1, which begins with the removal of the ammonium group of L-phenylalanine by the enzyme phenylalanine ammonia liase (PAL) to form trans-cinnamate, from here the pathway is branched out to give different flavonoids structures. In the case of anthocyanins and flavanones, the first step is catalyzed by chalcone synthase (CHS), which condenses malonyl-CoA and 4-coumaroyl-CoA to form tetrahydro chalcone. This compound is isomerized to flavanone naringenin. From naringenin, by the action of flavonol 3-hydroxylase and caffeoyl-CoA-O-methyltransferase is transformed into hesperetin and subsequently would give rise to hesperidin glucoside.

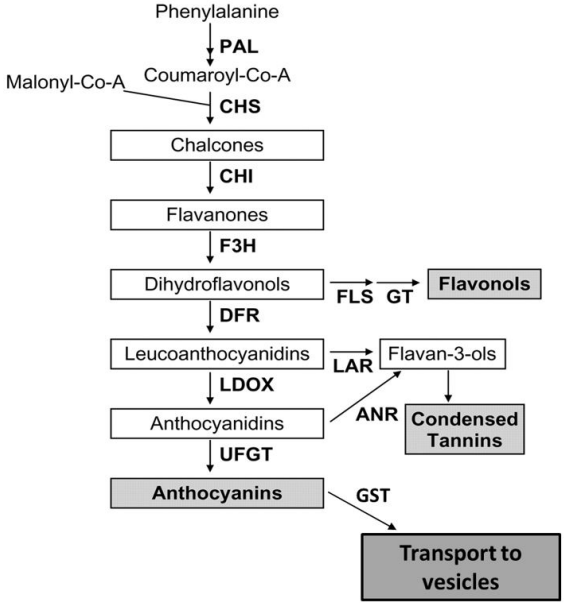

Figure 1. Schematic of the route of biosynthesis of flavonoids and anthocyanins. Framed in red the enzymes analyzed in this study. Chalcone synthase (CHS), flavanone-3-hydroxylase (F3H), dihydroflavonol reductase (DFR), glutathione-S-transferase (GST), the latter related to flavonoid oxidation processes, anthocyanidin synthase (ACS or LDOX), and anthocyanidin-3-glycosyl transferase.

On the other hand, naringenin is converted into dihydrokaempferol by hydroxylation. Anthocyanin 3-O-glycosides are synthesized by consecutive reactions catalyzed by dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS), and UDP-glucose flavonoid glucosyl transferase (UFGT) (Dixon & Paiva, 1995). In addition, these metabolites are usually accumulated in vacuoles, so they have to be activated by the enzyme glutathione-S-transferase (GST) for later transport to these accumulation organelles.

The biosynthesis of anthocyanins is regulated by various environmental stimuli such as light or water stress, osmotic or low temperatures, which can activate the transcription of these genes.

Therefore, the objectives proposed in this work are, on the one hand, to identify and quantify the main flavonoids present in the most common orange-blood variety in Spain (*C. sinensis* cv. Sanguinelli), and on the other hand to analyze the changes in expression levels of the major enzymes involved in anthocyanin biosynthesis such as CHS, F3H, DFR, ANS, UFGT and GST.

MATERIAL AND METHODS

Plant material

To perform this study, inflorescences and fruits of *C. sinensis* cv. Sanguinelli were used. The cultivar "Sanguinelli" was originated in Spain by spontaneous mutation of Doble Fina orange (Soler, 1999). For the realization of the present study, 5 adult trees of about 20 years of age were used. All of them are grafted on Cleopatra mandarin (*C. reshni* Hort. Ex Tanaka), to a planting frame of 5 × 4 m. All trial trees are located on the experimental farm of the Institute of Agricultural and Food Research (IMIDA), in La Alberca (Murcia). Monthly samples of the five trees were taken, each tree being considered as a sampling unit, during a natural year, to determine the changes in phenolic compounds (flavonoids and anthocyanins) during fruit development.

Samples of inflorescences and different stages of the flower (A: closed flower, B: open flower, C: petal fall and D: fruit set), immature and ripe fruits were taken from each tree or sample unit, gathered in all the heights of the

tree in a complete turn, in the exterior and interior of the same, to unify the zones sunnier and the less.

Extraction, identification and quantification of hesperidin

For the extraction of flavonoids, the plant material was dried immediately after being collected in a forced air stove at 50 °C. They were then ground and extracted with dimethylsulfoxide (DMSO) (Castillo et al., 1992) for 24 h in a ratio of 10 mg dw/mL. After decanting the residue, the liquid phase was filtered through 0.45 µm nylon filters and dispensed into vials for further analysis by HPLC.

For the quantification of hesperidin, the protocol described by Diaz et al. (2015) was used a Jasco Liquid Chromatography system equipped with a Jasco quaternary pump (model MD-2010Plus), a Jasco photodiode array detector (model MD-2010 Plus), and a Jasco autosampler (model AS-2055 Plus). The stationary phase was a LiChroCARTR C18 (Agilent, USA) analytical column with an average particle size of 5 mm (250 × 4 mm i.d.) at 30 °C. The flavonoids were separated using a binary gradient of water: methanol: acetonitrile: acetic acid (15: 2: 2: 1, v/v) as solvent (A) and acetonitrile (ACN) as solvent (B). The initial solvent composition consisted of 100% (A) for 40 min. Then, the solvent composition changed in a linear gradient to 20% (A) for 30 min. Between 70 and 80 min, the composition was maintained and then the solvent composition changed in a linear gradient to 100% (A). The eluent flow was 1 mL/min. Changes in absorbance were recorded in the UV / Vis diode array detector at 280 and 350 nm. The quantities of these secondary compounds are determined from the area given by the integrator using the response factor of the corresponding standards. Identification of these compounds was carried out by HPLC-MS/MS with an Agilent model VL ion trap spectrometer equipped with ESI interface coupled to HPLC (Agilent 1100). A 5 mm (250 × 4 mm i.d.) C18 Kromasil 100 (Tecnokroma) column was used for the separation, which was performed by means of a similar elution gradient to that described above to quantify the flavonoids. The column was maintained at 30 °C. ESI mass spectra were acquired in both positive and negative ion modes by scanning over the 50-1000 mass range. The ESI parameters were: source voltage 3.5 kV, dry temperature 350 °C, nebullizer 60 psi, and dry gas 9 L / min.

A mass spectrometer with time-of-flight analyzer (TOF) (MS-TOF 6220) with a mass range of 50-3000 m/z was used for the analysis of exact mass spectrometry (HPLC-DAD-TOF) Ionization by an Electrospray-APCI dual source in both positive and negative mode and with a resolution of 16,000, allowing analysis of masses with an error of less than 2 ppm coupled to an Agilent 1200 series (Meyrin, Switzerland) double pump HPLC unit Binary, degasser, automatic sampler and DAD.

Extraction, identification and quantification of anthocyanins

For the extraction of anthocyanins, 8 fruits were squeezed and the juice was homogenized. It was then centrifuged for 30 minutes at 1500 rpm and filtered through glass wool. Once the juice has been centrifuged and filtered, an aliquot of 500 µL of juice is taken and mixed with 9.5 mL of methanol with HCl (1% v/v). To quantify total anthocyanins, the absorbance of the filtrate was measured with a UNICAM UV500 spectrophotometer at 530 nm, corresponding to its maximum absorption (Rapisarda et al., 2000). To quantify the amount of anthocyanins present in the sample, a standard straight line was made with different concentrations of known sample and we checked the response obtained. The standard used was cyanidin 3-glucoside. In this way the corresponding conversion coefficients were obtained to quantify the amount of anthocyanin present in each sample.

Major anthocyanins were identified by reverse phase high pressure liquid chromatography (HPLC) with this elution method: solution A, formed by H_2O at pH 2 (reduced with formic acid) and solution B, composed by acetonitrile (ACN). The different anthocyanins were separated by gradient elution, with an initial composition of 100% (A) for 3 min, increasing the gradient to 70% (A) and 30% (B) during the next 30 minutes. Then, it is increased to 90% (B) for 5 min, to return to the initial conditions. The flow rate was 1 mL/min. The changes in absorbance at 280 nm and at 520 nm were detected by the diode array detector. Concentrations of the metabolites were determined by the area given the integrator using the response factor of the different standards. For the identification of the different compounds, they were based on the retention time, UV/Vis

absorption spectra, rupture pattern (HPLC-MS / MS) and exact mass (HPLC-DAD-TOF).

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from each sample, depending on the stage of development (inflorescence, immature fruit or mature fruit pulp). They were then ground mechanically after freezing with liquid nitrogen. The total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, The Netherlands) and was further treated with DNase (Recombinat.DNase I, Takara, Shiga, Japan) to remove any contamination with genomic DNA, and subsequently verified that the quality Of the RNA was the optimal (Abs 260 / 280 ≥ 1.9 and Abs 260 / 230 ≥ 1.9).

Expression analyses by RT-PCR

The cDNA was synthesized using the High Capacity RNA to cDNA Kit (Applied Biosystems, California, USA). In Table 1, the primers used for the analysis of the transcription levels of the different enzymes studied are shown. Quantitative PCR analyzes were performed, by triplicate, on 96-well plates using a 7500 Fast Real Time PCR System (Applied Biosystems, California, USA). Each reaction had a total volume of 20 μL, containing 10 μL of SYBR Green 2x (Takara), 100 ng of cDNA and each primer at a concentration of 0.4 μM. The program for the reaction was as follows: an initial stage of activation at 95 °C for 10 minutes, followed by 40 cycles consisting of three phases (95 °C for 5 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds) and one final elongation phase of 2 minutes at 72 °C.

The relative expression levels of each gene studied were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), using the elongation factor-1α as the endogenous reference gene.

RESULTS AND DISCUSSION

Changes in main flavonoids and anthocyanins during ripening of *C. sinensis* cv. Sanguinelli fruits

The study of phenolic compounds, by HPLC-MS/MS and HPLC-DAD-TOF, in *C. sinensis* cv. Sanguinelli reveal that hesperidin is the major flavonoid and cyanidin 3-glucoside and peonidin 3- (6" malonylglucoside) are the major anthocyanins present in these fruits.

Figure 2 shows the evolution of these metabolites during the fruit development and ripening period. For this, periodic samples in different stages of development (inflorescences, immature fruits and pulp of mature fruits) were analyzed during a year and the changes in the concentration of these compounds with the time were determined. It is observed that in the early stages of development, there is a high accumulation of hesperidin, after fruit setting, and it

Table 1. Sequences of primers used for the RT-PCR assays

Gene name	Primer name	Primer sequence	Reference
Chalcone synthase	CHS_for	**AATATTGCCCGATAGCGATG**	Bernardi et al. (2010)
	CHS_rev	**CCGAGATACCCAATGGTTTG**	
Flavanone 3-hydroxilase	F3H_for	**CCTCAACCCAAGCTTTGTCC**	Bernardi et al. (2010)
	F3H_rev	**AGTCCTCACACGCCTCAACA**	
Dihydroflavonol 4-reductase	DFR_for	**GCTGTTCGTGCTACTGTTC**	Lo Piero et al. (2006)
	DFR_rev	**GTTTCCCTCTTCGGCTAAATC**	
Anthocyanidin synthase	ACS_for	**GCCTAGCGACGAAAATTCTG**	Bernardi et al. (2010)
	ACS_rev	**CAGTTCTGGTTGAGGGCATT**	
UDP-glucose flavonoid glucosyl transferase	UFGT_for	**TCTTCAGCACTCCGCAATC**	Lo Piero et al. (2005)
	UFGT_rev	**TCCATCGGATACGTCGTAAG**	
Glutathione-S-transferase	GST_for	**TGTTAATGCTTGGTGGGACA**	Bernardi et al. (2010)
	GST_rev	**GCCAGCCGAAATAACAAAAA**	
Elongation factor 1 alpha	EF-551_for	**TCTCTGGTTTCGAGGGTGAC**	Bernardi et al. (2010)
	EF-696_rev	**AACATCCTGGAGTGGCAGAC**	

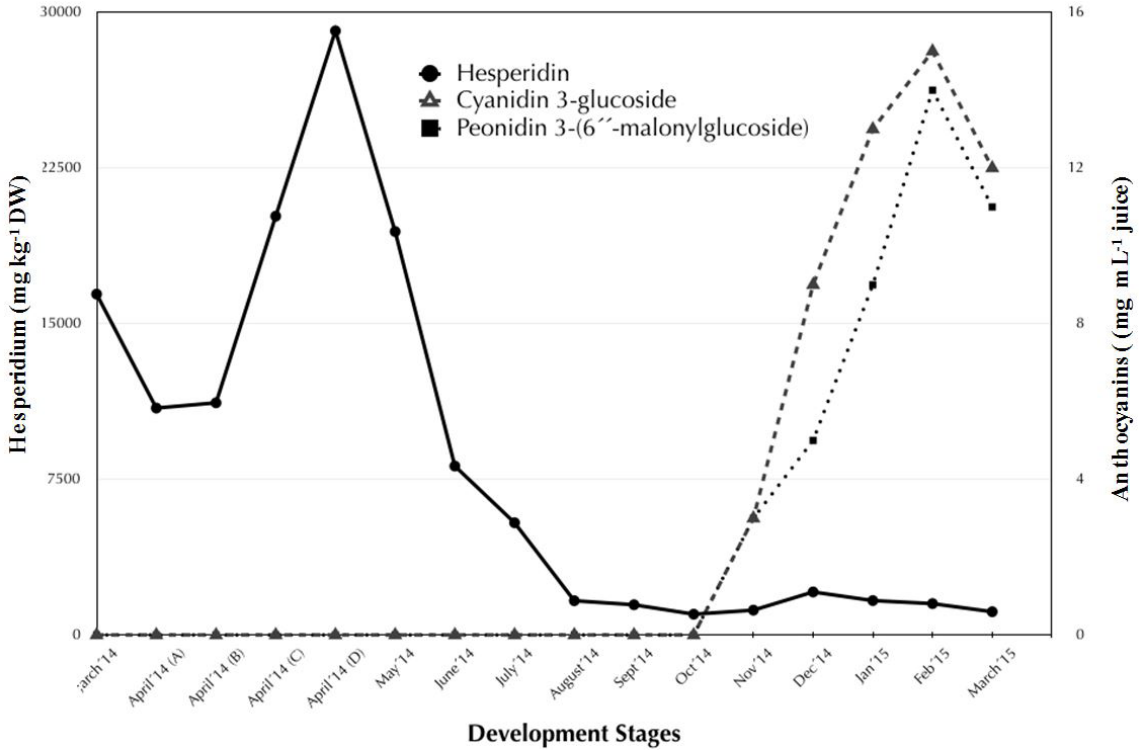

Figure 2. Accumulation of hesperidin and anthocyanins in *Citrus sinensis* cv. Sangunielli fruits during development and ripening stages.

decreases during the next development and maturation phases. In the case of anthocyanins, the biosynthesis increases in the winter period, when temperatures begin to decrease. In this sense, it is known that low temperatures induce the accumulation of anthocyanins in *Citrus* fruits (Lo Piero et al., 2005). It is the first time that is described the evolution of different flavonoids (flavanones and anthocyanins) in fruits of *C. sinensis* cv. Sanguinelli.

Expression analysis of implicated genes in biosynthesis pathway of hesperidin and anthocyanins in fruits during ripening phases

As for the analysis of the genes involved in anthocyanin biosynthesis, 6 enzymes directly related to their biosynthesis and accumulation has been selected. In Figure 3, the expression level of different genes and the anthocyanins content in fruits during ripening phases are shown.

Firstly, we can observe how these genes are activated in the winter stage, but not in the early stages of the development fruit, where the accumulation of hesperidin occurs. These results suggest that the accumulation of hesperidin comes from a source external to the fruit, and this occur in high intensity during first stages of fruit development. However, in the case of anthocyanins, the biosynthesis of these compounds occurs in the same tissues of the fruit.

On the other hand, all the genes studied show different increase in their transcription levels, being the highest for ACS, DFR and GST, which suffer increases with respect to their basal levels above 1200, 1700 and 10000 times. In contrast, the other genes studied showed very small increments, as in the case of CHS, increases occur only 14 times, for F3H of 14.3 and for UFGT of 14.6.

From these results, we can deduce that ACS, DFR and GST are key enzymes for control of the biosynthesis of anthocyanins in these fruits. Similar results have been observed for these enzymes in other species (Honda et al., 2002).

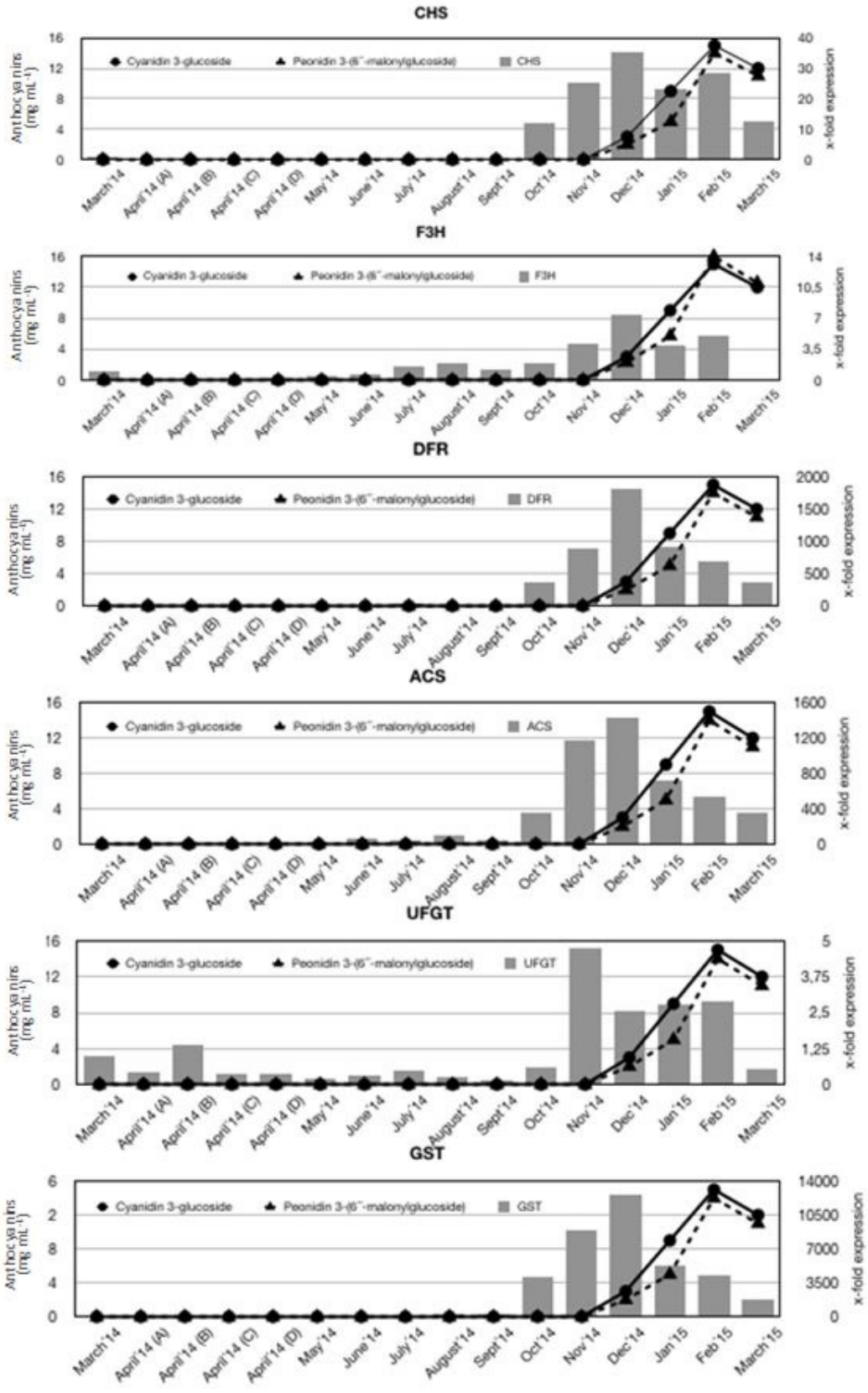

Figure 3. Expression levels of CHS, F3H, DFR, ACS, UFGT and GST in *Citrus sinensis* cv. Sanguinelli fruits during development and ripening stages, in comparison with accumulation levels of anthocyanins.

CONCLUSIONS

In conclusion, our results suggest that the fruits of the blood orange cv. Sanguinelli accumulate different secondary metabolites of interest in different phase of their development. In first stages of development, hesperidin is the principal phenolic compounds, and during the stage of maturation accumulates anthocyanins. Also, it is necessary to emphasize the importance of the enzymes DFR, ACS and GST, for being directly involved in the control of anthocyanin biosynthesis in these fruits. Deepen in knowledge in this genes and their induction would be of vital importance to increase their expression and obtain high contents in these healthy metabolites.

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Received: February 08, 2017
Accepted: October 26, 2017