**Characterization of the *Pale Yellow Petal/Xanthophyll esterase* gene family in citrus
as candidates for carotenoid esterification in fruits**

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Table 3

Concentration of carotenes, free and esterified xanthophylls and C30 apocarotenoid β -citraurin in flavedo and pulp of mature fruits of clementine mandarin (*Citrus clementina* cv. Clemenules), lemon (*Citrus limon* cv. Fino) and pummelo (*Citrus maxima* cv. Pink). Concentration given as mean $\pm$ standard deviation.

	Concentration [$\mu\text{g g}^{-1}$ FW]					
	Clementine		Lemon		Pummelo	
	<i>Flavedo</i>	<i>Pulp</i>	<i>Flavedo</i>	<i>Pulp</i>	<i>Flavedo</i>	<i>Pulp</i>
Carotenes	10.92 $\pm$ 0.89	3.68 $\pm$ 0.41	12.19 $\pm$ 0.24	0.05 $\pm$ 0.01	120.38 $\pm$ 9.12	0.24 $\pm$ 0.01
Free xanthophylls	6.55 $\pm$ 2.54	1.12 $\pm$ 0.08	2.29 $\pm$ 0.09	0.02 $\pm$ 0.00	2.57 $\pm$ 0.05	0.07 $\pm$ 0.01
Esters of xanthophylls ^a	123.90 $\pm$ 5.65	9.92 $\pm$ 0.05	0.38 $\pm$ 0.02	0.02 $\pm$ 0.00	1.40 $\pm$ 0.08	n.d.
Esters of β -citraurin ^a	8.31 $\pm$ 2.54	n.d	n.d	n.d	n.d	n.d
Total carotenoids	147.18 $\pm$ 17.90	14.56 $\pm$ 0.61	14.87 $\pm$ 0.42	0.09 $\pm$ 0.01	123.21 $\pm$ 14.60	0.31 $\pm$ 0.02

^aTentative identification. FW, fresh weight. n.d. Not detected.

Table 2

Concentrations of carotenes, free xanthophylls and xanthophyll esters in the flavedo and pulp of sweet oranges (*C. sinensis* cv. Navel) fruit harvested at four different maturity stages (*G*, green; *GB*, green-breaker; *M*, mature; *FM*, fully-mature). Concentration given as mean $\pm$ standard deviation ($n = 2$).

	Concentration [$\mu\text{g g}^{-1}$ FW]							
	Flavedo				Pulp			
	G	GB	M	FM	G	GB	M	FM
<i>Carotenes</i>								
Phytoene	0.84 $\pm$ 0.01	0.65 $\pm$ 0.03	2.68 $\pm$ 0.08	4.62 $\pm$ 0.11	n.d.	n.d.	0.06 $\pm$ 0.00	0.06 $\pm$ 0.00
Phytofluene	0.27 $\pm$ 0.00	0.21 $\pm$ 0.01	1.90 $\pm$ 0.12	3.98 $\pm$ 0.22	n.d.	n.d.	tr.	0.05 $\pm$ 0.00
α -Carotene	7.96 $\pm$ 0.30	1.22 $\pm$ 0.56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β -Carotene	5.88 $\pm$ 0.21	0.88 $\pm$ 0.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Free xanthophylls</i>								
Violaxanthin	11.84 $\pm$ 0.26	12.26 $\pm$ 0.87	3.48 $\pm$ 0.06	2.67 $\pm$ 0.17	0.12 $\pm$ 0.10	0.05 $\pm$ 0.01	0.23 $\pm$ 0.00	0.37 $\pm$ 0.02
(9Z)-Violaxanthin + (all-E)-antheraxanthin	4.79 $\pm$ 0.06	3.50 $\pm$ 0.12	2.88 $\pm$ 0.08	1.68 $\pm$ 0.10	n.d.	n.d.	0.19 $\pm$ 0.00	0.29 $\pm$ 0.02
Neoxanthin	3.93 $\pm$ 0.30	1.89 $\pm$ 0.28	n.d.	n.d.	tr.	n.d.	n.d.	n.d.
Zeaxanthin	3.47 $\pm$ 0.01	n.d.	tr.	1.69 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.
Lutein	57.95 $\pm$ 0.05	21.18 $\pm$ 1.22	tr.	n.d.	n.d.	n.d.	tr.	0.48 $\pm$ 0.01
<i>Esters of xanthophylls and β-citraurin</i>								
Vlx esters + UUX esters ^a (% Vlx esters)	n.d.	tr.	43.69 $\pm$ 1.15 (55%)	99.00 $\pm$ 2.54 (62%)	n.d.	1.59 $\pm$ 0.27 (72%)	4.37 $\pm$ 0.00 (61%)	11.05 $\pm$ 0.18 (63%)
Antheraxanthin esters	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.18 $\pm$ 0.00
β -Citraurin esters	n.d.	n.d.	4.65 $\pm$ 0.01	10.76 $\pm$ 0.34	n.d.	n.d.	n.d.	n.d.
<i>Total</i>								
Carotenes	14.95 $\pm$ 0.50	2.96 $\pm$ 1.11	4.58 $\pm$ 0.20	8.60 $\pm$ 0.33	n.d.	n.d.	0.06 $\pm$ 0.00	0.11 $\pm$ 0.00
Free xanthophylls	81.97 $\pm$ 0.04	38.83 $\pm$ 2.17	6.36 $\pm$ 0.14	6.04 $\pm$ 0.28	0.50 $\pm$ 0.02	0.05 $\pm$ 0.01	0.42 $\pm$ 0.00	1.14 $\pm$ 0.04
Esters of xanthophylls and β -citraurin	n.d.	n.d.	48.34 $\pm$ 1.14	109.76 $\pm$ 2.88	n.d.	1.59 $\pm$ 0.27	4.37 $\pm$ 0.00	12.23 $\pm$ 0.19

^a Percentage of violaxanthin esters represented in brackets. *FW*, fresh weight. *Vlx*, violaxanthin; *UUX*, unresolved and unknown xanthophyll; *n.d.*: not detected (below LOD at a signal-to-noise ratio of 1:20). *tr.*: traces (below LOQ at a signal-to-noise ratio of 1:30).

Table 1

Genomic and structural characteristics of *PYP/XES* gene family in sweet orange (*C. sinensis*).

Gene name	Genomic code ^a <i>Citrus sinensis</i>	Predicted Introns	Predicted protein length (aa) ^b	Chloroplast transit peptide ^c
<i>PYP1</i>	orange1.1g041641m	14	713	Yes (46)
<i>PYP2</i>	orange1.1g004396m	12	756	Yes (73)
<i>PYP3</i>	orange1.1g006325m	13	650	Yes (26)
<i>PYP4</i>	orange1.1g005300m	13	703	Yes (74)
<i>PYP5</i>	orange1.1g013862m	7	435	No
<i>PYP6</i>	orange1.1g005190m	13	709	Yes (45)
<i>PYP7</i>	orange1.1g016023m	8	396	No

^aJGI, Phytozome 12, Sweet Orange Genome (*Citrus sinensis* v1.1, 2014). ^b aa, amino acid.

^c ChloroP 1.1. Prediction Server, in parenthesis is indicated the predicted length of transit peptide.

Abstract

In orange-pigmented citrus fruits, the xanthophyll esters are the predominant carotenoids, but their biosynthetic origin is currently unknown. In this work seven *PYP/XES* (*Pale Yellow Petal/ Xanthophyll esterase*) genes were identified in *Citrus* genomes, but only PYP1-4 and 6 contained the structural domains essential for activity. The *PYP/XES* expression profiles in sweet orange and in other *Citrus* species such as lemon, mandarin and pummelo with marked differences in fruit pigmentation and content of xanthophylls esters, showed the upregulation of *PYP1,2* and *6* genes during ripening only in orange-pigmented fruits. Moreover, transcript levels of *PYP1*, *2* and *6* genes in peel and pulp of sweet orange were accompanied by the accumulation of xanthophyll esters during ripening. This work reports for the first time the *PYP/XES* gene family in *Citrus* and strongly suggests its involvement in xanthophyll esterification in citrus fruit tissues and its influence in carotenoid accumulation and fruit pigmentation.

Keywords:

Carotenoid; Citrus fruit; Coloration; Gene expression; Xanthophyll acyl-transferase; Esterification; Ripening; Sweet orange

1. Introduction

The colors of citrus fruit are imparted by carotenoids and chlorophylls, which are prone to dynamic accumulation and degradation during ripening (Tadeo, Terol, Rodrigo, Licciardello, & Sadka, 2020). The contribution of specific carotenoids to the characteristic fruit color of the different citrus species has been analyzed in detail (Fanciullino, Casanova, Morillon & Ollitrault, 2006; Matsumoto, Ikoma, Kato, Kuniga, Nakajima & Yoshida, 2007; Xu, Fraser, Wang, & Bramley, 2006). The main carotenoids in fruits of orange-pigmented citrus are the xanthophylls violaxanthin, β -cryptoxanthin and antheraxanthin, whereby the (all-*E*)- and (9*Z*)-isomers are prevailing. These xanthophylls are predominantly acylated with fatty acids in mature fruits (Eilati, Budowski, & Monselise, 1972; Philip, 1973). However, due to the high complexity of the carotenoid profile in citrus and technical challenges for an accurate identification of carotenoid esters, analytical investigations dedicated to the elucidation of the ester profiles are limited to the last years. The genuine carotenoid profile in sweet orange and mandarin indicated that the esterification pattern is species- and cultivar-specific, comprising mainly mono- or diesterified xanthophylls with saturated (capric C10:0, lauric C12:0, myristic C14:0, palmitic C16:0 and stearic C18:0) and monounsaturated (myristoleic C14:1, palmitoleic C16:1 and oleic C18:1) fatty acids (Lux, Carle, Zacarías, Rodrigo, Schweiggert & Steingass, 2019; Giuffrida et al., 2019; Ma et al., 2017; Petry & Mercadante, 2016 and 2017). In addition, the peel of mandarins and sweet oranges contains β -citraurin, a hydroxy-C30-apocarotenoid, which determines their orange-reddish tint (Rodrigo et al., 2013a; Ma et al., 2017; Zheng et al., 2019). Its accumulation also occurs preferentially in an esterified form (Lux et al., 2019; Ma et al., 2017).

75 Acylation of xanthophylls with fatty acids increases their lipophilic character. This
76 modification is a crucial requirement for their storage in the chromoplasts influences
77 xanthophylls stability and bioavailability (Mariutti & Mercadante, 2018). Xanthophylls
78 esters are believed to be primarily stored as nematic liquid crystals within tubular
79 structures and/or in the core of plastoglobuli chromoplasts (Schweiggert & Carle, 2017).
80 Recently, a novel xanthophyll acyl esterase (XAT), member of the GDGL
81 esterase/lipase (GELP) family, has been identified as responsible for lutein ester
82 formation in wheat grain (Ávila, Palomino, Hornero-Méndez, & Atienza, 2019;
83 Watkins et al., 2019). However, in flowers and fruits a protein related to the Phytol
84 Ester Synthase (PES) has been associated with xanthophyll esterification (Ariizumi et
85 al., 2014; Berry, Rickett, Baxter, Enfissi & Fraser, 2019; Kishimoto, Oda-Yamamizo &
86 Ohmiya, 2020). Tomato plants harboring non-functional *Pale Yellow Petal1* (*PYP1*)
87 gene, the homologous to *PES*, produced flowers with pale yellow color lacking
88 accumulation of xanthophyll esters and reduced free xanthophylls (Ariizumi et al.,
89 2014). In pepper fruit a good correlation between the expression of the *ripening-specific*
90 *Acyl-Transferase* (*rsAcT*) gene, the orthologue of *PYP1*, and capsanthin esters
91 accumulation has been reported (Berry et al., 2019). All these evidences strongly
92 suggest that xanthophyll esterification in flowers and fruits is catalyzed by PYP/rsAcT,
93 so called XES (Xanthophyll esterase), facilitating their accumulation in carotenoid rich
94 organs. All the PYP/XES/rsAcT sequences share homology with PES and conserve the
95 functional α/β hydrolase fold and acyltransferase domains (Lippold et al., 2012).

96 In citrus fruits only few studies have explored the changes in the profile and rate of
97 xanthophyll esterification during ripening (Eilati et al., 1972; Lux et al., 2019; Ma et al.,
98 2017). In green or immature citrus fruit, xanthophyll esters are absent and their
99 synthesis in the peel is initiated during fruit degreening and at the onset of carotenoid

accumulation in the pulp (Eilati et al., 1972; Lux 2019; Ma et al., 2017). Aside from the significant role of esterification in the accumulation and bioavailability of xanthophylls, information regarding the enzymes involved in this activity in citrus fruits is lacking. Thus, the aim of the present work was to characterize *PYP/XES* genes in sweet orange and to investigate their potential role in xanthophyll esterification in citrus fruits. To that end, the expression profile of *PYP* genes was analyzed in flavedo and pulp of sweet orange during ripening and in fruits of other citrus species with contrasting accumulation of xanthophyll esters and carotenoid content. The structural characteristics of the different citrus PYPs and the relationship between the transcriptional patterns and the occurrence of carotenoid esters may permit to identify the best PYP candidates, and to investigate their implications in pigmentation and carotenoid accumulation of citrus fruits.

2. Material and Methods

2.1. Plant material

Navel oranges (*C. sinensis* cv. Foios) were harvested from an experimental orchard in Museros (Valencia, Spain; 39° 34' 10.8" N, 0° 21' 28.8" W; 15 m elevation above the sea level) and fruits of clementine mandarins (*C. clementina* cv. Clemenules), lemon (*C. limon* cv. Fino) and pummelo (*C. maxima* cv. Pink) were obtained from the Citrus Germplasm Bank at the IVIA (Instituto Valenciano de Investigaciones Agrarias) located in Moncada (Valencia, Spain; 39° 35' 22" N, 0° 23' 40" W; 55 m elevation above the sea level). Fruits were harvested from adult trees (more than 10 years old) budded onto Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] rootstock, growing in a loamy-sand soil with drip irrigation. Fertilization, pruning, pest

management and other cultural conditions were performed according to the protocol established by IVIA for the Citrus Germplasm Bank. For each genotype and sampling date two biological replicates were collected. Each biological replicate consisted of at least 15 fruits, except for pummelo where 5-10 fruit per replicate were collected. Fruits were randomly selected from two trees around the outside of the canopy. Fruits were delivered to the laboratory and selected for color uniformity and free of any peel defect or damage (Table S1). For the different species, fruits of, at least, four developmental stages were collected: July-August (immature green, G), September-October (green-breaker, GB and breaker, B), November-December (mature, M), and January-February (fully mature, FM), which correspond, depending on the cultivar, approximately to 120, 180-210, 240-270, and 270-310 days after anthesis (DAA), respectively (Table S1). For analysis of gene expression in Navel sweet orange fruit, the sampling dates were extended up to six developmental and ripening stages (Table S1). Young fully-expanded leaves (about 3 month-old) and petals from flower at bloom were collected from sweet orange Navel trees (Table S1). Immediately after harvest, all plant material was transported to the laboratory, frozen and ground to fine powder with an electric grinder in liquid nitrogen, and stored at -80 °C until analysis.

2.2. Carotenoid extraction and analysis by HPLC-DAD-APCI-MSⁿ and HPLC-DAD

2.2.1 Sweet orange

Carotenoids were extracted from 2.0 and 0.5 g of orange pulp and flavedo, respectively, using a mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) as reported previously (Lux et al., 2019). HPLC-DAD-APCI-MSⁿ (High-Performance Liquid Chromatography coupled to photoDiode Array Detector and Atmospheric Pressure

Chemical Ionization Multistage Mass Spectrometry) analyses were performed as reported in our earlier study (Lux et al., 2019) on the identification of individual carotenoids in samples identical to those of the present work. Carotenoids were then classified as carotenes (phytoene, phytofluene, α - and β -carotene), free xanthophylls (violaxanthin, antheraxanthin, neoxanthin, zeaxanthin, lutein), and esters of xanthophylls and β -citaurin, including their geometrical isomers. The group of xanthophyll esters comprised esterified violaxanthin, antheraxanthin and β -citaurin. In some instances, unspecified esters (possibly geometrical isomers other than (all-*E*)- and (9*Z*)-violaxanthin, structurally related 5,8-epoxides, or esters of xanthophylls other than violaxanthin) were not baseline resolved from prevailing violaxanthin esters or their unambiguous assignment was not possible. The latter peaks were quantified as violaxanthin and summarized as unresolved and unknown xanthophyll esters. Limit of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio of 1:20 and 1:30, respectively.

2.2.2 Mandarin, lemon and pummelo

Clementine mandarins, lemon and pummelo fruits at mature stage were used for carotenoid analyses (Table S1). Carotenoids from 1.0 and 4.0 g were extracted from pulp and flavedo, respectively, as indicated elsewhere (Lado, Zacarías, Gurrea, Page, Stead & Rodrigo, 2015). To determine the ratio of esterified to free xanthophylls, each extract was divided in two equivalent aliquots and one aliquot was saponified in a methanolic solution of potassium hydroxide (6% w/v) overnight at room temperature (20-22 °C). The non-saponified aliquot was kept at -20 °C overnight under nitrogen atmosphere to avoid carotenoid oxidation. Saponified carotenoids were diluted with ultrapure water and extracted with a petroleum ether:diethyl ether (9:1,v/v) solution. Non-saponified samples were dissolved in petroleum ether:diethyl ether solution and

ultrapure water and proceed as for saponified extracts as described by Lado et al. (2015). The saponified and non-saponified extracts were reduced to dryness under nitrogen stream and kept at -20 °C until HPLC-DAD (high-performance liquid chromatography coupled to photodiode array detector) analysis. Carotenoids were separated by HPLC using a C30 YMC column (250 × 4.6 mm i.d., particle size 5 µm), coupled to a C30 guard column (10 × 4.0 mm i.d., particle size 5 µm) (Teknochroma, Barcelona, Spain) with ternary gradient elution of methanol, water and methyl *tert*-butyl ether (Lado et al., 2015). Carotenoids were identified by comparing their retention times, UV/vis absorption and fine spectra to those reported in literature (Lux et al., 2019; Rodrigo et al., 2019). For each elution, a Maxplot chromatogram was obtained and carotenoid contents were calculated using calibration curves as described elsewhere (Rodrigo et al., 2019). Esterified xanthophylls and β-citraurin were quantified by using the calibration curves of the corresponding free xanthophylls and β-apo-8-carotenal, respectively. All operations were carried out under dimmed light to prevent photodegradation, isomerization and structural changes of the carotenoids. Results were the average of 2 independent biological replicates and 2 technical replicates.

2.3 Gene expression analysis by RT-qPCR

The RNA isolation, cDNA synthesis and gene expression analyses were performed essentially as described by Rodrigo et al. (2013b). The absence of DNA contamination was checked by performing a no-reverse transcription assay using the *ACTIN* primers. The sweet orange sequences were identified at the Phytozome v12 portal (<https://phytozome.jgi.doe.gov/>) and the primers design for gene expression was carried out using Primer3Plus online tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) (Table S2). The specificity of each reaction was assessed by the melting curve analysis and amplicon sequencing. Quantitative real-time

PCR was carried out on a LightCycler 480 instrument (Roche Diagnostics, Barcelona, Spain) following conditions described (Rodrigo et al., 2013a). The cycling protocol used for all genes consisted of 10 min at 95 °C, then 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. The Relative Expression Software Tool (Pfaffl, Horgan & Dempfle 2002) was used to calculate the expression levels relative to values of Navel sweet orange pulp of fruits harvested 250 days after anthesis. Normalization was performed using the expression levels of the *ACTIN* gene (Rodrigo et al., 2013a). Results were the average of 2 independent biological replicates and 2 technical replicates. Significant differences on transcript levels between samples were detected using a pair-wise fixed reallocation randomization test ($p \leq 0.01$) (Pfaffl et al., 2002).

3. Results and Discussion

3.1. Identification of PYP/XES gene family in sweet orange

The tomato *PYP1* (*Pale Yellow Petal*) and the orthologous genes from pepper (*rsAcT*, *ripening-specific acyl-transferase*), *Tagetes erecta*. and *Ipomea obscura* (*XES*, xanthophyll esterase) have been proposed as the enzymes responsible for xanthophyll esterification in flowers and fruits (Ariizumi et al., 2014; Berry et al., 2019; Kishimoto et al., 2020). The PYP/XES/rsAcT proteins are closely related to the *Arabidopsis* PES, which catalyzes the fatty acid phytyl ester synthesis in *Arabidopsis* chloroplasts (Lippold et al., 2012). In order to identify the closest homologous *PYP/XES/rsAcT* genes from sweet orange, we searched in the 'Sweet Orange Genome' (Joint Genome Institute, Phytozome 12, *Citrus sinensis* v1.1) using the sequences of tomato *PYP1* (Solgenomics.net accession Solyc01g098110.2.1) and *Arabidopsis PES1* (TAIR

accession At1g54570.1) (Ariizumi et al., 2014; Lippold et al., 2012). A total of seven genes, denoted as *PYP1* to *PYP7*, were identified in the sweet orange genome (Table 1), with percentage of identity at the protein level with tomato PYP1 and Arabidopsis PES ranging from 48 to 66% (Table S3). It should be noted that similarity of the putative sweet orange PYPs and the homologous in other plants was high throughout the whole sequence and most of the variability was comprised in the first 100 amino acids at the N-terminal (Figure S1). Interestingly, all *PYP* genes were arranged in a cluster at chromosome 2 (Figure S2), with the exception of *PYP2* which was positioned at chromosome 3 (<http://citrus.hzau.edu.cn/orange/>). Analysis of the sweet orange *PYP* genes showed a common structure with 12 to 14 introns, except *PYP5* and 7 which contained 7 and 8 introns, respectively (Table 1). The elevated number of introns in the sweet orange *PYP* genes is a common feature to the tomato *PYP1* (13 introns) and pepper *rsAcT* (12 introns). A remarkable difference of the *PYP* sweet orange family is the large number of gene members (seven) compared to pepper and tomato with only two *PYP* genes (Ariizumi et al., 2014; Berry et al., 2019). The genomic organization and localization of *PYP* genes in *Citrus* suggests that the cluster comprising all *PYP* genes, except *PYP2*, originated by gene duplication and further divergence. Further search analysis of *PYP* genes in the genomes of the ancient citrus species *C. maxima/grandis* (pummelo), *C. reticulata* (mandarin) and *C. medica* (citron) available at the public databases (<http://citrus.hzau.edu.cn/>) revealed the presence of the seven *PYP* genes for the three species, indicating that the elevated number of *PYP* genes is a well conserved characteristic across the *Citrus* genus.

The length of the sweet orange PYP protein ranged between 756 to 650 amino acids, except PYP5 and 7 that were noticeably shorter with 435 and 396 amino acids, respectively (Table 1). Potential functional domains of PYPs were searched using the

InterProScan5 at EMBL-EBI (<https://www.ebi.ac.uk/interpro/about/interpro/>). The α/β hydrolase fold and the lysophospholipid acyltransferase (LPAT)-like domains were identified in all sweet orange PYPs; however, it should be noted that one or both domains were significantly shortened in PYP5 and 7 (Figure S1). The α/β hydrolase fold domain is characteristic of diacylglycerol transferases and the LPAT-like is found in enzymes that incorporate an acyl group from either acyl-CoAs or acyl-acyl carrier proteins (acyl-ACPs) into acceptors (Conserved Domain Architecture Retrieval Tool, NCBI). Both domains are consistently present in other plant PYP/XES/rsAcT and PES proteins (Figure S1; Ariizumi et al., 2014; Kishimoto et al., 2020; Lippold et al., 2012). Moreover, the LPAT domain is essential for xanthophyll acylation since the tomato *pyp1-1* mutant with a complete deletion of this motif fails to accumulate xanthophyll esters (Ariizumi et al., 2014). It has been proposed that sequence variability in this domain may be involved in substrate (fatty acid) specificity (Kishimoto et al., 2020).

The free and fatty acid acylated xanthophylls are synthesized and accumulated in plastids. Therefore, a plausible requirement for the esterification enzyme is the localization in this subcellular organelle (van Wyck & Kessler, 2017). Thus, the presence of plastid transit peptides in sweet orange PYPs and the potential cleavage sites was explored using the ChloroP 1.1 tool (Emanuelsson, Nielsen & von Heijne, 1999). The ChloroP predicted that PYP1, 2, 3, 4 and 6 contain chloroplast transit peptides at the N-terminus with a likely length from 26 to 74 amino acids (Table 1). The analysis failed to envisage the plastid localization of PYP5 and 7. Further evidence for plastid localization of the PYP1, 2 and 6 came from proteomic analysis since these proteins have been identified in the plastoglobuli and chromoplast proteomes of mature sweet orange juice vesicles (Zeng et al., 2015; Zhu et al., 2018). Therefore, the altered structural architecture of PYP5 and 7, lacking the plastid transit peptide and the full

acyltransferase domain, do not support their function as xanthophyll esterase in *Citrus* chromoplasts.

The relationship between the different PYP sweet orange proteins, the homologous tomato PYP and pepper rsAcT, and the related *Arabidopsis* PES1 was investigated by comparison of the sequences and a phylogenetic analysis (Fig. 1; Table S3). The percent of similarity and the phylogenetic tree revealed that orange PYP2 is the closest to tomato PYP1, pepper rsAcT and *Arabidopsis* PES1. The other citrus PYPs are clustered in an independent branch together with tomato PYP2. The sweet orange PYP2 shares between 62-65% identity with tomato PYP1, pepper rsAcT and *Arabidopsis* PES while similarity to other sweet orange PYPs was lower (49-56%). The similarity among citrus PYP1, 3, 4, 5, 6 and 7 proteins ranged between 62 to 77%. With the exception of PYP5 and 7, most of the sequence variability among the sweet orange proteins was restricted to the N-terminus region where the plastid transit peptide is predicted (Figure S1).

To investigate the degree of conservation of the PYP family in the genus *Citrus*, BLAST searches were performed against the genomes of pummelo (*C. grandis*), citron (*C. medica*), wild mandarin (*C. reticulata*) and kumquat (*Fortunella hindsii*), available at the '*Citrus sinensis* Annotation Project' (<http://citrus.hzau.edu.cn/>). The seven PYP genes were identified in all citrus and the percent of identity for PYP 2, 3, 4 and 6 among species was between 97.5 to 99.8% covering the whole sequence (Table S4), indicating a high conservation of these members across the *Citrus* genus. In the case of PYP5 and 7, the sequence variability was higher and the identity was between 83 and 86%. The analysis of PYP1 in the *Citrus* species revealed an interesting feature with potential functional implications. The predicted PYP1 from pummelo and citron, two of the ancient citrus species, lack the first 143 and 110 amino acids, respectively, at the N-terminus, which includes the predicted plastid signal peptide, while identity throughout

the rest of the protein sequence was very high to the PYP1 from sweet orange and mandarin (Table S4). The absence of the N-terminus domain in pummelo and citron PYP1 may result in a defective plastid import without access to xanthophylls for esterification. In the context of this study, it is important to note that the accumulation of xanthophylls is very low in pummelo and citron, in accordance with their low pigmented fruit tissues (Eilati et al., 1972; Fanciullino et al. 2006; Xu et al., 2006). Since the impairment of xanthophyll esterification is associated with a decrease in xanthophyll contents in chromoplastic tissues (Airizumi et al., 2014; Kishimoto et al., 2020), it is tempting to speculate that PYP1 mislocalization in the yellow-colored pummelo (*C. maxima/grandis*) and citron (*C. medica*) may directly affect the xanthophyll accumulation in these species.

3.2 Expression pattern of sweet orange PYP genes during fruit ripening and their correlation with carotenoid esterification profile.

Based on the protein structural characteristics, the PYP1, 2, 3, 4 and 6 are candidates to catalyze the esterification of xanthophylls in sweet orange. The PYP5 and 7 lack the full domains required for the activity (Ariizumi et al., 2014; Kishimoto et al., 2020) and the plastid signal peptide. Then PYP5 and 7 are unlikely functional acyltransferases and were not considered for further analysis. In order to characterize the genes *PYP1*, 2, 3, 4 and 6, their expression patterns were analyzed in during ripening of sweet orange, and related to the dynamic changes in individual free and esterified carotenoids.

The *PYP* genes showed different transcriptional profiles in both flavedo and pulp during fruit development and maturation (Fig. 2). All *PYP* genes showed the lowest expression level in the flavedo of immature green (G) fruits while the highest was reached at full-

mature (FM). However, different patterns were observed among them along ripening (Fig. 2). The expression of *PYP1* and *6* remained very low at G and green-breaker (GB) stages, and were enhanced after GB (Fig. 2) with the initiation of natural degreening and the accumulation of β,β -xanthophylls in the flavedo (Alquézar, Zacarías & Rodrigo, 2009; Lux et al., 2019). At full-mature (FM) stage, the relative transcript levels of *PYP1* and *PYP6* were 7- and 15-times higher than in G fruit, respectively. The *PYP3* and *4* showed similar expression profiles. Their transcripts remained at basal levels fairly constant during ripening and experienced a 4-fold increase at FM stage (Fig. 2). The *PYP2* showed a unique expression pattern in flavedo with a sustained increase in transcripts accumulation throughout fruit ripening (Fig. 2). In general, *PYP1*, *2* and *6* showed expression profiles similar to those reported for other carotenoid biosynthetic genes in the flavedo of sweet orange, such as phytoene synthase, ζ -carotene isomerase, chromoplastic lycopene β -cyclase and β -carotene hydroxylase, which play a key role regulating the production of β,β -xanthophylls (Alquézar et al., 2009; Kato, 2012; Rodrigo et al., 2019). These results suggest a coordinated regulation between the biosynthesis of β,β -xanthophylls and the putative enzymes involved in their esterification, which may favor the massive xanthophyll accumulation in the esterified form. In red chili pepper, the expression of *rsAcT* gene, orthologous of tomato and citrus *PYPs*, is also enhanced at the onset ripening and, similarly, paralleled the expression of *PSY-1* and capsanthin capsorubin synthase (*CCS*) (Berry et al., 2019), essential for the biosynthesis of keto-xanthophylls.

The *PYP* genes showed differential expression patterns in the pulp. The maximum transcript level for all genes in the pulp was between 3 and 10 times lower than the in the corresponding flavedo samples (Fig. 2). This observation has been also reported for most of the carotenoid biosynthetic genes in citrus fruits and correlates with the

concentration of total carotenoids which usually is between 5 and 10-times lower in pulp than in the flavedo (Kato, 2012; Tadeo et al., 2020). The lowest *PYP* transcript levels in the pulp were observed at G stage (Fig. 2). The *PYP1*, 2 and 6 showed the highest transcript levels at M or FM stages; the expression of *PYP2* and 6 displayed a progressive increase during ripening, while *PYP1* transcript levels remained low until breaker (approximately 220 DAA) and experienced a marked increment at M and FM stages (Fig. 2). The expression profiles of *PYP3* and 4 were similar with a transient peak in the expression at breaker stage and returning to low levels at M and FM (Fig. 2).

Recently, the in-depth profiling of genuine carotenoids in flavedo and pulp of Navel sweet orange fruit at different developmental stages has been described. The profile was highly complex and 77 carotenoids were identified by HPLC-DAD-APCI-MSⁿ, of which 59 were mono- and di-esters of β,β -xanthophylls and β -citraurin (Lux et al., 2019). Based on this previous information, in the present work the main carotenoids and their esters were quantified at four ripening stages (Table 2).

In flavedo at G stage, the main carotenoids resembled those commonly found in chloroplastic tissues, with lutein (about 60% of the total carotenoids) being the most abundant constituent, followed by (all-*E*)-violaxanthin, α - and β -carotene, (all-*E*)-antheraxanthin (not baseline resolved from (9*Z*)-violaxanthin detected as a minor constituent in G flavedo) and zeaxanthin. Noteworthy, esters were not detected at the G stage (Table 2). At the GB stage, the profile was similar to that of the previous stage (G), but concentrations of the typical carotenoids of photosynthetic tissues (lutein, α - and β -carotene and neoxanthin) were reduced by 2.1-6.8 times, while the concentrations of violaxanthin and antheraxanthin did not change substantially. Again, carotenoid esters were not detected at the GB stage in the flavedo. These results were in

agreement with previous works in citrus (Eilati et al., 1972) and other fruits, since unripe fruits and green tissues do usually not accumulate xanthophyll esters (Hornero-Méndez, 2019). The decline of carotenoid concentrations from G to GB stage reflects the initiation and progress of the degreening process in the flavedo, being associated with the transformation of chloroplasts to chromoplasts (Tadeo et al., 2020). This is also evidenced by the total chlorophylls, decreasing from 320 ± 15 to $165 \pm 12 \mu\text{g g}^{-1}$ FW at G and GB stage, respectively. At M and FM stages, important qualitative and quantitative differences were noticed. The chloroplast-specific carotenoids decreased to traces or were not detected. Hereby, concentrations of free (all-*E*)-violaxanthin and (9*Z*)-violaxanthin/(all-*E*)-antheraxanthin were reduced between 2 and 3-fold (Table 2). The colorless phytoene and phytofluene were detected at all stages analyzed and their concentrations increased upon progressing from GB to M stage, reaching the highest contents at FM (Table 2). The most relevant characteristic in carotenoid composition of the flavedo at M and FM stages was the emergence of considerable amounts of xanthophyll and β -citraurin esters, representing about 80% of the total carotenoids. The esters of (all-*E*)- and (9*Z*)-violaxanthin were the most abundant ones at both advanced ripening stages, being not always baseline resolved from other minor unidentified carotenoid esters. Noteworthy, although violaxanthin isomers represented the prevailing xanthophylls after saponification (not shown; Rodrigo et al., 2019), all violaxanthin esters were quantified together with these unresolved and unknown compounds (UUX, Table 2). Concentrations of all ester categories (violaxanthin, UUX and β -citraurin) doubled from M to FM stage, and the fraction of esterified carotenoids reached a total of $109.76 \mu\text{g g}^{-1}$ FW in FM flavedo (Table 2). The occurrence of esters in citrus fruit flavedo has been scarcely explored (Eilati et al., 1972; Lux et al., 2019; Ma et al., 2017; Philip, 1973) and, in line with previous data, our results suggest that the acylation of

xanthophylls with fatty acids is triggered once the degreening process initiates and remains a highly active process throughout fruit maturation. In pepper (*Capsicum* sp.) fruits esterification of xanthophylls is a ripening-regulated process and triggered at the onset of ripening (Berry et al., 2019; Hornero-Mendez & Minguez-Mosquera, 2000).

The comparison of *PYP1*, 2 and 6 expression patterns and the changes in esters accumulation in flavedo showed a good association. Relatively low gene expressions at green (G and GB) stages coincide with the absence of carotenoid ester, regardless of the significant pool of xanthophylls available for esterification. At the onset of peel degreening (GB stage), the *PYP 1* and 6 transcripts levels were greatly enhanced in parallel with the increase in ester contents. Highest expression rates were reached at FM, when also the concentration of esters reached their maximum (Table 2 and Fig. 2). The increase expression of *PYP2* through ripening is also compatible with a role in xanthophyll esterification in the flavedo; however, the low expression of *PYP3* and *PYP4* at M stage suggests that their contributions to xanthophyll esterification in flavedo may be restricted to FM fruit.

In the pulp sweet oranges carotenoid profile and content differed from that of the flavedo. The concentration of total carotenoids in the pulp was between 700 to 10 times lower than in the flavedo (Table 2). At the G stage, the content of carotenoids was reduced and the main carotenoid was free violaxanthin (Table 2). At GB stage, despite the low amounts of carotenoids (less than 2 $\mu\text{g g}^{-1}$ FW), 97% of the carotenoids corresponded to xanthophyll esters (Table 2). These results are showing that esterification occurs earlier in the pulp than in flavedo. At M and FM stage, the concentration of violaxanthin and other xanthophyll esters increased nearly 3- and 8-times, respectively, compared to GB stage. In contrast to flavedo, esters of antheraxanthin (1.18 $\mu\text{g g}^{-1}$ FW), representing ~10% of the ester fraction, were detected

in the pulp at FM stage. The content of free xanthophylls also increased as pulp matured, and at FM stage, the concentration of (all-*E*)- and (9*Z*)-violaxanthin, (all-*E*)-antheraxanthin and lutein ranged from 0.29 to 0.48 $\mu\text{g g}^{-1}$ FW. Phytoene and phytofluene, the xanthophyll precursors, were only detected in pulp at M and FM stage at very low concentrations (Table 2). The ester fraction represents more than 85% of the total carotenoid levels in the pulp across all ripening stages. This is in line with results obtained in juices of different sweet orange cultivars at commercial maturity (Giuffrida et al., 2019; Petry & Mercadante, 2016). However, at FM stage, the concentration of carotenoids esters in the pulp (12.23 $\mu\text{g g}^{-1}$ FW) was substantially higher than those reported in juices of Navel sweet orange cultivars (between 2 and 4 $\mu\text{g g}^{-1}$ FW) (Giuffrida et al., 2019). The fact that the reduction alters specifically the ester contents in juice compared to pulp suggests that acylated xanthophylls may be preferentially retained in the membranes of juice vesicles and poorly released to the aqueous phase during juicing process.

The relatively low expression of *PYP* genes in the pulp is in agreement with the lower carotenoid biosynthetic flux and ester content in this tissue compared to flavedo. The reduced expression of genes in the pulp has been reported for most of the carotenoid biosynthetic genes (Rodrigo et al., 2013b; Kato, 2012), suggesting again a coordinated regulation between the biosynthetic pathway of xanthophylls and their esterification. The increment in acylated xanthophylls in the pulp from G to GB stage was associated with the moderate increase in transcript levels of *PYP2* and 6 (Fig. 2 and Table 2). Compared to flavedo, the expression of *PYP2* and 6 in the pulp at GB is rather low, despite more than 95% of the xanthophylls are esterified. This can be explained since the biosynthetic flux of xanthophylls is reduced in the pulp and *PYP* enzyme levels may be sufficient for an efficient esterification. The highest increments in xanthophyll esters

were observed at M and FM stages (4 and 12-fold, respectively, compared to GB), which were linked to the marked enhancement of *PYP1*, 2 and 6 expression. The patterns of *PYP3* and 4 did not correlate with changes in the esterification profile and their potential involvement in xanthophyll esterification in the pulp is questionable.

In citrus, xanthophyll esters have been exclusively reported in fruit tissues, but the expression of *PYP* genes were also detected in sweet orange leaves and petals but, in general, at lower levels than in flavedo of mature fruit (Fig. S1). The relative expression levels of *PYPs* in leaves were similar to those found in green flavedo (Fig. 2). In photosynthetic tissues PES/PYP activities may modulate contents of free phytol, a harmful product of chlorophyll breakdown (Lippold et al., 2012). Thus, in citrus leaf tissue PYP may have a role for phytol detoxification. The moderate expression of *PYPs* in petal is difficult to explain since do not contain detectable amounts of xanthophylls or chlorophylls; therefore, the PYPs functionality in this tissue remains an open question to be explored.

3.3 Expression of PYP genes in Citrus species with diversity in total carotenoid and xanthophyll esters content in fruit

Based on citrus fruit pigmentation and carotenoid composition three different species were selected: clementine mandarin (*C. clementina*) with deep orange colored flavedo and pulp associated with high content of β,β -ring xanthophylls; lemon (*C. limon*), with yellow peel and pulp, and low content of xanthophylls; and pink pummelo (*C. maxima/grandis*) with pale yellow peel and pulp, and small proportion of pink juice vesicles containing lycopene (Rodrigo et al., 2013b; Xu et al., 2006). The carotenoid profile of mature fruits of these three citrus species was analyzed with and without

saponification of the extracts to determine the proportion of esterified xanthophylls, and related to the expression of *PYPI*, 2 and 6. The flavedo of clementine mandarins contained high concentrations of carotenoids ($147.18 \pm 17.90 \mu\text{g g}^{-1}$ FW) and showed values similar to that of sweet orange fruit (Table 3). The total carotenoid content in the flavedo of pummelo, despite its pale yellow color, was also high and only 16% lower than that of mandarins. The flavedo of lemon showed the lowest carotenoid content, which was 10-times lower than in clementine (Table 3). As expected, nearly 90% of carotenoids were esterified in the flavedo of clementine, representing 95% of the total xanthophylls. Carotenes (phytoene and phytofluene) accounted for nearly 7% (Table 3). In the flavedo of lemon and pummelo, the colorless carotenes phytoene and phytofluene represented the main category, and the concentration in pummelo was 10-times higher reaching $120 \mu\text{g g}^{-1}$ FW. The content of free xanthophylls was similar in both pummelo and lemon flavedo (about $2 \mu\text{g g}^{-1}$ FW). However, the xanthophyll esters corresponded to 14 and 35% of the total xanthophylls in lemon and pummelo flavedo, respectively (Table 3). The low esterification degree of xanthophylls in lemon flavedo is also in agreement with previous studies (Eilati et al., 1972; Yokoyama & Vandercook, 1967). Works reporting the analysis of carotenoids in the flavedo of pummelos are scarce and the carotenes are predominant with minor amounts of lutein and violaxanthin (Matsumoto et al., 2007; Xu et al., 2006). In the pulp of the three citrus species selected the total carotenoid content and concentration of xanthophyll esters were markedly different. Clementine pulp showed the highest carotenoid concentration (nearly $15 \mu\text{g g}^{-1}$ FW) and about 90% of the xanthophylls were esterified (Table 3). In lemon and pummelo pulp the content of carotenoids was reduced 160 and 48-fold, respectively, compared to clementine (Table 3). In both lemon and pummelo, the xanthophyll contents were very low (0.04 and $0.07 \mu\text{g g}^{-1}$ FW, respectively), but in lemon 50% of

the xanthophylls were esterified while such esters were not detected in pummelo (Table 3). In lemon pulp or juice, carotenoid contents are usually very low ($<0.5 \mu\text{g g}^{-1}$ FW), with the colorless carotenes and β -cryptoxanthin being the main components (Fanciullino et al., 2006; Yokoyama & Vandercook, 1967), but information regarding the carotenoid ester profile is lacking. The carotenoid composition in pummelo has been poorly studied and the pulp of red or pink pigmented cultivars accumulate carotenes (phytoene, lycopene and β -carotene) and very small proportions of xanthophylls (Table 3; Fanciullino et al., 2006; Xu et al., 2006; Matsumoto et al., 2007).

The expression of *PYP1*, 2 and 6 in flavedo and pulp of the three species during ripening showed a differential pattern (Fig. 3). In both tissues of clementine mandarin, the expression in green immature fruit was very low, and notably increased during ripening, reaching the highest transcript levels at mature stage. This enhancement in the expression of *PYP* genes in clementine flavedo occurred after breaker stage (250 DAA) and at least one month earlier in the pulp (210 DAA) (Fig. 3). An earlier induction in the pulp compared to flavedo has been reported previously for most of the carotenoid biosynthetic genes in mandarins and parallels with the massive xanthophyll accumulation in both tissues (Kato et al., 2004). Thus, *PYP* genes seem to display a coordinated expression pattern with xanthophyll biosynthetic genes in both flavedo and pulp. The relative expression levels and the patterns of *PYP1*, 2 and 6 in clementine fruit tissues were comparable to those in sweet orange (Fig. 2), in agreement with their similar contents of carotenoid esters. Interestingly, all *PYP* genes, with the exception of *PYP2* in flavedo, showed a much lower expression in pummelo and lemon than in clementine. In general, the expression of *PYPs* in pummelo and lemon remained at relatively low and basal levels during fruit ripening and only experienced a slight increment at mature stage (Fig. 3). The reduced expression of *PYPs* in pummelo and

lemon is in accordance with their lower xanthophyll levels and, more importantly, with their ester contents (Table 3). It is worth to note that in flavedo of pummelo the expression of *PYP6* showed a moderate increase at breaker stage and that was sustained during ripening, while in lemon this increment is more attenuated and occurred later (Fig. 3). The enhanced expression of *PYP6* in pummelo flavedo could be related with higher proportion of esterified xanthophylls (35% of the total) compared to lemon flavedo (14%) (Table 3). Interestingly, the expression of *PYP6* showed a moderate increment in the pulp of lemon at ripe stage, which may explain the accumulation of xanthophyll esters, although at very low levels. The expression of *PYP2* in flavedo of clementine and lemon increased during maturation, and in pummelo did not follow a clear trend (Fig. 3). Overall, considering the expression patterns of the *PYP* genes in these *Citrus* species during fruit ripening and the xanthophyll ester contents at ripe stage, it can be suggested that *PYP1* and 6 in flavedo, and *PYP1*, 2 and 6 in pulp may have a role in the esterification of carotenoids in these *Citrus* species. It can be suggested that the low expression of *PYPs* in yellow pigmented citrus such as lemon or pummelo, specifically at mature stage, may result in low esterification. Fatty acid acylation of xanthophylls is a mechanism that prevents their degradation in flowers and fruits (Aziirumi et al., 2014; Kishimoto et al., 2020; Ma et al., 2017). Therefore, low *PYP/XES* activity in citrus fruit may severely affect xanthophyll contents and, in turn, fruit pigmentation. It is also possible that reduced biosynthesis of xanthophylls in yellow pigmented citrus, as has been proposed in lemon (Kato, 2012), may affect the expression of *PYP* genes by a negative feedback regulatory mechanism.

4. Conclusions

This is the first study addressing the characterization of the *PYP/XES* genes likely responsible for carotenoids esterification in citrus fruits. A multigene family of seven members was identified in sweet orange, showing a high degree of conservation across other plants and *Citrus* species. The analysis of citrus *PYP/XES* genes structure and protein domains, their expression patterns, and the levels of free and esterified carotenoids has led to postulate PYP1, 2 and 6 as the most promising candidates for β,β -xanthophyll esterification in citrus. Comparison of the expression profile of *PYP* genes in fruit tissues of citrus species with marked differences in the contents of carotenoids and xanthophyll esters supported the potential role of PYP1 and 6 in the esterification of carotenoid in the flavedo, and PYP1, 2 and 6 in the pulp. In view of these results, future work will focus to design further functional studies to demonstrate the fatty acid acyl transferase activity of the citrus PYP and to investigate their substrate specificity. The understanding of the mechanism involved in carotenoids esterification should provide new insights into the regulatory processes governing the complex pattern of carotenoids in citrus fruits and their diversity of pigmentation.

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569 **Conflict of interest statement**

570 The authors declare that there are no conflicts of interest.

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Figures legends

Figure 1. The phylogenetic tree was generated based on the alignment of deduced amino acid sequences of PYP/PES from tomato (SlPYP1 and 2; Ariizumi et al., 2014), Arabidopsis (AtPES1; Lippold et al., 2012), chili pepper (CaPYP/rsAcT; Berry et al., 2019) and sweet orange (CsPYP1 to CsPYP7; this work, Table 1). The tree was constructed on the basis of the Neighbour-Joining method (Saitou & Nei, 1987) in MEGA6 software (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). The percentage of replicate trees in which the associated protein clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The bootstrap values on the nodes indicate the number of times that each group occurred with 1000 replicates.

Figure 2. Relative expression levels of *PYP1*, 2, 3, 4 and 6 in sweet orange flavedo and pulp during fruit development and ripening. The immature green (G), green-breaker (GB), mature (M) and fully-mature (FM) fruit ripening stages are labelled in *PYP1* graph. Results are normalized to the expression value of pulp of sweet orange cv. Navel at M stage which was set to 1. Data are means $\pm$ S.E. DAA, Days after anthesis. Note the different scale used for flavedo and pulp graphs.

Figure 3. Relative expression levels of *PYP1*, 2 and 6 in flavedo and pulp of mandarin clementine (*Citrus clementina* cv. Clemenules) (●), lemon (*Citrus limon* cv. Fino) (▽) and pummelo (*Citrus maxima* cv. Pink) (○) during fruit development and ripening. The green (G), breaker (B), mature (M) fruit ripening stages are labelled in *PYP1* panel. For all species the G stage corresponded approximately to 125 days after anthesis (DAA) and B to 210 DAA; M stage corresponded to 280 DAA for clementine and lemon, and 310 DAA for pummelo. Results are normalized to the expression value of pulp of sweet orange Navel at M stage which was set to 1. Data are means $\pm$ S.E. Note the different scale used for flavedo and pulp graphs.

Table 1

Genomic and structural characteristics of *PYP/XES* gene family in sweet orange (*C. sinensis*).

Gene name	Genomic code ^a <i>Citrus sinensis</i>	Predicted Introns	Predicted protein length (aa) ^b	Chloroplast transit peptide ^c
<i>PYP1</i>	orange1.1g041641m	14	713	Yes (46)
<i>PYP2</i>	orange1.1g004396m	12	756	Yes (73)
<i>PYP3</i>	orange1.1g006325m	13	650	Yes (26)
<i>PYP4</i>	orange1.1g005300m	13	703	Yes (74)
<i>PYP5</i>	orange1.1g013862m	7	435	No
<i>PYP6</i>	orange1.1g005190m	13	709	Yes (45)
<i>PYP7</i>	orange1.1g016023m	8	396	No

^aJGI, Phytozome 12, Sweet Orange Genome (*Citrus sinensis* v1.1, 2014). ^b aa, amino acid.

^c ChloroP 1.1. Prediction Server, in parenthesis is indicated the predicted length of transit peptide.

Table 2

Concentrations of carotenes, free xanthophylls and xanthophyll esters in the flavedo and pulp of sweet oranges (*C. sinensis* cv. Navel) fruit harvested at four different maturity stages (*G*, green; *GB*, green-breaker; *M*, mature; *FM*, fully-mature). Concentration given as mean $\pm$ standard deviation ($n = 2$).

	Concentration [$\mu\text{g g}^{-1}$ FW]							
	Flavedo				Pulp			
	G	GB	M	FM	G	GB	M	FM
<i>Carotenes</i>								
Phytoene	0.84 $\pm$ 0.01	0.65 $\pm$ 0.03	2.68 $\pm$ 0.08	4.62 $\pm$ 0.11	n.d.	n.d.	0.06 $\pm$ 0.00	0.06 $\pm$ 0.00
Phytofluene	0.27 $\pm$ 0.00	0.21 $\pm$ 0.01	1.90 $\pm$ 0.12	3.98 $\pm$ 0.22	n.d.	n.d.	tr.	0.05 $\pm$ 0.00
α -Carotene	7.96 $\pm$ 0.30	1.22 $\pm$ 0.56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β -Carotene	5.88 $\pm$ 0.21	0.88 $\pm$ 0.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Free xanthophylls</i>								
Violaxanthin	11.84 $\pm$ 0.26	12.26 $\pm$ 0.87	3.48 $\pm$ 0.06	2.67 $\pm$ 0.17	0.12 $\pm$ 0.10	0.05 $\pm$ 0.01	0.23 $\pm$ 0.00	0.37 $\pm$ 0.02
(9Z)-Violaxanthin + (all-E)-antheraxanthin	4.79 $\pm$ 0.06	3.50 $\pm$ 0.12	2.88 $\pm$ 0.08	1.68 $\pm$ 0.10	n.d.	n.d.	0.19 $\pm$ 0.00	0.29 $\pm$ 0.02
Neoxanthin	3.93 $\pm$ 0.30	1.89 $\pm$ 0.28	n.d.	n.d.	tr.	n.d.	n.d.	n.d.
Zeaxanthin	3.47 $\pm$ 0.01	n.d.	tr.	1.69 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.
Lutein	57.95 $\pm$ 0.05	21.18 $\pm$ 1.22	tr.	n.d.	n.d.	n.d.	tr.	0.48 $\pm$ 0.01
<i>Esters of xanthophylls and β-citraurin</i>								
Vlx esters + UUX esters ^a (% Vlx esters)	n.d.	tr.	43.69 $\pm$ 1.15 (55%)	99.00 $\pm$ 2.54 (62%)	n.d.	1.59 $\pm$ 0.27 (72%)	4.37 $\pm$ 0.00 (61%)	11.05 $\pm$ 0.18 (63%)
Antheraxanthin esters	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.18 $\pm$ 0.00
β -Citraurin esters	n.d.	n.d.	4.65 $\pm$ 0.01	10.76 $\pm$ 0.34	n.d.	n.d.	n.d.	n.d.
<i>Total</i>								
Carotenes	14.95 $\pm$ 0.50	2.96 $\pm$ 1.11	4.58 $\pm$ 0.20	8.60 $\pm$ 0.33	n.d.	n.d.	0.06 $\pm$ 0.00	0.11 $\pm$ 0.00
Free xanthophylls	81.97 $\pm$ 0.04	38.83 $\pm$ 2.17	6.36 $\pm$ 0.14	6.04 $\pm$ 0.28	0.50 $\pm$ 0.02	0.05 $\pm$ 0.01	0.42 $\pm$ 0.00	1.14 $\pm$ 0.04
Esters of xanthophylls and β -citraurin	n.d.	n.d.	48.34 $\pm$ 1.14	109.76 $\pm$ 2.88	n.d.	1.59 $\pm$ 0.27	4.37 $\pm$ 0.00	12.23 $\pm$ 0.19

^a Percentage of violaxanthin esters represented in brackets. *FW*, fresh weight. *Vlx*, violaxanthin; *UUX*, unresolved and unknown xanthophyll; *n.d.*: not detected (below LOD at a signal-to-noise ratio of 1:20). *tr.*: traces (below LOQ at a signal-to-noise ratio of 1:30).

Table 3

Concentration of carotenes, free and esterified xanthophylls and C30 apocarotenoid β -citraurin in flavedo and pulp of mature fruits of clementine mandarin (*Citrus clementina* cv. Clemenules), lemon (*Citrus limon* cv. Fino) and pummelo (*Citrus maxima* cv. Pink). Concentration given as mean $\pm$ standard deviation.

	Concentration [$\mu\text{g g}^{-1}$ FW]					
	Clementine		Lemon		Pummelo	
	<i>Flavedo</i>	<i>Pulp</i>	<i>Flavedo</i>	<i>Pulp</i>	<i>Flavedo</i>	<i>Pulp</i>
Carotenes	10.92 $\pm$ 0.89	3.68 $\pm$ 0.41	12.19 $\pm$ 0.24	0.05 $\pm$ 0.01	120.38 $\pm$ 9.12	0.24 $\pm$ 0.01
Free xanthophylls	6.55 $\pm$ 2.54	1.12 $\pm$ 0.08	2.29 $\pm$ 0.09	0.02 $\pm$ 0.00	2.57 $\pm$ 0.05	0.07 $\pm$ 0.01
Esters of xanthophylls ^a	123.90 $\pm$ 5.65	9.92 $\pm$ 0.05	0.38 $\pm$ 0.02	0.02 $\pm$ 0.00	1.40 $\pm$ 0.08	n.d.
Esters of β -citraurin ^a	8.31 $\pm$ 2.54	n.d	n.d	n.d	n.d	n.d
Total carotenoids	147.18 $\pm$ 17.90	14.56 $\pm$ 0.61	14.87 $\pm$ 0.42	0.09 $\pm$ 0.01	123.21 $\pm$ 14.60	0.31 $\pm$ 0.02

^aTentative identification. FW, fresh weight. n.d. Not detected.

Zacarias et al. Figure 1

Zacarias et al. Figure 2.

Figure 3

Table S1. Samples used for experiments of gene expression and carotenoid analysis. Fruit harvesting dates and peel color in the four citrus genotypes used in this study. Data of colour index for each cultivar are the means $\pm$ SD of at least 15 fruits, except for pummelo where 4 fruits were used. Peel fruit colour was measured using a Minolta CR-400 chromameter on three locations around the equatorial plane of the fruit. Hunter parameters a (negative to positive correspond from green to red, respectively), b (negative to positive, from blue to yellow, respectively) and L (0 to 100, black to white) were used and colour was expressed as the Hunter *a/b* ratio, a classical relationship for colour measurement in citrus fruits. The *a/b* ratio is negative for green fruits, the zero value correspond to yellow fruits at the midpoint of the colour break period and is positive for orange fruit. For each sample the analysis performed is indicated: gene expression (GE); carotenoid analysis (CAR).

Genotype	Harvesting date	Ripening stage	Fruit peel color (Hunter <i>a/b</i> ratio)	Analysis	
				Flavedo	Pulp
Fruit Sweet orange cv. Navel Foios (<i>C.sinensis</i>)	3.August.2016	Immature Green	-0.79±0.05	GE, CAR	GE, CAR
	14.October.2016	Mature Green-Breaker	-0.60±0.03	GE, CAR	GE, CAR
	5.November.2016		-0.39±0.06	GE	GE
	19.November.2016		-0.19±0.05	-	GE
	15.December.2016	Mature	0.52±0.03	GE, CAR	GE, CAR
	3.January.2017		0.61±0.02	GE	GE
	23.January.2017	Fully Mature	0.72±0.04	GE,CAR	GE,CAR
Fruit Pummelo cv. Pink (<i>C.maxima</i>)	25.July.2016	Immature green	-0.71±0.02	GE	GE
	5.October.2016		-0.67±0.01	GE	GE
	28.October.2016	Breaker	-0.40±0.06	GE	GE
	15.December.2016		-0.21±0.04	GE	GE
	1.February.2017	Mature	-0.06±0.02	GE,CAR	GE,CAR
Fruit Clementine cv.Clemenules (<i>C.clementina</i>)	14.July.2016	Immature Green	-0.89±0.02	GE	GE
	2.September.2016		-0.70±0.01	-	GE
	6.October.2016	Breaker	-0.31±0.01	GE	GE
	2.November.2016		0.22±0.05	GE	GE
	5.December.2016	Mature	0.81±0.04	GE,CAR	GE,CAR
Fruit Lemon cv.Fino (<i>C.limon</i>)	25.July.2016	Immature Green	-0.72±0.02	GE	GE
	22.October.2016	Breaker	-0.35±0.02	GE	GE
	19.November.2016		-0.02±0.01	GE	GE
	15.December.2016	Mature	0.02±0.01	GE,CAR	GE,CAR
				Analysis	
Leaf cv. Navel Foios (<i>C.sinensis</i>)	14.October.2016			GE	
Petals cv. Navel Foios (<i>C.sinensis</i>)	15.April.2016			GE	

Table S2. Sequences of the primers used for real-time PCR. The genes *PYP1*, 2, 3, 4 and 6 are described in Table 1. The *ACTIN* was used as constitutive gene for data normalization.

Primer	Orientation	Gene	Sequence (5'-3')	Sweet orange Genomic Sequence ^a
689	F	<i>PYP1</i>	GTCAGGATGGCAGCTAGA	orange1.1g041641m
690	R	<i>PYP1</i>	CAACTTGATA GTCTCGCCTGTT	orange1.1g041641m
691	F	<i>PYP2</i>	GTGAGGATGGCAGCACGG	orange1.1g004396m
692	R	<i>PYP2</i>	TATGTTTACAGTGTGTCACGAGCC	orange1.1g004396m
693	F	<i>PYP3</i>	ATCAGGATGGCGGCTAGG	orange1.1g006325m
694	R	<i>PYP3</i>	TAGATTCCCAACTGAATTCGTA	orange1.1g006325m
695	F	<i>PYP4</i>	GTCAGGATGTCATCTACA	orange1.1g005300m
696	R	<i>PYP4</i>	CAGCTTAACAACCCTTTTGTTTC	orange1.1g005300m
699	F	<i>PYP6</i>	GTCAGGATGGCGACTACA	orange1.1g005190m
700	R	<i>PYP6</i>	CAGCCTAGCAGCTGTTACTGTC	orange1.1g005190m
ACTF	F	<i>ACTIN</i>	TTAACCCCAAGGCCAACAGA	orange1.1g037845m
ACTR	R	<i>ACTIN</i>	TCCCTCATAGATTGGTACAGTATGAGAC	orange1.1g037845m

^a JGI, Phytozome 12, Sweet Orange Genome (*Citrus sinensis* v1.1, 2014)

Table S3 Percent identity matrix of deduced amino acid sequences of PYP/PES from tomato (SlPYP1 and 2; Ariizumi et al., 2014), Arabidopsis (AtPES1; Lippold et al., 2012), chili pepper (CaPYP/rsAcT; Berry et al., 2019) and sweet orange (CsPYP1 to CsPYP7; this work, Table 1). The identity matrix was created using CLUSTAL Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

	SlPYP1	SlPYP2	AtPES1	CaPYP	CsPYP1	CsPYP2	CsPYP3	CsPYP4	CsPYP5	CsPYP6	CsPYP7
SlPYP1	100.00	49.08	60.15	85.82	52.36	64.58	51.69	49.85	48.39	50.60	53.03
SlPYP2	49.08	100.00	46.74	48.67	60.68	50.37	57.34	53.26	53.00	58.24	60.35
AtPES1	60.15	46.74	100.00	57.31	50.07	62.21	48.91	48.24	47.92	48.83	51.02
CaPYP	85.82	48.67	57.31	100.00	50.43	63.12	52.10	48.62	50.00	49.78	53.67
CsPYP1	52.36	60.68	50.07	50.43	100.00	51.52	65.07	64.27	65.21	68.52	69.95
CsPYP2	64.58	50.37	62.21	63.12	51.52	100.00	50.15	49.35	50.92	51.94	56.35
CsPYP3	51.69	57.34	48.91	52.10	65.07	50.15	100.00	62.40	59.72	62.87	63.75
CsPYP4	49.85	53.26	48.24	48.62	64.27	49.35	62.40	100.00	67.37	70.82	72.42
CsPYP5	48.39	53.00	47.92	50.00	65.21	50.92	59.72	67.37	100.00	69.12	71.52
CsPYP6	50.60	58.24	48.83	49.78	68.52	51.94	62.87	70.82	69.12	100.00	77.53
CsPYP7	53.03	60.35	51.02	53.67	69.95	56.35	63.75	72.42	71.52	77.53	100.00

Table S4. Percentage of sequence coverage and identity of PYP/XES genes alignments between *C.sinensis* (Table 1) and other citrus specie (*C.maxima*, *C.medica*, *C.reticulata* and *Fortunella hindsii*). Coding sequences were obtained and analysis performed at the platform <http://citrus.hzau.edu.cn/orange/index.php> by using the BLAST tool.

Gene	Species	Genomic code	Sequence coverage (%)	Identity (%)
PYP2	<i>C.maxima</i>	Cg3g012010	98	99.5
	<i>C.medica</i>	Cm248450	98	99.5
	<i>C.reticulata</i>	MSYJ010160	100	99.8
	<i>Fortunella hindsii</i>	sjg242700	98	99.0
PYP3	<i>C.maxima</i>	Cg2g010050	100	98.0
	<i>C.medica</i>	Cm137560	100	98.0
	<i>C.reticulata</i>	MSYJ061200	95	99.1
	<i>Fortunella hindsii</i>	Sjg102270	100	97.5
PYP4	<i>C.maxima</i>	Cg2g010110	100	100
	<i>C.medica</i>	Cm137610	100	99.1
	<i>C.reticulata</i>	MSYJ61260	100	100
	<i>Fortunella hindsii</i>	sjg098200	100	99.3
PYP6	<i>C.maxima</i>	Cg2g010070	100	99.5
	<i>C. medica</i>	Cm137580	100	98.0
	<i>C.reticulata</i>	MSYJ061220	100	99.0
	<i>Fortunella hindsii</i>	sjg102060	100	98.2

Figure S1. Alignment of deduced amino acid sequences of PYP/PES genes from tomato (SlPYP1 and 2; Ariizumi et al., 2014), Arabidopsis (AtPES1; Lippold et al., 2012), chilli pepper (CaPYP/rsAcT; Berry et al., 2019) and sweet orange (CsPYP1 to CsPYP7; this work, Table 1). The alignment of proteins was created using CLUSTAL Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Numbers on the right denote the number of amino acid residue. Residues identical for all the sequences in a given position are in white text on a black background and 75 to 100% homologue residues are presented on grey background. In Arabidopsis AtPES1 and chill pepper CaPYP/rsAcT is marked in red letters the domain alpha-beta hydrolase-1 fold (Diacylglycerol acyltransferase) and in blue letters the PLAT_MGAT-like domain that belong to superfamily Lysophospholipid Acyltransferases (LPLATs) of glycerophospholipid biosynthesis.

SlPYP1	1	-----
SlPYP2	1	-----MEAISAGTYTAAGVSPLL
AtPES1	1	-----MATCSSLLVLPNL
CaPYP	1	-----MASFVQTFWAAPRF
CsPYP1	1	-----MATIPASCIFSADLSAIY
CsPYP2	1	MYFCRSIKGVSVSVKSILWNFQLSLVIAVQPVNSGKILIFIPLLFKTMASVINFPVSPSF
CsPYP3	1	-----MATTAGACFFAAGSFQAF
CsPYP4	1	-----MTTLGASIFSTDISTAF
CsPYP5	1	-----
CsPYP6	1	-----MATVGACIFSAVSSPVF
CsPYP7	1	-----
SlPYP1	1	-----MGTSDL-VNSVIVNGVSSIEEKEKSSTIIDVKNS
SlPYP2	19	QS-----RNSCLRITSA---RFKIA---STAATTGRSTASI-----
AtPES1	15	RLS-----SNQRRNFKVRAQISGENK-KATS-LEPVNNNGSVSLST-----TVQNQ
CaPYP	15	ALT-----PEYKPRCIARIARLPSRDSTFLP-SDSVIVNSVSSIEEREKSSPIADVQNG
CsPYP1	19	RRDKTSSFGERRRNPVLKRVAVTTEQTSSNSGTAVT--TSGRSFVQEK--AQRSSEAEA-
CsPYP2	61	VIN-----SQYKTSFRARAQSVGGGDSTVLS-SGSIAVNGDPVSREKEKNGALIDVGNG
CsPYP3	19	HPSPR-----RVAA-----
CsPYP4	18	HPEMTSLFWNQRRNPILKRLAVSTEQLASTATTVTSKTTPKRNFEVEKE--SSEAAAFST-
CsPYP5	1	-----
CsPYP6	18	RRQITSSSEKPKRNPILKRVAVSTERTSAATTTVMTEETTPTRIFVEKK--SSELVEDEA-
CsPYP7	1	-----
SlPYP1	34	HLAPAI-KEK--NKEDIQNKLETLWDDGYGTQTVKDYLEIGSEI IKP--DGGPPRWFTPI
SlPYP2	49	-----GQQPPTVPMSIVDSSELEQ--TTTLNMKDFMKWSKDMIASGSEGGPPRWFSPL
AtPES1	59	KGANEVNGKGSKRKIVSDEIELLWDDGYGSKSVKDYFAAAKEILKA--DGGPPRWFSPL
CaPYP	68	HLVSTI-KEE--SKKDQVKKLEPLWDDGYGTQTVKDYLEIASEI IKP--DGGPPRWFTPI
CsPYP1	74	-----ETS---LR---RVNVEGDWE--ESRKS LKDYFDEAKDMIRS--DGGPPRWFSPL
CsPYP2	114	TLKPRV-EKKKLKINVISSELEVLWDDGYGTDVSKDYLDAAKEI IKP--DGGPPRWFCPV
CsPYP3	28	-----TTKTTARFAEMNVE--GTRKGLRDYFEESKVMIKS--DGGPPRWFSPL
CsPYP4	75	-----ATAVSKTTSTGTTYLSEES--GNRKS LKDYFDEAKDMIKA--DGAPPRWFSPL
CsPYP5	1	-----
CsPYP6	75	-----ETK---QRVNVREYSFEES--GNGKSLKDYFDEAEDMIKSSSGGGPPRWFSPL
CsPYP7	1	-----
SlPYP1	89	SAGPPLEDSPLLLFLPGMDGTGMGLVLHEKALGKVFQVWCLHIPVYDRTPFDELVKFVGR
SlPYP2	100	DCAAPLKDSPLLLYLPGIDGVLGLIKHHKRLGKIFNIWCLHVPVTDRTSFDLVYLVEA
AtPES1	117	DCCRPVEDAPTLLFLPGMDGTGMGLVPHKALGKAHVSLCHIPVLDRTPFEGLLKVVED
CaPYP	123	SAGPPLEDSPLLLFLPGMDGTGMGLVLHEKALGKVFQVWCLHIPVYDRTPFVELVKFVER
CsPYP1	118	ECCAHSPPSPLLLYLPGIDGVLGLIMQHQRVGQIFDVWCLHIPVKDRTSFTGLVKLVER
CsPYP2	171	DCGRPLKGSPTLLFLPGIDGLGLILHHKPLGKAFEVRCLHIPVYDRTPFEGLVKFEVEE
CsPYP3	72	ETGARSHPDSPLLLFLPGIDGVLGLVRHHYSLGKIFDIWCLHIPVKDRTSFAGLIKLVK
CsPYP4	126	ECGSHSPDSPLLLFLPGIDGVLGLTRQHQRGLKIFDVWSLHIPVKDRTSFTGLVQLIER
CsPYP5	1	-----MYGACI--FQLQIGHHFTGLLKLIER
CsPYP6	124	ECGSHTRDSPLLLFLPGIDGVLGLTRQHQRGLKIFDIWCLHIPVKDRTSFTGLVKLVES
CsPYP7	1	-----

SlPYP1	149	TVRMKHA	SSPNKPIYL	VGDSFGG	CLALAA	IAAHNP	ETDLVL	ILANPAT	SFDR	TQLO	QPLLI	PLI
SlPYP2	160	TVRSEHH	APRRPIYL	LGESFGG	CLALAVA	ARNPHI	DALILAN	PATRLR	ESQLEN	LI	TL	
AtPES1	177	VLROEQ	ATRPNKPIYL	VGDSFGG	CLALAVA	ARNRSL	DLVLIL	VNPATS	FD	RSPL	QPLLI	PLI
CaPYP	183	TVRMKHA	SSPNKPIYL	VGDSFGG	CLALAVA	AHNPKI	DLVLIL	ANPAT	SFG	STQL	QSLL	PLI
CsPYP1	178	TVRSENY	LPNRP IYL	VGESLG	ACALAVA	AQNPD	IDVLIL	ANPAT	SF	SKS	QLO	PLI
CsPYP2	231	TVRREH	ASSPEKPIYL	VGDSFGG	CLALAVA	ARNPTI	DLILIL	SNPAT	SF	GRS	QLO	PLI
CsPYP3	132	TVRSEV	KRSPNRP IYL	VGESLG	ACIALAVA	SCNPD	VDVLIL	ANPAT	SF	SKS	QLO	PLI
CsPYP4	186	TIRSEH	NHSPNKPIYL	VGESLG	ACFALAVA	ARNPHI	DLVLIL	SNPAT	SF	SMS	VLO	STISL
CsPYP5	25	TIRSENC	RSSNRPVYL	VGESLG	ACALAVA	ARNPD	MDVLIL	ANPAT	SF	RKS	LLQ	TIMPI
CsPYP6	184	TVRSE	SNRSPKRPVYL	VGESLG	ACIALAVA	ARNPD	IDVLIL	VNPAT	SF	NKS	SVLQ	STIPL
CsPYP7	1	-----										

SlPYP1	209	L-ESLP	DEFHVT	VPY	-----	LLSF	IMGD	PLKMA	MVNID	SMLPP	GQIT	QRLAG	NLT					
SlPYP2	220	C-EVI	PEQLH	PSMVK	-----	LLSV	TTGV	PARLA	VAIP	GS	GHS	LQQA	VAELFR	GDV				
AtPES1	237	L-EMV	PEELH	FTVPY	-----	ALSF	IMGD	PIKMA	TGL	IDN	QLPT	GVKIE	KUROR	IT				
CaPYP	243	L-ESLP	DEFHVT	VPY	-----	LLSF	IMGD	PMKMA	MVNID	SMLPP	GQIS	QRLS	SNLI					
CsPYP1	238	L-Q	ITPD	QSD	EELR	LYVM	FVKFQ	ENGK	TRIG	DPLR	MAVD	ILVK	GLPL	QOKAGE	V	SQDLV		
CsPYP2	291	L-K	AMP	DELH	CAVPY	-----	LLSY	VMGD	PIKMA	MVNIE	NRLP	PPRI	KLEQ	LS	NNLP			
CsPYP3	192	L-E	VIPD	HFL	TLTRY	-----	VLSS	LTGD	LLKRV	SGIL	VRG	QTLQ	QTV	GGLC	QDSV			
CsPYP4	246	L-E	FIPG	QMTL	TLCH	-----	LLSS	MTGD	PLKMA	IDNV	VKGIS	VPPT	TIQD	L	-----			
CsPYP5	85	PAEL	MSG	QMTL	TL	SV	-----	LLSL	LTGD	PLKMA	DSIV	KGLF	LQ	STIQ	ERS	QDFV		
CsPYP6	244	L-E	FIPG	QMTL	TL	SS	-----	TL	SLMT	GD	PLKMA	DNV	AKRL	SLQ	PTIQ	ERS	QDLV	
CsPYP7	1	-----											MVMD	NVVK	GLSP	QPTTIE	QLS	QDLV

SlPYP1	258	D-L	LAHLY	GLADI	IPKET	LLWK	LKLL	RSASS	SY	SNR	LHAV	NAEVL	VIA	SGK	D	NMLP	SENE					
SlPYP2	269	A-F	SSYLS	VLA	DVLP	VETL	WR	LKIL	KSAA	SF	SSR	LHAV	RSQ	TLV	LS	SGK	DHLIS	SPEE				
AtPES1	286	K	TMLP	LLSEL	GGIIP	RET	LLWK	LKLL	RS	GCAY	ANSRI	HAVQ	AEVL	V	LA	SGK	D	NMLP	SQEE			
CaPYP	292	D-L	LVHLS	GLADI	IPKET	LLWK	LKLL	RSASS	SY	SNR	LHAV	NAEVL	V	LA	SGK	D	NMLP	SGNE				
CsPYP1	297	V-M	SSYHS	VAD	IMP	KET	LLWK	LEML	KSAS	AYAN	SR	LHAV	KAQ	TLI	I	SSG	KD	QLE	PSQEE			
CsPYP2	340	A-L	PLRL	SVMS	DIIPK	D	LLWK	LKLL	KSAS	AYAN	SR	LHAV	KA	AEVL	V	LA	SGK	D	NMLP	SEDE		
CsPYP3	241	A-L	PLVLS	VLT	DILP	QETL	WK	LQML	KTAS	TFV	NAR	LHAVE	AQ	TLI	I	SSG	R	D	QLLP	SLEE		
CsPYP4	290	---	S	TYLS	VLA	DILP	NET	LLWK	LELL	KSAS	AYAN	AR	LH	SVKA	Q	TLI	I	LSG	KD	Q	MMP	SEEE
CsPYP5	135	A-M	SSYLP	VLAN	ILP	KET	LLWK	LELL	KSAS	AYAN	AR	LDAV	KA	Q	TLI	I	LSG	R	D	QLLP	SEEE	
CsPYP6	293	A-L	SSYLP	VLA	DILP	KET	LLWK	LELL	KAAS	AYAN	SR	LHAV	KA	Q	MLV	LC	SGK	D	Q	LMP	SQEE	
CsPYP7	25	T-V	SSYLP	VLA	DILP	KET	LLWK	LELL	KAAS	AYAN	AR	LHAV	KA	Q	TLV	LS	SGK	D	QLLP	SQEE		

SlPYP1	317	AQRL	GN	SLRN	CTV	RYFK	DNGHT	ILLED	G	NLLS	IIKAT	SKYRR	SKRR	DYV	KD	FL	PP	SKSE																															
SlPYP2	328	SEKL	HKML	PNC	EIRK	FNN	S	CHALL	LEAD	FN	LV	TM	GAN	FYRR	GR	HL	D	V	R	D	V	P	P	STSE																									
AtPES1	346	AKRL	HGL	LKNC	SR	CFK	DNGHT	ILLED	S	ISLL	TVIK	GT	GKYRR	SWRY	D	V	S	D	F	L	P	P	SKGE																										
CaPYP	351	SQRL	KNS	LRNCK	VRYF	KDNGHT	ILLED	G	VNLLS	IIKY	TNKY	RRSK	RH	D	F	V	M	D	F	V	P	P	SRSE																										
CsPYP1	356	GERL	RHAL	SKCQ	IRK	FND	NGH	FLF	LED	D	IDL	V	TI	I	KGT	S	FYRR	G	KYH	D	V	S	D	F	I	P	P	T	P	DE																			
CsPYP2	399	AKRL	NN	SLON	CI	VRN	F	KDNGHT	ILLED	G	ISLL	TI	I	KGT	C	KYRR	SR	K	L	D	S	V	A	D	F	L	P	P	SRQE																				
CsPYP3	300	GERL	FHAL	PNGE	IR	RAG	D	S	GHFL	F	LED	G	I	D	L	S	A	I	K	S	Y	FYRR	G	KY	L	D	C	V	S	D	V	P	L	A	P	SE													
CsPYP4	347	GQRL	S	REL	PNC	Q	TRR	F	D	NGH	F	L	L	E	G	V	D	L	V	T	I	I	K	G	A	Y	Y	R	R	G	K	C	I	N	V	S	D	F	I	P	L	T	T	E					
CsPYP5	194	GQRL	C	RAL	PNC	Q	TRR	F	G	G	G	H	F	L	F	L	E	D	G	V	D	L	V	T	I	I	K	G	A	Y	Y	R	R	G	R	I	V	D	V	S	D	F	I	P	P	T	T	E	
CsPYP6	352	GERL	SSAL	HK	CEPR	N	FYGH	G	H	F	L	L	E	D	G	V	D	L	V	T	I	I	K	G	A	S	Y	Y	R	R	G	R	N	H	D	V	S	D	F	M	P	P	T	S	S				
CsPYP7	84	GQRL	T	SAL	PKS	Q	LRS	F	ED	H	G	H	F	L	F	L	E	D	G	V	D	L	V	T	I	I	K	G	A	S	Y	Y	R	R	G	S	L	D	I	S	D	F	M	P	P	T	A	T	E

SlPYP1	377	E	KNA	I	KNN	S	WYLN	-IT	G	P	V	M	L	S	T	M	E	N	G	K	I	V	R	G	L	A	G	V	P	R	E	G	P	V	L	L	V	G	Y	H	M	L	G	L	E	I	V	P	L	V	Q									
SlPYP2	388	F	D	S	V	Y	Q	P	Y	R	W	M	E	V	-A	F	N	P	V	M	I	S	T	L	E	N	G	I	V	R	G	L	T	G	I	P	S	E	G	P	V	L	L	V	G	Y	H	M	L	G	L	E	L	V	P	L	V	S		
AtPES1	406	L	A	Y	A	L	D	E	V	L	G	F	L	R	N	A	V	G	S	V	F	F	S	T	H	E	D	G	K	I	V	R	G	L	A	G	I	P	D	K	G	P	V	L	L	V	G	Y	H	M	L	G	L	E	I	V	P	L	V	Q
CaPYP	411	E	K	N	T	L	K	N	R	F	Y	L	D	-C	T	S	P	V	M	L	S	T	M	E	N	G	K	I	V	R	G	L	A	G	I	P	C	E	G	P	V	L	L	V	G	Y	H	M	L	G	L	E	I	V	P	L	V	Q		
CsPYP1	416	E	R	K	I	Y	E	S	N	R	G	I	C	V	-A	T	G	P	V	M	L	S	T	L	E	D	G	K	I	V	R	G	L	A	G	I	P	S	E	G	P	V	L	F	V	G	Y	H	M	L	G	L	E	L	T	P	L	V	C	
CsPYP2	459	E	K	Y	A	F	D	Q	V	V	G	L	L	R	V	A	S	S	V	M	L	S	T	L	E	D	G	K	I	V	R	G	L	A	G	I	P	N	E	G	P	V	L	L	V	G	Y	H	M	L	G	F	E	L	Y	S	L	V	E	
CsPYP3	360	E	N	K	I	N	-----	L	-L	S	P	V	T	L	S	T	L	E	D	G	M	I	V	R	G	L	G	I	P	M	E	G	P	V	L	I	V	G	Y	H	M	L	G	I	E	L	I	P	L	V	C									
CsPYP4	407	E	N	K	F	C	E	E	I	R	L	R	S	D	-L	T	S	P	V	M	L	S	T	L	E	D	G	K	I	V	A	D	L	S	G	I	P	S	E	G	P	V	L	V	G	Y	H	N	L	L	G	L	E	A	F	P	M	V	Q	
CsPYP5	254	V	N	K	I	Y	E	E	Y	R	W	M	V	D	-L	T	S	S	V	M	L	S	T	L	E	D	G	K	I	V	R	G	L	S	G	I	P	S	E	G	P	V	L	L	V	G	Y	H	N	L	L	G	L	E	A	L	P	M	V	
CsPYP6	412	E	N	K	I	C	E	D	F	R	W	M	R	V	-L	S	S	P	V	M	L	S	T	L	E	D	G	K	I	V	R	G	L	S	G	I	P	S	E	G	P	V	L	F	V	G	Y	H	N	L	L	G	D	V	L	T	L	I	P	
CsPYP7	144	E	N	E	N	E	N	R	W	M	S	V	-L	M	S	P	V	M	L	S	T	L	E	D	G	K	I	V	R	G	L	S	G	I	P	S	E	G	P	V	L	L	V	G	Y	H	N	L	M	G	F	E	V	H	T	M	V	P		

SlPYP1 436 EYMMQTKILLRGIAHPSLFTQLVESRPDASSFIDMLKLYGATPVTASNFFKLLATKSHVL
SlPYP2 447 RLWNEHQIVLRGIAHPLMFKKRREGKMPVLSMYDDYRFMGAVPVSATNFYKLLSSKSHVL
AtPES1 466 AFKEKNILFRGMAHPVLYSDN--DPAKAFDYGDWIKVFGAYPVTATNLFKLLDSKSHVL
CaPYP 470 EYIRLRKILLRGIAHPTLFTQLVESQTNETSFFDTLRLYGATPVASASNFFKLLATKSHVL
CsPYP1 475 QFMIERNILLRGIAHPLMFIRLRDGLLPDLATFDTHRIMGAVPVSGTNFYKLLSSKSHVL
CsPYP2 519 EFLREKNIMVHGIAHPEIFLGRLENSNEFGMTDWLKVMGAVPVAARNLFKLLSTKSHVL
CsPYP3 412 QFFIQRKIVLRGMAHPMLFVKLKDGRLLDSFPEDQIGIEGGVPVS AVNFYKLLSLKSHIL
CsPYP4 466 QFMIQRNVLRCAVHPM-FFESKDGGLPDFEGNDTLRIVGGVPASAVNLYKLLSSKSHVM
CsPYP5 313 TFVIERNILVRAIAHPMVFFNAKDGGLPDLVTYDTFRIMGVSPVSAINFYKLVSSKAHVL
CsPYP6 471 EFMIESNILLRGLAHPMMYFKSKEGGLSDLSPYDVMRIMGAVPVSGINLYKLMSSKSHVL
CsPYP7 203 QFTLIERKILLRGLTHPIILFVDSKDGGLPDLGPYDKFRIMGAVPVSAVNLYKLMSSKSHAL

SlPYP1 496 LYPGGAREALHRKGEEYKVIWPDQPEFIRMAAKFGATIVPFGVVGEDDIAQLVLDYDDLK
SlPYP2 507 LYPGGAREALHRKGEEYKLFWPEQSEFVRMAARFGAKIIPFGTVGEDDIGQMLLDYDDMM
AtPES1 524 LYPGGAREALHNGEYKLIWPEQSEFVRMAARFGATIVPFGTVGEDDIAQLVLDYNDLM
CaPYP 530 LYPGGAREALHRKGEEYKVIWPDQPEFIRMAAKFGATIVPFGVVGEDDIAQLVLDYEDDK
CsPYP1 535 LYPGGAREALHRKGEEYKLPWDSEFVRMAARFGAKIIPFGVGEDDIGQVVDYDLM
CsPYP2 579 LYPGGAREALHRKGEEYKLFWPEQSEFVRMAARFGATIVPFGAVGEDDIAQLVLDYKDLM
CsPYP3 472 LYPGGAREALHRKGEEYKLFWPEQSEFIRMAARFGAKIVPFGVVGEDDFGDVLLDYDDQI
CsPYP4 525 LYPGGAREALHRKGEEYKLFWPESSEFVRMSSTFGAKIIPFGAVGEDDIAQLVLDYNDQM
CsPYP5 373 LYPGGVREALHRKGEEYKLFWPESSEFVRVAAAFGGKIVPFGVVGEDDLAQVIK-----
CsPYP6 531 LYPGGVREALHRKGEEYKLFWPESSEFVRMATTFGAKIVPFGAVGEDDLAQIVLDYNDQM
CsPYP7 263 LYPGGAREALHRKGEEYKLFWPETSEFVRTAATFGATIIPFGTVGEDDVAQDWY-----

SlPYP1 556 SIPILGDRIRSENEEAARRGLAVRADMDGEIANQMLYIPGLLPKIPGRFYFFFGKPIHTK
SlPYP2 567 KVPYLKALIEE----LTGEVEKLRDTEGEVSNQDVHLPILPKVPGRFYFYFGKPIETA
AtPES1 584 KIPILNDYITEVTRD--TKQFKLRESEGEVANQPLYLPGLPKVPGRFYFLFGKPIETK
CaPYP 590 SIPILGDRIRHDNELATRNGLTIRTEMNGEVANQALYIPGLLPKIPGRFYFLFGKPFHTK
CsPYP1 595 KIPYFKAQIEA----LTSETIKLRDANGEIQNODIHLPGILPKLPGRFYFYFGKPIETE
CsPYP2 639 SIPVINDCVRE----LARDTVNIRDDTRGEVANQALFFPGLLPKVPGRFYFLFGKPIQTK
CsPYP3 532 KIPFLKSIIEE----FTNSVGNLRDTETRGEVANQDLHFFMFLPKVPGRFYFYFGKPIETE
CsPYP4 585 KIPFLKSQIEE----MNKRVVKLRDITGEVANQPVHLPPLPKIPGRFYFYFGKPIETK
CsPYP5 427 -LEMFHFSIQ*-----
CsPYP6 591 KIPYFKSQIEE----LTVTAARLRDITKGEVANQDMHPYPVPKVPGRFYFYFGKPIETK
CsPYP7 317 -----PRRGRFYFYFGKPIETK

SlPYP1 616 GRQDLVKDREKARELYLOVKSEVQNNMNYLLKKREEDPYRNFIIDRTMYRAFSATSQDVPT
SlPYP2 623 GRKEELKSKEKAHELYLEVKSEVERCIDYLKEKRESDSYRNIMARLPYQASHGFDSEVPT
AtPES1 642 GRPELVDMEEANQVYLEVKADEVNSLAYLLKKREEDPYRSVLDRLNYSLTHTTATHVPS
CaPYP 650 GRQDLVKDREKARELYLOVKSEVQNNMNYLLKKREEDPYRSVIDRTAYRAFSATFDDIPT
CsPYP1 651 GRKQELRDREKCNELYLEVKSEVEKCLAYLKEKRETDPYRNLLARLTYQATHGSTNEVPT
CsPYP2 695 GREVSLKDKENANELYLHKSQVERCIDYLKKREEDPYRNIIIDRTAYRAVYG--DEVPT
CsPYP3 588 GRKQELRDKGAHELYLOVQDEIKKNIAFLKEKREKDPYRSVLSRLAYQAAHGVTSEIPT
CsPYP4 641 GRKQELRDKKSQKLYLOVKGEVENCLAYLKEKRQNDPYRNILPRLIYQATHGFRAQVPT
CsPYP5 -----
CsPYP6 647 GRKRELDRDREKAHELYLEIKSEVEKCLAYLKEKRENDPYRNILPRLIYQATHGFTSQVPT
CsPYP7 334 GRKQELRDKKKAHELYLEIKSEVENCLAYLKEKRENDPYRNILARLIYQATHGFTSQVPT

SlPYP1 676 FDF-
SlPYP2 683 FDL-
AtPES1 702 FEP-
CaPYP 710 FD--
CsPYP1 711 FQI-
CsPYP2 753 FKLD
CsPYP3 648 FEI-
CsPYP4 701 FEL-
CsPYP5 ----
CsPYP6 707 FEP*
CsPYP7 394 FDL*

Figure S2. Structural organization and direction of transcription of sweet orange *PYP1,3,4,5,6 and 7* genes on chromosome 2. These genes are organized in a cluster and may be originated by gene duplication and further divergence. Genes are represented in orange boxes and in blue are intergenic spaces where no genes are predicted. Blue arrows indicate direction of transcription. Distance is indicated in kb.

Figure S3. Relative expression of sweet orange *PYP1*, 2, 3, 4 and 6 genes in leaves, petals and flavedo of mature sweet orange fruit.