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Genetic Regulation of Citrus Fruit Ripening

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Sommario

La maturazione è considerata un processo complesso a causa dei numerosi cambiamenti fisiologici e biochimici che coinvolgono sia la parte esterna che quella interna durante le ultime fasi di sviluppo dei frutti. Le modificazioni metaboliche che avvengono precedentemente alla raccolta hanno un impatto signiicativo sulle caratteristiche di qualità dei frutti stessi. La maturazione determina l'incremento di zuccheri solubili, la degradazione degli acidi e la biosintesi di composti bioattivi come la vitamina C. I pigmenti come i carotenoidi e gli antociani giocano un ruolo fondamentale nel definire le proprietà nutrizionali e salutistiche degli agrumi. Al fine di ottenere nuove informazioni a proposito dei sistemi di regolazione genica coinvolti nella maturazione dei frutti, sono stati realizzati due studi su due diverse specie di agrumi. Il primo lavoro ha riguardato l'analisi del processo di sintesi dei carotenoidi a livello genico e chimico di un mutante di limone caratterizzato da un accumulo considerevole di licopene. Mentre, il secondo lavoro ha riguardato la determinazione dell'interazione portinnesto-nesto sui principali tratti qualitativi della polpa di una varietà di arancia rossa. Nonostante ulteriori studi siano necessari per chiarire un fenomeno biologico tanto complesso come la maturazione, i risultati forniscono nuove ed importanti informazioni sui meccanismi di regolazione di diversi aspetti qualitatitvi coinvolti nella produzione di frutti dalle migliorate proprietà sensoriali e nutraceutiche.

Abstract

Ripening is deemed a complex process due to the drastic physiological and biochemical changes involved in both internal and external parts of the fruit. The metabolic modifications which take place before the harvesting have a significant impact on the fruit quality traits. Maturation consists of the increase of soluble sugars, the degradation of acids and the biosynthesis of bioactive compounds as vitamin C, carotenoids and anthocyanin play a key role in defining the nutritional and health-related properties of citrus fruits. In order to get new insight about the gene regulatory networks involved in fruit maturation, two studies were carried out on two different citrus species. The first experiment focused on the analysis of the flesh pigmentation of a spontaneous lemon mutant which accumulates remarkable amounts of lycopene. While the second work was aimed to evaluate the influence of rootstock-scion interaction on the main aspects of flesh quality in blood-sweet orange. Although further studies are required to elucidate such a complex biological phenomenon as ripening, the results supply new important informations related to fruit quality aspects involved in enhanced sensorial and nutraceutical properties.

Keywords: anthocyanin, blood orange, carotenoids, fruit quality, gene expression analysis, lycopene, rootstock-scion interaction, lemon mutant.

General introduction

The study of *Citrus* species has always aroused great interest not only for their agronomical and economic importance, but also for the peculiar scientific aspects which characterize many biological processes of the Citrus genera. Among these, the physiological and biochemical modifications that occur during maturation of their fruits have been some of the most investigated. Furthermore, citrus fruits are deemed a useful model to study the accumulation of sugars, organic acids and secondary metabolites. Then, considerable efforts have been made along the years to elucidate the complex series of events and biological phenomena related to fruit ripening (Cercós et al., 2006).

Citrus fruit is classified as hesperidium, a special kind of berry composed by an external part called epicarp (peel) and an interior portion called endocarp (pulp). The peel is further distinguished into mesocarp (albedo), the white portion in contact with the pulp, and exocarp (flavedo), the external pigmented layer. The pulp is divided into segments covered by a membrane and filled by juice vesicles. The fully ripe flesh is composed for the main part by water (85-90%) and many other compounds like carbohydrates, organic acids, aminoacids, vitamin C, small amounts of lipids and proteins, carotenoids, flavonoids and volatile compounds (Iglesias et al., 2007). The development of citrus fruits is divided into three phases: the first stage is characterized by an active cell division and a slow growth; the second stage is the one of major increase in size and weight of the fruits, while during the last phase the rate of growth decreases and modifications typical of maturation take place (Lado et al., 2018).

The physiological and anatomical changes that occur during ripening are the result of a complex interplay of hormonal signals and are highly influenced by environmental conditions. Citrus is generally defined as a non-climacteric fruit because of the low production of ethylene during ripening and the progressive reduction of respiration rate. However, recent findings suggest that ethylene is strictly involved in several processes associated with maturation. In particular, ethylene seems to be involved in citrus peel degreening by stimulating several molecular responses (Distefano et al., 2009; Alós et al., 2014a). Indeed, the application of exogenous ethylene represses

chlorophyll biosynthesis, stimulates chlorophyllase activity and enhances the transcription of carotenoid biosynthetic genes which happens concomitantly with the transformation of chloroplasts to chromoplasts (Alós et al., 2006; Alquézar et al., 2008).

The accumulation of soluble sugars and the degradation of organic acids are some of the main modifications taking place during the ripening process and leading the fruits to achieve the quality traits needed for their commercialization. The biosynthesis of the primary metabolites (sugar, acids, vitamin C) takes place simultaneously to the changes in bioactive compounds content. During the ripening process a wide variety of biochemical components such as carotenoids and anthocyanin, which confer citrus fruits their nutraceutical properties, are synthesized (Lado et al., 2014).

Recent studies have highlighted that a regular consumption of food reach in bioactive compounds can have positive effects on human health preventing neurodegenerative, cardiovascular and aging-related diseases, as well as reducing cancer risk (Grosso et al., 2013; Pojer et al., 2013; Fiedor and Burda, 2014; Woodside et al., 2015; Mozos et al., 2018). These findings have led modern consumers to expect fruits not only tasty and attractive, but also safe and healthy. In order to meet the increasing demand for nutraceutical food and to meet consumers' expectations, plant breeding strategies are focusing not only to the improvement of fruit quality traits such as shape, size, ripening time, etc., but also to their biochemical composition, with special emphasis to nutraceutical components.

Environmental conditions and cultural practices are the most important external factors which affect several maturation processes related to fruit quality. Climatic conditions as temperature and light exposure play a fundamental role in fruit ripening. Although *Citrus* are moderately resistant to water stress, an adequate irrigation is essential to promote vegetative growth, fruit setting and fruit production. Furthermore, fertilization is one of the main effective means to improve fruit market appreciability. Among the agronomic factors, the selection of rootstock represents a

key choice since it controls numerous parameters related to fruit quality as sugar, organic acid and phenols content (Emmanouilidou and Kyriacou, 2017; Lado et al., 2018; Legua et al., 2018)

Citrus fruits are deemed excellent functional food because of their high content in antioxidant and nutritional compounds (Goldenberg et al., 2018). Then, a deeper knowledge of the mechanisms that control the biosynthesis of these molecules is crucial to understand the physiological basis regulating phenotypes with enhanced quality traits. Although numerous aspects about the genetic regulation of maturation are still unclear, recent advancements in this field have provided new insight into some of the regulatory networks involved in citrus fruit ripening. Numerous genes belonging to different biosynthetic pathways have been identified and their expression studied also in *Citrus* species (Alquézar et al., 2008; Rodrigo et al., 2013a; Lo Piero, 2015).

Unraveling the modifications occurred in mutant phenotypes is the prerequisite to identify new genetic markers associated with traits of agronomical relevance. Indeed, molecular markers are an irreplaceable tool for breeders to reduce the amount of time and economic resources that a breeding project usually requires. For this reason, a study was carried out on Pink lemon, a spontaneous bud mutation of lemon (*Citrus limon* L. Burm. *f.*) characterized by the production of pink-flesh fruits due to the unusual accumulation of lycopene. To elucidate the basis behind the altered pigmentation, a comparative study was carried out between the pink mutant and its wild type. The carotenoid content and the regulation of the main genes involved in carotenoid biosynthesis and storage were evaluated through all the ripening process.

The adoption of new citrus rootstocks tolerant to *Citrus Tristeza Virus* (CTV) is a needful tool to cope with this disease and to adapt the crop to environmental conditions. As reported in literature, the rootstock could influence the biochemical composition of the fruits affecting their qualitative characteristics (Cardeñosa et al., 2015). Then, in order to get new insight about the influence of rootstock-scion interaction in citrus fruit quality traits, a comparative study was conducted on a

selection of blood orange (*C. sinensis* (L.) Osb. cv Tarocco Sciré) grafted onto three different rootstocks: Citrange Carrizo [*C. sinensis* (L.) Osb. cv. Washington navel x *Poncirus trifoliata* (L.) Raf.], Bitters and Furr [*C. sunki* Hort. ex Tan. x *P. trifoliata* (L.) Raf.]. Anthocyanins, sugars, acids, and vitamin C content, which contribute to the sensory and nutritional quality of citrus flesh, were assayed by metabolic analysis. While, qPCR analysis was employed to investigate whether the transcription of twelve genes (*PAL, CHS, DFR, ANS, UFGT, Ruby, SPS, ACL, NADP-IDH1, CS, GGP, GalUR-12*) involved in the citrus fruit maturation process was differentially expressed among the three rootstock-scion combinations.

Transcriptional analysis of carotenoids accumulation and metabolism in a pink-fleshed lemon mutant

1 Introduction

Carotenoids are isoprenoids-derived molecules playing essential functions in plant cells. they are part of the photosynthetic system and participate to light capture. Carotenoids play important roles in photo-protection, increasing tolerance to light and heat stresses and preventing membranes from lipid peroxidation. These pigments are also precursors of important phytohormones such as abscisic acid (ABA) and strigolactones; carotenoids constitute the substrates for the formation of apocarotenoids-derived volatiles. In addition, they are involved plant-animal interaction (Rodriguez-Concepcion et al., 2018).

Carotenoids are not only responsible for the attractive colour of flowers, fruits and other organs in many plant species, but they are also known for their benefits on human health. These properties are mainly due to the antioxidant activity and the fact that α-carotene, β-carotene and β-cryptoxanthin are precursors of vitamin A, an essential dietary component (Eggersdorfer and Wyss, 2018). Recent studies highlighted that a regular intake of carotenoids plays a positive effect on human health preventing neurodegenerative, cardiovascular and aging-related diseases, as well as reducing cancer risk (Fiedor and Burda, 2014; Woodside et al., 2015; Eggersdorfer and Wyss, 2018).

Citrus fruit pigmentation is characterized by a wide variability: the peel and pulp colour ranges from the pale yellow of lemons, pummelos and grapefruits to the light and deep orange respectively of oranges and mandarins until the reddish shades of red-grapefruits and some orange mutants (Rodrigo et al., 2013a; Lado et al., 2015a). This variability in pigmentation is mainly due to the differences in carotenoid accumulation and composition, which is responsible for the species-specific colour according to the ripening stages (Lado et al., 2015a; Tadeo et al., 2020). Citrus fruits are consumed worldwide for their organoleptic characteristics as well as for their nutraceutical value (Ma et al., 2020). In light of this, unrevealing the modifications occurring in mutant

phenotypes is a prerequisite to identify new genetic markers associated with improved commercial and nutritional traits. Indeed, molecular markers are a fundamental tool for breeders to reduce the amount of time and money required to develop a new cultivar through traditional breeding.

In plants, carotenoids are generally formed by the condensation of eight C5 isoprenoid units forming a C40 polyene backbone that contains a variable number of conjugated double bonds. This particular chemical structure provides to carotenoids the capacity to absorb visible light at different wavelengths. Carotenoids are classified in carotenes and xanthophylls; the first ones are composed exclusively by carbon and hydrogen atoms, while the second ones contain at least one oxygenated group (Gross, 1987; Rodríguez-Concepción, 2010). During the last decades, several genes encoding for enzymes involved in the main steps of carotenoid biosynthesis pathway have been isolated and their molecular and biochemical regulation has been clarified (Rodríguez-Concepción, 2010; Yuan et al., 2015). Moreover, other processes related to the storage of carotenoids in chromoplasts and how they are catabolized by a family of enzymes known as carotenoid cleavage dioxygenases (CCDs) have also been addressed (Ahrazem et al., 2016; Sun and Li, 2020). The expression of a large number of carotenoid biosynthetic genes has been studied in the peel and pulp of many citrus varieties during all the ripening process (Tadeo et al., 2020; Tatmala et al., 2020).

The initial substrate for carotenoid biosynthesis, geranylgeranyl diphosphate (C20, GGPP), is produced by the condensation of one dimethylallyl diphosphate (DMAP) and three isopentenyl diphosphate (IPP) molecules (Figure 1). The synthesis of these precursors takes place through the so-called methylerythritol 4-phosphate (MEP) pathway and involves several enzymes like 1-deoxy-D-xylulose-5-phosphate synthase (DXS), located upstream, and the hydroxymethylbutenyl diphosphate synthase (HDS) and reductase (HDR) that are located downstream the pathway. At the end of the MEP pathway, the formation of GGPP is catalysed by the geranyl geranyl pyrophosphate synthase (GGPPS) enzyme which is coded by a multigene family (Rodriguez-Concepcion et al., 2018). During the first two steps of carotenoid formation, phytoene synthase

(*PSY*) and phytoene desaturase (*PDS*) catalyses the head-to-head condensation of two molecules of GGPP to form the colourless phytoene (C40) and phytofluene. Subsequently, desaturation and isomerization by ζ-carotene desaturase (*ZDS*) and ζ-carotene isomerase (*Z-ISO*) produce lycopene, through the intermediates ζ-carotene and neurosporene. At this point, the pathway splits into two branches. Lycopene ε-cyclase (*ε-LCY*) and lycopene β-cyclase (*β-LCY*) are responsible for the addition of one or two β-ionone rings producing δ-carotene and β-carotene, respectively. Subsequently, *β-LCY* introduces a second β-ionone ring on δ-carotene to produce α-carotene (Ikoma et al., 2016; Tadeo et al., 2020). Two subfamilies of β-lycopene cyclases have been identified in citrus fruits and named as *β-LCY1* and *β-LCY2*. The first of these two genes shows a constant expression during all the ripening process and is expressed in a large variety of organs and tissues, while the second one is chromoplast-specific and is typically expressed in fruit tissues and it is highly up-regulated during the fruit maturation phase (Mendes et al., 2011). Two different alleles of *β-LCY2* have been isolated: *β-LCY2a*, and *β-LCY2b.* The studies carried out on both variants revealed a differential tissue and temporal expression, other than a different enzymatic efficiency to convert lycopene into β-carotene (Alquézar et al., 2009; Zhang et al., 2012). δ-carotene is converted into α-carotene and then in lutein by β-carotene hydroxylase, while, β-carotene is hydroxylated to β-cryptoxanthin and zeaxanthin by β-carotene hydroxylase (*β-CHX*) (Ma et al., 2016). In citrus fruits these two last carotenoids can be catabolized to C30-apocarotenoids by a class of enzymes generally recognized as carotenoids cleavage dioxygenases (*CCDs*) (Ma et al., 2013; Rodrigo et al., 2013a; Zhang et al., 2019). Zeaxanthin epoxidase (*ZEP*) adds to antheraxanthin and subsequently to violaxanthin epoxy groups resulting in neoxanthin formation. The last reaction of the pathway is catalyzed by neoxanthin synthase (*NSY*), which turns violaxanthin into neoxanthin. The 9-cis-isomers of these last two xanthophylls are then utilized as substrates by 9-cis epoxycarotenoid dioxygenase (*NCED*) to produce ABA (Rodrigo et al., 2006; Agustí et al., 2007).

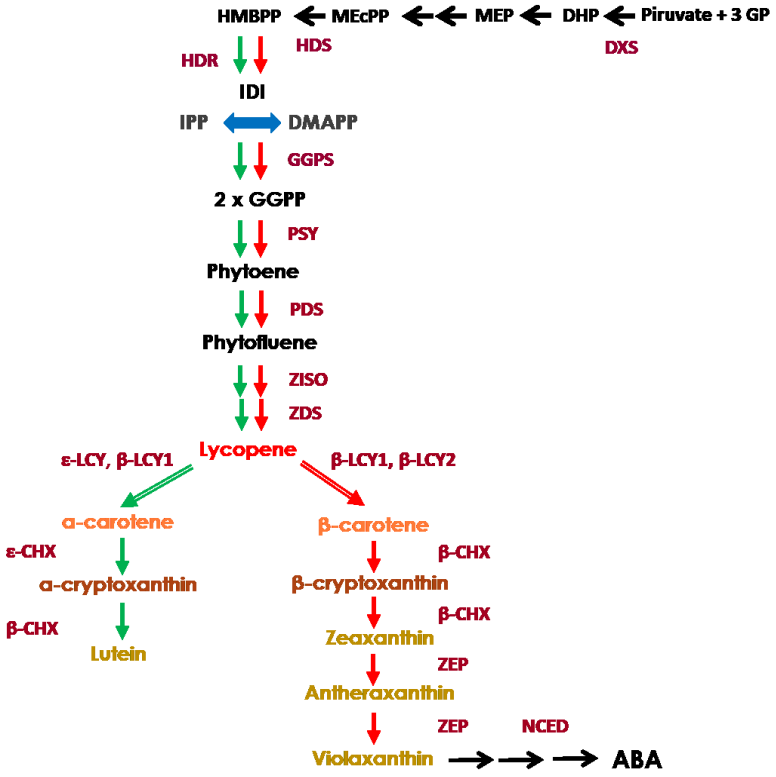

Figure 1. Schematic representation of carotenoid biosynthesis in citrus fruits, indicating main enzymes and genes of the pathway. 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*), hydroxymethylbutenyl diphosphate synthase (*HDS*) and reductase (*HDR*), geranylgeranyl diphosphate synthase (*GGPSS*), phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene isomerase (*ZISO*), ζ-carotene desaturase (*ZDS*), ε-lycopene cyclase (*ε -LCY*), β-lycopene cyclase (*β-LCY1/2*), ε-carotene hydroxylase (*ε-CHX*), β-carotene hydroxylase (*β-CHX*), zeaxanthin epoxidase (*ZEP*) and 9-cis-epoxy-carotenoid

Carotenoid synthesis takes place concurrently to the differentiation of chromoplasts leading to the development of diverse sink structures organized to storage carotenoid just produced (Sun et al., 2018). The ultrastructural changes that occur during this phase involve several proteins. Among them, the most important are the small heat shock proteins (sHSPs), fibrillins (FIBs or PAPs) and Orange gene protein (*OR*). *HSP21* transcript level has been correlated with carotenoid accumulation in tomato fruit (Neta-sharir et al., 2005). Fibrillins play a structural role on fibrils, organizing carotenoids in lipoprotein complexes (Simkin et al., 2007). Several studies have demonstrated that OR protein promotes PSY activity and chromoplasts biogenesis

which leads to the enhancement of carotenoid accumulation (Zhou et al., 2015; Sun et al., 2018; Welsch et al., 2018).

The large variety of pigmentation showed by both rind and flesh of mature citrus fruits is strictly related to the differences in the total amount and composition of carotenoids typical of each species and cultivar (Ikoma et al., 2016; Tadeo et al., 2020). In the case of ordinary lemon (*Citrus limon*), the light-yellow colouration is due to a very low accumulation of carotenoids (Gross, 1987; Kato et al., 2004). Comparative transcriptomic analysis has highlighted a reduced expression of most of the carotenoids biosynthetic genes in both flavedo and juice sacs of lemon fruits compared to what found in oranges and mandarins (Kato et al., 2004).

Accumulation of lycopene in *Citrus* is relatively uncommon and characterize few varieties and mutants of pummelo, grapefruit and sweet orange. Despite the extensive efforts to investigate carotenoid biosynthesis and metabolism in several red-fleshed citrus mutants, the molecular basis of lycopene accumulation has not been completely elucidated yet (Ikoma et al., 2016; Tadeo et al., 2020). In the case of Cara Cara orange mutant, it has been proposed that the red pigmentation is amenable to an enhanced flow of carotenoids precursors through the MEP pathway (Alquezar et al., 2008; Lu et al., 2017). In addition, it has been found that alterations in the expression of the two alleles of *β-LCY2* might lead to the accumulation of lycopene (Lu et al., 2006; Alquézar et al., 2009; Xu et al., 2009; Yu et al., 2012; Alquezar et al., 2013). Lycopene cyclase activity is a rate-limiting step in the biosynthesis of carotenoids, then a partial blockage in the conversion of lycopene to β-carotene may increase the accumulation of lycopene and repress the production of downstream metabolites like xanthophylls (Alquézar et al., 2009; Xu et al., 2009; Yu et al., 2012; Zhang et al., 2012). Interestingly the comparison between white and red pummelos indicated that lycopene accumulation is associated with a reduced expression of genes encoding for enzymes which operate downstream lycopene production (Liu et al., 2016; Yan et al., 2018; Promkaew et al., 2020; Tatmala et al., 2020), reinforcing the hypothesis that a

reduction of activity of lycopene cyclase might contribute to the onset of a bottleneck along the carotenoid pathway.

A pink-fleshed lemon was described in 1932 in California as a spontaneous bud mutation of Eureka lemon. The peel of pink lemon is variegated, with green stripes, which turn into yellow when mature, while the yellow sector becomes light-pink (Figure 2A). The pulp has a light-pink colouration due to lycopene accumulation with few seeds and a sour taste when full mature (Shamel, 1932). Although, the mutant is known by long time and is commercially available in specialized markets, no information is still available about the transcriptomic and metabolic changes behind the pink pigmentation.

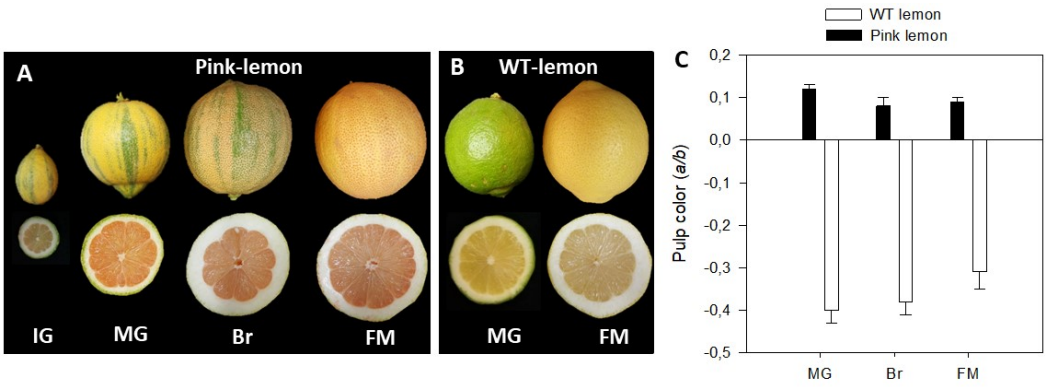

Figure 2. External and internal appearance of Pink lemon (PL) (A) and wild-type (WT) (B) lemon fruit during development and maturation. Immature green (IG), mature green (MG), breaker (BR) and full mature (FM) stages. Changes in colour (a/b Hunter) of the pulp of PL and WT (C) during fruit maturation. Data are the mean ± SD of at least 10 fruits.

The aim of the present work was to carry out a comparative analysis of carotenoids biosynthesis between the pulp of the pink-fleshed lemon and its wild type (WT), in order to elucidate the metabolic and molecular changes at the basis of the pigmentation of the red-fleshed mutant. To this end, carotenoids identification and quantification was performed employing a HPLC-PDA technique, while the regulation of the genes involved in carotenoids biosynthesis and the production of proteins related to carotenoid-sequestering structures was detected through qPCR. An

increased understanding of the genetic determinism of the pink-fleshed lemon phenotype could be of great interest to identify candidate genes for the development of molecular markers to be employed in fruit quality breeding programmes.

2 Materials and Methods

2.1 Plant material

Fruits of Pink lemon (PL) and Fino (*Citrus limon*, cv. Fino), referred as wild type (WT), were harvested from adult trees grafted on Citrange carrizo (*Poncirus trifoliate* L. Raf x *Citrus sinensis* L. Osb) rootstocks cultivated at The Citrus Germplasm Bank (Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain) and subjected to standard cultural practices. Samples were collected at four developmental stages: immature-green (IG), mature-green (MG), breaker (BR) and full-mature (FM) (Figure 2). Trees of both genotypes were located in the same orchards and samples of each genotype were collected at the same time. Fruits were quickly delivered to the laboratory, where the pulp was separated from flavedo, frozen in liquid nitrogen, ground to a fine powder and stored at -80°C until analysis. Colour of the pulp was measured using a CR-400 Minolta chromameter on three different locations around the equatorial plan of the fruits. The Hunter parameters *a* (negative to positive, from green to red) and *b* (negative to positive, from blue to yellow) were measured, and colour was expressed as the *a/b* Hunter ratio, a colour index that has been widely used for colour measurement in citrus fruits [40]. Data of colour index for each cultivar are the means ± SD of at least 10 fruits. Fruits were harvested and colour determined in two consecutive crop seasons.

2.2 Carotenoid extraction and quantification by HPLC-PDA

Carotenoids were extracted from frozen flesh, following the protocol described by Rodrigo et al. (Rodrigo et al., 2015). Extracts were dried and kept at −20°C until further

analysis. Each sample was extracted in triplicate and results were expressed as mean ± SD. In order to prevent photodegradation, isomerizations and structural changes of carotenoids all the operations were carried out on ice under dim light.

Individual carotenoid analysis of each sample was carried out by HPLC-PDA as previously described by Lado et al. (Lado et al., 2015b) and Rodrigo et al. (Rodrigo et al., 2015). Carotenoids were identified by their absorption, fine spectra and retention time. Then, they were quantified integrating each one of them at its corresponding maximum absorbance wavelength and using the corresponding calibration curves as reported by Rodrigo et al. (Rodrigo et al., 2015).

2.3 Gene expression analysis by quantitative real-time PCR

The RNA isolation, cDNA synthesis and gene expression analyses were performed essentially as described by Rodrigo et al. (Rodrigo et al., 2013b), and subsequently treated with DNA free, DNase treatment and removal (Ambion, Madrid, Spain) to eliminate any residual trace of DNA. Total RNA was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) and absence of DNA was checked by gel electrophoresis.

Briefly, 2 µg of total RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Madrid, Spain) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBRGreen I Master kit (Roche). The primers employed for the amplification of each gene are listed in Table S2. 20 ng of cDNA was used for each amplification reaction in a total volume of 10 µl. The cycling protocol consisted of 10 min at 95°C for pre-incubation, followed by 35 cycles of 10 s at 95°C for denaturation, 10 s at 59°C for annealing and 10 s at 72°C for extension. Fluorescence data were acquired at the end of extension phase and reactions specificity was checked by post-amplification dissociation curve. For expression measurements, we used the LightCycler 480

Software release 1.5.0, version 1.5.0.39 (Roche) and calculated expression levels relative to the values of a reference sample using the Relative Expression Software Tool (Pfaffl et al., 2002). Actin gene expression was chosen to normalize raw Cp's based on a previous selection of reference genes (Alós et al., 2014b). The results were the average of three independent sample replicates.

2.4 Statistical analysis

The outputs of both HPLC-PDA and qPCR analysis were processed using the R software (R Core Team, 2016). An ANOVA test was employed to determine significant differences (*p* value <0.01) between Pink lemon and its wild type. A Shapiro-Wilk test was performed before the ANOVA test.

3 Results

3.1 Phenotypic characteristics of the pink lemon fruit

Pink-fleshed lemon trees are characterized by variegated leaves and fruits, both featured by green and white sectors variable in shape and size. The typical green stripes were unevenly distributed on the fruit skin and even if they were evident from early developmental stages, during the maturation process their colour changed to the characteristic yellow, while the white areas turned into light pink (Figure 2A). The red tone of the pulp was evident already in IG and increased in intensity with maturation, reaching an intense red colour at MG. The reddish colouration of the pulp at early stages moved to clearer shades, probably due to the dilution effect caused by the substantial growth of the pulp along the maturation process (Figure 2A). Remarkable differences in pulp colour (determined as *a/b* Hunter ratio) were found between the two genotypes (Figure 2C). Colour of Pink lemon (PL) pulp assumed positive values at all developmental stages, although they slightly decreased as the maturation progressed. By contrast, the colour of wild-type (WT) lemon pulp assumed negative

values typical of the light-yellow tone and it showed an increasing trend over maturation (Figure 2B).

3.2 Carotenoids content and composition in Pink lemon fruit

Carotenoids content and composition were analysed in the pulp of PL and WT fruits at four developmental stages from immature-green to full maturity as outlined in the material and methods section (Table 1). HPLC-PDA analysis allowed the detection and quantification of eleven carotenoids. Carotenoids content and composition in PL fruits were markedly different from WT (Table 1; Figure S2) at all the four stages analyzed. Total carotenoids content was much higher in PL fruits than in WT (from 100 to 1000 fold higher) (Table 1). Total carotenoids content was very low (<0.4 μg/g FW) in WT at all developmental stages, while in PL reached a maximum at MG (53.3 μg/g FW) and declined subsequently. The colourless phytoene and phytofluene were the major carotenes detected in PL flesh composing respectively the 82-86% and 11-16% of total carotenoids, while these carotenes were only detected in traces or at extremely low levels in the pulp of WT fruits. In addition to phytoene and phytofluene, low amounts of lycopene, neurosporene, ζ- and δ-carotene were detected in the pulp of PL. In the pulp of mature WT fruits, only low levels of β-cryptoxanthin and traces of other carotenoids were detected (Table 1).

Table 1. Carotenoid content and composition (μg/g FW) in the pulp of the Pink lemon and wild type at four developmental and ripening stages. The amount of violaxanthin represents the sum of all-trans and 9-cis isomers. Traces indicate amount lower than 0.01 μg/g FW. nd: not detected. Tr: traces. Data are expressed as mean ± SD. [α] Total carotenoids are the sum of the main carotenoids identified and quantified

	Pink lemon				Wild type			
Carotenoids (μg/g FW)	**IG**	**MG**	**BR**	**FM**	**IM**	**MG**	**BR**	**FM**
Phytoene	17.59±1.12	44.01±0.90	4.98±0.30	8.81±0.07	tr.	tr.	0.04±0.01	0.04±0.01
Phytofluene	2.27±0.17	8.93±1.75	1.00±0.01	1.79±0.01	nd	nd	nd	tr.
ζ-carotene	nd	0.05±0.01	nd	nd	nd	nd	nd	nd
Neurosporene	0.06±0.01	0.24±0.03	nd	nd	nd	nd	nd	nd
Lycopene	0.24±0.01	0.49±0.13	tr.	0.02±0.01	nd	nd	nd	nd
δ-carotene	tr.	0.04±0.01	nd	nd	nd	nd	nd	nd
Lutein	0.06±0.01	nd	nd	nd	tr.	tr.	tr.	tr.
β-carotene	nd	nd	nd	nd	nd	tr	nd	tr.
β-cryptoxanthin	0.02±0.01	nd	0.02±0.01	nd	nd	0.03±0.01	0.02±0.01	0.02±0.01
Anteraxanthin	nd	nd	nd	nd	nd	nd	tr.	tr.
Violaxanthin	nd	tr	nd	nd	nd	tr.	nd	tr.
Total carotenoids[α]	**20.24±1.54**	**53.33±2.17**	**6.01±0.29**	**10.61±0.03**	**tr.**	**0.05±0.01**	**0.06±0.01**	**0.08±0.01**

3.3 Expression of the genes involved in the biochemical pathway of carotenoids

The expression levels of eleven genes related to carotenoids biosynthesis were tested through a qRT-PCR assay to explore the possible causal relation between the increased carotenoid accumulation in PL and the transcripts abundance of such candidate genes.

Noticeable differences were highlighted in the expression of several genes on the two genotypes (Errore. L'origine riferimento non è stata trovata.-6). In general, the expression of the three genes belonging to the MEP pathway (*DXS*, *HDS* and *HDR*) increased progressively in the pulp of WT. Differently than in WT *DXS* and *HDR* were up-regulated at early development stage in PL, while they were down-regulated during the last stages of development. The accumulation of the transcripts corresponding to the plastid-associated *GGPS11* was higher in the pulp of mutant lemon at IG stage and gradually declined during maturation (Figure 3).

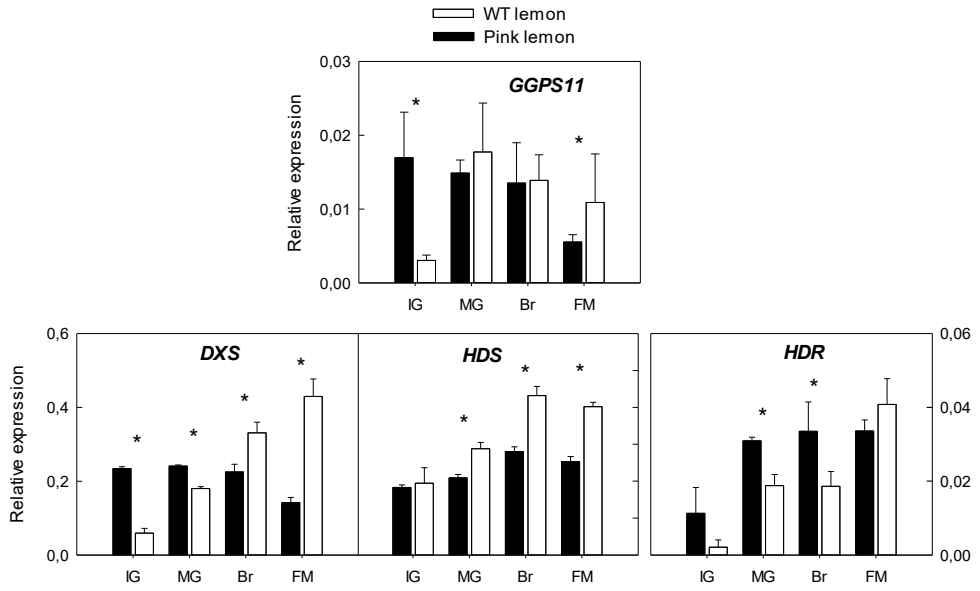

Figure 3. Changes in the expression of genes involved in the MEP pathway: *GGPS11*, geranylgeranyl diphosphate synthase 11; *DXS*, 1-deoxy-D-xylulose-5-phosphate synthase; *HDS,* hydroxymethylbutenyl diphosphate synthase; *HDR,* hydroxymethylbutenyl diphosphate reductase; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage (p<0.01) by one-way ANOVA (*p*<0.01).

Expression of genes involved in early desaturation and isomerization steps of carotenoid biosynthesis, with the exception of *PSY3a*, were up-regulated during maturation in the pulp of WT lemon fruits. Expression of the *PSY*, *PDS*, *ZDS* and *ZISO* genes experienced minor increases in PL mutant fruit and after MG transcripts accumulation were significantly lower than in WT pulp (Figure 4). The transcription of genes involved in lycopene cyclization also showed important differences between the genotypes under evaluation. The expression of *β-LCY1* remained relatively constant during WT lemon maturation, conversely to PL in which the gene was down-regulated. Despite the expression of *β-LCY2* increased in both genotypes during the four ripening stages, it was consistently lower in the pulp of PL mutant than in WT lemon. No significant differences were observed in the accumulation of ε-LCY transcript between the two varieties. *β-CHX* was up-regulated in both WT and PL, following a similar trend

(Figure4).

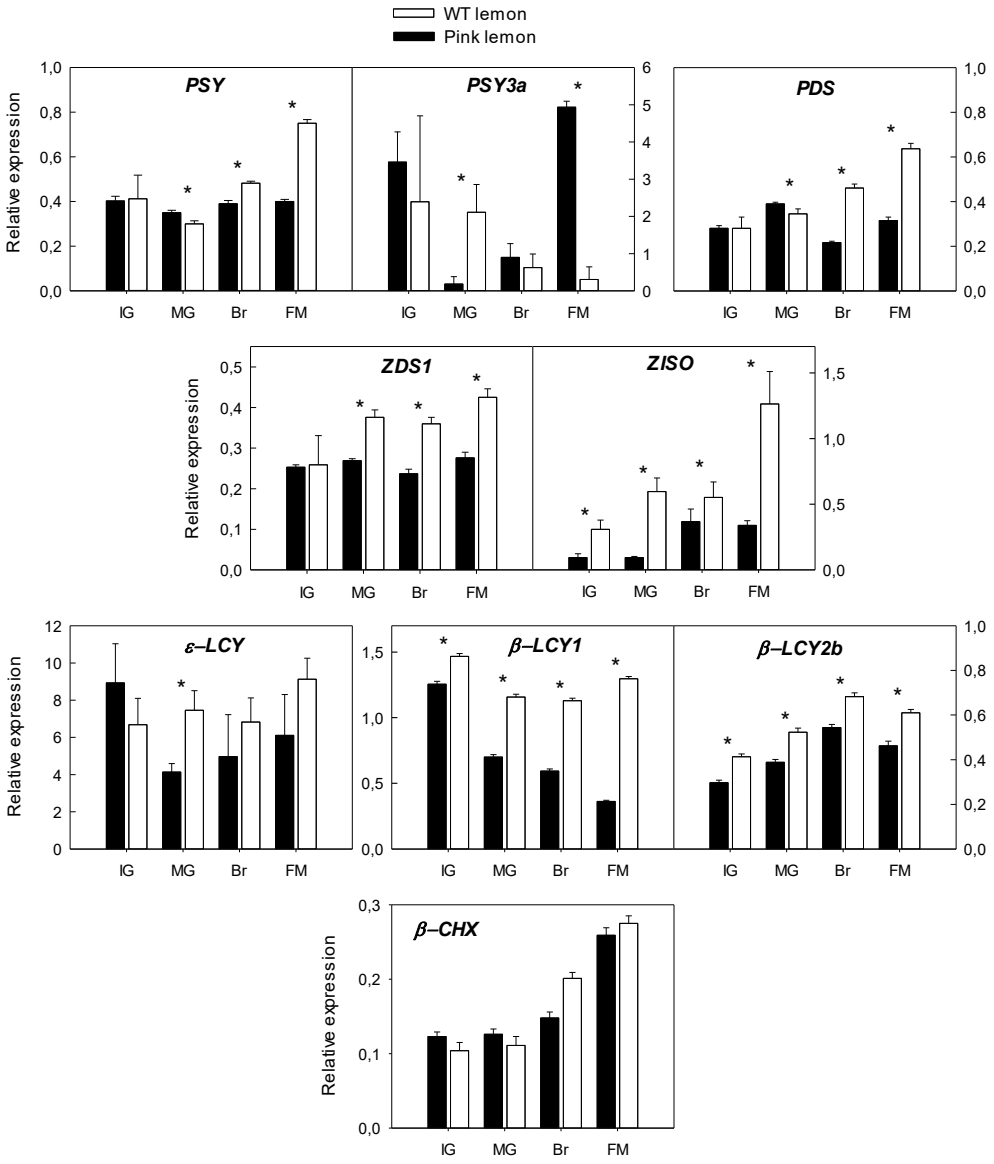

Figure 4. Changes in the expression of genes involved in carotenoids biosynthesis: *PSY*, phytoene synthase; *PSY3a*, phytoene synthase 3a; *PDS*, phytoene desaturase; *ZDS1*, Ƶ-carotene desaturase 1; *ZISO*, Ƶ-carotene isomerase; ε-*LCY*, ε-cyclase ; *β-LCY1*, β-lycopene cyclase 1; *β-LCY2b*, β-lycopene cyclase 2b; *β-CHX*, β-carotene hydroxylase; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage (p<0.01) by one-way ANOVA (*p*<0.01).

3.4 Expression of genes involved in the biosynthesis of abscisic acid

Regarding the genes encoding for 9-cis epoxycarotenoid dioxygenase (*NCED*), which are involved in the production of ABA, both *NCED1* and *NCED2* were up-regulated in WT and PL. However, the level of transcript of *NCED1* and *NCED2* accumulated in PL was respectively 6- to 9-times and 2.3 to 7-times higher than in WT (Figure 5).

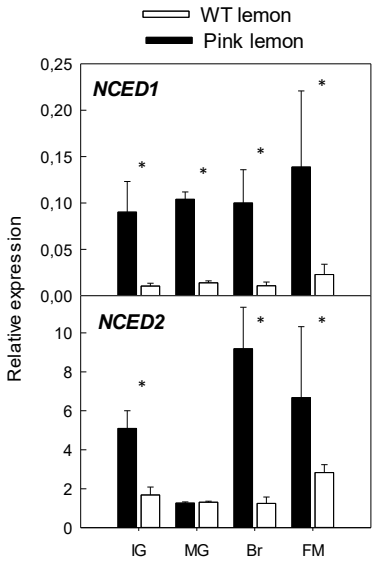

Figure 5. Changes in the expression of genes involved in abscisic acid biosynthesis: *NCED1,* 9-cis-epoxy-carotenoid dioxygenase 1; *NCED2*, 9-cis-epoxy-carotenoid dioxygenase 2; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage (p<0.01) by one-way ANOVA (*p*<0.01).

3.5 Expression of accessory genes involved in the accumulation of carotenoids

In order to clarify whether the massive accumulation of carotenoid in the PL is associated with alterations in the expression of genes related to chromoplast differentiation and carotenoids-sequestering structures, accumulation of mRNAs corresponding to three *HSP* (*HSP20_3, HSP20_4, HSP21*), two fibrillins (*FIB1, FIB2*) and an *ORANGE* (*OR*) gene was investigated. The relative expression pattern of the *HSP* genes underwent to a noticeable up-regulation in WT during the last ripening stages. The most pronounced differences were highlighted in the transcription of *HSP21*, which was over-expressed in the pulp of the PL mutant during three of the four developmental stages analyzed. Although the transcription of *FIB1* and *FIB2* genes followed a more constant trend in WT lemon, the level of transcript accumulated in the PL was considerably higher except for a slight decline at MG. The expression of the *Or* gene followed the same pattern of *FIB1* and *FIB2*. Accumulation of the *O*r transcript was 3.4 to 11 times higher in the PL than in WT (Figure 6).

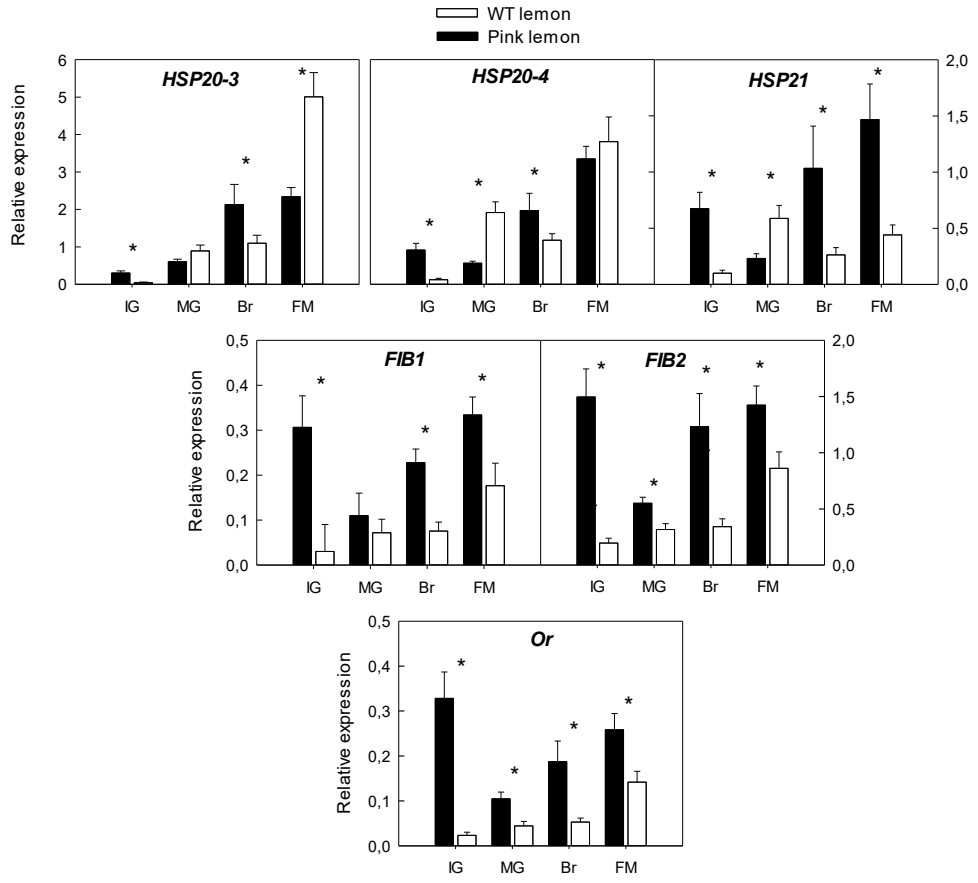

Figure 6. Changes in the expression of genes of carotenoid-associated proteins: *HSP20-3*, heat shock protein 20-3; *HSP20-4*, heat shock protein 20-4; *HSP21*, heat shock protein 21; *FIB1*, fibrillin 1; *FIB2*, fibrillin 2; *Or*, orange protein gene; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage (p<0.01) by one-way ANOVA (*p*<0.01).

4 Discussion

Although variegated pink-fleshed lemon was identified already in 1932 and its fruits are cultivated and purchased in many countries, the biochemical and molecular alteration behind its characteristic pigmentation is still unknown. A detailed observation of the pulp colouration and a comparative analysis of carotenoid content

and composition are reported here for the first time revealing interesting features of this mutant in comparison with the other red-fleshed citrus mutants.

The pulp of WT lemon contained negletable amounts of carotenoids at the four developmental stages analysed (Table 1) in agreement with previous investigations [9,29] reinforcing the classification of lemon as low-carotenoid accumulating *Citrus* genotypes [15]. The red colouration of the pulp was clearly distinguishable in IG fruits (Figure 2A) and lycopene, even at moderated amount (Table 1), was already detectable. These observations indicate that the accumulation of the red pigment is not a ripening-related event like in other red-fleshed pummelos, grapefruits and oranges mutants. Indeed, lycopene biosynthesis was initiated very early in the fruit development, at the beginning of the second phase of fruit growth during the cell enlargement. Accumulation of lycopene in the pulp of PL was associated with a high concentration of phytoene and phytofluene, two colourless carotenes that were virtually absent in WT lemon (Table 1). The concentration of these two linear carotenes reached a maximum in MG fruits and declined immediately after (Table 1). It is worth to note that except for Pinalate orange mutant (Rodrigo et al., 2019) accumulation of phytoene and phytofluene is very unusual in lemon and in other citrus fruits, in particular the concentrations found in the PL pulp are among the highest ever reported in citrus fruits (Lado et al., 2016; Tadeo et al., 2020). Moreover, HPLC-PDA analysis enabled the detection of small amounts of neurosporene and δ-carotene at early development stages, both carotenes were hardly identified in the pulp of other citrus fruits (Gross, 1987; Lado et al., 2016). This unusual accumulation of δ-carotene, which is a carotene characterized by a ε-ring at one end while the other end is linear, may suggest a defect in the β-cyclization of lycopene that would lead to the accumulation of upstream carotenes, and explaining also the large amount of phytoene and phytofluene found in the PL (Table 1).

The pattern of carotenoid accumulation in the pulp of PL is different to the ones found in other lycopene accumulating citrus fruits. In the case of orange fruits such as Hong Anliu or Cara Cara (Xu et al., 2006; Liu et al., 2007; Alquezar et al., 2008; Lu et al., 2017)

or red-pummelos (Yan et al., 2018; Promkaew et al., 2020; Tatmala et al., 2020), lycopene is accumulated progressively during maturation and the highest concentration is reached in fully ripe. Similarly, low amounts of phytoene accumulated in immature fruits of some mutants gradually increased along maturation (Alquezar et al., 2008; Tatmala et al., 2020). In lemon, the expression of genes involved in the biochemical pathway of carotenoid is different from other citrus species (Kato et al., 2004; Ikoma et al., 2016), and non-pigmented lemons have a low capability to accumulate carotenoids. Then, it is reasonable to assume that the genetic alteration responsible of the accumulation of lycopene in the pink-fleshed lemon may be different to that occurred in the other citrus mutants characterized by increased levels of lycopene.

The transcriptomic analysis, which included the study of 4 genes of the MEP pathway (Figure 3) and 9 genes of the biosynthesis of carotenoids (Figure 4), highlighted important differences between the two accessions under evaluation that might clarify the cause of the mutation. In ordinary lemons the transcription of MEP pathway genes increases with ripening in response to enhanced demand for precursors due to the formation of downstream products (Nisar et al., 2015; Rodriguez-Concepcion et al., 2018). However, in immature PL fruits the transcription of *GGPS11*, *HDS* and *DXS* was higher than in the WT. These results are in accordance with the extensive differences in total carotenoids content between the two genotypes (20.2 µg/g FW in PL vs traces in the WT) and may increase the availability of GGPP to be converted into phytoene and other carotenoids. Similar results have been observed in the pulp of red-fleshed oranges Hong Anliu and Cara Cara, where the accumulation of early carotenes appears to be associated with an increased production of isoprenoid precursors (Liu et al., 2007; Alquezar et al., 2008).

The expression patterns of carotenoid biosynthetic genes in the pulp of WT lemon was similar to the ones characterizing other white-fleshed varieties (Kato et al., 2006; Liu et al., 2016). The low carotenoid content in non-pigmented lemons is generally associated with an up-regulation of the upstream carotenoid genes like *PSY*, *PDS*, *ZDS*,

ZISO, β-LCY2b and *β-CHX*, while *ε-LCY*, *β-LCY1* are usually down-regulated. In the pulp of PL, however, differences in the pattern of expression of carotenoid biosynthetic genes were not consistent with the alterations in carotenoid content and composition (Figure 4, Table 1). Genes synthetizing the precursors of lycopene were not up-regulated during maturation, on the contrary, the genes related to the β-cyclization of lycopene *β-LCY1* and *β-LCY2b* showed reduced transcript levels in PL than in WT from early stages of development (Figure 4). In particular, during the major development of the fruit (IG to MG), when carotenoids reached their maximum concentration in the PL (Table 1). In addition, the expression of *β-LCY1* was severely down-regulated in PL mutant respect to WT (Figure 4). Transcripts of *β-LCY2a*, which is the allele with the highest *in vitro* activity, were not detected in the pulp of both lemon genotypes at any developmental stages (data not shown) indicating a reduced capability of lemon fruits to convert early carotenes to xanthophylls. These alterations combined with the lower expression of the *β-LCY2b* in PL might be responsible for the onset of a bottleneck along the carotenoid pathway that could lead to the massive accumulation of lycopene, phytoene and phytofluene (Figure 4). According to this hypothesis, the *PSY* transcription would not be a limiting factor at early developmental stages but it might be more critical during the latest stages. These data are in agreement with those reported in red pummelo, in which the balance between the transcription of genes located upstream and downstream lycopene, together with the reduced LCY activity, led to the accumulation of lycopene (Liu et al., 2016; Promkaew et al., 2020; Tatmala et al., 2020). Our results suggest that *β-CHX* is not a limiting factor for the accumulation of carotenoids in the PL, although the levels of the transcripts are very low compared to other citrus fruits (Kato et al., 2004; Ikoma et al., 2016).

A further key node in carotenoid accumulation is the balance between biosynthesis and degradation of metabolites, indeed the pool of carotenoids present in the tissues is related to their degradation rate (Nisar et al., 2015; Rodriguez-Concepcion et al., 2018). It is reasonable to assume that the remarkable alteration in the carotenoid pool occurred in the PL could modify the regulatory network operating in ordinary lemons.

Thus, besides carotenoid biosynthetic genes, the expression of *NCED1* and *NCED2* were up-regulated in PL respect to WT (Figure 5). These two genes are related to ABA synthesis and they operate downstream the xanthophylls production. Therefore, an enhancement in their transcription might suggest an altered homeostasis of the pathway. Despite, ordinary lemons contain very low amounts of xanthophylls in their flesh, they accumulate considerable quantities of ABA, indicating that flux of metabolites producing for this hormone is pretty active (Norman et al., 1991). Then, the altered carotenoid composition in the pulp of the PL, likely due by a reduced lycopene cyclization, might de-regulated the normal genetic network of the pathway originating a positive feedback of the genes involved in ABA formation. These results are similar to those found in the Cara Cara orange mutant, where lycopene accumulation in the flesh was accompanied by a reduction in ABA content and enhanced expression of both *NCED1* and *NCED2* genes (Alquezar et al., 2008). In other plant tissues, it has been also shown that alterations in carotenoid composition originate a coordinated regulation of *NCED* genes and ABA content (Norman et al., 1991).

Accumulation of carotenoid in specialized structures is a stable storage system and an alternative mechanism to regulate carotenoids availability (van Wijk and Kessler, 2017; Wurtzel, 2019). The transcriptional analysis of genes encoding for carotenoid-associated proteins highlighted significant alterations in PL. *HPS21*, *FIB1, FIB2* and *OR* genes were consistently over-expressed in the pulp of PL mutant than in WT (Figure 6). The carotenoid associated-proteins encoded by these three genes were associated with numerous processes involved in carotenoid storage, in addition, they contribute to carotenoid stabilization in plastoglobuli especially during the transition phase from chloroplast to chromoplast (Wurtzel, 2019). It has been found that *HSP21* chaperone stimulates accumulation of lycopene in tomato and protects fruit pigmentation from heat-stress demonstrating its close relation with carotenoid content (Neta-sharir et al., 2005). Fibrillin is a family of proteins playing structural functions in the packaging and organization of carotenoids in plant tissues and constitute a key element for their

storage and metabolism (Singh and McNellis, 2011). The carotenoid content and composition in tomato and pepper fruits have been correlated with the transcript abundance of fibrillins (Kilcrease et al., 2015). Both *FIB* genes displayed a similar expression pattern during the massive accumulation of carotenoids in the PL with a high mRNA accumulation at IG and a slight decline at MG (Figure 6). These evidences support the involvement of fibrillins in the unusual accumulation of carotenoids in the pulp of the pink-fleshed lemon mutant, where they probably increase the storage capacity of structures that are not usually differentiated in ordinary lemons.

The *OR* gene, firstly described in cauliflower, enhances carotenoids accumulation and chromoplasts differentiation (Lu et al., 2006). OR is considered one of the main post-translation regulator of PSY (Osorio, 2019) since recent studies carried out in several plant species has come out that OR protein interacts directly with PSY stabilizing the enzyme and increasing its activity (Zhou et al., 2015; Welsch et al., 2018; Yazdani et al., 2019). Moreover, OR plays a crucial role in the formation of carotenoid-sequestering complexes and the stabilization of carotenoids in plant tissues (Osorio, 2019). The transcription of *Or* gene followed the same pattern of *FIB* in PL (Figure 6) suggesting for both proteins may share critical function in the stabilization of the large amount of carotenoid accumulated in the mutant. It is tempting to speculate that the overexpression of the *Or* gene in PL may increase PSY stability enhancing the flow of carotenes into the pathway. In that case, a reduced enzymatic activity or a lower transcription of genes encoding for LYCs would favour the accumulation of lycopene and other upstream carotenes like phytoene and phytofluene in PL. In transgenic potato tuber overexpressing the *Or* gene the accumulation of β-carotene was significantly higher than in control samples (Li et al., 2012). On the whole, our results suggest that the over expression of *OR* gene might be strictly involved in the events connected with the stabilization of the massive amount of carotenoids accumulated by PL. Unfortunately, it is not possible to establish if the up-regulation of carotenoid-associated protein genes was the cause or just the consequence of the lycopene and the others upstream carotenoids accumulation in PL. However, these findings provide

novel insights on the metabolic changes occurred in the mutant that might support further studies aimed at the identification of molecular markers related with the accumulation of lycopene, which is currently one of the most demanded quality trait in modern fruit crop.

Table S1 qPCR primer sequences.

	Gene name	Forward 5'-3'	Reverse 5'-3'	Reference
XM_006464503.3	ACTINA	TTAACCCCAAGGCCAACAGA	TCCCTCATAGATTGGTACAGTATGAGAC	Alos et al. 2014
XM_006466273.3	DXS1	CGTGTTTTCAACACACCTGACG	AAGCCCCGAAGTCTTCCTCAT	Alos et al. 2006
XM_006488044.3	HDS	CTGCCGGAAATTGGACTTCC	CCATCCTGAAGAAGGGTACC	Alquezar et al. 2008
XM_006487143.3	HDR1	AGACCGTGGAATTCCCTCATACG	AGGCACCGGCTGTCACC	Alquezar et al. 2008
XM_006486607.3	GGPPS1	CCGAGGTCAGCCCTCAAACC	CTCAGGCACGAGATGGGGG	Lado et al. 2015
XM_006481880.3	PSY1	GGTCGTCCATTTGATATGCTTG	CCTAAGGTCCATCCTCATTCCT	Carmona et al. 2012
XM_006492653.3	PSY3a	AATGCATTTTGTGTAAGCCCTGCT	TGTCCCTAAAAGGCTTGATGTGTAAATTG	Manzi et al. 2016
NM_001288862.1	PDS	TCCCTTCTAAGTGTGTATGCC	TGCAAGCTCCTTCATTGTAGC	Carmona et al. 2012
AF372617.1	ZDS	ACAATCTGTTTGAGGCGCAG	CATAGGTATTGGAAACCCTTACTCC	Carmona et al. 2012
MG492005.1	BLCY1	GAACCAGGAGCTTAGGTCTG	GCTAGGTCTACAACAAGGCC	Carmona et al. 2012
MG492007.1	BLCY2a	GAGCAAGTCTCATCGCGTCATAGTG	ACTTTAGCCTTATGAAACTTAACTCCATTTG	Alquezar et al. 2013
MG492008.1	BLCY2b	GCAAGTCTCATCGCGTCATGGTA	ACTTTAGCCTTATGAAACCTAACGCCATTTA	Alquezar et al. 2013
XM_006475429.2	ε-LCY	AAGGTGTGTCGAGTCAGGTGTTT	CCTGCAGGGGACAATCATATCATGTT	Alquezar et al. 2008
XM_025102457.1	BCHX	GGCTCATAAAGCTCTGTGGC	CCAGCACCAAAACAGAGACC	Carmona et al. 2012
XM_006478830.3	ZISO	GCAGCGTCACTGGGTTTAAT	GTTCGCCTCTTCACAGCTTC	
AB219179.1	NCED1	CCACGATGATAGCTCATCCG	CCACTTGCTGGTCAGGCACC	Rodrigo et al. 2006
AB219172.1	NCED2	CTTCCCAACGAAGTCCATAG	GGATTCCATTGTGATTGCTG	Rodrigo et al. 2006
XM_006436998.2	Or	GATGTTGATGTGTTGCGGCGG	AAGTCCTGCACTGTTTCAGGACC	Lado et al. 2015
AB011797.1	FIB1 CitPAP	GGTGGCAGAGGAGGAGAG	GGCATTTAGCAGAGTTAAGGC	Lado et al. 2015
AB011797.1	FIB2 CitPAP	CCATTGGCGAGGGTGGAGG	CGAACTTGATCTGCACACGCTTG	Lado et al. 2015
XM_006469901.3	HSP21	GGGGAAGAAGAAGAGTGGCC	TGTCGACGATTTTGGCAGTGG	Lado et al. 2015
XM_006480857.3	HSP20-3	ACGTCTGGGCGCCCCTTGG	CTCACCGCTGATCTGAAGGACTC	Lado et al. 2015
XM_006424900.2	HSP20-4	TCCGGTTATTCGCCTGCGC	TGACCGCTTATCTGAAGCACCC	Lado et al. 2015

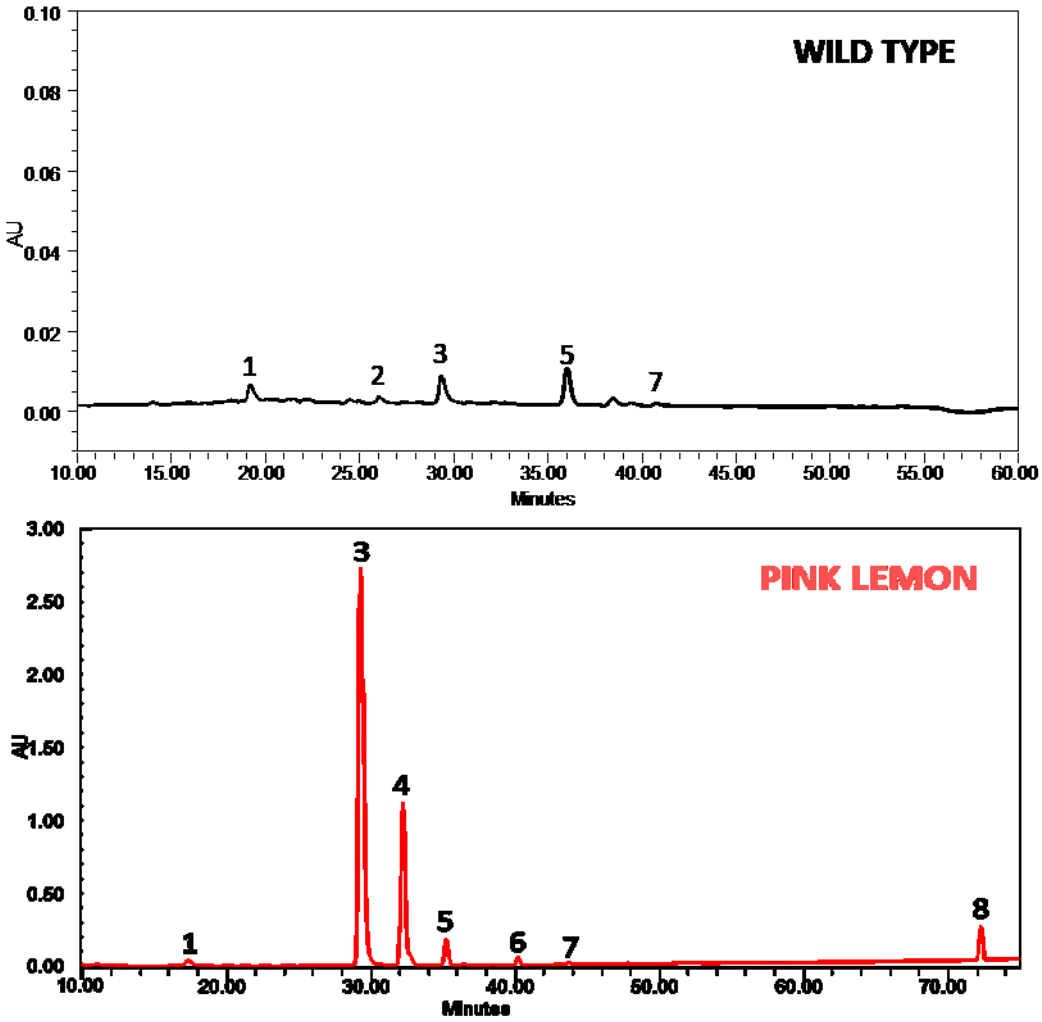

Figure S1. HPLC profiles of saponified carotenoid extracts in pulp of fruits of wild type lemon and Pink lemon at mature green stage (MG). All profiles are MaxPlot chromatograms (each carotenoid shown at its individual λ maxima). Note the high concentration of phytoene (peak no. 3), phytofluene (peak no. 4) and lycopene (peak no. 8) in Pink lemon extract. AU, Absorption units. The compounds correspond to (1) Violaxanthin; (2) Lutein; (3) Phytoene; (4) Phytofluene; (5) β-cryptoxanthin; (6) Neurosporene; (7) β-carotene; (8) Lycopene.

New insights about the effects of rootstock on citrus fruit quality traits

1 Introduction

Grafting is a widespread and almost indispensable technique in modern fruit culture. The use of rootstocks can provide several advantages, such as better adaptation to limiting soil conditions, improved tolerance to temperature and water stress, and especially resistance/tolerance to pests and plant diseases. The strict relationship between the rootstock and the scion can deeply modify the performance of the whole plant (Gainza et al., 2015). The main fruit quality traits are highly dependent on the scion genotype, however it seems that some of the fruit characteristics can be strongly influenced by rootstocks. The combination of the same scion with different rootstocks can lead to very different results in terms of fruit size, shape, pigmentation, juice content, and soluble solid concentration (Forner-Giner et al., 2003). For this reason rootstock choice represents a key step for a productive and profitable orchard (Castle, 1995). Although, it is known that some rootstocks can provide fruits of better internal quality rather than others, the current knowledge show that the influence of the rootstock on fruit flavour and aroma depends on the specific interaction established between a specific variety and the rootstock itself (Lin et al., 2015). In addition, the increasing interest for functional food has led the attention of breeders not only to the fruit organoleptic properties, but also to their nutritional quality in order to meet the current consumer expectations. Indeed, the combination scion/rootstock has strong effects on bioactive compounds concentration and antioxidant activity in the marketed products (Cardeñosa et al., 2015; Aguilar-Hernández et al., 2020; Ordóñez-Díaz et al., 2020).

Color is the first characteristic perceived by customers and one of the major factors influencing fruit aesthetic value and market appreciation. Citrus pigmentation is regulated by several factors such as environmental conditions, cultivation practices, water and nutrient availability and depends on the maturity of the fruits (Morales et al., 2020) and on postharvest storage temperature (Carmona et al., 2020). The changes of color that take place along the ripening process are related to the biosynthesis and

degradation of three main class of biochemical compounds: chlorophylls, carotenoid and anthocyanins (Abouzari and Nezhad, 2016). Chlorophylls are the predominant pigments in the green peel of unripe fruits, while the yellow-orange colour typical of mature fruits is provided by carotenoids. The biosynthesis of anthocyanins is limited to the group of blood orange which presents purple-red shades in both their flesh and rind (Rodrigo et al., 2013a; Lo Piero, 2015).

The consumption of blood oranges has been related with numerous positive effects on human health, thanks to their high content of vitamin C, carotenoids and the antioxidant properties of anthocyanins (Grosso et al., 2013). Like other foods rich in anthocyanins they prevent cardiovascular diseases, cancers, protect tissues from oxidative stress and they would counteract obesity reducing the negative effects of high-fat diets (Pojer et al., 2013). Most of the genes that encode for enzymes involved in anthocyanin biosynthesis have been identified and characterized in various species, Citrus species included. Phenylalanine ammonia-lyase (PAL) is deemed a key gene in anthocyanin metabolism because phenylalanine is the main precursor of these pigments. The first enzyme of the anthocyanin biosynthetic pathway is chalcone synthase (CHS) which is also involved in the production of other phenols. In the next steps dihydroflavonol 4-reductase (DFR) reduces the dihydroflavonols to leucoanthocyanidins, these colorless compounds are then transformed into colored anthocyanins by anthocyanidin synthase (ANS). The last step of biosynthesis is driven by UDPglucose-flavonoid glucosyl transferase (UFGT), which increases the stability and water solubility of the anthocyanins just produced through the addition of one glucose molecule in the 3-OH positions. In addition anthocyanin biosynthetic genes are regulated by a complex system of transcription factors like *Ruby* (Butelli et al., 2012; Lo Piero, 2015).

The taste of citrus flesh is highly dependent on its sugar and acid content and in particular on the ratio between total soluble solids (TSS) and titratable acidity (TA), which has a strong impact on the perception of sweetness and sourness (Benjamin et al., 2013). This is the reason why the ratio TSS/TA is deemed one of the most

important fruit quality traits and it is also the most common parameter utilized to define the ripening index. Sugars progressively accumulate in juice sacs going toward maturation, while organic acids gradually undergo degradation processes. However, some citrus fruit might not reach an adequate sugar to acid ratio when fully ripe with a consequent loss of consumer acceptance. For this reason controlling the fruit acidity level is of relevant economic importance (Terol et al., 2010; Lado et al., 2014; Lado et al., 2018).

Recent studies have identified and characterized several structural genes that are involved in the process of biosynthesis and catabolism of organic acids in citrus flesh. Among these citrate synthase (*CS*) participates to the biosynthesis of citrates, while isocitrate dehydrogenase (*IDH*) and ATP citrate lyase (*ACL*) encode for enzymes involved in citrate utilization (Guo et al., 2016). The transcription of sucrose phosphate synthase (*SPS*) gene has been pointed out as one of the major factors that regulates sucrose synthesis and storage in mature fruit. The enzyme produced by this gene catalyses the conversion of fructose-6-phosphate and UDP glucose to sucrose-6-phosphate and it is responsible for sucrose synthesis in plants (Komatsu et al., 1999; Langenkamper et al., 2002).

Citrus fruits are a great source of vitamin C for humans, who are not capable to synthesize it by their self and are obliged to cover their requirements through the daily dietary intake. L-ascorbic acid (Asa) is produced in large amounts in plants, where it protects tissues from oxidative damages related to numerous biotic and abiotic stresses (Giovannoni, 2007). Due to its free radical scavenging activity and its role as cofactor in many chemical reactions AsA is an essential component of human diet. The reaction catalyzed by the enzyme encoded by GDP-L-galactose phosphorylase (GGP) is deemed the first committed step of the main pathway and also the most important of the AsA biosynthesis. A second key gene in AsA biosynthesis is D-galacturonate reductase (GalUR), since it regulates one of the alternative routes of AsA synthesis (Mellidou and Kanellis, 2017).

Rootstocks affect fruit quality both in deciduous and evergreen crops such as citrus, either directly on carbon allocation or indirectly through crop load (Castle, 1995). Evidences of the influence of the rootstock on qualitative traits in *Citrus* species were largely investigated (Legua et al., 2014; Cano-Lamadrid et al., 2018). Trifoliate orange (*Poncirus trifoliata* L. Raf.) enhances fruit quality and anthocyanin content, citranges - widely spread in US and Europe - confer good internal qualitative traits; *C. volkameriana* produces very vigorous and productive trees, but fruit quality is poor. Citrumelo [*C. paradisi* (Macfadyen) x *P. trifoliata* (L.) Raf.] is highly productive, however it performs poorly in heavy and calcareous soils (Forner-Giner et al., 2020).

Despite the great efforts made, it is still unclear how rootstocks can exert their influence on fruit quality parameters like anthocyanin, sugar, acids, and vitamin C content. In the present work we attempted to elucidate whether rootstock performances were related to any change in the regulation of key quality trait genes. To this aim, metabolic and transcriptomic studies were carried out on fruits of a blood orange selection (*C. sinensis* (L.) Osb. cv Tarocco Sciré) grafted on three different rootstocks tolerant to *Citrus Tristeza Virus* (CTV).

2 Materials and methods

2.1 Plant material

Fruits of Tarocco Sciré sweet orange were harvested from 9 years old trees grafted on three different rootstocks: Citrange Carrizo (CAR) [*C. sinensis* (L.) Osb. cv. Washington navel x *P. trifoliata* (L.) Raf.], Bitters (BIT) and Furr (FUR) [*C. sunki* Hort. ex Tan. x *P. trifoliata* (L.) Raf.]. Plants were cultivated in an experimental field located in Lentini (Siracusa, Italy) and subjected to standard cultural practice. Three biological replicates for each one of the rootstocks were selected in a randomized block design. Samplings were done monthly, from November 2018 to March 2019 for a total of five developmental stages (NOV, DEC, JAN, FEB, MAR) ranging from the onset of ripening

until the full mature stage (**Errore. L'origine riferimento non è stata trovata.**). For each block 28 randomized fruits were collected from seven trees and quickly transferred to the laboratory. Fruit juice was extracted with a commercial juice extractor (Kenwood Citrus Juicer JE290, UK) filtered quickly frozen in liquid nitrogen and stored at -80°C until processed.

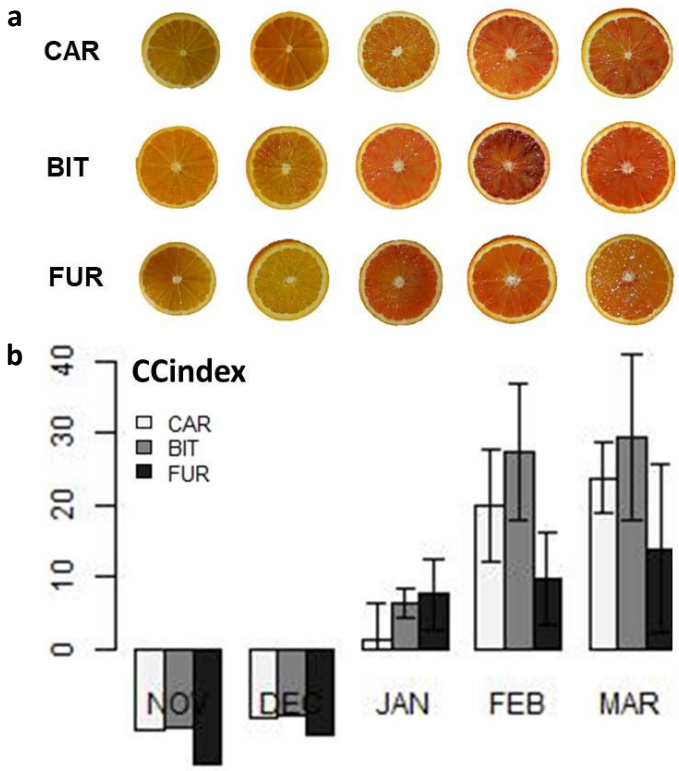

Figure 1. (a) Aspect of the flesh of sweet orange 'Tarocco Scirè' grafted onto Carrizo (CAR), Bitters (BIT) and Furr (FUR) rootstocks during all the ripening process. (b) Citrus color index (CCI) changes of Tarocco Scirè juice during the months of November, December, January, February, March.

2.2 Biocemical analysis

Total anthocyanin content (TAC) was calculated by the pH differential method (NanoDrop 2000, Thermo Scientific) (Rapisarda et al., 2001). Samples absorbance was measured in buffers at pH 1.0 and 4.5 and setting the wavelength at 510 and 700 nm. The results were expressed as the mg of cyanidin-3-glucoside equivalents per liter of

fresh juice. The Total Soluble Solids (TSS) content was measured using a digital refractometer (Atago CO., LTD, model PR-32 α, Tokyo, Japan) and expressed as °Brix. According to the AOAC method (AOAC, 1995), titratable acidity (TA) was determined by potentiometric titration (Hach, TitraLab AT1000 Series) of the juice using 0.1 N NaOH at pH over 8.1, results are expressed as g L^{-1} of citric acid equivalent. Vitamin C (L-ascorbic acid, AsA) was determined using an automatic titration apparatus (702 SM Titrino, Metrohm, Herisau, Switzerland) with 0.001 M I_2, and the results were expressed as mg L^{-1}. Ripening index (RI) represents the ratio between TSS and TA. Juice color was recorded with a Minolta CR-400 chroma-meter (Minolta Corp., Osaka, Japan) as described by Caruso et al. (2020). The results were expressed as citrus color index (CCI= a*1000/L*b), a maturation index widely utilized in the citrus industry (DOGV, 2006).

2.3 Transcriptomic analysis

The plant material used for total RNA extraction was the same as that used for metabolic studies. Total RNA was isolated from 3 ml of juice as described by Butelli and colleagues (Butelli et al., 2019) and subsequently treated with DNA free (DNase treatment and removal, Ambion, Madrid, Spain). Total RNA concentration and purity were assessed using a spectrophotometer (NanoDrop-2000, Thermo Scientific, USA). The absence of DNA was checked by gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, UK) following the procedure indicated by the manufacturer.

Quantitative real-time polymerase chain reaction (qPCR) was performed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) in 10 µL total reaction volume containing 1x PCR buffer II, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of forward and reverse primer (Life Technologies), 1.5 µM SYTO9 (Life Technologies, UK), 20 ng of cDNA and 1U of MyTaq DNA polymerase (Bioline, UK). Thermal cycling conditions included a pre-incubation at 95°C for 5 min, followed by 35 cycles at 95 °C for 10'' for denaturation and 60 °C for 60'' for a single annealing-elongation step. The ΔΔCt

method was utilized to normalize raw Ct data, the Elongation Factor 1-alpha (EF-1α) was chosen as reference gene, while CAR gene expression of November was taken as calibrator. The showed results are the average of three independent sample replicates.

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

Accession number	Gene name	Forward 5'-3'	Reverse 5'-3'	Reference
XM_006481431.3	PAL	GATTTGAGACATTTGGAGGA	ATGGATGAAGCTCTCCACTA	
XM_006420545.2	CHS	TCTATCGACGGGCATCTTC	TGCCTCGGTTAGGCTTTT	Lo Piero et al. 2005
NM_001288931.1	DFR	GCTGTTCGTGCTACTGTTC	GGCTAAATCGGCTTTCCATA	
XM_025097974.1	ANS	GGGTGACTGCTAAATGTGTT	CAAGTCCCCTGTGAAGAATA	
NM_001320060.1	UFGT	TCTTCAGCACTCCGCAATC	TCCATCGGATACGTCGTAAG	
NM_001288889.1	R2R3Myb	ACAATCCACCCCGTCTGATC	CTGGCCTGCTTCAATGACTC	
XM_006483335.2	SPS	TTGTAACTAGCACCCGACAGG	CAACCATACGAGGCATAAACC	Wang et al. 2015
XM_006480234.2	ACL	GATACTGTTGGAGACTTGGG	GCTCTCTTACGACCATCAGG	Guo et al. 2016
XM_006494513.2	NADP-IDH1	GAAAATTGGGGATTGGGATT	CAACAGAGGTGCAGCTCAAA	Guo et al. 2016
XM_006482744.2	CS	GGTGCCCCCAATATTAACAA	AGAGCTCGGTCCCATATCAA	Guo et al. 2016
XM_006474957.2	GGP	TACCAAAGTGGGGCAAGAAG	TGGCAACAACACTTGGAGAA	Alos et al.2014
XM_006492225.2	GalUR-12	CCCAGGTTTCTTTGAGGTGGGTTTATC	TACTGTGGAATTTGTTCGATCTTTTGCAGC	Alos et al.2014
AY498567	EF	CACCACCCCCAAGTACTC	GTTGTCACCCTCGAAACC	

2.4 qPCR data and statistical analysis

The outputs of the different analyses were processed and visualized using the R software (R core team, 2016). The barplots were produced through base package.

qPCR data were normalized using 'HTqPCR' package (Dvinge and Bertone, 2009) and the outputs were visualized thank to 'heatmap3' package (Zhao et al., 2014). ANOVA test was conducted with 'aov' function of the base package and a Tukey test was applied to the results, samples were previously submitted to Shapiro-Wilk test to check their normal distribution. Statistically significant differences in metabolic data were represented on plots by letters a, b, c (*p value<0.01*).

3 Results

3.1 Biochemical analysis

The content of total anthocyanins (TAC) recorded was very low during the first two months of sampling in all of the three scion/rootstock combinations under evaluation (**Errore. L'origine riferimento non è stata trovata.**a). However, it raised pretty quickly on JAN and followed a growing trend until MAR. The rootstock that reached the highest concentrations during the last three months was BIT, which accumulated higher amounts of anthocyanins since JAN. While FUR assumed the lowest values in comparison with the others two genotypes, except for JAN when the lowest content of anthocyanins was recorded in CAR. The fruit of CAR seemed to be the last ones to achieve the red typical pigmentation of blood varieties, conversely to BIT and FUR that showed an intense pigmentation since JAN, the month during which anthocyanin accumulation took over. Nevertheless, the differences recorded among the rootstocks on MAR were not statistically significant.

TSS values increased progressively until JAN, subsequently they assumed quite constant values; no significant fluctuations were recorded from JAN to MAR (Figure b). No substantial difference was recorded in TSS between the three rootstocks, however the statistical analysis highlighted a lower content of TSS in the fruits of FUR during NOV and DEC.

Remarkable differences were found in TA, which was constantly higher in FUR than in the other two rootstocks (Figure c). Although TA values were similar in CAR and BIT, the ones of BIT were slightly higher during all the stage analysed. Even in this case the largest changes about the trend in metabolite accumulation were observed in JAN, when TA decreased substantially in all samples.

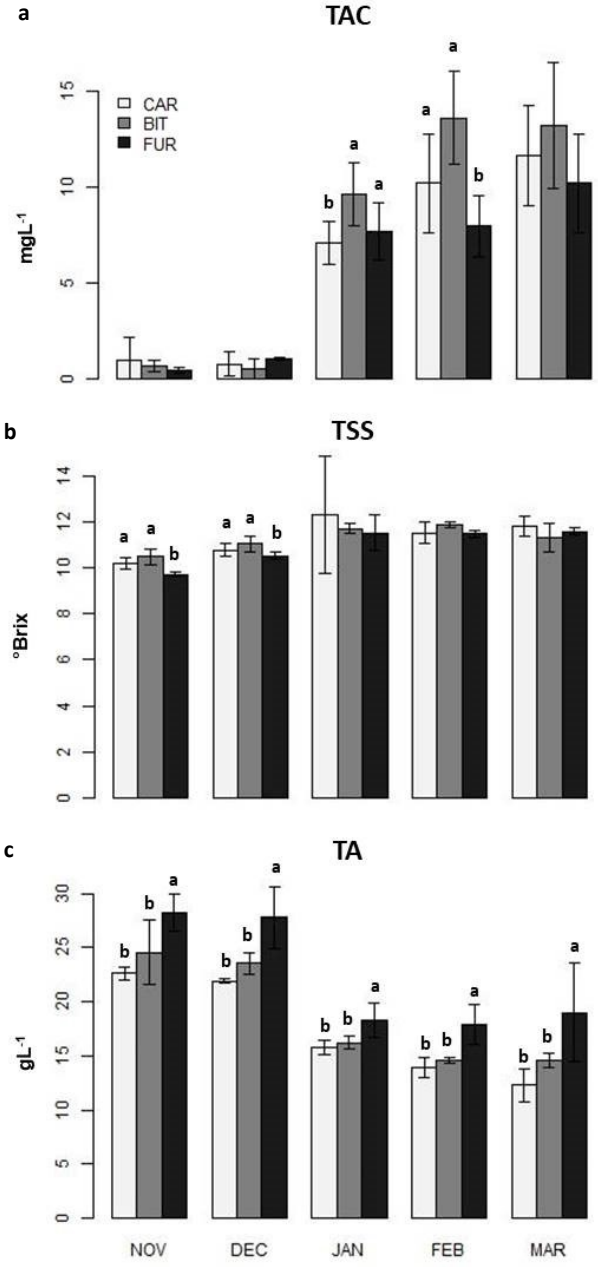

Figure 2. Histograms of biochemical data recorded on juice of sweet orange 'Tarocco Sciré' grafted onto Carrizo (CAR), Bitters (BIT) and Furr (FUR) during the five sampling stages. (a) TAC, Total anthocyanin content (mgL^{-1}). (b) TSS, total soluble solids (°Brix). (c) TA, titratable acidity (gL^{-1}). Vertical bars indicate standard deviation. Statistically significant differences are represented by letters a,b,c (*p value<0.01*).

pH measurements were in accordance with TA data; FUR assumed the lowest values among the three rootstocks during all the monthly surveys except for MAR, while the comparison between BIT and CAR did not show any noteworthy difference since their values were quite leveled except on NOV and MAR (Figure a).

Since no large differences were recorded in TSS content between the samples, TSS/TA ratio was more influenced by TA (Figure b). CAR assumed the higher TSS/TA values during all the five stages of ripening because of its low values of TA, while the slightly lower content of TSS jointly with the high TA led FUR to acquire the lowest TSS/TA ratios. Intermediate values were recorded in the case of BIT that had shown moderate levels of TA.

The AsA content slightly decreased along the maturation until to reach the lowest values on MAR (Figure c). Among the samples the lowest AsA contents were recorded in CAR, showing levels constantly lower than in BIT and FUR. The variations between these last two rootstocks were not statistically significant except in FEB when FUR was significantly lower than BIT.

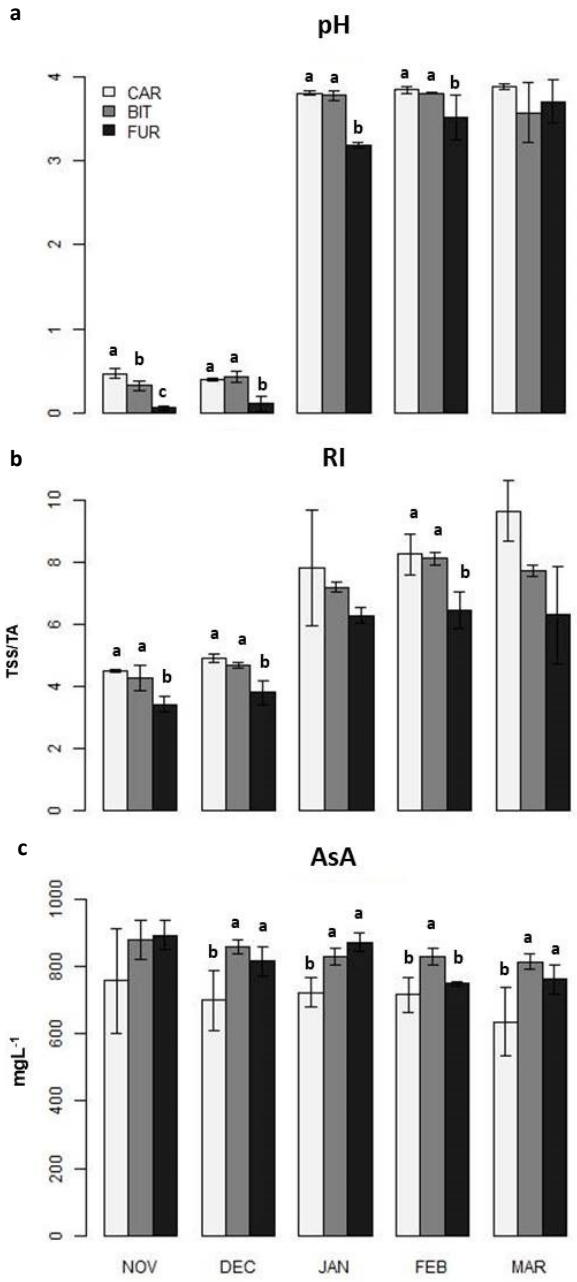

Figure 3. Histograms of biochemical data recorded on juice of 'Tarocco Sciré' grafted onto Carrizo (CAR), Bitters (BIT) and Furr (FUR) during the five sampling stages. (a) pH. (b) RI, ripening index expressed as total soluble solids/titratable acidity (TSS/TA). (c) AsA, ascorbic acid (mgL^{-1}). Vertical bars indicate standard deviation. Statistically significant differences are represented by letters a,b,c (*p value<0.01*).

3.2 Transcriptomic analysis

PAL transcription was clearly higher in BIT than in the others two rootstocks during all the inspection times analysed, except for JAN and FEB when the values of FUR and CAR, respectively, were very close to the ones of BIT (Figure). Similarly to *PAL*, *CHS* reached a peak of regulation in BIT on DEC and in FUR on JAN. On DEC and FEB, CAR values were significantly down regulated compared to BIT. The highest transcription levels of *DFR* were recorded on DEC and FEB, in BIT and FUR respectively. The expression of the gene was not statistically different in the two rootstocks during NOV and MAR, while it was more elevated on DEC and FEB in BIT and on JAN in FUR. In comparison with CAR, *DFR* showed an increased transcription in BIT on DEC, while the differences during JAN, FEB, MAR were not significant. *ANS* was up-regulated during DEC and JAN in both BIT and FUR, however, we found that in BIT the peak of expression was anticipated to DEC, whereas in FUR the highest FC value was reached on JAN. It is worth to note that the regulation of *ANS* was generally lower in CAR than in BIT and FUR and the highest FC value was reached only on FEB. *UFGT* transcription was relatively constant in all of the three rootstocks under evaluation, slight differences were highlighted just on NOV when the accumulation of gene transcripts in CAR and FUR was higher than in BIT and FEB when it was higher than both BIT and FUR. The only differences about *RUBY* expression were recorded on FEB, when CAR exhibited a higher accumulation of mRNA in comparison with BIT and FUR.

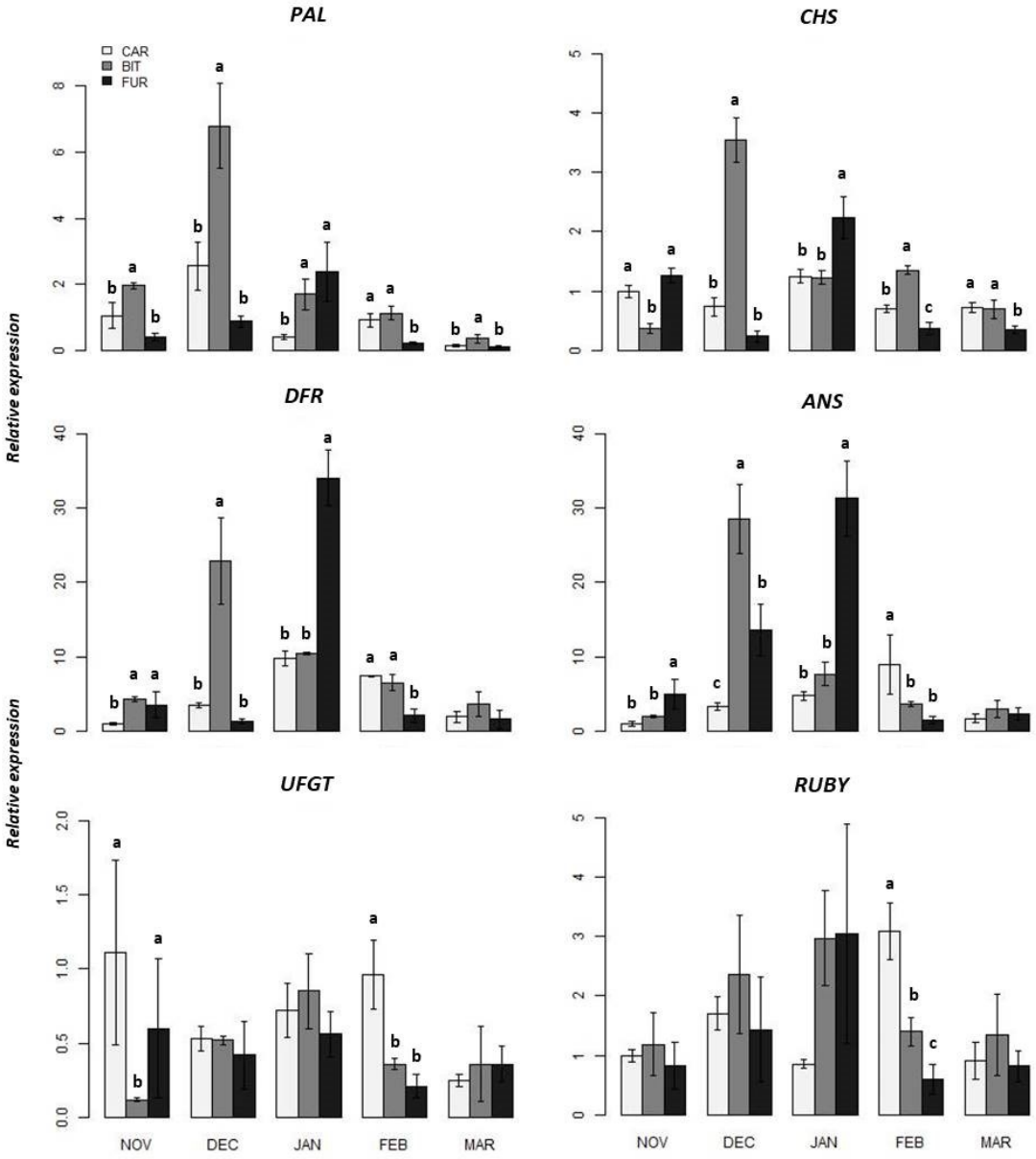

Figure 4. Anthocyanin gene regulation. Histograms of qPCR data (FC, fold change) detected on juice of 'Tarocco Sciré' grafted onto Carrizo (CAR), Bitters (BIT) and Furr (FUR) during the five sampling stages. *PAL*, Phenylalanine ammonia-lyase. *CHS*, chalcone synthase. *DFR*, dihydroflavonol 4-reductase. *ANS*, anthocyanidin synthase. *UFGT*, UDPglucose-flavonoid glucosyl transferase. *RUBY*, R2R3Myb. Vertical bars indicate standard deviation. Statistically significant differences are represented by letters a,b,c (*p value<0.01*).

The expression of *SPS* showed no significant differences among the rootstocks during the whole process of ripening except for DEC, when the gene was sensibly up-regulated in CAR than in the others two ones (Figure).

On NOV and FEB, *CS* was down-regulated in BIT and FUR in comparison with CAR; on DEC, *CS* was more expressed in both CAR and BIT than in FUR (Figure). The gene was also up-regulated in CAR on FEB. During the other months no remarkable differences were found. A sensitive down-regulation of *NADP-IDH1* was noted in FUR on NOV, JAN and FEB if compared with BIT and CAR. Any significant divergence was highlighted by statistical analysis about the accumulation of *ACL* transcript levels except for FEB, when CAR accumulated higher amounts of mRNA in comparison with BIT and FUR.

The expression of *GPP* was relatively variable along the five sampling stages. CAR assumed the highest expression values on DEC and FEB, conversely to FUR that acquired the lowest ones (Figure). *GPP* was up-regulated in FUR in comparison with both CAR and BIT on JAN. On DEC and FEB, the highest FC values were registered in CAR, while the lower one by FUR. An early up-regulation of *GalUR-12* was evident in FUR since the first stages of development. Although *GalUR-12* transcription was lower in BIT and CAR than in FUR during NOV, DEC, MAR, it increased significantly on FEB and MAR. The highest peak of expression was recorded in FUR on NOV, before the FC value dropped on DEC still being higher than in BIT. On JAN *GalUR-12* was up-regulated in FUR in comparison with BIT and CAR, but on FEB it was CAR the rootstock with the highest regulation of the gene and FUR the one assuming the lowest values of the three.

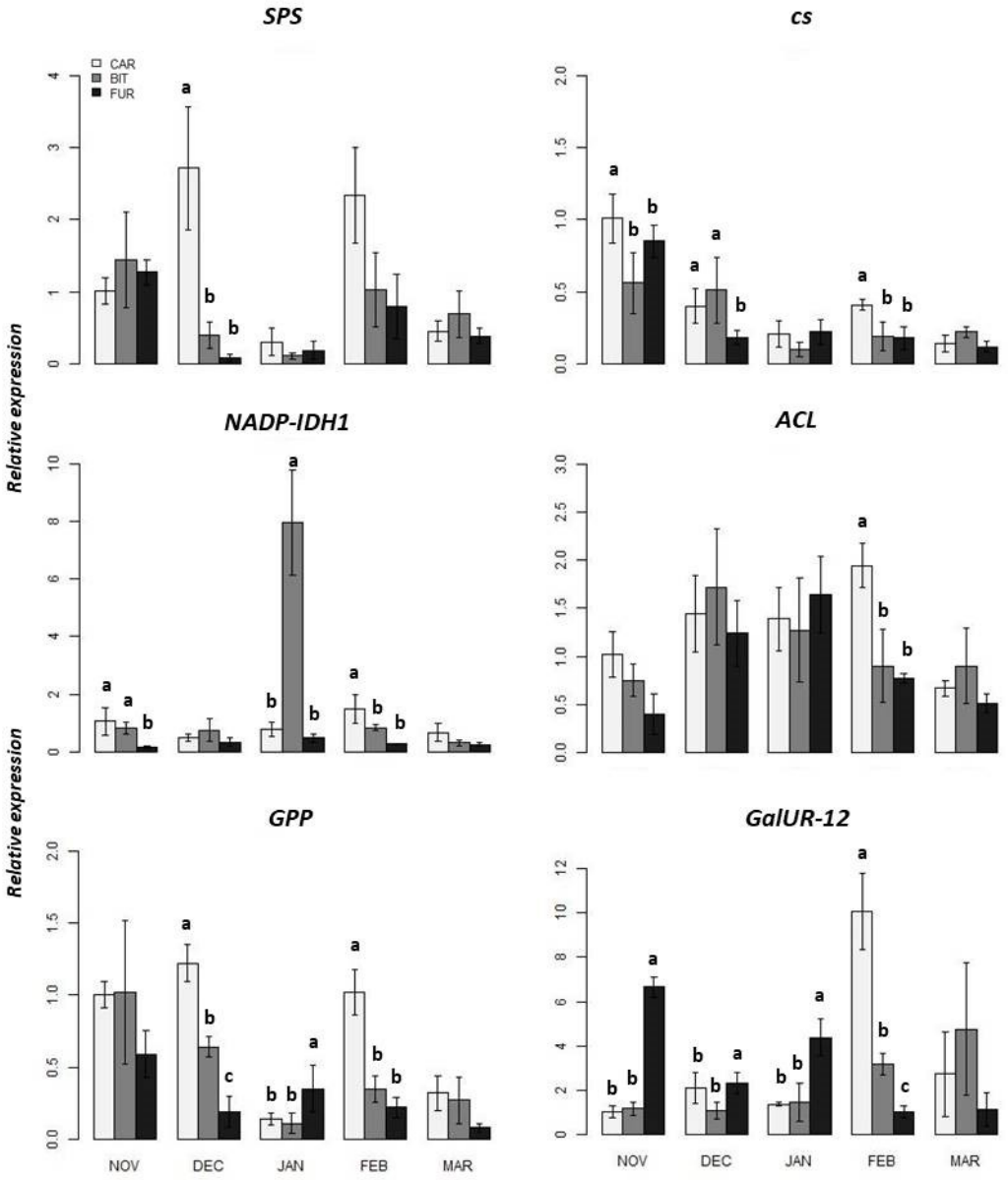

Figure 5. Histograms of qPCR data (FC, fold change) detected on juice of 'Tarocco Sciré' grafted onto Carrizo (CAR), Bitters (BIT) and Furr (FUR) during the five sampling stages. *SPS*, sucrose phosphate synthase. *CS*, citrate synthase. *NADP-IDH1*, isocitrate dehydrogenase. *ACL*, ATP citrate lyase. *GGP*, GDP-L-galactose phosphorylase. *GalUR-12*, D-galacturonate reductase. Vertical bars indicate standard deviation. Statistically significant differences are represented by letters a,b,c (*p value<0.01*).

4 Discussion

A recent study has demonstrated that some of the modern rootstocks tolerant to *Citrus Tristeza Virus* (CTV) are more suitable than others for the production of high pigmented fruits because they are able to enhance the biosynthesis and accumulation of anthocyanins in citrus fruit flesh (Continella et al., 2018). Monitoring the anthocyanin accumulation during the citrus fruit ripening, our results seem to support these findings since the metabolic data highlighted clear differences in anthocyanin content among the three rootstocks tested.

The higher TAC recorded in BIT (Figure a) matched with an early up-regulation of anthocyanin biosynthetic gene (Figure). qPCR data highlighted that *PAL*, CHS, *DFR* and *ANS* showed their highest peak of expression in BIT during DEC, while in FUR, *DFR* and *ANS* exhibit their highest expression on JAN, one month later than in BIT (Figure). In pistachio, it has been found that the rootstock can influence the activity of Phenylalanine ammonia-lyase (PAL), which plays a pivotal role in the production of phenolic compounds, flavonoid and anthocyanin (Nadernejad et al., 2013). While, in *Arabidopsis* a mutation of *CHS* led to a phenotype with flavonoid biosynthesis deficiency (Shirley et al., 1995).

DFR is deemed a key gene for the biosynthesis of anthocyanin because its transcription is generally not detected in blond oranges (Lo Piero et al., 2006) and it is highly up-regulated in the flesh of pigmented citrus fruits (Catalano et al., 2020). *DFR* and *ANS* were generally down-regulated in CAR if compared to BIT and FUR, and seemed to reach their higher expression only on FEB, two months later than BIT and one later than FUR (Figure). This delayed activation of the gene transcription might explain why in CAR fruits anthocyanin accumulation began later than BIT and FUR. Indeed, both *DFR* and *ANS* are part of the downstream gene of the pathway and for this reason are called late biosynthetic genes (LBGs). Conversely to early biosynthetic genes (EBGs) like *PAL* and *CHS,* which encode for precursors in common with several other pathways,

LBGs encode specifically for enzyme involved into the synthesis of anthocyanins (Xu and Dubos, 2015).

Although fruits of FUR reached a higher TAC earlier than the ones of CAR, at the end of the ripening process FUR resulted the rootstock with the lowest TAC of the three studied (Figure a). Conversely to BIT where the regulation of anthocyanin biosynthetic genes decreased progressively after the peak of DEC, in FUR the transcription of these genes dropped suddenly on FEB (Figure). The amount of pigments accumulated into the tissues of citrus pulp was positively correlated with the expression of anthocyanin biosynthetic genes (Cotroneo et al., 2006). Then, the lower TAC registered in the ripe fruit of FUR might be connected with the early down-regulation of these genes.

The accumulation of anthocyanins in citrus fruit pulp is regulated not only by the structural genes just discussed, but also by *Ruby,* a transcriptional factor belonging to MYB family. The transcription of this gene is activated in blood orange by the insertion of a retrotransposon called Tcs1, which is cold-dependent and for this reason it is also responsible for temperature-dependent anthocyanin biosynthesis (Lo Piero et al., 2005; Butelli et al., 2012; Carmona et al., 2017). During the experiment none remarkable difference in Ruby expression was detected among the three rootstocks (Figure).

A slightly lower content of TSS was found in FUR on NOV and DEC (Figure b), nevertheless it was not possible to correlate this event to alteration of SPS transcription (Figure). The regulatory network that controls sugar accumulation is strictly related to the sink strength, the competitive ability of fruits to attract assimilates with respect to others organs of the plant (Komatsu et al., 1999). Thus, the lower values assumed by FUR during the first two stage of development might be related to other factors involved in sugar accumulation rather than *SPS* expression.

Although TA was constantly higher in FUR than in BIT and CAR (Figure c), *CS* was generally down-regulated in FUR samples (Figure). The acidity of citrus fruit largely depends on citrate accumulation since the 90% of the organic acids contained in juice

vesicles is represented by citrates. However, the reduction of acidity that takes place during citrus fruit ripening seems to be due to an increased catabolism of citrate rather than a reduced biosynthesis of them (Cercós et al., 2006). As suggested by several studies, the main regulator of citrate content is a group of genes related to citrate degradation pathways and not *CS*, which takes place to the synthesis of these compounds (Chen et al., 2012; Chen et al., 2013; Lin et al., 2015; Guo et al., 2016).

NADP-IDH1 is one of the key genes involved in citric acid catabolism and its up-regulation has been related to a reduced accumulation of citrate (Guo et al., 2016). Clear down-regulation of this gene was noted in FUR in comparison with BIT and CAR (Figure), then it is possible to speculate that the higher TA values recorded in FUR (Figure c) were likely related to a lower citrate degradation. A second important gene participating to acidity reduction is *ACL*, which catalyzes citrate to oxaloacetate. Although this gene plays a fundamental role in citric acid utilization (Hu et al., 2014), no noteworthy changes of its transcription were highlighted during the experiment.

During the last years the regulation of AsA has been subjected to in-depth studies for its positive effects to both plant and human health. AsA accumulation depends on the balance between biosynthesis and oxidation rate, which is characteristic of each genotype and tissue. The regulation of *GPP* has been strongly related to AsA concentration in the tissues of several plants (Mellidou and Kanellis, 2017). However, the steadily higher content of AsA in FUR (Figure c) might not be related to the transcription of this gene, since it was generally down-regulated in comparison with CAR and BIT (Figure). More likely the higher level of AsA registered in FUR was caused by an early up-regulation of *GalUR-12*. The expression of the gene in CAR and BIT increased during FEB and MAR, while in FUR the transcription of *GalUR-12* was quite high from NOV to JAN before to undertake a decreasing trend (Figure). *GalUR-12* is highly expressed on citrus fruits and represents the rate-limiting step of galacturonate pathway, an alternative biosynthetic route to the main L-Galactose pathway for AsA accumulation. However, recent evidences suggest that GalUR genes may be the main responsible for the high accumulation of vitamin C in citrus fruit (Xu et al., 2013).

Despite it is well documented that grafting can affect fruit organoleptic and nutritive qualities such as the content of bioactive compound (Sharma et al., 2015; Dubey and Sharma, 2016; Sharma et al., 2016; Suriano et al., 2016; Font i Forcada et al., 2019), it is still unclear how rootstock exerts its influence on these characteristics. The results just discussed suggest that the performances of grafted trees are related to a more specific interaction between scion and rootstock and not only factors like the water and nutrient plant status or the crop yield (Castle, 1995). The higher accumulation of anthocyanins in BIT and of AsA in FUR was connected to the up-regulation of genes encoding for key enzymes involved in the biosynthesis of these metabolites. While, the higher level of acidity recorded in FUR was correlated to the down-regulation of a gene that activates the degradation of citrates. Not much it is known about the effects of rootstock at molecular level, however recent advancements have shown that the use of a rootstock can affect scion gene regulation (Liu et al., 2017). Moreover, it seems that scion gene expression might be induced by the movement of proteins and small RNA through the grafting point (Tzarfati et al., 2013; Wu et al., 2019). In conclusion, it is possible to assert that rootstock plays a fundamental role in the control of the gene regulatory networks involved in citrus fruit quality traits.

General conclusion

Remarkable differences were found between Pink lemon and its wild type, HPLC-PAD analysis revealed that the pink mutant produced massive amounts of phytoene and lycopene from early developmental stages. While qPCR data highlighted a slightly up-regulation of carotenoids substrates encoding genes and an evident down-regulation of *LCYs* gene. Moreover, lycopene accumulation matched with the over-expression of several genes involved in the synthesis of ABA and the production of carotenoid-sequestering structures.

Despite several similarities with the other citrus red-mutants, as the huge production of uncolored carotenoids, the mutation confers to Pink lemon flesh few singular traits. Among these, the lack of xanthophylls is without doubt the most interesting feature. The characteristic pink color is the result of a complex cascade of events, which leads to an altered balance in upstream carotenoid biosynthesis. In addition, the massive pigment accumulation was coordinated by the up-regulation of genes that encode for plastid-associated proteins. Although, little information is still available about these proteins, they showed an important involvement in the process of carotenogenesis in citrus fruits. On the whole, these results provide information that might support further studies aimed at the identification of molecular markers related to the accumulation of lycopene, which is currently one of the most demanded quality trait in the modern fruit crop.

The grafting technique is an important mean in fruit cultivation since the use of a rootstock has an important impact on horticultural and pathological traits of citrus cultivars. The combination of scion and rootstock can have important effects on tree vigor, nutrition, stress resistance and can also exert a positive influence on fruit quality traits (Liu et al., 2017).

The comparative study carried on the three combinations of scion/rootstock pointed out a positive correlation between the regulation of quality-related genes and the accumulation of bioactive compounds as well as acid degradation. Monitoring the anthocyanin accumulation during the ripening season showed as some combination of

scion/rootstock can enhance the biosynthesis of these compounds as well as vitamin C accumulation and acid degradation. Then, it is possible to conclude that rootstock genotype can exert an important influence on citrus fruit quality by affecting scion gene expression.

Although the study of rootstock effects has been a relevant topic in citrus research during the last decades, the main part of these studies has been conducted at the biochemical level, while little efforts have been dedicated to the study of the influence of citrus rootstock at the molecular level. Getting new insights about the molecular interaction between scion and rootstock might unravel the system through the rootstock exert its influence on the regulatory networks involved with traits of agronomical relevance. This information could be beneficial in order to improve rootstock breeding selection and define scion/rootstock combinations able to enhance quality traits in fruits.

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Published papers and posters

Transcriptional analysis of carotenoids accumulation and metabolism in a pink-fleshed lemon mutant

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Abstract: Pink Lemon is a spontaneous bud mutation of lemon (*Citrus limon*, L. Burm. f) characterized by the production of pink-flesh fruits due to the unusual accumulation of lycopene. To elucidate the genetic determinism of the altered pigmentation, a comparative carotenoid profiling and transcriptional analysis of both genes involved in carotenoid precursors and metabolism, and proteins related to carotenoid-sequestering structures have been performed in both pink-fleshed lemon and its wild-type. The carotenoid profile of Pink lemon pulp is characterized by an increased accumulation of linear carotenoids such as lycopene, phytoene and phytofluene from the early stages of development, reaching its maximum in mature-green fruits. The distinctive phenotype of Pink lemon is associated with an up-regulation and down-regulation of the genes upstream and downstream the lycopene cyclase, respectively. In particular, 9-cis epoxycarotenoid dioxygenase genes resulted overexpressed in pink lemon compared to the wild-type, suggesting an altered regulation of abscisic acid biosynthesis. Similarly, during the early development of the fruits, genes of the carotenoid-associated proteins heat shock protein 21, fibrillin 1 and 2 and Orange gene were overexpressed in the pulp of the pink-fleshed lemon compared to the wild-type, indicating an increased capacity for sequestration, stabilization or accumulation of carotenes in mutant lemon. Altogether, results showed significant changes at the transcriptomic level between the pink-fleshed lemon and its wild-type highlighting a differential carotenoid metabolism and capacity of stabilization in storage structures between the two accessions. Such changes may be either a responsible for the altered carotenoid accumulation or a metabolic consequence.

Keywords: citrus; gene expression; fruit quality; *Citrus limon* L. Burm. f; lycopene

1. Introduction

Carotenoids are isoprenoids-derived molecules playing essential functions in plant cells, they are part of the photosynthetic system and participate to light capture. Carotenoids play important roles in photo-protection, increasing tolerance to light and heat stresses and preventing membranes from lipid peroxidation. These pigments are also precursors of important phytohormones such as abscisic acid (ABA) and strigolactones; carotenoids

constitute the substrates for the formation of apocarotenoids-derived volatiles. In addition, they are involved plant-animal interaction (Rodriguez-Concepcion et al., 2018).

Carotenoids are not only responsible for the attractive colour of flowers, fruits and other organs in many plant species, but they are also known for their benefits on human health. These properties are mainly due to the antioxidant activity and the fact that α-carotene, β-carotene and β-cryptoxanthin are precursors of vitamin A, an essential dietary component (Eggersdorfer and Wyss, 2018). Recent studies highlighted that a regular intake of carotenoids plays a positive effect on human health preventing neurodegenerative, cardiovascular and aging-related diseases, as well as reducing cancer risk (Fiedor and Burda, 2014; Woodside et al., 2015; Eggersdorfer and Wyss, 2018).

Citrus fruit pigmentation is characterized by a wide variability, the peel and pulp colour ranges from the pale yellow of lemons, pummelos and grapefruits to the light and deep orange respectively of oranges and mandarins until the reddish shades of red-grapefruits and some orange mutants (Rodrigo et al., 2013a; Lado et al., 2015a). This variability in pigmentation is mainly due to the differences in carotenoid accumulation and composition, which is responsible for the species-specific colour according to the ripening stages (Lado et al., 2015a; Tadeo et al., 2020). Citrus fruits are consumed worldwide for their organoleptic characteristics as well as for their nutraceutical value (Ma et al., 2020). In light of this, unrevealing the modifications occurring in mutant phenotypes is a prerequisite to identify new genetic markers associated with improved commercial and nutritional traits. Indeed, molecular markers are a fundamental tool for breeders to reduce the amount of time and money required to develop a new cultivar through traditional breeding.

In plants, carotenoids are generally formed by the condensation of eight C5 isoprenoid units forming a C40 polyene backbone that contains a variable number of conjugated double bonds. This particular chemical structure provides to carotenoids the capacity to absorb visible light at different wavelengths. Carotenoids are classified in carotenes and xanthophylls, the first ones are composed exclusively by carbon and hydrogen atoms, while the second ones contain at least one oxygenated group (Gross, 1987; Rodríguez-Concepción, 2010). During the last decades, several genes encoding for enzymes involved in the main steps of carotenoid biosynthesis pathway have been isolated and their molecular and biochemical regulation has been clarified (Rodríguez-Concepción, 2010; Yuan et al., 2015). Moreover, other processes related to the storage of carotenoids in chromoplasts and how they are catabolized by a family of enzymes known as carotenoid cleavage dioxygenases (CCDs) have been also addressed (Ahrazem et al., 2016; Sun and Li, 2020). The expression of a large number of carotenoid biosynthetic genes has been studied in the peel and pulp of many citrus varieties during all the ripening process (Tadeo et al., 2020; Tatmala et al., 2020).

The initial substrate for carotenoid biosynthesis, geranylgeranyl diphosphate (C20, GGPP), is produced by the condensation of one dimethylallyl diphosphate (DMAP) and three isopentenyl diphosphate (IPP) molecules (Figure 1). The synthesis of these precursors takes place through the so-called methylerythritol 4-phosphate (MEP) pathway and involves several enzymes like 1-deoxy-D-xylulose-5-phosphate synthase (DXS), located upstream, and the hydroxymethylbutenyl diphosphate synthase (HDS) and reductase (HDR) that are located downstream the pathway. At the end of the MEP pathway, the formation of GGPP is catalysed by the geranyl geranyl pyrophosphate synthase (GGPPS) enzyme which is coded by a multigene family (Rodriguez-Concepcion et al., 2018). During the first two steps of carotenoid formation, phytoene synthase (*PSY*) and phytoene desaturase (*PDS*) catalyses the head-to-head condensation of two molecules of GGPP to form the colourless phytoene (C40) and phytofluene. Subsequently, desaturation and isomerization by ζ-carotene desaturase (*ZDS*) and ζ-carotene

isomerase (*Z-ISO*) produce lycopene, through the intermediates ζ-carotene and neurosporene. At this point, the pathway splits into two branches. Lycopene ε-cyclase (*ε-LCY*) and lycopene β-cyclase (*β-LCY*) are responsible for the addition of one or two β-ionone rings producing δ-carotene and β-carotene, respectively. Subsequently, *β-LCY* introduces a second β-ionone ring on δ-carotene to produce *α*-carotene (Ikoma et al., 2016; Tadeo et al., 2020). Two subfamilies of β-lycopene cyclases have been identified in citrus fruits and named as *β-LCY1* and *β-LCY2*. The first of these two genes shows a constant expression during all the ripening process and is expressed in a large variety of organs and tissues, while the second one is chromoplast-specific and is typically expressed in fruit tissues and it is highly up-regulated during the fruit maturation phase (Mendes et al., 2011). Two different alleles of *β-LCY2* have been isolated: *β-LCY2a*, and *β-LCY2b.* The studies carried out on both variants revealed a differential tissue and temporal expression, other than a different enzymatic efficiency to convert lycopene into β-carotene (Alquézar et al., 2009; Zhang et al., 2012). δ-carotene is converted into α-carotene and then in lutein by β-carotene hydroxylase, while, β-carotene is hydroxylated to β-cryptoxanthin and zeaxanthin by β-carotene hydroxylase (*β-CHX*) (Ma et al., 2016). In citrus fruits these two last carotenoids can be catabolized to C30-apocarotenoids by a class of enzymes generally recognized as carotenoids cleavage dioxygenases (*CCDs*) (Ma et al., 2013; Rodrigo et al., 2013a; Zhang et al., 2019). Zeaxanthin epoxidase (*ZEP*) adds to antheraxanthin and subsequently to violaxanthin epoxy groups resulting in neoxanthin formation. The last reaction of the pathway is catalyzed by neoxanthin synthase (*NSY*), which turns violaxanthin into neoxanthin. The 9-cis-isomers of these last two xanthophylls are then utilized as substrates by 9-cis epoxycarotenoid dioxygenase (*NCED*) to produce ABA (Rodrigo et al., 2006; Agustí et al., 2007).

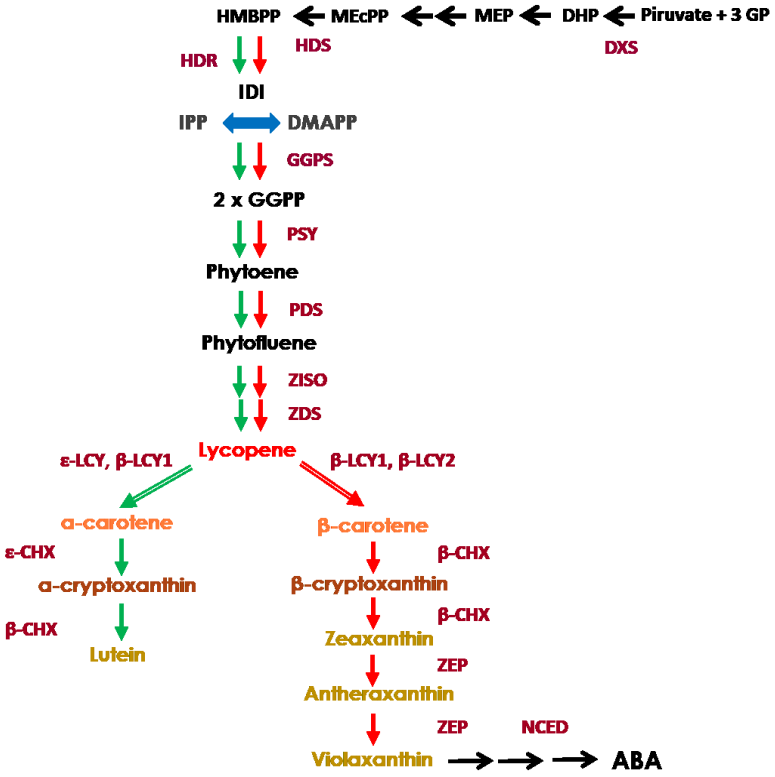

Figure 3. Schematic representation of carotenoid biosynthesis in citrus fruits, indicating main enzymes and genes of the pathway. 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*), hydroxymethylbutenyl diphosphate synthase (*HDS*) and reductase (*HDR*), geranylgeranyl diphosphate synthase (*GGPSS*), phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene isomerase (*ZISO*), ζ-carotene desaturase (*ZDS*), ε-lycopene cyclase (*ε -LCY*), β-lycopene cyclase (*β-LCY1/2*), ε-carotene hydroxylase (*ε-CHX*), β-carotene hydroxylase (*β-CHX*), zeaxanthin epoxidase (*ZEP*) and 9-cis-epoxy-carotenoid dioxygenase (*NCED*). Green and red arrows represent carotenoid biosynthesis flux in green and mature

Carotenoid synthesis takes place concurrently to the differentiation of chromoplasts leading to the development of diverse sink structures organized to storage carotenoid just produced (Sun et al., 2018). The ultrastructural changes that occur during this phase involve several proteins. Among them, the most important are the small heat shock proteins (sHSPs), fibrillins (FIBs or PAPs) and Orange gene protein (*OR*). *HSP21* transcript level has been correlated with carotenoid accumulation in tomato fruit (Neta-sharir et al., 2005). Fibrillins play a structural role on fibrils, organizing carotenoids in lipoprotein complexes (Simkin et al., 2007). Several studies have demonstrated that OR protein promotes PSY activity and chromoplasts biogenesis which leads to the enhancement of carotenoid accumulation (Zhou et al., 2015; Sun et al., 2018; Welsch et al., 2018).

The large variety of pigmentation showed by both rind and flesh of mature citrus fruits is strictly related to the differences in the total amount and composition of carotenoids typical of each specie and cultivar (Ikoma et al., 2016; Tadeo et al., 2020). In the case of ordinary lemon (*Citrus limon*), the light-yellow colouration is due to a very low accumulation of carotenoids (Gross, 1987; Kato et al., 2004). Comparative transcriptomic analysis has highlighted a reduced

expression of most of the carotenoids biosynthetic genes in both flavedo and juice sacs of lemon fruits compared to what found in oranges and mandarins (Kato et al., 2004).

Accumulation of lycopene in *Citrus* is relatively uncommon and characterize few varieties and mutants of pummelo, grapefruit and sweet orange. Despite the extensive efforts to investigate carotenoid biosynthesis and metabolism in several red-fleshed citrus mutants, the molecular basis of lycopene accumulation has not been completely elucidated yet (Ikoma et al., 2016; Tadeo et al., 2020). In the case of Cara Cara orange mutant, it has been proposed that the red pigmentation is amenable to an enhanced flow of carotenoids precursors through the MEP pathway (Alquezar et al., 2008; Lu et al., 2017). In addition, it has been found that alterations in the expression of the two alleles of *β-LCY2* might lead to the accumulation of lycopene (Lu et al., 2006; Alquézar et al., 2009; Xu et al., 2009; Yu et al., 2012; Alquezar et al., 2013). Lycopene cyclase activity is a rate-limiting step in the biosynthesis of carotenoids, then a partial blockage in the conversion of lycopene to β-carotene may increase the accumulation of lycopene and repress the production of downstream metabolites like xanthophylls (Alquézar et al., 2009; Xu et al., 2009; Yu et al., 2012; Zhang et al., 2012). Interestingly the comparison between white and red pummelos indicated that lycopene accumulation is associated with a reduced expression of genes encoding for enzymes which operate downstream lycopene production (Liu et al., 2016; Yan et al., 2018; Promkaew et al., 2020; Tatmala et al., 2020), reinforcing the hypothesis that a reduction of activity of lycopene cyclase might contribute to the onset of a bottleneck along the carotenoid pathway.

A pink-fleshed lemon was described in 1932 in California as a spontaneous bud mutation of Eureka lemon. The peel of pink lemon is variegated, with green stripes, which turn into yellow when mature, while the yellow sector becomes light-pink (Figure 2A). The pulp has a light-pink colouration due to lycopene accumulation with few seeds and a sour taste when full mature (Shamel, 1932). Although, the mutant is known by long time and is commercially available in specialized markets, no information is still available about the transcriptomic and metabolic changes behind the pink pigmentation.

The aim of the present work was to carry out a comparative analysis of carotenoids biosynthesis between the pulp of the pink-fleshed lemon and its wild type (WT), in order to elucidate the metabolic and molecular changes at the basis of the pigmentation of the red-fleshed mutant. To this end, carotenoids identification and quantification was performed employing a HPLC-PDA technique, while the regulation of the genes involved in carotenoids biosynthesis and the production of proteins related to carotenoid-sequestering structures was detected through qPCR. An increased understanding of the genetic determinism of the pink-fleshed lemon phenotype could be of great interest to identify candidate genes for the development of molecular markers to be employed in fruit quality breeding programmes.

2. Materials and Methods

2.1 Plant material

Fruits of Pink lemon (PL) and Fino (*Citrus limon*, cv. Fino), referred as wild type (WT), were harvested from adult trees grafted on Citrange carrizo (*Poncirus trifoliate* L. Raf x *Citrus sinensis* L. Osb) rootstocks cultivated at The Citrus Germplasm Bank (Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain) and subjected to standard cultural practices. Samples were collected at four developmental stages: immature-green (IG), mature-green (MG), breaker (BR) and full-mature (FM) (Figure 2). Trees of both genotypes were located in the same orchards and samples of each genotype were collected at the same time. Fruits were quickly delivered to the laboratory, where the pulp was separated from flavedo, frozen in

liquid nitrogen, ground to a fine powder and stored at -80°C until analysis. Colour of the pulp was measured using a CR-400 Minolta chromameter on three different locations around the equatorial plan of the fruits. The Hunter parameters *a* (negative to positive, from green to red) and *b* (negative to positive, from blue to yellow) were measured, and colour was expressed as the *a/b* Hunter ratio, a colour index that has been widely used for colour measurement in citrus fruits [40]. Data of colour index for each cultivar are the means ± SD of at least 10 fruits. Fruits were harvested and colour determined in two consecutive crop seasons.

2.2 Carotenoid extraction and quantification by HPLC-PDA

Carotenoids were extracted from frozen flesh, following the protocol described by Rodrigo et al. (Rodrigo et al., 2015). Extracts were dried and kept at −20°C until further analysis. Each sample was extracted in triplicate and results were expressed as mean ± SD. In order to prevent photodegradation, isomerizations and structural changes of carotenoids all the operations were carried out on ice under dim light.

Individual carotenoid analysis of each sample was carried out by HPLC-PDA as previously described by Lado et al. (Lado et al., 2015b) and Rodrigo et al. (Rodrigo et al., 2015). Carotenoids were identified by their absorption, fine spectra and retention time. Then, they were quantified integrating each one of them at its corresponding maximum absorbance wavelength and using the corresponding calibration curves as reported by Rodrigo et al. (Rodrigo et al., 2015).

2.3 Gene expression analysis by quantitative real-time PCR

The RNA isolation, cDNA synthesis and gene expression analyses were performed essentially as described by Rodrigo et al. (Rodrigo et al., 2013b), and subsequently treated with DNA free, DNase treatment and removal (Ambion, Madrid, Spain) to eliminate any residual trace of DNA. Total RNA was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) and absence of DNA was checked by gel electrophoresis.

Briefly, 2 μg of total RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Madrid, Spain) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBRGreen I Master kit (Roche). The primers employed for the amplification of each gene are listed in Table S2. 20 ng of cDNA was used for each amplification reaction in a total volume of 10 μl. The cycling protocol consisted of 10 min at 95°C for pre-incubation, followed by 35 cycles of 10 s at 95°C for denaturation, 10 s at 59°C for annealing and 10 s at 72°C for extension. Fluorescence data were acquired at the end of extension phase and reactions specificity was checked by post-amplification dissociation curve. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and calculated expression levels relative to the values of a reference sample using the Relative Expression Software Tool (Pfaffl et al., 2002). Actin gene expression was chosen to normalize raw Cp's based on a previous selection of reference genes (Alós et al., 2014b). The results were the average of three independent sample replicates.

2.4 Statistical analysis

The outputs of both HPLC-PDA and qPCR analysis were processed using the R software (R Core Team, 2016). An ANOVA test was employed to determine significant differences (*p*

value <0.01) between Pink lemon and its wild type. A Shapiro-Wilk test was performed before the ANOVA test.

3. Results

3.1 Phenotypic characteristics of the pink lemon fruit

Pink-fleshed lemon trees are characterized by variegated leaves and fruits, both characterized by green and white sectors variable in shape and size. The typical green stripes were unevenly distributed on the fruit skin and even if they were evident from early developmental stages, during the maturation process their colour changed to the characteristic yellow, while the white areas turned into light pink (Figure 2A). The red tone of the pulp was evident already in IG and increased in intensity with maturation, reaching an intense red colour at MG. The reddish colouration of the pulp at early stages moved to clearer shades, probably due to the dilution effect caused by the substantial growth of the pulp along the maturation process (Figure 2A). Remarkable differences in pulp colour (determined as *a*/*b* Hunter ratio) were found between the two genotypes (Figure 2C). Colour of Pink lemon (PL) pulp assumed positive values at all developmental stages, although they slightly decreased as the maturation progressed. By contrast, the colour of wild-type (WT) lemon pulp assumed negative values typical of the light-yellow tone and it showed an increasing trend over maturation (Figure 2B).

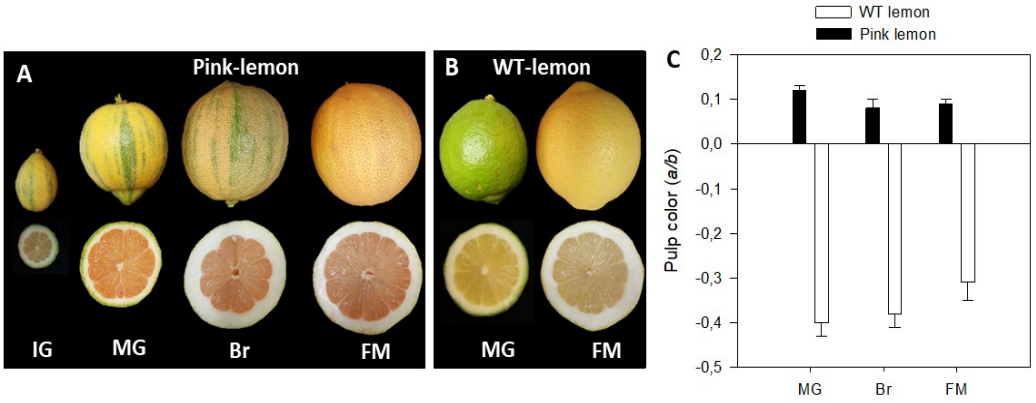

Figure 4. External and internal appearance of Pink lemon (PL) (A) and wild-type (WT) (B) lemon fruit during development and maturation. Immature green (IG), mature green (MG), breaker (BR) and full mature (FM) stages. Changes in colour (a/b Hunter) of the pulp of PL and WT (C) during fruit maturation. Data are the mean ± SD of at least 10 fruits.

3.2. Carotenoids content and composition in Pink lemon fruit

Carotenoids content and composition were analysed in the pulp of PL and WT fruits at four developmental stages from immature-green to full maturity as outlined in the material and methods section (Table 1). HPLC-PDA analysis allowed the detection and quantification of eleven carotenoids. Carotenoids content and composition in PL fruits were markedly different from WT (Table 1; Figure S2) at all the four stages analyzed. Total carotenoids content was much higher in PL fruits than in WT (from 100 to 1000 folds higher) (Table 1). Total carotenoids

content was very low (<0.4 μg/g FW) in WT at all developmental stages, while in PL reached a maximum at MG (53.3 μg/g FW) and declined subsequently. The colourless phytoene and phytofluene were the major carotenes detected in PL flesh composing respectively the 82-86% and 11-16% of total carotenoids, while these carotenes were only detected in traces or at extremely low levels in the pulp of WT fruits. In addition to phytoene and phytofluene, low amounts of lycopene, neurosporene, ζ- and δ-carotene were detected in the pulp of PL. In the pulp of mature WT fruits, only low levels of β-cryptoxanthin and traces of other carotenoids were detected (Table 1).

Table 1. Carotenoid content and composition (μg/g FW) in the pulp of the Pink lemon and wild type at four developmental and ripening stages. The amount of violaxanthin represents the sum of all-trans and 9-cis isomers. Traces indicate amount lower than 0.01 μg/g FW. nd: no detected. Tr: traces. Data are expressed as mean ± SD. [α] Total carotenoids are the sum of the main carotenoids identified and quantified

	Pink lemon				**Wild type**			
Carotenoids (μg/g FW)	**IG**	**MG**	**BR**	**FM**	**IM**	**MG**	**BR**	**FM**
Phytoene	17.59±1.12	44.01±0.90	4.98±0.30	8.81±0.07	tr.	tr.	0.04±0.01	0.04±0.01
Phytofluene	2.27±0.17	8.93±1.75	1.00±0.01	1.79±0.01	nd	nd	nd	tr.
ζ-carotene	nd	0.05±0.01	nd	nd	nd	nd	nd	nd
Neurosporene	0.06±0.01	0.24±0.03	nd	nd	nd	nd	nd	nd
Lycopene	0.24±0.01	0.49±0.13	tr.	0.02±0.01	nd	nd	nd	nd
δ-carotene	tr.	0.04±0.01	nd	nd	nd	nd	nd	nd
Lutein	0.06±0.01	nd	nd	nd	tr.	tr.	tr.	tr.
β-carotene	nd	nd	nd	nd	nd	tr	nd	tr.
β-cryptoxanthin	0.02±0.01	nd	0.02±0.01	nd	nd	0.03±0.01	0.02±0.01	0.02±0.01
Anteraxanthin	nd	nd	nd	nd	nd	nd	tr.	tr.
Violaxanthin	nd	tr	nd	nd	nd	tr.	nd	tr.
Total carotenoids[α]	**20.24±1.54**	**53.33±2.17**	**6.01±0.29**	**10.61±0.03**	**tr.**	**0.05±0.01**	**0.06±0.01**	**0.08±0.01**

3.3. Expression of the genes involved in the biochemical pathway of carotenoids

The expression levels of eleven genes related to carotenoids biosynthesis were tested through a qRT-PCR assay to explore the possible causal relation between the increased carotenoid accumulation in PL and the transcripts abundance of such candidate genes.

Noticeable differences were highlighted in the expression of several genes on the two genotypes (**Errore. L'origine riferimento non è stata trovata.**-6). In general, the expression of the three genes belonging to the MEP pathway (*DXS*, *HDS* and *HDR*) increased progressively in the pulp of WT. *DXS* and *HDR* were up-regulated at early development stage in PL than in WT, while they were down-regulated during the last stages of development. The accumulation of the transcripts corresponding to the plastid-associated *GGPS11* was higher in the pulp of IG mutant lemon and gradually declined during maturation (Figure 3).

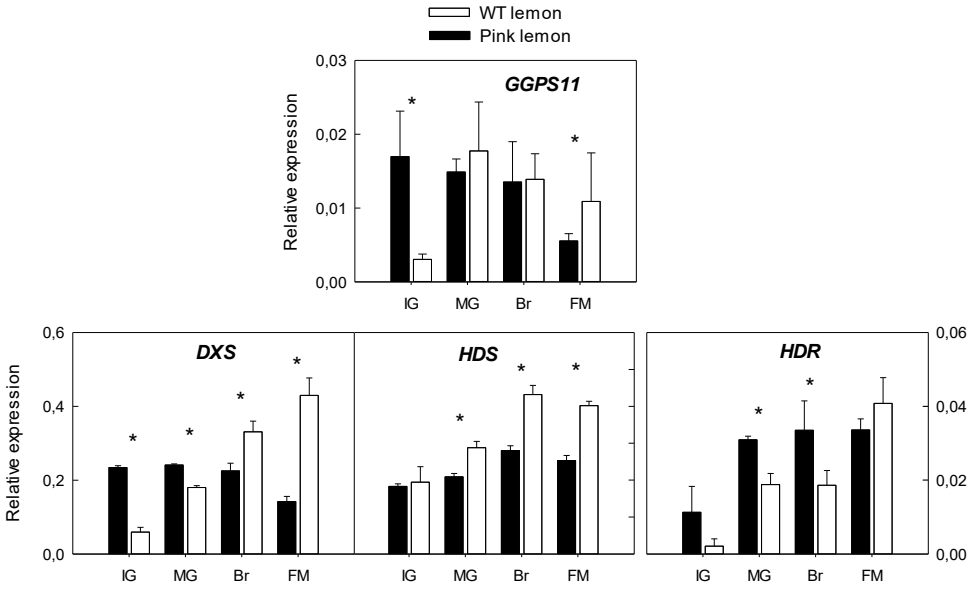

Figure 3. Changes in the expression of genes involved in the MEP pathway: *GGPS11*, geranylgeranyl diphosphate synthase 11; *DXS*, 1-deoxy-D-xylulose-5-phosphate synthase; *HDS*, hydroxymethylbutenyl diphosphate synthase; *HDR*, hydroxymethylbutenyl diphosphate reductase; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage ($p<0.01$) by one-way ANOVA ($p<0.01$).

Expression of genes involved in early desaturation and isomerization steps of carotenoid biosynthesis, with the exception of *PSY3a*, were up-regulated during maturation in the pulp of WT lemon fruits. Expression of the *PSY*, *PDS*, *ZDS* and *ZISO* genes experienced minor increases in PL mutant fruit and after MG transcripts accumulation were significantly lower than in WT pulp (Figure 4). The transcription of genes involved in lycopene cyclization also showed important differences between the genotypes under evaluation. The expression of *β-LCY1* remained relatively constant during WT lemon maturation, conversely to PL in which the gene was down-regulated. Despite the expression of *β-LCY2* increased in both genotypes during the four ripening stages, it was consistently lower in the pulp of PL mutant than in WT lemon. No significant differences were observed in the accumulation of ε-LCY transcript between the two varieties. *β-CHX* was up-regulated in both WT and PL following a similar trend (Figure 4).

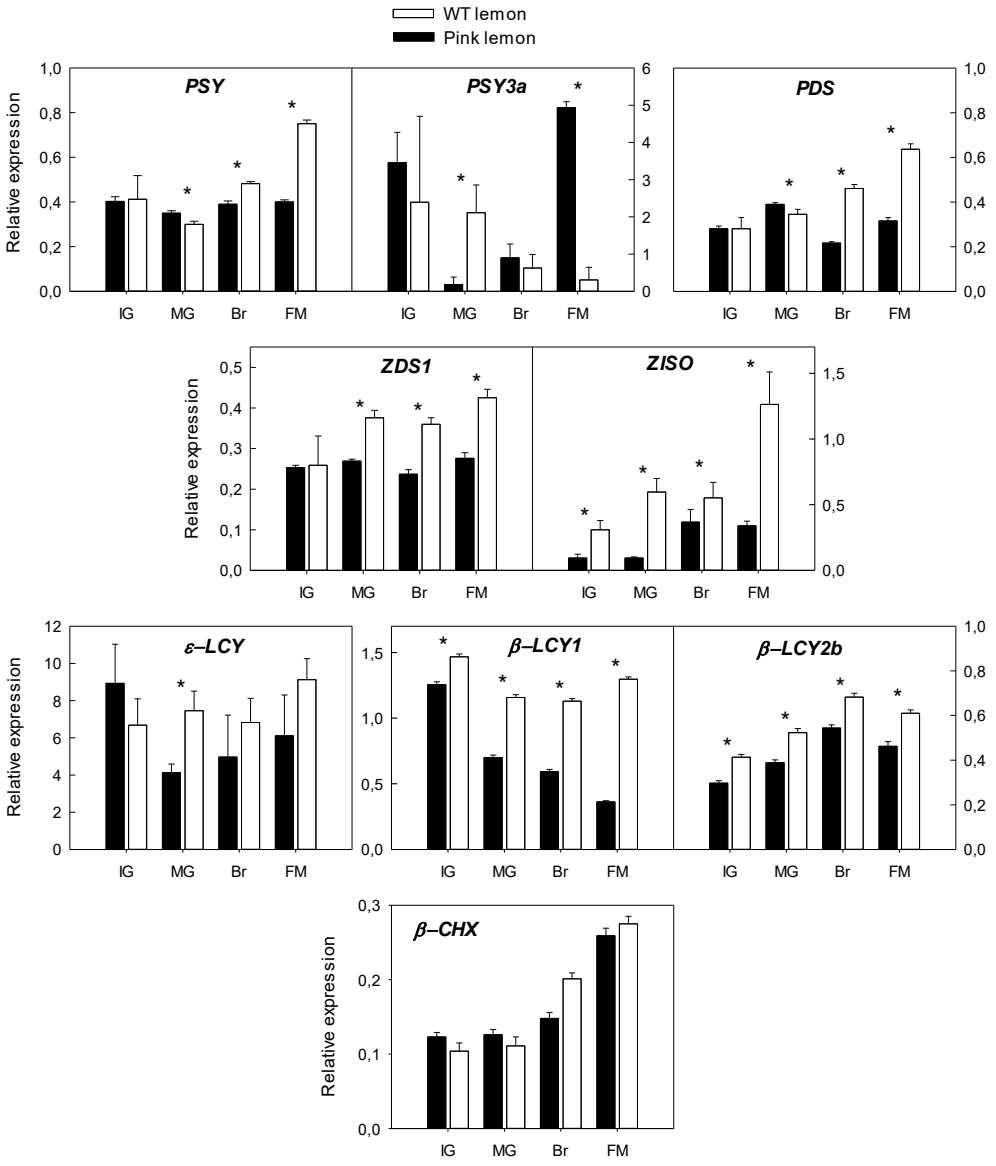

Figure 4. Changes in the expression of genes involved in carotenoids biosynthesis: *PSY*, phytoene synthase; *PSY3a*, phytoene synthase 3a; *PDS*, phytoene desaturase; *ZDS1*, ζ-carotene desaturase 1; *ZISO*, ζ-carotene isomerase; ε-*LCY*, ε-cyclase ; *β-LCY1*, β-lycopene cyclase 1; *β-LCY2b*, β-lycopene cyclase 2b; *β-CHX*, β-carotene hydroxylase; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage ($p<0.01$) by one-way ANOVA ($p<0.01$).

3.4 Expression of genes involved in the biosynthesis of abscisic acid

Regarding the genes encoding for 9-cis epoxycarotenoid dioxygenase (*NCED*), which are involved in the production of ABA, both *NCED1* and *NCED2* were up-regulated in WT and PL.

However, the level of transcript of *NCED1* and *NCED2* accumulated in PL was respectively 6- to 9-times and 2.3 to 7-times higher than in WT (Figure 5).

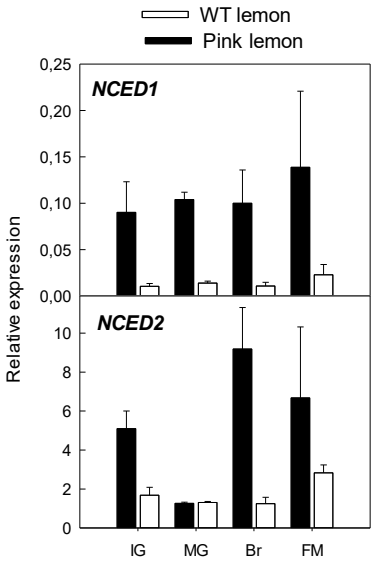

Figure 5. Changes in the expression of genes involved in abscisic acid biosynthesis: *NCED1*, 9-cis-epoxy-carotenoid dioxygenase 1; *NCED2*, 9-cis-epoxy-carotenoid dioxygenase 2; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage ($p<0.01$) by one-way ANOVA ($p<0.01$).

3.5 Expression of accessory genes involved in the accumulation of carotenoids

In order to clarify whether the massive accumulation of carotenoid in the PL is associated with alterations in the expression of genes related to chromoplast differentiation and carotenoids-sequestering structures, accumulation of mRNAs corresponding to three *HSP* (*HSP20_3*, *HSP20_4*, *HSP21*), two fibrillins (*FIB1*, *FIB2*) and an *ORANGE* (*OR*) gene was investigated. The relative expression pattern of the *HSP* genes underwent to a noticeable up-regulation in WT during the last ripening stages. The most pronounced differences were highlighted in the transcription of *HSP21*, which was over-expressed in the pulp of the PL mutant during three of the four developmental stages analyzed. Although the transcription of *FIB1* and *FIB2* genes followed a more constant trend in WT lemon, the level of transcript accumulated in the PL was considerably higher except for a slight decline at MG. The expression of the *Or* gene followed the same pattern of *FIB1* and *FIB2*. Accumulation of the *Or* transcript was 3.4 to 11 times higher in the PL than in WT (Figure 6).

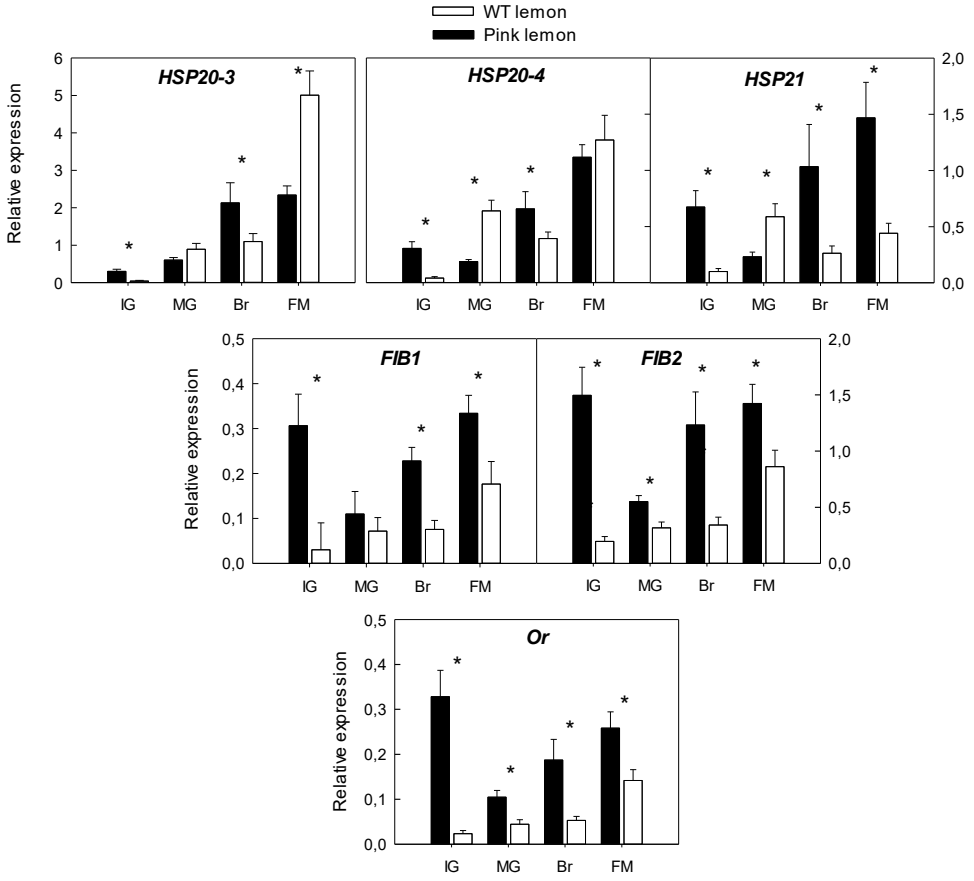

Figure 6. Changes in the expression of genes of carotenoid-associated proteins: *HSP20-3*, heat shock protein 20-3; *HSP20-4*, heat shock protein 20-4; *HSP21*, heat shock protein 21; *FIB1*, fibrillin 1; *FIB2*, fibrillin 2; *Or*, orange protein gene; in the pulp of the PL and WT lemon fruit at four developmental stages: IG (immature green), MG (mature green), BR (breaker), FM (full mature). Asterisks indicate significant differences between genotypes for each developmental stage ($p<0.01$) by one-way ANOVA ($p<0.01$).

4. Discussion

Although variegated pink-fleshed lemon was identified already in 1932 and its fruits are cultivated and purchased in many countries, the biochemical and molecular alteration behind its characteristic pigmentation is still unknown. A detailed observation of the pulp colouration and a comparative analysis of carotenoid content and composition are reported here for the first time revealing interesting features of this mutant in comparison with the other red-fleshed citrus mutants.

The pulp of WT lemon contained negletable amounts of carotenoids at the four developmental stages analysed (Table 1) in agreement with previous investigations [9,29] reinforcing the classification of lemon as low-carotenoid accumulating *Citrus* genotypes [15]. The red colouration of the pulp was clearly distinguishable in IG fruits (Figure 2A) and

lycopene, even at moderated amount (Table 1), was already detectable. These observations indicate that the accumulation of the red pigment is not a ripening-related event like in others red-fleshed pummelos, grapefruits and oranges mutants. Indeed, lycopene biosynthesis was initiated very early in the fruit development, at the beginning of the second phase of fruit growth during the cell enlargement. Accumulation of lycopene in the pulp of PL was associated with a high concentration of phytoene and phytofluene, two colourless carotenes that were virtually absent in WT lemon (Table 1). The concentration of these two linear carotenes reached a maximum in MG fruits and declined immediately after (Table 1). It is worth to note that except for Pinalate orange mutant (Rodrigo et al., 2019) accumulation of phytoene and phytofluene is very unusual in lemon and in other citrus fruits, in particular the concentrations found in the PL pulp are among the highest ever reported in citrus fruits (Lado et al., 2016; Tadeo et al., 2020). Moreover, HPLC-PDA analysis enabled the detection of small amounts of neurosporene and δ-carotene at early development stages, both carotenes were hardly identified in the pulp of citrus fruits (Gross, 1987; Lado et al., 2016). This unusual accumulation of δ-carotene, which is a carotene characterized by a ε-ring at one end while the other end is linear, may suggest a defect in the β-cyclization of lycopene that would lead to the accumulation of upstream carotenes, and explaining also a large amount of phytoene and phytofluene found in the PL (Table 1).

The pattern of carotenoid accumulation in the pulp of PL is different to the ones found in other lycopene accumulating citrus fruits. In the case of orange fruits such as Hong Anliu or Cara Cara (Xu et al., 2006; Liu et al., 2007; Alquezar et al., 2008; Lu et al., 2017) or red-pummelos (Yan et al., 2018; Promkaew et al., 2020; Tatmala et al., 2020), lycopene is accumulated progressively during maturation and the highest concentration is reached in fully ripe. Similarly, low amounts of phytoene accumulated in immature fruits of some mutants gradually increased along maturation (Alquezar et al., 2008; Tatmala et al., 2020). In lemon, the expression of genes involved in the biochemical pathway of carotenoid is different from other citrus species (Kato et al., 2004; Ikoma et al., 2016), and non-pigmented lemons have a low capability to accumulate carotenoids. Then, it is reasonable to assume that the genetic alteration responsible of the accumulation of lycopene in the pink-fleshed lemon may be different to that occurred in the other citrus mutants characterized by increased levels of lycopene.

The transcriptomic analysis, which included the study of 4 genes of the MEP pathway (Figure 3) and 9 genes of the biosynthesis of carotenoids (Figure 4), highlighted important differences between the two accessions under evaluation that might clarify the cause of the mutation. In ordinary lemons the transcription of MEP pathway genes increases with ripening in response to enhanced demand for precursors due to the formation of downstream products (Nisar et al., 2015; Rodriguez-Concepcion et al., 2018). However, in immature PL fruits the transcription of *GGPS11*, *HDS* and *DXS* was higher than in the WT. These results are in accordance with the extensive differences in total carotenoids content between the two genotypes (20.2 μg/g FW in PL vs traces in the WT) and may increase the availability of GGPP to be converted into phytoene and other carotenoids. Similar results have been observed in the pulp of red-fleshed oranges Hong Anliu and Cara Cara, where the accumulation of early carotenes appears to be associated with an increased production of isoprenoid precursors (Liu et al., 2007; Alquezar et al., 2008).

The expression patterns of carotenoid biosynthetic genes in the pulp of WT lemon was similar to the ones characterizing other white-fleshed varieties (Kato et al., 2006; Liu et al., 2016). The low carotenoid content in non-pigmented lemons is generally associated with an up-regulation of the upstream carotenoid genes like *PSY*, *PDS*, *ZDS*, *ZISO*, *β-LCY2b* and *β-CHX*, while *ε-LCY*, *β-LCY1* are usually down-regulated. In the pulp of PL, however, differences in the

pattern of expression of carotenoid biosynthetic genes were not consistent with the alterations in carotenoid content and composition (Figure 4, Table 1). Genes synthetizing the precursors of lycopene were not up-regulated during maturation, on the contrary, the genes related to the β-cyclization of lycopene *β-LCY1* and *β-LCY2b* showed reduced transcript levels in PL than in WT from early stages of development (Figure 4). In particular, during the major development of the fruit (IG to MG), when carotenoids reached their maximum concentration in the PL (Table 1). In addition, the expression of *β-LCY1* was severely down-regulated in PL mutant respect to WT (Figure 4). Transcripts of *β-LCY2a*, which is the allele with the highest *in vitro* activity, were not detected in the pulp of both lemon genotypes at any developmental stages (data not shown) indicating a reduced capability of lemon fruits to convert early carotenes to xanthophylls. These alterations combined with the lower expression of the *β-LCY2b* in PL might be responsible for the onset of a bottleneck along the carotenoid pathway that could lead to the massive accumulation of lycopene, phytoene and phytofluene (Figure 4). According to this hypothesis, the *PSY* transcription would not be a limiting factor at early developmental stages but it might be more critical during the latest stages. These data are in agreement with those reported in red pummelo, in which the balance between the transcription of genes located upstream and downstream lycopene, together with the reduced LCY activity, led to the accumulation of lycopene (Liu et al., 2016; Promkaew et al., 2020; Tatmala et al., 2020). Our results suggest that *β-CHX* is not a limiting factor for the accumulation of carotenoids in the PL, although the levels of the transcripts are very low compared to other citrus fruits (Kato et al., 2004; Ikoma et al., 2016).

A further key node in carotenoid accumulation is the balance between biosynthesis and degradation of metabolites, indeed the pool of carotenoids present in the tissues is related to their degradation rate (Nisar et al., 2015; Rodriguez-Concepcion et al., 2018). It is reasonable to assume that the remarkable alteration in the carotenoid pool occurred in the PL could modify the regulatory network operating in ordinary lemons. Thus, besides carotenoid biosynthetic genes, the expression of *NCED1* and *NCED2* were up-regulated in PL respect to WT (Figure 5). These two genes are related to ABA synthesis and they operate downstream the xanthophylls production. Therefore, an enhancement in their transcription might suggest an altered homeostasis of the pathway. Despite, ordinary lemons contain very low amounts of xanthophylls in their flesh, they accumulate considerable quantities of ABA, indicating that flux of metabolites producing for this hormone is pretty active (Norman et al., 1991). Then, the altered carotenoid composition in the pulp of the PL, likely due by a reduced lycopene cyclization, might de-regulated the normal genetic network of the pathway originating a positive feedback of the genes involved in ABA formation. These results are similar to those found in the Cara Cara orange mutant, where lycopene accumulation in the flesh was accompanied by a reduction in ABA content and enhanced expression of both *NCED1* and *NCED2* genes (Alquezar et al., 2008). In other plant tissues, it has been also shown that alterations in carotenoid composition originate a coordinated regulation of *NCED* genes and ABA content (Norman et al., 1991).

Accumulation of carotenoid in specialized structures is a stable storage system and an alternative mechanism to regulate carotenoids availability (van Wijk and Kessler, 2017; Wurtzel, 2019). The transcriptional analysis of genes encoding for carotenoid-associated proteins highlighted significant alterations in PL. *HPS21*, *FIB1*, *FIB2* and *OR* genes were consistently over-expressed in the pulp of PL mutant than in WT (Figure 6). The carotenoid associated-proteins encoded by these three genes were associated with numerous processes involved in carotenoid storage, in addition, they contribute to carotenoid stabilization in plastoglobuli especially during the transition phase from chloroplast to chromoplast (Wurtzel, 2019). It has

been found that *HSP21* chaperone stimulates accumulation of lycopene in tomato and protects fruit pigmentation from heat-stress demonstrating its close relation with carotenoid content (Neta-sharir et al., 2005). Fibrillin is a family of proteins playing structural functions in the packaging and organization of carotenoids in plant tissues and constitute a key element for their storage and metabolism (Singh and McNellis, 2011). The carotenoid content and composition in tomato and pepper fruits have been correlated with the transcript abundance of fibrillins (Kilcrease et al., 2015). Both *FIB* genes displayed a similar expression pattern during the massive accumulation of carotenoids in the PL with a high mRNA accumulation at IG and a slight decline at MG (Figure 6). These evidences support the involvement of fibrillins in the unusual accumulation of carotenoids in the pulp of the pink-fleshed lemon mutant, where they probably increase the storage capacity of structures that are not usually differentiated in ordinary lemons.

The *OR* gene, firstly described in cauliflower, enhances carotenoids accumulation and chromoplasts differentiation (Lu et al., 2006). OR is considered one of the main post-translation regulator of PSY (Osorio, 2019) since recent studies carried out in several plant species has come out that OR protein interacts directly with PSY stabilizing the enzyme and increasing its activity (Zhou et al., 2015; Welsch et al., 2018; Yazdani et al., 2019). Moreover, OR plays a crucial role in the formation of carotenoid-sequestering complexes and the stabilization of carotenoids in plant tissues (Osorio, 2019). The transcription of *Or* gene followed the same pattern of *FIB* in PL (Figure 6) suggesting for both proteins may share critical function in the stabilization of the large amount of carotenoid accumulated in the mutant. It is tempting to speculate that the overexpression of the *Or* gene in PL may increase PSY stability enhancing the flow of carotenes into the pathway. In that case, a reduced enzymatic activity or a lower transcription of genes encoding for LYCs would favour the accumulation of lycopene and other upstream carotenes like phytoene and phytofluene in PL. In transgenic potato tuber overexpressing the *Or* gene the accumulation of β-carotene was significantly higher than in control samples (Li et al., 2012). On the whole, our results suggest that the over expression of *OR* gene might be strictly involved in the events connected with the stabilization of the massive amount of carotenoids accumulated by PL. Unfortunately, it is not possible to establish if the up-regulation of carotenoid-associated protein genes was the cause or just the consequence of the lycopene and the others upstream carotenoids accumulation in PL. However, these findings provide novel insights on the metabolic changes occurred in the mutant that might support further studies aimed at the identification of molecular markers related with the accumulation of lycopene, which is currently one of the most demanded quality trait in modern fruit crop**.**

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S2. qRT-PCR primer sequences, Figure S1. HPLC profiles of saponified carotenoid extracts in pulp of fruits of wild type lemon and Pink lemon at mature green stage (MG). All profiles are MaxPlot chromatograms (each carotenoid shown at its individual λ maxima). Note the high concentration of phytoene (peak no. 3), phytofluene (peak no. 4) and lycopene (peak no. 8) in Pink lemon extract. AU, Absorption units. The compounds correspond to (1) Violaxanthin; (2) Lutein; (3) Phytoene; (4) Phytofluene; (5) β-cryptoxanthin; (6) Neurosporene; (7) β-carotene; (8) Lycopene.

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	Gene name	Forward 5'-3'	Reverse 5'-3'	Reference
XM_006464503.3	ACTINA	TTAACCCCAAGGCCAACAGA	TCCCTCATAGATTGGTACAGTATGAGAC	Alos et al. 2014
XM_006466273.3	DXS1	CGTGTTTTCAACACACCTGACG	AAGCCCCGAAGTCTTCCTCAT	Alos et al. 2006
XM_006488044.3	HDS	CTGCCGGAAATTGGACTTCC	CCATCCTGAAGAAGGGTACC	Alquezar et al. 2008
XM_006487143.3	HDR1	AGACCGTGGAATTCCCTCATACG	AGGCACCGGCTGTCACC	Alquezar et al. 2008
XM_006486607.3	GGPPS1	CCGAGGTCAGCCCTCAAACC	CTCAGGCACGAGATGGGGG	Lado et al. 2015
XM_006481880.3	PSY1	GGTCGTCCATTTGATATGCTTG	CCTAAGGTCCATCCTCATTCCT	Carmona et al. 2012
XM_006492653.3	PSY3a	AATGCATTTTGTGTAAGCCCTGCT	TGTCCCTAAAAGGCTTGATGTGTAAATTG	Manzi et al. 2016
NM_001288862.1	PDS	TCCCTTCTAAGTGTGTATGCC	TGCAAGCTCCTTCATTGTAGC	Carmona et al. 2012
AF372617.1	ZDS	ACAATCTGTTTGAGGCGCAG	CATAGGTATTGGAAACCCTTACTCC	Carmona et al. 2012
MG492005.1	BLCY1	GAACCAGGAGCTTAGGTCTG	GCTAGGTCTACAACAAGGCC	Carmona et al. 2012
MG492007.1	BLCY2a	GAGCAAGTCTCATCGCGTCATAGTG	ACTTTAGCCTTATGAAACTTAACTCCATTTG	Alquezar et al. 2013
MG492008.1	BLCY2b	GCAAGTCTCATCGCGTCATGGTA	ACTTTAGCCTTATGAAACCTAACGCCATTTA	Alquezar et al. 2013
XM_006475429.2	ε-LCY	AAGGTGTGTCGAGTCAGGTGTTT	CCTGCAGGGGACAATCATATCATGTT	Alquezar et al. 2008
XM_025102457.1	BCHX	GGCTCATAAAGCTCTGTGGC	CCAGCACCAAAACAGAGACC	Carmona et al. 2012
XM_006478830.3	ZISO	GCAGCGTCACTGGGTTTAAT	GTTCGCCTCTTCACAGCTTC	
AB219179.1	NCED1	CCACGATGATAGCTCATCCG	CCACTTGCTGGTCAGGCACC	Rodrigo et al. 2006
AB219172.1	NCED2	CTTCCCAACGAAGTCCATAG	GGATTCCATTGTGATTGCTG	Rodrigo et al. 2006
XM_006436998.2	Or	GATGTTGATGTGTTGCGGCGG	AAGTCCTGCACTGTTTCAGGACC	Lado et al. 2015
AB011797.1	FIB1 CitPAP	GGTGGCAGAGGAGGAGAG	GGCATTTAGCAGAGTTAAGGC	Lado et al. 2015
AB011797.1	FIB2 CitPAP	CCATTGGCGAGGGTGGAGG	CGAACTTGATCTGCACACGCTTG	Lado et al. 2015
XM_006469901.3	HSP21	GGGGAAGAAGAAGAGTGGCC	TGTCGACGATTTTGGCAGTGG	Lado et al. 2015
XM_006480857.3	HSP20-3	ACGTCTGGGCGCCCCTTGG	CTCACCGCTGATCTGAAGGACTC	Lado et al. 2015
XM_006424900.2	HSP20-4	TCCGGTTATTCGCCTGCGC	TGACCGCTTATCTGAAGCACCC	Lado et al. 2015

Table S3. qPCR primer sequences.

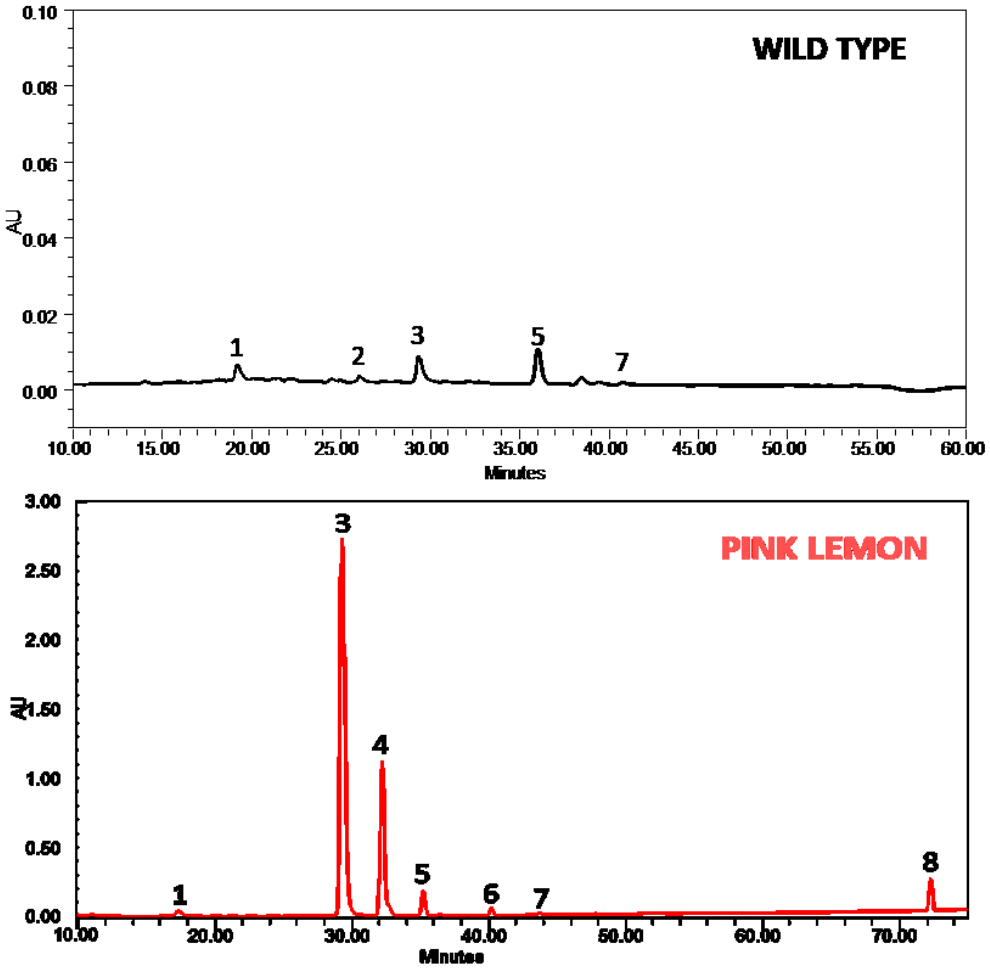

Figure S2. HPLC profiles of saponified carotenoid extracts in pulp of fruits of wild type lemon and Pink lemon at mature green stage (MG). All profiles are MaxPlot chromatograms (each carotenoid shown at its individual λ maxima). Note the high concentration of phytoene (peak no. 3), phytofluene (peak no. 4) and lycopene (peak no. 8) in Pink lemon extract. AU, Absorption units. The compounds correspond to (1) Violaxanthin; (2) Lutein; (3) Phytoene; (4) Phytofluene; (5) β-cryptoxanthin; (6) Neurosporene; (7) β-carotene; (8) Lycopene.

CAROTENOIDS BIOSYNTHESIS IN THE FLESH OF CITRUS FRUIT WITH CONTRASTING COLOR DIVERSITY

4th International & 5th National Student Congress of Food Science and Technology

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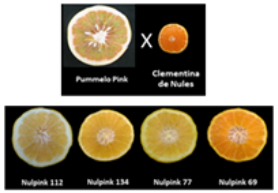
INTRODUCTION

- Carotenoids are isoprenoid compounds which play an important role in plant photosynthesis and photoprotection, and are precursors of phytohormones and volatiles. They also provide an attractive pigmentation to many fruits and flowers (Rodriguez-Concepcion et al., 2018).
- Citrus fruit pigmentation is a key quality trait and is due to their carotenoid content and composition.
- Carotenoids biosynthesis has been studied in different citrus species: mandarins and their hybrids usually produce moderate to high concentration of carotenoids in their flesh. Moreover, they accumulate β-cryptoxanthin, a provitamin A carotenoid and other health-related properties. Conversely, pumelo (*Citrus maxima*), an ancestral specie of Citrus, accumulates little amount of carotenoids in the pulp (Rodrigo et al., 2013).

OBJECTIVE

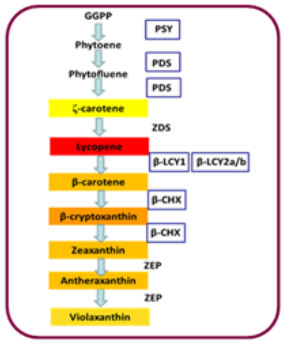
- In the present work we attempted to increase the current knowledge on the biosynthesis and accumulation of carotenoids in the citrus fruit flesh.
- To that end, four hybrids, denominated Nulpink, derived from an interspecific cross between Pummelo Pink (*Citrus maxima*; male parent) and Clementina de Nules (*Citrus clementina*; female parent), were selected (Ollitrault et al., 2015). The Nulpink hybrids used in this study displayed a wide range of color from light-yellow, similarly to the male parent, to deep orange, likewise the female one.



MATERIALS AND METHODS

- Carotenoid content and composition were determined by HPLC-PAD technique. Samples of flesh were collected from fully ripen fruits of the two parental species: Clementina de Nules (CN) and Pumelo Pink (PP), and from the four Nulpink hybrids (NP112, NP134, NP77 and NP69). Carotenoids were extracted and quantified as described in *Lado et al., 2015*.
- The transcription profile of five key genes belonging to the carotenoid biosynthetic pathway: phytoene synthase (PSY), phytoene desaturase (PDS), lycopene β-cyclases (β-LCY1, β-LCY2a/2b) and β-ring hydroxylase (β-CHX), was analysed by RT-qPCR technique. Samples of hybrids and parental flesh were collected and analysed at three ripening stages: green (GR); breaker (BK) and Fully ripen (FR). RNA extraction and RT-qPCR were performed as described in *Lado et al., 2015*.

Carotenoid Biosynthetic Pathway in Citrus



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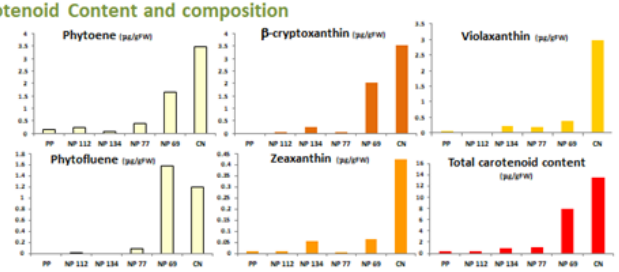
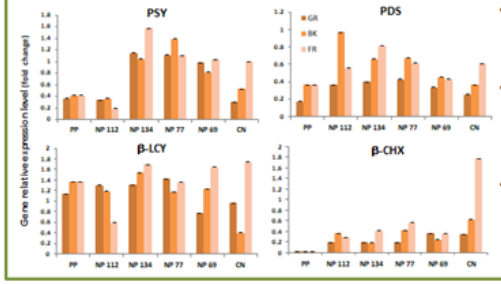
RESULTS

Carotenoid Content and composition



A significant amount of colourless (phytoene and phytofluene) and coloured (β-xanthophylls) carotenoids was found in CN and NP69, whereas PP, NP112-134-77 showed a reduced content of all carotenoids (>1µg/g). Violaxanthin and β-cryptoxanthin (β-cx) were the most abundant carotenoids in CN, while in NP69 were β-cx and colourless carotenes. The CN flesh displayed the highest total carotenoid concentration (13 µg/g) followed by NP69 which was 30% lower. Total carotenoids content reflected the trend observed by colorimetric analysis (not reported).

Carotenoid Biosynthetic Genes Expression



- Nulpink hybrids 134, 77 and 69 showed a relative PSY expression similar or higher to that of CN, while in NP112 and PP was much lower.
- No relevant differences were observed between hybrids and parental β-LCY1 and PDS genes expression. An important upregulation of β-CHX was found only in CN during ripening.
- Although β-LCY2b transcription was upregulated in CN (data not shown), no expression was detected in PP and NPs, except for NP69, which showed a slight transcription only at green stage.

CONCLUSIONS

- The intense pigmentation of Clementine de Nules was associated to the accumulation of coloured carotenoids, particularly of β-cryptoxanthin. This was linked to an upregulation of PSY, β-CHX and β-LCY2a/b genes.
- The pale yellow colour of Pummelo Pink is due to a low production of upstream carotenes, as well as an almost nil content of coloured carotenoids. This scarce pigmentation, was probably correlated with the reduced expression of β-LCY2 and β-CHX genes, which are the main genes involved in xanthophyll production.
- Although Nulpink 112, 134 and 77 showed a very low concentration of coloured carotenoids, the expression levels for most of the genes analysed were similar to Clementine and Nulpink 69, which display a higher carotenoid content. The expression of βLCY2b (only detected in NP69) might enhance the flux trough the pathway leading to an increased biosynthesis of downstream coloured carotenoids.

List of publications and participations to congresses

G Lana, J Zacarias-Garcia, G Distefano, A Gentile, MJ Rodrigo, L Zacarias. Transcriptional analysis of carotenoids accumulation and metabolism in a pink-fleshed lemon mutant.*Genes* **2020**, *11*(11),1294.

https://doi.org/10.3390/genes11111294

Bennici S, Distefano G, Las Casas G, Di Guardo M, Lana G, Pacini E, La Malfa S, Gentile A. Temperature stress interferes with male reproductive system development in clementine (Citrus clementina Hort. ex. Tan.). Ann Appl Biol. 2019;175:29–41.

https://doi.org/10. 1111/aab.12508

G. Lana, G. Distefano, P. Aleza, L. Zacarias, M.J. Rodrigo. Carotenoid biosynthesis in the flesh of citrus fruit with contrasting color diversity. VI International Student Congress of Food Science and Technology 21-22/02/2019, Valencia (Spain). (poster)

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