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A xanthophyll-derived apocarotenoid regulates carotenogenesis in tomato chromoplasts

Caterina D'Ambrosio [a], Adriana Lucia Stigliani [a], José L. Rambla [b,c], Sarah Frusciante [d], Gianfranco Diretto [d], Eugenia M.A. Enfissi [e], Antonio Granell [b], Paul D. Fraser [e], Giovanni Giorio [a,*]

[a] Centro Ricerche Metapontum Agrobios, Agenzia Lucana di Sviluppo e di Innovazione in Agricoltura (ALSIA), Metaponto, MT, Italy
[b] Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain
[c] Universitat Jaume I., Departamento de Biología, Bioquímica y Ciencias Naturales, Avda Sos Baynat s/n, 12071 Castellón de la Plana, Spain
[d] Italian National Agency for New Technologies Energy and Sustainable Development (ENEA), Casaccia Research Centre, Rome, Italy
[e] School of Biological Sciences, Royal Holloway University of London (RHUL), Egham, Surrey, UK

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ABSTRACT

Carotenoids possess important biological functions that make them essential components of the human diet. β-Carotene and some other carotenoids have vitamin A activity while lutein and zeaxanthin, typically referred to as the macular pigments, are involved in good vision and in delaying the onset of age-related eye diseases. In order to create a zeaxanthin-producing tomato fruit, two transgenic lines, one with a high β-carotene cyclase activity and the other with a high β-carotene hydroxylase activity, have been genetically crossed. Ripe fruits from the resulting progeny contained significant levels of violaxanthin, antheraxanthin, and xanthophyll esters. However, their zeaxanthin content was not as high as expected, and the total level of carotenoids was only 25% of the carotenoids found in ripe fruits of the comparator line. Targeted transcript analysis and apocarotenoids determinations indicated that transcriptional regulation of the pathway or degradation of synthesized carotenoids were not responsible for the low carotenoid content of hybrid fruits which instead appeared to result from a substantial reduction of carotenoid biosynthesis. Notably, the content of an unidentified hydroxylated cyclic (C13) apocarotenoid was 13 times higher in the hybrid fruits than in the control fruits. Furthermore, a GC-MS-based metabolite profiling demonstrated a perturbation of carotenogenesis in ripening hybrid fruits compatible with a block of the pathway. Moreover, carotenoid profiling on leaf, fruit, and petal samples from a set of experimental lines carrying the *hp3* mutation, in combination with the two transgenes, indicated that the carotenoid biosynthesis in petal and fruit chromoplasts could be regulated. Altogether the data were consistent with the hypothesis of the regulation of the carotenoid pathway in tomato chromoplasts through a mechanism of feedback inhibition mediated by a xanthophyll-derived apocarotenoid. This chromoplast-specific post-transcriptional mechanism was disclosed in transgenic fruits of HU hybrid owing to the abnormal production of zeaxanthin and antheraxanthin, the more probable precursors of the apocarotenoid signal. A model describing the regulation of carotenoid pathway in tomato chromoplasts is presented.

1. Introduction

Carotenoids are a widespread class of natural pigments synthesized by plants, fungi, algae, and many bacteria, but not by animals that cannot synthesize de novo these compounds (Walter and Strack, 2011). The vast majority of carotenoid molecules possess a skeleton of 40 carbons, with a system of several conjugated double bonds, the chromophore, and hydrocarbon rings on one or both ends of the molecule. The biological functions and activities of carotenoids result from the physical and chemical properties of their molecular structures (Britton, 1995). Plant carotenoid biosynthesis occurs in the plastid organelle (chloroplasts and chromoplasts), even if biosynthetic enzymes are encoded by nuclear genes. In photosynthetic tissues, they act as ancillary pigments in light-harvesting complexes while in flowers and

* Corresponding author.
E-mail address: giovanni.giorio@alsia.it (G. Giorio).

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fruits they are responsible for the bright yellow, orange, and red colouration, which attract insects and other animals for pollination and seed dispersal (Walter and Strack, 2011). The enzymatic and non-enzymatic oxidative breakdown of carotenoids produces an extensive array of essential metabolites called apocarotenoids which can be active as phytohormones (abscisic acid and strigolactones), signaling molecules and growth regulators. Some apocarotenoids are essential components of rhizospheric communication networks with symbionts (mycorrhizae) or parasite plants, while others have roles in abiotic stress response or in plant-to-plant and plant-herbivore interactions (Moreno et al., 2021; Wang et al., 2021). Lycopene, β-carotene, zeaxanthin and lutein are important for human health as they are involved in many physiologic processes and in the prevention of some diseases. Lycopene, due to its strong power to quench singlet oxygen can prevent many diseases in human (Di Mascio et al., 1989) while β-carotene and other carotenoids with unsubstituted β-ionone rings are vitamin A precursors (O'Connor et al., 2022). Finally, lutein and zeaxanthin, having the peculiar characteristic of being accumulated in the macula of eye, have been shown to be indispensable for maintaining a good eye vision in humans and animals and in preventing many age-related macular degenerative diseases (Ma et al., 2012). Plant carotenoid metabolism has been studied in many species and the genes, the enzymes and the cofactors involved in most of pathway reactions have been characterized (Gupta and Hirschberg, 2022; Moise et al., 2014; Nisar et al., 2015; Sun and Li, 2020). The first reaction in the pathway (Fig. 1), catalyzed by phytoene synthase (PSY) enzyme, is the synthesis of 15-*cis*-phytoene through the condensation of two molecules of geranylgeranyl diphosphate (Dogbo et al., 1988).

In tomato (*Solanum lycopersicum* L.) almost all carotenoid biosynthetic genes have been isolated and characterized (Bartley et al., 1992; Bartley and Scolnik, 1993; Burbidge et al., 1997a; Burbidge et al., 1997b; D'Ambrosio et al., 2011; Fray and Grierson, 1993; Galpaz et al., 2006; Isaacson et al., 2002; Pecker et al., 1996; Ronen et al., 2000). In this species the pathway is highly diversified, as shown by carotenoid profiles of leaf, petal, and fruit at different development and ripening stages.

Fruit ripening in tomato is a complex process requiring the coordination of multiple genetic pathways. Its activation follows a climacteric burst of ethylene biosynthesis and depends on an elaborate combination of epigenetic and transcriptional processes involving some well-known transcription factors like *RIN*, and *CNR* (Eriksson et al., 2004; Giovannoni et al., 2017; Ito et al., 2017). Like in all fleshy fruits, ripening in tomato typically starts when the fruit is fully developed and the seeds are mature. The fruit undergoes drastic changes in color, texture, sugar content, aroma and flavor in order to become appealing to seed-dispersal vectors. Carotenoids have been thought to represent not active players in the control of fruit ripening, albeit in recent studies it has been shown that the over-expression of some carotenoid genes, specifically lycopene cyclases, can directly affect the fruit shelf-life properties (Diretto et al., 2020; Mi et al., 2022a). The huge amount of lycopene of ripe tomato fruits, mainly stored in the long needle-like thylakoid-derived crystalloid bodies inside chromoplasts (Camara et al., 1995; Mohr, 1979), is thought to be a consequence of a metabolic block along the pathway derived by the concurrent down-regulation of the two lycopene cyclases genes (Ronen et al., 1999, 2000) and the up-regulation of *Psy1* following its promoter demethylation (Gapper et al., 2013; J. Giovannoni et al., 2017). However, lycopene biosynthesis in ripening fruits reaches a peak after 10–15 days from the breaker stage, then degrades gradually and terminates at the Red Ripe stage under the control of an unknown mechanism. Available evidence suggests that the regulation of carotenogenesis in tomato fruit is through the transcriptional control of the biosynthetic genes mediated by transcription factors (Fraser et al., 2009; Giliberto et al., 2005; Giovannoni, 2004; Shinozaki et al., 2018; Stanley and Yuan, 2019) and through some post-transcriptional mechanisms (Sun et al., 2022). However, the capability of fruit chromoplasts to store huge amounts of lycopene, β-carotene or non-endogenous compounds like ketocarotenoids (Enfissi et al., 2019; Giorio et al., 2007; Mi et al., 2022; Orchard et al., 2021) is the result of the remarkable structural modifications happening in the plastids during the process of chloroplast-to-chromoplast transition (Barsan et al., 2012; Egea et al., 2010; Camara et al., 1995). Several works with mutant and transgenic tomato lines have demonstrated that carotenogenesis in ripening fruits can be regulated by other mechanisms, like forward-feed regulation, feedback inhibition, and substrate channeling (Sun and Li, 2020). However, it appears that the homeostasis of carotenoid metabolism in tomato is maintained by concealed regulatory mechanisms, which are difficult to disclose unless a strong perturbation is induced by a transgene over-expression or by a mutation in one of the key biosynthetic genes (Fraser et al., 2009).

The original purpose of this work was the generation of a tomato line capable of synthesizing and storing xanthophylls, ideally zeaxanthin, in fruits. In order to reach this objective, a hybrid line was produced by crossing the HighCaro and the UO transgenic lines (D'Ambrosio et al., 2015) showing, respectively, a high β-cyclase and a high β-hydroxylase enzymic activities in ripening fruits (Fig. S1). Ripe fruits of HU hybrid line contained significant levels of violaxanthin and antheraxanthin though their zeaxanthin content was not as high as expected, and the total amount of carotenoids was strikingly low as compared to fruits of control RS line. Results of a complex characterization of carotenoid synthesis and degradation in leaf, fruits and petals of a set of experimental lines carrying the two transgenes combined to the *high-pigment 3* (*hp3*) mutation of *Zep* gene (Galpaz et al., 2008) allowed us to demonstrate that the low content of carotenoids in HU fruits resulted from a block of the carotenoid biosynthesis. We hypothesized the existence of an endogenous regulation mechanism of the carotenoid pathway in tomato chromoplasts mediated by a xanthophyll-derived apocarotenoid. The knowledge of this mechanism, once it will be confirmed and fully characterized, would pay the way to new approaches of metabolic engineering in tomato and close species for selecting lines with increasing contents of nutritionally active carotenoids.

2. Results

2.1. Carotenoids in HU and RS leaves

The phenotype of HU plants was visibly different from that of control RS plants since the early stages of growth. Transgenic plants showed lower growth rates, curled leaves, with many sectors of the lamina of a pale yellow instead of green color, and stems with longer internodes (Fig. S2). Significant changes in the carotenoid contents of transgenic leaves with respect to the control leaves (Table 1) demonstrated the effects of the constitutive over-expression of *Lcyb* and *CrtRb2* transgenes in the HU plants. HU leaves were shown to have higher contents of total carotenoids (3304 vs 2493 μg per g of DW) and violaxanthin (1937 vs 495 μg per g of DW) and a lower content of lutein (297 vs 931 μg per g of DW) with respect to RS leaves. Interestingly, the contents of β-carotene, neoxanthin and chlorophylls were not different for the two lines. These results demonstrated a diversion of metabolite flux toward the synthesis of β,β-xanthophylls in transgenic leaves. As a result of the higher content of violaxanthin, HU leaves contained a significantly higher content of ABA than RS leaves (17.5 vs 7.2 pmol of ABA per g of DW).

2.2. Carotenoids in fruits of HU and RS at IG, MG, TR and RR stages

HU fruits were different from control RS fruits, particularly in color. Indeed, the fruits were yellow during the early stages of fruit development, became pale green at the mature green stage, turned to lemon yellow at the turning stage, and finally appeared light orange at the end of ripening (Fig. 2). Carotenoid contents of fruits sampled at four stages (Immature Green, IG; Mature Green, MG; Turning, TR and Red Ripe, RR) were determined by HPLC (Table 2). Pigment profiles (chlorophylls and carotenoids) of RS fruits at the four stages reflected the typical process of

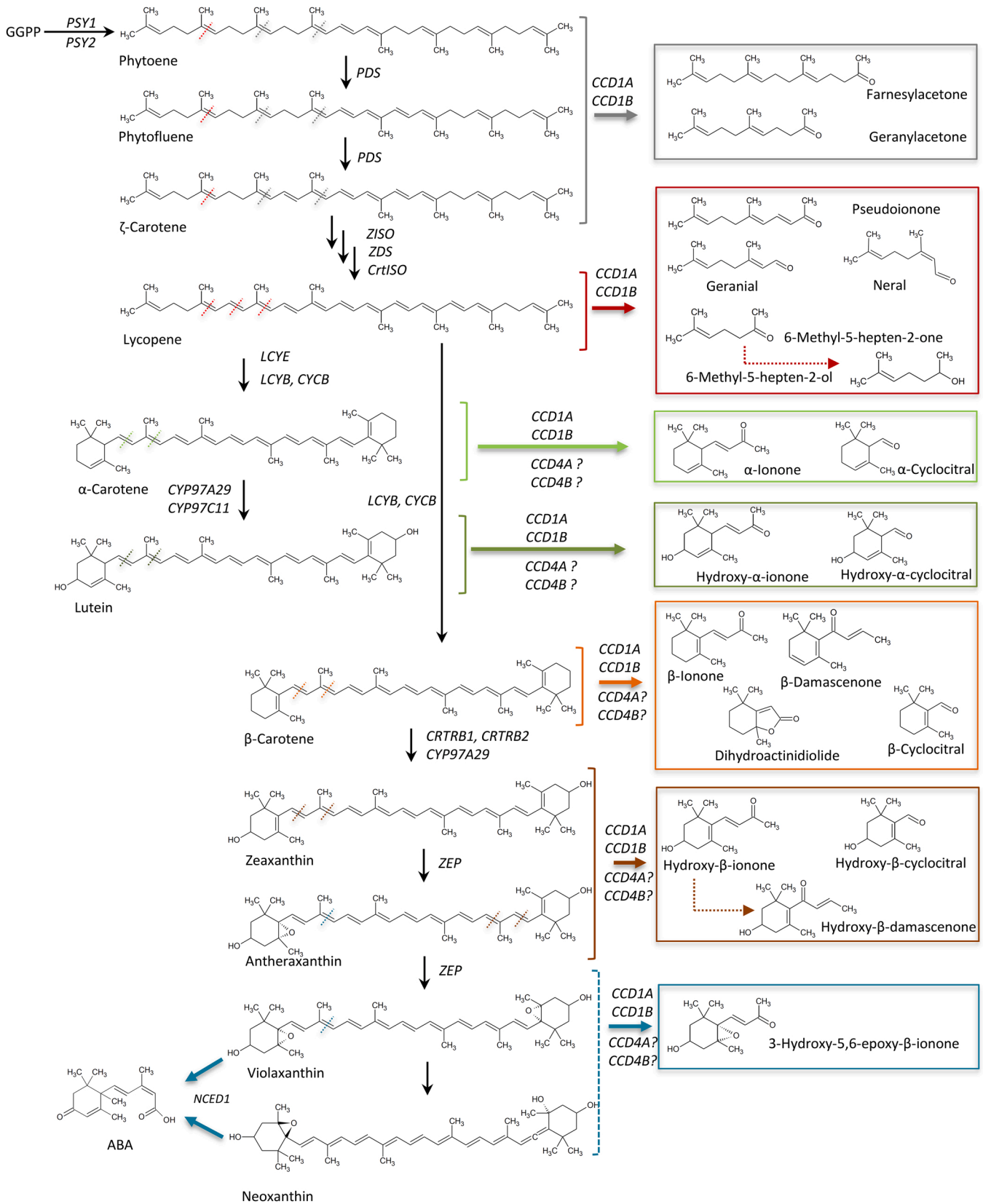

Fig. 1. Carotenoid biosynthetic pathway in tomato (*Solanum lycopersicum* L.). Apocarotenoids described in the text are depicted in rectangular boxes with the indication of their precursor carotenoids. The enzymes involved in the biosynthetic step or in the degradation are indicated near the reaction arrow. GGPP (Geranylgeranyl diphosphate); PSY1, PSY2 (Phytoene synthase 1, 2); PDS (Phytoene desaturase); ZISO (15-cis-ζ-Carotene isomerase); ZDS (ζ-Carotene desaturase); CRTISO (Carotenoid isomerase); LCYB (Lycopene β-cyclase); CYCB (Chromoplastic-specific lycopene β-cyclase); LCYE (Lycopene ε-cyclase); CRTRB1, CRTRB2 (Non-heme di-iron β-carotene hydroxylase 1, 2), CYP97A29 (Cyt P450 carotenoid β-hydroxylase); CYP97C11 (Cyt P450 carotenoid ε-hydroxylase); ZEP (Zeaxanthin epoxidase); NCED1 (9-cis-Epoxycarotenoid dioxygenase); CCD1A, CCD1B, CCD4A, CCD4B (Carotenoid cleavage dioxygenase 1A, 1B, 4A, 4B).

Table 1
Leaf carotenoid and chlorophyll contents of RS and HU lines.

Genotype	Violaxanthin	Neoxanthin	Lutein	β-Carotene	Others	Total carotenoids	Total chlorophylls
RS	495.0[a]	375.9[a]	931.3[a]	644.1[a]	46.9[a]	2493[a]	16,456[a]
HU	1937.5[b]	350.1[a]	297.0[b]	678.9[a]	27.8[a]	3304[a]	16,826[a]

Contents are reported as μg of compound per g of DW. The values shown are the mean of at least three biological replications. Compound means with the same superscript letter were not significantly different (*t*-test, α = 0.05).

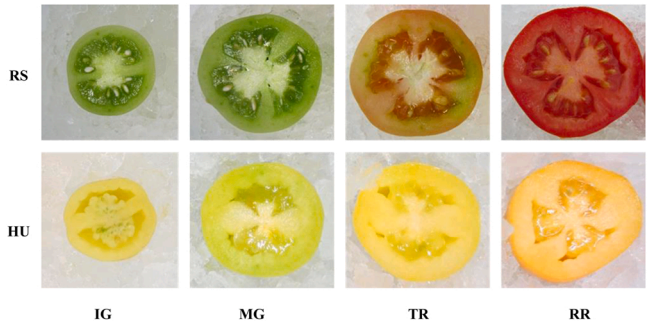

Fig. 2. Fruits of Red Setter (RS) and HU lines sampled at four stages (Transversal sections). Fruit stages: **IG**, Immature Green; **MG**, Mature Green; **TR**, Turning; **RR**, Red Ripe.

biosynthesis and storage in developing and ripening tomato fruits. Conversely, pigment profiles of HU fruits revealed a strong perturbation to carotenoid metabolism induced by the action of two transgenes whose effects were the diversion of the flux of metabolites in the carotenoid pathway toward the synthesis of the β-β-xanthophylls. Differences in the color of fruits at the stages IG and MG were due to a very low content of chlorophyll *a* in HU fruits as compared to the content in fruits of the control. Contents of violaxanthin, antheraxanthin, zeaxanthin and derived esters were higher in HU than in control fruits at all the stages while those of lutein were lower. Total carotenoid contents were similar between the two fruit types at IG, MG and TR stages. Conversely, at the end of ripening HU fruits were shown to contain only a fourth of the carotenoids of RS fruits (387.7 vs 1549.5 μg per g of DW). At this stage HU fruits were shown to contain 182.1 μg/g DW of free violaxanthin, which was undetectable in RS fruits, and 5.5 times more xanthophylls esters than control fruits, while lycopene content was significantly lower in transgenic fruits than in RS fruits (27.6 vs 1427.4 μg per g of DW). Finally, the contents of ABA of HU and RS fruits at the RR stage were not significantly different (12.2 vs 9.9 pmoles of ABA per g of DW).

Since differences in fruit carotenoid contents between hybrid and control fruits could be either due to a reduced biosynthesis or to an increased enzymatic degradation, we conducted a transcription analysis of a set of genes encoding enzymes of carotenoids, ethylene and ABA pathways on the same fruit samples used for HPLC analysis. In addition, the contents of volatile, semi-volatile, and non-volatile apocarotenoids were also determined on fruits sampled at the RR stage.

2.3. Transcription analysis of genes involved in carotenoid, ethylene and ABA metabolisms

The transcript contents of genes encoding enzymes involved in the biosynthesis (*DXS1, Psy1, Lcyb, CrtRb2, Zep*) and in the enzymatic cleavage (*CCD1A, CCD1B, CCD4B*) of carotenoids as well as in the synthesis of ethylene (*ACS2, ACS4, ACO1*) and ABA (*NCED1*) were determined by reverse- transcription quantitative PCR (RT qPCR) analysis in fruits of HU and control RS lines sampled at the four stages. Transcription patterns of the twelve genes in RS fruits (Fig. S3) were similar to patterns described in prior investigations (Giorio et al., 2008; Stigliani et al., 2011).

As for transgenic fruits, it is worth highlighting that the transcript contents of *Lcyb* and *CrtRb2* were significantly higher in HU than in RS fruits at all stages due to the over-expression of the two transgenes. However, the expression of both transgenes appeared to be developmentally regulated. The *ACO1* gene, involved in the ethylene synthesis, was shown to be down-regulated in the HU compared to RS fruits at the TR and RR stages though the difference between transcript contents was only statistically significant at the former stage. Interestingly, the transcript contents of genes encoding the carotenoid cleavage dioxygenases CCD1A, CCD1B and CCD4B were, almost always, significantly higher in transgenic as compared to control fruits. In particular, *CCD1B* transcript was higher in HU fruits than in RS fruits at all the four stages, though the differences were statistically significant only at MG and RR stages. Similarly, the *CCD1A* gene was more expressed in HU than in RS fruits at MG, TR and RR stage, albeit the difference between transcript contents was statistically significant only at the end of ripening. Finally, transcript contents of the *CCD4B* gene were higher, though not significantly, in transgenic fruits at IG and MG.

Altogether these results showed that: a) the two transgenes were highly transcribed in transgenic fruits; b) the transcription of genes involved in the biosynthesis of carotenoids (*DXS1*, *Psy1*, *Zep*) did not differ between HU and RS fruits at the four stages; c) the genes encoding enzymes involved in the cleavage of carotenoids were upregulated in HU fruits during the development (*CCD4B*) or the ripening (*CCD1A*, *CCD1B*).

2.4. Apocarotenoid analysis

The extent of oxidative degradation and CCDs enzymatic cleavage of carotenoids during the ripening of RS and HU fruits was assessed

Table 2
Carotenoid contents of RS and HU fruits sampled at four stages.

Stage	Line	Viola	Neo	Anthera	Lutein	Zea	β-Caro	Lyco	Xantho esters	Others	Total carot	Chl *a*
IG	RS	23.5[a]	19.7[a]	1.4[a]	7	1.8[a]	34.4[a]	nd	2.5[a]	nd	131.0[a]	683.7[a]
	HU	77.5[b]	5.4[b]	9.3[b]	4.0[b]	9.2[b]	5.0b	nd	3.2[b]	nd	113.5[b]	63.6[b]
MG	RS	17.4[a]	12.2[a]	0.6[a]	26.9[a]	1.8[a]	21.3[a]	nd	0.3[a]	0.7[a]	81.1[a]	405.7[a]
	HU	41.8[b]	5.5[b]	4.0[b]	3.8[b]	9.4[b]	7.3[b]	nd	2.6[b]	0.2[b]	74.6[a]	71.7[b]
TR	RS	6.6[a]	5.4	nd	26.7[a]	1.6[a]	31.2[a]	101.6[a]	2.7[a]	6.6[a]	182.3[a]	68.7[a]
	HU	91.6[b]	nd	6.4	6.4[b]	6.7[b]	28.6[a]	6.9[b]	37.9[b]	2.9[b]	187.4[a]	118.5[a]
RR	RS	nd	nd	nd	18.1[a]	nd	46.3[a]	1427.4[a]	14.5[a]	43.2[a]	1549.5[a]	nd
	HU	182.1	nd	13.9	5.4[b]	7.4	60.0[b]	27.6[b]	79.5[b]	11.8[b]	387.7[b]	nd

Contents are reported as μg of compound per g of DW. The values shown are the mean of five biological replications. For each stage, compound means with the same superscript letter were not significantly different (*t*-test, α = 0.05). Viola, violaxanthin; Neo, neoxanthin; Anthera, antheraxanthin; Zea, zeaxanthin; β-Caro, β-carotene; Lyco, lycopene; Total carot, Total carotenoids; Chl *a*, chlorophyll *a*; nd, not detected.

through the determination of contents of volatile, semi-volatile and non-volatile apocarotenoids (Fig. 1).

2.4.1. Volatile and semi-volatile apocarotenoids

Volatile and semi-volatile apocarotenoids derived from carotenes were determined by HS-SPME/GC-MS analysis. For each compound, contents were expressed relative to Red Ripe RS fruit content. Six linear (6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, neral, geranial, geranylacetone, and pseudoionone) and four cyclic (β-cyclocitral, β-damascenone, β-ionone, and dihydroactinidiolide) apocarotenoids were detected (Fig. S4).

As expected, linear apocarotenoids deriving from phytoene and lycopene were almost absent in fruits of both lines at IG, MG and TR stages. Between the TR and the RR stage, the contents of these compounds increased only in RS fruits concurrently with the rise of lycopene content. As for the four cyclic apocarotenoids, their contents increased strongly between the TR and the RR stage in fruits of both lines though the contents of all compounds were lower in HU fruits than in RS fruits at all stages, particularly at the end of ripening (RR). At this experimental point, the contents of β-cyclocytral, β-ionone, β-damascenone and dihydroactinidiolide in transgenic fruits were only the 36%, the 40%, the 51% and the 30%, respectively, of contents in RS fruits. The determinations of semi-volatile apocarotenoid content were repeated on fruits sampled at the Red Ripe stage rising the incubation temperature from 50 to 80 °C. Results of this second analysis were in solid agreement with prior results (Fig. S5). Moreover, with the new protocol, it was possible to detect and quantify the farnesylacetone, a semi-volatile linear C18 apocarotenoid, and a new unidentified compound that, using its mass spectrum, was classified as a hydroxylated cyclic C13 apocarotenoid (OH-C13-Apo). Interestingly, the content of this new compound was 13 times higher in HU than in RS fruits.

2.4.2. Non-volatile compounds: aglycons and glycosylated apocarotenoids

To rule out the possibility that the low carotenoid contents in red ripe fruits of HU line could be due to enzymatic or oxidative degradation of xanthophylls, the contents of non-volatile apocarotenoids, as aglycons and as glycosylated compounds, were determined on the same set of fruit samples.

Contents of hydroxylated apocarotenoids in the form of aglycons were determined by LC-APCI-MS in the non-polar fraction, while contents of glycosylated hydroxyl-apocarotenoids were determined by LC-ESI-MS in the polar fraction of fruit extracts (Fig. S6). The following six hydroxy-apocarotenoids were quantified as aglycons in fruits of both lines: hydroxy-β-cyclocitral, hydroxy-β-damascenone1 (or 3-oxo-α-ionone1), hydroxy-β-damascenone2 (or 3-oxo-α-ionone), hydroxy-β-ionone (or 3-oxo-α-ionol), 3-hydroxy-5,6-epoxy-β-ionone, and 6-hydroxy-3-oxo-α-ionone.

As expected from xanthophyll profiles of fruits, the contents of these six apocarotenoids were almost always higher in HU fruits than in RS fruits at IG, MG and TR stages. At the end of ripening, the levels of all aglycons but hydroxy-β-damascenone2 were significantly higher in RS fruits respect to HU fruits because these compounds presented a marked increase in RS fruits between the TR and the RR stages. Conversely, only minimal increases (hydroxy-β-damascenone1, hydroxy-β-damascenone2, 3-hydroxy-5,6-epoxy-β-ionone, 6-hydroxy-3-oxo-α-ionone,) or no change (hydroxy-β-cyclocitral and hydroxy-β-ionone) in contents were observed between the two stages in HU fruits. Additionally, six glycosylated hydroxy-apocarotenoids compounds were detected and quantified in fruits: hydroxy-β-cyclocitral-glucoside, hydroxy-β-damascenone-glucoside (or 3-oxo-α-ionone-glucoside), hydroxy-β-ionone-glucoside1 (or 3-oxo-α-ionol-glucoside1), hydroxy-β-ionone-glucoside2 (or 3-oxo-α-ionol-glucoside2), 3-hydroxy-5,6-epoxy-β-ionone-glucoside1, 3-hydroxy-5,6-epoxy-β-ionone-glucoside2 (Fig. S7). Among the six compounds, only two showed patterns of fruit accumulation different between the two lines. The content of hydroxy-β-ionone-glucoside was higher in the RS fruits than in HU fruits at TR and RR stages. This finding could be explained by noting that the content of lutein, the most probable precursor of this compound, was much higher in RS than in HU fruits. Finally, the content of the 3-hydroxy-5,6-epoxy-β-ionone-glucoside2, which can derive from the cleavage of violaxanthin or antheraxanthin, was significantly higher in HU fruits, which were enriched with these xanthophylls, than in RS fruits, at all the four stages. Altogether these results ruled out the possibility that the low content of carotenoids in HU fruits at the end of ripening could be the result of a consistent degradation of lycopene, β-carotene or xanthophylls occurring after their biosynthesis. Conversely, these results lent support to the hypothesis of a substantial reduction of the carotenoid biosynthesis in HU fruits through an endogenous mechanism of regulation.

2.5. Metabolomic analysis of RS and HU fruits sampled at four stages

In order to evaluate the intermediary metabolism changes during the development and the ripening of RS and HU fruits, a GC-MS-based metabolite profiling was carried out on fruits sampled at four stages. Among the 42 metabolites, belonging to four classes (amino acids, organic acids, sugars, fatty acids and isoprenoids), many significant differences between contents of HU and RS fruits were shown (Table S1). Overall, HU fruits showed higher contents of almost all the amino acids, particularly at mature green, turning, and ripe stages. Similarly, increased levels in transgenic fruits than in control fruits were observed for the organic acids across all the stages. In the case of fatty acids and isoprenoids, most of the differences between contents of the two fruit types were not statistically significant. A Principal Component Analysis (PCA) was performed using the whole dataset of the individual metabolite contents. PCA results (Fig. S8) showed that the two genotypes and the four stages of growth could be easily separated in the plane of the first and second principal components, which explained, respectively, 21% and 15% of the total compositional data variation. Moreover, while the trajectory of fruit ripening was clearly defined for RS samples, since they clustered in small groups according to the stage, the ripening of HU fruits was not clearly depicted in the plane of first two PCs because TR and RR fruits clustered in the same group and not in two different groups like for RS fruits. These results clearly demonstrated a modification of the ripening process in HU fruits resulting from the perturbation of metabolism due to the transgenes over-expression.

2.6. The effect of the Lcyb and CrtrB2 transgenes and the hp3 mutation on the carotenoid biosynthesis in leaves, fruits and petals

In order to look for evidence supporting the existence of a post-transcriptional regulation mechanism of carotenoid metabolism in tomato, a set of experimental lines was generated crossing the HU hybrid with an RS-derived line carrying the *high-pigment 3* (*hp3*) mutation.

The *hp3* is a mutant allele of *Zep* locus encoding a not fully functional isoform of the zeaxanthin epoxidase (ZEP) enzyme. Leaves of mutant plants have a higher content of zeaxanthin and lower contents of violaxanthin and neoxanthin then leaves of control plants, while ripe fruits of *hp3* lines show higher contents of carotenoids than fruits of control lines, possibly due to the increase of plastid size and the number of plastids per cell (Galpaz et al., 2008). The following six tomato lines (Fig. S9) were characterized: RS, HU, HC^{hp3} carrying the *Lcyb* transgene, UO^{hp3} carrying the *CrtRb2* transgene, HU^{hp3} carrying both transgenes and RS^{hp3}. Carotenoid contents were determined in leaves, petals and fruits, at the IG and RR stages while the transcript levels of *DXS1*, *Psy1*, *Lcyb*, *CrtRb2*, *CCD1A* and *CCD1B* genes were determined only in fruits. Leaves of the four *hp3* lines were shown to have significantly higher contents of zeaxanthin and antheraxanthin and significantly lower contents of violaxanthin and neoxanthin as compared to leaves of RS and HU lines, carrying a functional ZEP enzyme (Table 3). Interestingly, HU^{hp3} leaves did not show the curling margins and the yellowing of lamina observed in HU leaves, probably due to a reduction of ABA synthesis. As for the effects of the two transgenes, the overexpression of

CrtRb2 appeared to have a more significant impact, in comparison to the overexpression of the *Lcyb*, on the carotenoid profiles of transgenic leaves. IG fruits of *hp3* plants were shown to have greater contents of total carotenoids than fruits of RS and HU lines (Table 4A). In particular, IG fruits of HU^{hp3} line were shown to have the highest contents of zeaxanthin, antheraxanthin and total carotenoids (271.8 μg per g of DW) than IG fruits of all the other lines. Conversely, RR fruits of HU and HU^{hp3} lines contained only 21% and 24%, respectively, of carotenoids contained in fruits of RS control (237 and 272 vs 1137 μg per g of DW). Interestingly, HU^{hp3} fruits were shown to have the highest contents of zeaxanthin at both the IG and the RR stage (139 and 194 μg per g of DW). Notably, in these fruits the total amount of carotenoids did not change between the two stages (incidentally, the values were strikingly identical, 272 μg). Fruits of the control RS^{hp3} line were shown to have the highest content of lycopene (978 μg per g of DW) and a significantly higher content of total carotenoids respect to fruits of transgenic HC^{hp3} and UO^{hp3} lines. As for the effect of transgenes, UO^{hp3} fruits contained more lycopene and zeaxanthin, while HC^{hp3} fruits, in turn, were enriched in β-carotene. As for the transcription pattern of the six analysed genes, the *DXS1* the *Psy1* showed the typical fruit expression patterns in all transgenic and control lines. The *Lcyb* and *CrtRb2* were highly expressed in fruits of the lines carrying the corresponding transgenes compared to fruits of non-transgenic lines. Finally, the transcript contents of *CCD1A* and *CCD1B* genes were significantly higher in RR fruits of HU and HU^{hp3} lines then in RR fruits of RS and RS^{hp3} control lines, respectively (Fig. S10).

Results of HPLC determinations in petals (Table 4B) clearly showed a smaller effect of transgenes on the carotenoid metabolism in contrast to the effect of the *hp3* mutation. The contents of violaxanthin and neoxanthin, mainly present as esters (92%), were much higher in petals of HU and RS, where they accounted for roughly 99% of all carotenoids, than in petals of all the other lines. Notably, petals of lines carrying the *hp3* mutation (RS^{hp3}, HC^{hp3}, UO^{hp3} and HU^{hp3}) were shown to contain on average only the 22% of carotenoids stored in petals of HU and RS lines. The inhibition of carotenoid biosynthesis in *hp3* petals was thus probably due to the impairment of ZEP and not to the action of two transgenes. In petals of *hp3* lines, the contents of zeaxanthin and its esters accounted for 40% and 23%, respectively, of all carotenoids. In contrast, the contents of violaxanthin, neoxanthin, and their esters, accounted for roughly 29% of all pigments stored. Moreover, zeaxanthin was undetectable in HU petals and barely present in RS petals. Notably, the ratio between free and esterified violaxanthin and neoxanthin contents ranged between 0.08 and 0.10 in all the six lines. It is also interesting that the petal carotenoid contents of RS^{hp3} and RS lines were quite similar to petal contents of *hp3–1* and M82 lines characterized by Galpaz et al. (2008). Altogether gene transcription and carotenoid data of transgenic and control *hp3* lines provided new clues supporting the hypothesis of a regulatory mechanism controlling the flux of metabolites toward the carotenoid pathways in tomato chromoplasts. Furthermore, the substantial reduction of carotenoid contents in petals of all *hp3* lines in comparison to petals of RS and HU lines and in RR fruits of HU, HC^{hp3}, UO^{hp3}, and HU^{hp3} lines in comparison to fruits of RS and RS^{hp3} indicated that the zeaxanthin and the antheraxanthin, rather than violaxanthin, could be the precursors of the apocarotenoid involved in the regulation of carotenoid metabolism in fruit and petal chromoplasts.

3. Discussion

Results presented in this study provide evidence for a post-transcriptional mechanism regulating the carotenoid pathway in tomato chromoplasts. The mechanism was disclosed because of the significant perturbation of the carotenoid metabolism in ripening fruits of the HU line determined by the combined effects of *Lcyb* and the *CrtRb2* transgenes (D'Ambrosio et al., 2004, 2011). Although transgenic HU fruits contained significant levels of violaxanthin and antheraxanthin, their carotenoid contents at the RR stage unexpectedly amounted to only 25% of that of Red Setter comparator line. Detailed transcription analysis of genes involved in the synthesis and the catabolism of carotenoids and the determination of apocarotenoids in fruit samples gave unequivocal indications that neither a transcriptional block of the pathway nor the oxidative degradation or enzymatic cleavage of the newly formed xanthophylls could be blamed for the low contents of carotenoids in HU fruits. This reduction in hybrid fruits seemed, instead, the result of a block of carotenoid biosynthesis due to a negative feedback regulation mediated by a xanthophyll-derived apocarotenoid. The hypothesis of a mechanism regulating the carotenoid metabolism in tomato chromoplasts was also supported by the carotenoid profiles of petals and fruits of a set of experimental tomato lines carrying the two transgenes and the *hp3* mutation. The latter data also indicated that the putative signal involved in the regulation could be an apocarotenoid derived from the enzymatic cleavage of zeaxanthin or antheraxanthin. We postulated that the high rates of synthesis and cleavage of xanthophylls in developing HU fruits, fueled by two transgenes, could have abnormally increased the content of the apocarotenoid signal, which, in turn, could have short-circuited the regulation mechanism up to the complete block of the carotenoids biosynthesis. A post-transcriptional regulation mechanism of carotenoid biosynthesis, like that described in this study, was suggested by Arango et al. (2014) to explain the reduction of carotenoid contents in the root of a transgenic carrot line overexpressing the Arabidopsis carotenoid ε-hydroxylase *CYP97A3*. Through the reanalysis of all data from this study, it was possible to outline a model (Fig. 3) of the mechanism regulating the carotenoid pathway in tomato chromoplasts.

3.1. Premises of the model

The model is based on two main assertions. The first states that the carotenoid pathway operating during the development of the fruit (green stages) and involving the PSY2 and the other enzymes required for the synthesis of xanthophylls continues to be active throughout the fruit ripening in order to sustain the production of ABA (Schwarz et al., 2015), which can be synthesized through the ZEP-dependent or, the recently discovered, ZEP-independent pathways (Jia et al., 2022). Instead, the carotenoid pathway involving the PSY1 operates from the breaker until the end of ripening to sustain the massive synthesis of lycopene. Although it was known that the *Psy2* gene is transcribed along

Table 3
Leaf carotenoid contents of RS, HU, RS^{hp3} HC^{hp3}, UO^{hp3} and HU^{hp3}.

Genotype	Viola	Neo	Anthera	Lutein	Zea	β-Caro	Others	Total carot	Total Chls
RS	524.5^{a}	416.7^{a}	20.1^{a}	1094.2^{a}	12.6^{a}	780.7^{a}	93.5^{a}	2942.3^{a}	19,901^{a}
HU	1627.0^{b}	375.3^{a}	126.2^{b}	241.2^{b}	69.7^{b}	523.2^{b}	68.6^{a}	3031.3^{a}	12,830^{b}
RS^{hp3}	22.5^{c}	67.4^{b}	193.7^{b}	1085.1^{a}	730.9^{c}	877.6^{a}	92.8^{a}	3070.2^{a}	21,398^{a}
HC^{hp3}	29.8^{c}	93.1^{c}	313.5^{b}	387.5^{c}	1176.8^{c}	970.2^{a}	92.6^{a}	3063.4^{a}	21,962^{a}
UO^{hp3}	67.2^{d}	265.6^{d}	530.5^{c}	598.8^{d}	1369.9^{d}	664.2^{b}	113.2^{a}	3609.4^{b}	21,168^{a}
HU^{hp3}	130.0^{e}	277.2^{d}	674.1^{d}	142.1^{e}	1754.9^{e}	731.5^{ab}	117.2^{a}	3826.9^{b}	20,938^{a}

Contents are reported as μg of compound per g of DW. The values shown are the mean of three biological replications. For each compound, genotype means with different superscript letters were significantly different (Tukey's pairwise *post-hoc* test, $p \leq 0.05$). Viola, violaxanthin; Neo, neoxanthin; Anthera, antheraxanthin; Zea, zeaxanthin; β-Caro, β-carotene; Total carot, Total carotenoids; Total Chls, Total chlorophylls; nd, not detected.

Table 4
Carotenoid contents in fruits (A) and petals (B) of RS, HU, RS^{hp3}, HC^{hp3}, UO^{hp3} and HU^{hp3} lines.

A											
Stage	**Line**	**Viola**	**Neo**	**Anthera**	**Lutein**	**Zea**	**β-Caro**	**Lyco**	**Others**	**Total carot**	**Total Chls**
IG	RS	23.9^{a}	12.5^{a}	2.4^{a}	36.3^{a}	3.1^{a}	28.0^{a}	nd	3.9^{a}	110.1^{ab}	582.71^{a}
	HU	77.6^{b}	4.4^{b}	14.0^{b}	2.9^{b}	9.7^{b}	2.6^{b}	nd	nd	111.2^{a}	54.0^{b}
	RS^{hp3}	nd	3.2^{b}	6.8^{c}	37.1^{a}	43.6^{c}	36.4^{a}	nd	4.2^{a}	131.2^{b}	722.2^{a}
	HC^{hp3}	nd	3.2^{b}	9.9^{c}	16.2^{ac}	54.3^{c}	37.4^{ac}		7.1^{ab}	128.1^{ab}	752.1^{ac}
	UO^{hp3}	15.1^{d}	11.5^{ac}	32.2^{d}	22.2^{c}	109.4^{d}	30.9^{a}		8.8^{b}	230.1^{c}	784.9^{c}
	HU^{hp3}	18.4^{ad}	15.4^{c}	43.5^{e}	8.1^{d}	139.1^{d}	38.4^{c}	nd	8.9^{b}	271.8^{c}	879.2^{c}
RR	RS	8.1^{a}	nd	0.6^{a}	15.0^{a}	0.5^{a}	38.0^{ad}	929.2^{a}	145.9^{a}	1137.4^{a}	nd
	HU	110.8^{b}	nd	8.39^{b}	3.2^{b}	6.6^{b}	49.8^{a}	7.9^{b}	50.5^{a}	237.0^{b}	nd
	RS^{hp3}	nd	nd	2.8^{a}	25.3^{c}	98.4^{c}	58.4^{a}	977.6^{a}	168.8^{a}	1331.3^{a}	nd
	HC^{hp3}	nd	nd	3.0^{a}	6.7^{b}	117.9^{c}	433.9^{b}	13.8^{b}	70.7^{a}	646.0^{c}	nd
	UO^{hp3}	1.5^{a}	nd	8.6^{a}	17.2^{a}	148.6^{d}	8.9^{c}	252.9^{c}	47.6^{a}	485.2^{c}	nd
	HU^{hp3}	4.1^{a}	nd	12.5^{b}	3.4^{b}	194.3^{d}	30.6^{d}	nd	26.9^{a}	271.8^{b}	nd
B											
Tissue	**Line**	**Viola + Neo**	**Xantho esters**	**Anthera**	**Zea**	**Zea esters**	**Lutein**	**β-Caro**		**Total carot**	
Petals	RS	484.0^{a}	6311.4^{a}	8.6^{a}	9.8	nd	39.0^{a}	nd		6853^{a}	
	HU	655.0^{a}	5955.1^{a}	nd	nd	nd	20.8^{b}	nd		6632^{a}	
	RS^{hp3}	39.8^{b}	472.1^{b}	91.4^{b}	723.7^{a}	457.9^{a}	35.6^{ab}	24.0^{a}		1844^{b}	
	HC^{hp3}	38.5^{b}	373.9^{b}	79.8^{b}	587.8^{b}	378.2^{a}	18.4^{b}	17.0^{a}		1494^{b}	
	UO^{hp3}	34.9^{b}	335.1^{b}	81.2^{b}	542.0^{b}	263.1^{a}	22.1^{b}	17.3^{a}		1296^{b}	
	HU^{hp3}	39.0^{b}	392.9^{b}	90.1^{b}	578.2^{b}	283.2^{a}	13.8^{b}	16.3^{a}		1414^{b}	

Contents are reported as μg of compound per g of DW. The values shown are the mean of three biological replications. For each compound, genotype means with different superscript letters were significantly different (Tukey's pairwise *post-hoc* test, $p \leq 0.05$). Viola, violaxanthin; Neo, neoxanthin; Xantho esters, esters of xanthophylls; Zea esters, zeaxanthin esters; Anthera, antheraxanthin; Zea, zeaxanthin; β-Caro, β-carotene; Total carot, Total carotenoids; Total Chls, Total chlorophylls; nd, not detected.

all fruit development and ripening (Fraser et al., 1999; Giorio et al., 2008), the pathway involving the PSY2 has always been considered irrelevant to carotenoid biosynthesis in ripening fruits. This assumption stemmed from the observation that fruits of *yellow flesh* lines, carrying a knocked-out *Psy1* gene, do not contain any appreciable amount of lycopene. The operativity of the PSY2-mediated carotenoid pathway during the ripening of tomato fruits has been recently reported by Gupta et al. (2022). This study demonstrated that ripening *yellow flesh* (*r*) fruits treated with the CPTA, a known inhibitor of lycopene cyclase enzymes, accumulate lycopene. Since *yellow flesh* fruits lack a functional PSY1 enzyme, the lycopene could only derive from the PSY2 pathway. Another convincing demonstration of the operativity of a PSY2-driven carotenoid pathway in ripening tomato fruits has been provided recently by Karniel et al. (2022) who studied the biosynthesis of carotenoids in fruits of the *yellow flesh/ tangerine* (*r/t*) double mutant using the virus-induced gene silencing assay (VIGS). Interestingly, the hypothesis concerning the existence of two spatially distinct pathways of carotenoid biosynthesis in tomato fruit chromoplasts was formulated many years ago by Goodwin and Jamikorn (1952) and later by many other authors interested in the effects of DMSO and CPTA on carotenoid biosynthesis in ripening fruits (Baqar and Lee, 1978; Raymundo, 1971; Sink et al., 1974). Although at that time the reactions of the pathway were still debated and the enzymes were unknown, the results of several experiments brought most authors to the conclusion that two spatially distinct carotenoid biosynthesis pathways had to be active in tomato fruits during the ripening. The first pathway was thought to be active in fruit since the onset of fruit development to sustain the synthesis of photosynthetic carotenes and xanthophylls, while the second pathway was thought to be triggered at the onset of ripening to produce lycopene as the end-product (Camara et al., 1995; Raymundo, 1971).

The second assertion of the model states that the enzyme specificity of tomato PYP, a classical acyltransferase catalyzing the esterification of xanthophylls in petal and fruit chromoplasts, is much higher for violaxanthin and neoxanthin than for zeaxanthin (Ariizumi et al., 2014; Lewis et al., 2021). This assertion was recently demonstrated by Kishimoto et al. (2020) who evaluated the expression of the tomato PYP in Petunia petals. The authors concluded that the tomato PYP has a higher esterification activity for neoxanthin and violaxanthin than for zeaxanthin and antheraxanthin. The differential specificity of tomato PYP is not usually a problem in petals because the zeaxanthin is quickly converted to violaxanthin by the ZEP enzyme (D'Ambrosio et al., 2011). Conversely, when the zeaxanthin cannot be converted efficiently to violaxanthin or stored in photosynthetic apparatuses it become an easy substrate of CCDs.

Finally, it is interesting to consider that from the structural point of view, the carotenoid metabolism in plants could be seen as an array of three-dimensional modular apparatuses located in the different plastid compartments. These apparatuses are made of biosynthetic enzymes, mainly embedded in membranes, that interact with substrates, cofactors and products in spatially and developmentally changing combinations (Ruiz-Sola and Rodríguez-Concepción, 2012; Shinozaki et al., 2018; Shumskaya et al., 2012; Shumskaya and Wurtzel, 2013; Welsch et al., 2000; Wurtzel, 2019). Most of the enzymes involved in the biosynthesis and the oxidative cleavage of carotenoids in tomato are organized in protein complexes or, possibly, in metabolons localized in various plastid compartments (Cunningham and Gantt, 1998; Nogueira et al., 2013; Ruiz-Sola and Rodríguez-Concepción, 2012; Zhang and Fernie, 2020). Given the above assertions and assuming that the flux of metabolites toward the carotenoid pathway in tomato plastids is regulated through a mechanism involving a xanthophyll-derived apocarotenoid, it was possible to interpret the carotenoid profiles of leaves, fruits, and petals of the experimental tomato lines analysed in this study.

3.2. Carotenoids metabolism in chloroplasts

Carotenes and xanthophylls synthesised in chloroplasts are usually readily stored inside the membranes as free molecules or in complexes with proteins. They are, for this reason, not degraded until the onset of the senescence process (Rottet et al., 2016). The carotenoid pathway in the chloroplast is not downregulated since the apocarotenoid signal is not produced. When the carotenoid biosynthesis is boosted, as with the overexpression of a transgene encoding a biosynthetic enzyme, the chloroplasts can sustain high rates of xanthophyll synthesis and storage (Enfissi et al., 2019; Mi et al., 2022).

3.2.1. Leaves and developing fruits

The leaves of the transgenic lines analysed in this study, in particular those of HU^{hp3} and UO^{hp3} plants (Table 3), showed similar or significantly higher contents of carotenoids compared to the leaves of the RS control line.

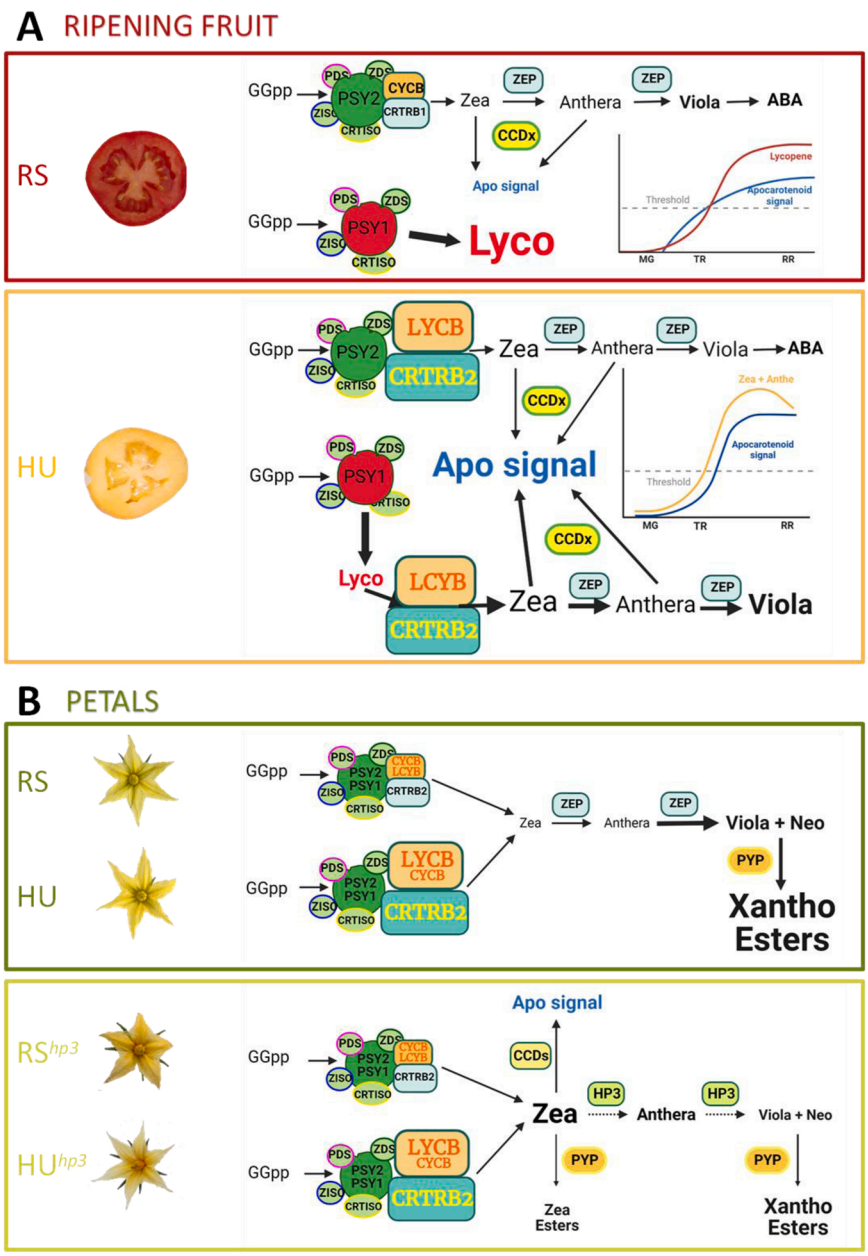

Fig. 3. Model of carotenogenesis regulation in ripening fruit of RS and HU (A) and in petals of RS, HU, RS^{hp3} and HU^{hp3} (B). Enzyme names: **CCD**, carotenoid cleavage dioxygenase; **CRTISO**, carotenoid isomerase; **CRTRB1**, **CRTRB2**, carotenoid β-hydroxylase; **DXS**, 1-D-deoxyxylulose 5-phosphate synthase; **LCYB**, **CYCB**, lycopene β-cyclase; **PDS**, phytoene desaturase; **PSY1**, **PSY2**, phytoene synthase; **PYP**, xanthophyll esterase; **ZDS**, ζ-carotene desaturase; **ZEP**, zeaxanthin epoxidase; **HP3**, mutated zeaxanthin epoxidase; **ZISO**, 15-*cis*-ζ-carotene isomerase. Metabolite names: **ABA**, Abscisic acid; **Anthera**, Antheraxanthin; **Apo**, Apocarotenoid; **GGpp**, Geranylgeranyl diphosphate; **Lyco**, Lycopene; **Neo**, Neoxanthin; **Viola**, Violaxanthin; **Zea**, Zeaxanthin.

As in leaves, the xanthophylls in chloroplasts of developing fruits were not cleaved by the CCDs and the carotenoid pathway appeared to be not downregulated. IG fruits of HU^{hp3} and UO^{hp3} plants (Table 4A and Fig. S9) were shown to contain significantly higher contents of total carotenoids and zeaxanthin than fruits of all the other lines. HU^{hp3} fruits were green as the control fruits since their violaxanthin and chlorophyll contents were similar to those of RS fruits. Conversely, HU fruits were yellow, with a low chlorophyll *a* content and high levels of zeaxanthin, antheraxanthin and violaxanthin. The high content of xanthophylls in these fruits probably boosted the synthesis of ABA, which, in turn, activated the precocious degradation of the chlorophylls (Gao et al., 2016; Hussain et al., 2000; Tung et al., 2008; Zhao et al., 2016).

3.3. Carotenoids metabolism in chromoplasts

3.3.1. Ripening fruits

Two carotenoid pathways are active in fruit chromoplasts (Fig. 3A). All the enzymes, as well as the co-factors of the PSY2 pathway, also active in chloroplasts, are probably organized in multienzyme assemblies which are localized in the membranous structures derived from the reorganization of thylakoids, in the plastoglobuli, and, possibly, in the envelope (Bartley and Scolnik, 1993; Lütke-Brinkhaus et al., 1982; Ruiz-Sola and Rodríguez-Concepción, 2012; Welsch et al., 2000). The second carotenoid pathway involving the PSY1 enzyme, committed to the biosynthesis of lycopene, is activated at the breaker stage and operates along all the fruit ripening. Our data lent support to the hypothesis that the rate of lycopene biosynthesis in ripening fruits of tomato is regulated by a mechanism involving an apocarotenoid derived from the enzymatic cleavage of the zeaxanthin or the antheraxanthin (Fig. 3A). According to the model, the content of the signal apocarotenoid start to rise at the onset of the chloroplast-chromoplast transition because the xanthophylls released from the plastidial membranes are cleaved by the CCDs similarly to what happens in senescent leaves (Rottet et al., 2016). The signal apocarotenoid content continues to increase throughout the ripening because a portion of xanthophylls derived from the PSY2-involving pathway is also cleaved. Consequently,

the flux of metabolites through the PSY1 pathway is gradually reduced. It is possible to explain the evolution of carotenoid biosynthesis in HU fruits keeping in mind this hypothetic mechanism. Up to the Turning stage, carotenoid biosynthesis in HU fruits was not much perturbed by the over-expression of the two transgenes whose main effects on the pigment profiles of fruits were a partial degradation of chlorophylls and the diversion of lycopene toward the production of β,β-xanthophylls (Table 2). With the rise of carotenoid biosynthesis, due to the up-regulation of *Psy1*, most of the newly synthesised β,β-xanthophylls underwent a rapid cleavage by CCDs. Consequently, the increase of the apocarotenoid signal strongly reduced the flux of metabolites through the carotenoid pathway ending up with its complete downregulation. This mechanism could explain why at the end of ripening, the HU fruits contained only a fifth of the carotenoids of RS fruits (Tables 2 and 4A).

The downregulation of carotenoid metabolism was also observed in the lines carrying one or both transgenes combined with the *hp3* mutation of *Zep* gene (Table 4A). All transgenic lines were shown to produce ripe fruits with significantly lower contents of carotenoids than fruits produced by control RS and RS^{hp3} lines. As in HU fruits, the over-expression of the transgenes permitted a constant production of xanthophylls and, as a consequence of their cleavage, of the signal acting as a quencher of the carotenoid biosynthesis.

Finally, though at the RR stage the zeaxanthin in RS^{hp3} fruits was higher than in RS fruits, their total carotenoid contents were not significantly different. This could be due to the fact that the zeaxanthin present in RS^{hp3} fruits at the end of ripening was mainly synthesised and stored in the chloroplasts during the development of fruits thus escaping the cleavage by the CCDs. Indeed, 44% of zeaxanthin present in RS^{hp3} fruits at the RR stage was already present at IG stage and together to that synthesized until the onset of ripening remained stored in the many rod-shaped membranous structures still present in fruit plastids at the end of ripening (Karniel et al., 2020).

3.3.2. Petals

In contrast to what was observed in leaves and fruits, petals of HU and RS lines showed similar carotenoid profiles. Moreover, their total carotenoid contents were significantly higher than those of all *hp3* line petals, including those of RS^{hp3} (Table 4B). These results can be easily interpreted in light of the proposed model if we hypothesize that also in petal chromoplasts the apocarotenoid signal could activate the down-regulation of the carotenoid pathway (Fig. 3B). In our interpretation, the carotenogenesis was inhibited in the petals of lines carrying the *hp3* mutation because the petal acyl-transferase enzyme (PYP), as demonstrated by Kishimoto et al. (2020) and Lewis et al. (2021), could not efficiently esterify the zeaxanthin and the antheraxanthin that, consequently, were degraded by CCDs. The anomalous production of the apocarotenoid signal triggered the downregulation of the carotenoid biosynthesis. Consequently, the total carotenoid contents in petals of *hp3* lines were, on average, 22% of those of RS and HU petals in which the carotenogenesis was not downregulated because the ZEP enzyme efficiently converted all zeaxanthin to violaxanthin which was, in turn, readily esterified by the endogenous acyl-transferase (PYP) enzyme. Following this premise, it is clear why 99% of all carotenoids stored in petals of HU and RS plants were represented by violaxanthin, neoxanthin, and their esters, while these compounds accounted for only 28% of all carotenoids, on average, in petals of all *hp3* lines. As for the rate of xanthophyll esterification, 90% of violaxanthin and neoxanthin were esterified in HU and RS petals while, on average, only 36% of zeaxanthin was esterified in petals of all *hp3* lines. Altogether these results support the conclusions of Kishimoto et al. (2020) on the xanthophyll differential specificity of PYP and also indicate that the apocarotenoid signal involved in the regulation of the carotenoid biosynthesis could derive from the cleavage of zeaxanthin or antheraxanthin rather than by the cleavage of violaxanthin.

3.4. Evidence from other experimental data of the regulation of carotenoid metabolism in tomato chromoplasts

3.4.1. Zeaxanthin-rich tomato fruits

A recent paper by Karniel et al. (2020) reported the generation of a set of transgenic tomato lines, producing fruits enriched in zeaxanthin, which carried the mutations *B* and *hp3* in combination with the *CcBCH2* transgene, encoding the fruit carotene β-hydroxylase of *Citrus clementina*. These lines can be considered analogous to line HU^{hp3} since the elevation of zeaxanthin content was attempted through the increase of its biosynthesis and the reduction of its conversion. The increase of biosynthesis was reached through the enhancement of lycopene β-cyclization and β-carotene hydroxylation, obtained, respectively, with the *B* mutation and the *CcBCH2* transgene. The reduction of zeaxanthin conversion was obtained using the *hp3* allele of the *Zep* gene, which encodes an impaired ZEP enzyme. It is worth highlighting that the fruits of all the *B/hp3/CcBCH2* transgenic lines showed consistent reductions in the content of total carotenoids. In particular, the fruits of the best-performing line only contained 37% of carotenoids present in fruits of the control lines, similarly to the reduction we observed in ripe fruits of the HU and HU^{hp3} lines. These results further support the hypothesis that the enhancement of carotenoid hydroxylation can result in the downregulation of the pathway when the plastids cannot efficiently esterify the xanthophylls or store them in suitable membranous structures.

3.4.2. Ketocarotenoid producing transgenic tomato lines

An interesting example of fruits escaping the downregulation of the carotenoid pathway is that of engineered tomato plants for ketocarotenoid production. In all reported cases (Enfissi et al., 2017, 2019; Huang et al., 2013), transgenic tomato lines produced fruits and leaves with significantly higher amounts of carotenoids than control plants. The results of Enfissi et al. (2019) are particularly interesting because one of the ketocarotenoid-producing lines, the HU/MM:CrtZW, was obtained by crossing the HU hybrid, described in this study, with the transgenic MM:CrtZW line carrying the 3,3' carotenoid hydroxylase and 4,4' carotenoid oxygenase of *Brevundimonas* sp. SD-212 (Mortimer et al., 2017). These authors observed that ripe fruits of the HU/MM:CrtZW line contained 1.8 more carotenoids than ripe fruits of the MM:CrtZW line, which, in turn, produced fruits with 3.5 more carotenoids than control MM fruits. Interestingly, the non-endogenous ketocarotenoids were mainly stored in the plastoglobuli inside the chromoplasts. It can be hypothesized that the carotenogenesis was not downregulated in ketocarotenoid-producing fruits for the concurrent action of two factors: the less efficient cleavage of free ketocarotenoids by the CCDs and the reduced or completely abolished ability of the apocarotenoid to act as a signal molecule due to presence of a keto group on the hydroxylated β-ring.

3.5. Apocarotenoids as a signal of the carotenoid regulation mechanism

The data presented in this study seem to point to an apocarotenoid molecule derived from the enzymatic cleavage of zeaxanthin or antheraxanthin as the signal regulating the carotenoid pathway in tomato chromoplast. In contrast to carotenoids, which are too lipophilic to be transported out of the plastid, apocarotenoids and their derivatives are more suitable to act as signaling metabolites. The roles of apocarotenoids, derived from CCD-mediated cleavages of carotenoids, have been demonstrated in various physiological plant processes (Moreno et al., 2021; Simkin, 2021). β-Cyclocitral, deriving from the cleavage of β-carotene, is directly involved in the regulation of root growth (Dickinson et al., 2019), while its acidic derivative, β-cyclogeranic, can enhance tolerance to drought (D'Alessandro et al., 2019). The role of β-cyclocitral in the reduction of MEP pathway flux under herbivory insect damage was demonstrated in Arabidopsis (Mitra et al., 2021). More interestingly, apocarotenoids derived from the cleavage of zeaxanthin

are involved in the symbiosis of plants with arbuscular mycorrhizae (mycorradicin, blumenols) and the interaction of plants with insects (liolide). In rice, as an example, zaxinone has been shown: to regulate plant growth and development, suppress strigolactones biosynthesis, and modulate biotic interactions with arbuscular mycorrhizae (Wang et al., 2019). Recently, it has been shown that the β-apo-11-carotenoids (C15 apocarotenoids) derived from the cleavage of β-carotene and zeaxanthin are involved in a ZEP-independent ABA biosynthesis pathway (Jia et al., 2022). In light of all these examples, our hypothesis of a possible role of a zeaxanthin- or an antheraxanthin-derived apocarotenoid in the regulation of carotenogenesis appears reasonable. Many examples reported in the literature have demonstrated the role of apocarotenoids in secondary metabolism regulation. It has been recently shown that the ectopic expression of the saffron CCD2 and UGT in tomato fruits results in the accumulation of crocins at the expense of carotenoids (Ahrazem et al., 2022). Since zeaxanthin is a known substrate of CCD2, and crocins are stored in the vacuoles, it would be interesting to understand if the endogenous and exogenous CCDs are competing for the same substrate and if this unknown xanthophyll-derived apocarotenoid could be inactivated by glycosylation. Finally, it is interesting to note that since ripe fruits of HU were shown to lack precursors of lycopene as well as their cleavage products and the expression of *Psy1* was not downregulated, the regulation mechanism has to involve the phytoene synthase enzyme as it was observed in transgenic carrot plants overexpressing the carotenoid P450 ε-hydroxylase gene (Arango et al., 2014). Moreover, in Arabidopsis, it has been shown that the PSY cell amount is controlled by of the translational elements located in the 5′ UTR of *Psy* mRNAs (Álvarez et al., 2016). In tomato, the steady-state level of PSY1 protein is modulated by the cochaperone-like OR protein (Yazdani et al., 2019) and by the Clp protease (D'Andrea et al., 2018). A post-transcriptional regulation mechanism targeting the precursor of PSY1 outside the plastid and involving the E3 ubiquitin ligase PPSR1 has also been recently reported (Wang et al., 2020). Finally, the down-regulation of the carotenoid metabolism could also be indirect through a transcriptional or post-transcriptional control of the MEP pathway. DXS protein content can be regulated post-translationally through a mechanism involving the Clp protease complex and ClpC chaperones (Pulido et al., 2016) and transcriptionally by β-cyclocitral, derived from the photo-oxidation of β-carotene following insect tissue damage (Mitra et al., 2021).

Altogether results obtained in this study provide evidence of a post-transcriptional mechanism regulating the carotenoid pathway in tomato chromoplasts involving an apocarotenoid derived, most probably, from the enzymatic cleavage of the zeaxanthin or the antheraxanthin. Several questions remain to be answered before the model presented could be considered demonstrated: the identity of the compound acting as the signal, the xanthophyll precursor, the CCD enzyme involved in the cleavage and the precise mechanism through which the signal can relay information to other cells or plastid compartments to reduce the flux of metabolites toward the carotenoid pathway. In order to demonstrate the actual existence of this mechanism and how it operates in tomato chromoplasts, a more specific study involving both genetic and biochemical approaches must be conducted.

Furthering knowledge on the regulation of carotenoid metabolism in tomato will help researchers to interpret experimental results better and to devise more efficient programs of metabolic engineering in tomato and in close species for selecting lines with increasing contents of nutritionally active carotenoids.

4. Materials and methods

4.1. Plant materials and growth conditions

All the tomato plants were grown in the glasshouse facilities at the Metapontum Agrobios Research Centre of ALSIA in Metaponto (ITALY).

4.1.1. First glasshouse trial

The HU hybrid line was obtained by crossing the HC and the UO (D'Ambrosio et al., 2004, 2011; Giorio et al., 2007) transgenic lines.

In the first trial the control Red Setter (RS) and the hybrid HU lines were evaluated using a Randomized block design (RBD) with 5 replicates, each made of 5 different plants for a total of 25 plants for single genotype. Plants were grown in pots of 3.3 litres of capacity filled with a compost made of equal volumes of a clay soil and peat (Flora Gard Sub Professional, Type S 0.5, Floragard,Villa Lagarina, TN, Italy). The temperature was set at 20–22 °C during the day and 15–16 °C during the night for the whole cultivation period. Light supplementation of three hours was provided in the morning and in the afternoon from the transplant until flowering. The third or the fourth leaf from each plant was collected for analyses. Fruits were sampled during the development and the ripening at the following four stages as defined in Gillaspy et al. (1993). Immature Green (IG); Mature Green (MG); Turning (TR); and Red Ripe (RR). Fruits were harvested from all the 5 plants of each replicate and processed. Sampled fruits were separated from seeds, cut in small parts and divided in four sub-samples and stored at − 80 °C until further treatment. Three subsamples were lyophilized and used for HPLC determinations, RT-qPCR analysis and metabolite analysis while the fourth was stored at − 80 °C until apocarotenoid determinations.

4.1.2. Second glasshouse trial

The *hp3–1* (e1472m1 line) mutant line (Galpaz et al., 2008), kindly provided by Prof. Dani Zamir (The Hebrew University of Jerusalem, Israel), was crossed to the RS line. RS^{hp3} line was breed through three backcrosses to RS and the self-pollination of plants carrying the *hp3–1* mutation. RS^{hp3} line was then crossed to HU line and the progeny plants carrying both the transgenes were backcrossed to parental RS^{hp3} line. Following a PCR screening for the presence of transgenes and the HRM analysis for the zygosity at the *Zep* locus it was possible to select plants homozygous for the *hp3* mutation and carrying the *Lcyb* transgene (HC^{hp3}), the *CrtRb2* transgene (UO^{hp3}), or both the transgenes (HU^{hp3}).

In the second trial, the following six lines were evaluated: Red Setter (RS), HU, RS^{hp3}, HC^{hp3} UO^{hp3} HU^{hp3}. Plants were analysed according to a Randomized block design (RBD) with 3 replicates, each made of 2 different plants for a total of 6 plants for single genotype. Plants were grown and leaf samples were collected as described above. Petals were collected just after the complete opening of the flowers while the fruits were sampled at the Immature Green (IG) and the Red Ripe (RR) stages.

4.2. Pigment analyses

Pigment content determinations were performed by HPLC as previously described (Fraser et al., 2000) with modifications (D'Ambrosio et al., 2011). Pigment separation was performed with an Agilent 1200 ChemStation HPLC system (Agilent Technologies, Inc., Santa Clara, USA) equipped with a DAD system using a C30 reverse-phase column (C30-YMC; 250 9 4.6 mm, S-5 lm, YMC, Milford, MA). Data acquisition and analysis were carried out with the ChemStation for LC 3D system software (Rev.B.03.02; Agilent Technologies, Santa Clara, CA). Quantification of carotenoids was carried out using calibration curves derived from analysis with authentic standards as previously described (Giorio et al., 2013). Peak identification of violaxanthin, neoxanthin, antheraxanthin, lutein, zeaxanthin, β-carotene, lycopene, chlorophyll *a* and chlorophyll *b* were based on retention times and spectral properties of authentic standards. Lycopene, β-carotene, lutein, zeaxanthin, chlorophyll *a*, chlorophyll *b* and 8′-apo-*β*-carotenal were purchased from Sigma (Sigma–Aldrich, St. Louis, MO), violaxanthin, neoxanthin, and antheraxanthin were purchased from CaroteNature GmbH (Lupsingen, Switzerland).

4.3. Real-time qPCR analysis

Total RNAs were extracted from fruits of transgenic and control

plants using the protocol described in Giorio et al. (2013). Three out of the five available replicates were used for the analysis. RNA samples were treated with DNAse (TURBO DNA-*free*™ Kit, Thermo Fisher Scientific Inc., Waltham, USA) before cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad Laboratories, Hercules, USA). Transcription analysis of twelve genes (*DXS1*, *Psy1*, *Lcyb*, *CrtRb2*, *Zep*, *CCD1A*, *CCD1B*, *CCD4B*, *NCED*, *ACO1*, *ACS2* and *ACS4*) involved in the biosynthesis and degradation of carotenoids and apocarotenoids, and in the ABA and ethylene metabolism was carried out using an RT-qPCR assay (Bustin et al., 2009) with gene-specific hydrolysis probes (TaqMan). Primer and probe sequences are reported in Table S2. Reactions were conducted in 96-well reaction plates in a volume of 25 µl containing 12.5 µl Platinum™ Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific), 300 nM forward primer, 300 nM reverse primer, 150 nM TaqMan probe and 1 µl of cDNA sample for each reaction. Amplification reactions were performed using a LightCycler® 480 II-96 (Roche Diagnostics Ltd., Basel, Switzerland) with the following thermal cycle: 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Calibration curves for 12 genes and for the endogenous reference gene (Actin) were prepared using a unique cDNA stock solution derived from the reverse transcription of a mix of leaf and fruit RNAs. Each single data point was derived from three reactions both in the construction of the standard curves and for sample quantification. Relative quantification of gene transcripts was carried out using the Standard Curve method (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (2004) Applied Biosystems Part Number 4371095 Rev A).

4.4. Zygosity of hp3 plants using high resolution melting (HRM) analysis

High Resolution Melting (HRM) analysis was used to discriminate between *hp3–1* heterozygous and homozygous plants. Genomic DNA was isolated from leaf samples using the Nucleospin Tissue kit (Macherey-Nagel). HRM analysis was carried out using the LightCycler® 480 II-96 (Roche Diagnostics Ltd., Basel, Switzerland). HRM primers (For:TGCTATCAGAGGAGAGGGACA, Rev:CAGTTGCCAGAAATACCATCAA) were designed using the Primer3 software on the DNA regions flanking the *hp3–1* mutation on the first exon of *Zep* gene (GenBank: EU004202.1) (Galpaz et al., 2008) using the recommendations provided in the LightCycler® 480 manual. DNA samples from RS and RS^{hp3} were used as homozygous negative and homozygous positive controls, respectively. Moreover, a mixed 1:1 DNA solution using DNAs of the two controls was used as the heterozygous control. PCR reactions were performed with the Precision Melt Supermix for High Resolution Melt (HRM) Analysis kit (Bio-Rad Laboratories, Inc), following the manufacturer instructions. Following the amplification, the PCR products were first denatured at 95 °C for 1 min and then rapidly cooled to 40 °C to allow heteroduplex formation. PCR samples were then subjected to increasing temperatures to allow dissociation of DNA double-stranded molecules. The fluorescent signal was measured continuously from 70° to 85°C, with a ramp rate of 0.01 °C/sec and 60 acquisitions per degree. High Resolution Melting curve data were analysed with the LightCycler® 480 Gene Scanning software (Roche Diagnostics GmbH, Germany).

4.5. Measurement of ABA concentration

Quantitative determination of abscisic acid (ABA) in leaves and fruits were performed by the enzyme linked immunosorbent assay (ELISA) with the Phytodetek® ABA Test Kit (Agdia Inc.).

ABA determinations were carried out on three different biological replicates for each genotype using freeze-dried leaf or fruit samples following the kit instructions. Color absorbances were detected with the Spectramax 250 microplate reader (Molecular Devices) at 405 nm. Two independent experiments were performed and for each extract two technical replicates were analysed.

4.6. Metabolite analysis

Metabolites were extracted from finely milled tomato powder using methanol, with ribitol as an internal standard for relative quantification, and derivatized as described in (Enfissi et al., 2010). GC-MS analysis was carried out on an Agilent HP6890 GC with a 5973MSD and components identified using a mass spectral (MS) library constructed from in-house standards as well as the NIST 98 mass spectral library.

4.7. Volatile and semi-volatile apocarotenoid determination by HS-SPME-GC/MS analysis

Volatile compounds were captured by means of headspace solid phase microextraction (HS-SPME) and separated and detected by means of gas chromatography coupled to mass spectrometry (GC/MS). Samples were processed similarly as described in Rambla and Granell (2020). Roughly, five hundred milligrams of frozen tomato powder were introduced in a 15 mL glass vial and incubated at 37ºC for 10 min in a water bath. Five hundred mL of an EDTA 100 mM, pH 7.5 solution and 1.1 g of $CaCl_2 0.2\ H_2O$ were added, mixed gently and sonicated for 5 min. One mL of the resulting paste was transferred to a 10 mL screw cap headspace vial with silicon/PTFE septum and analysed within 12 h. Volatile compounds were extracted from the headspace by means of a 65 µm PDMS/DVB solid phase microextraction fiber (Supelco, Merck KGaA, Darmstadt, Germania). Volatile extraction was performed automatically by means of a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Two different incubation temperatures were used for volatile extraction. First, more conservative conditions were used, incubating the vials at 50ºC for 10 min with agitation at 500 rpm. Then, the fiber was exposed to the headspace of the vial for 20 min under the same conditions of temperature and agitation. Then, all the samples were analysed again under the same extraction conditions with the only exception of an incubation temperature of 80ºC, to facilitate the detection of semi-volatile apocarotenoids. The rest of the methodology was identical in both cases after this step. Desorption was performed at 250ºC during 1 min in splitless mode in the injection port of a 6890 N gas chromatograph (Agilent Technologies, Inc., Santa Clara, USA). After desorption, the fiber was cleaned in an SPME fiber conditioning station (CTC Analytics AG) at 250ºC for 5 min under a helium flow. Chromatography was performed on an Agilent J&W DB-5 ms (60 m, 0.25 mm, 1.00 µm) capillary column with helium as carrier gas at a constant flow of 1.2 mL/min. The GC interface and MS source temperatures were 260ºC and 230ºC respectively. Oven programming conditions were 40ºC for 2 min, 5ºC/min ramp until 250ºC, and a final hold at 250ºC for 5 min. Data was recorded in a 5975B mass spectrometer (Agilent Technologies, Inc.) in the 35–250 *m/z* range at 6.2 scans/s, with electronic impact ionization at 70 eV. Chromatograms were processed by means of the Enhanced ChemStation E.02.02 software (Agilent Technologies, Inc.).

Identification of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, neral, geranial, geranylacetone, pseudoionone, β-cyclocitral, β-damascenone and β-ionone was performed by the comparison of both retention time and mass spectrum with those of pure standards purchased from Sigma-Aldrich (Merck KGaA). The other compounds were tentatively identified based on mass spectra and Kovats retention index similitude with those in the NIST05 mass spectral database. For quantitation, one specific ion was selected for each compound and the corresponding peak from the extracted ion chromatogram was integrated. An admixture reference sample was prepared for each season by mixing thoroughly equal amounts of each sample. A 500 mg aliquot of the admixture was analysed every six samples and processed as any other sample as part of the injection series. This admixture was used as a reference to normalize for temporal variation and SPME fiber aging.

4.8. Glycosylated and aglycon apocarotenoid determination by LC-ESI-MS and LC-APCI-MS analyses

LC-ESI-MS analysis of apocarotenoid glycosylated forms was performed as previously described (Ahrazem et al., 2022). Briefly, semi-polar metabolites were extracted from 10 mg lyophilized, homogeneously ground tomato fruit tissues with 0.75 mL of cold 75% (v/v) methanol, 0.1% (v/v) formic acid, spiked with 10 μg/mL formononetin as internal standard. After shaking for 40' at 20 Hz using a Mixer Mill 300 (Qiagen), samples were centrifuged for 15 min at 20,000 g at 4ºC. Then, 0.6 mL of supernatant were removed and transferred to HPLC tubes. For each experimental point, 3 independent biological replicates, were analysed. For each biological replicate, at least one technical replicate was carried out. Liquid chromatography (LC) was carried out using a Phenomenex C18 Luna column (100 × 2.0 mm, 2.5 μm) and the mobile phase was composed by water-0.1% formic acid (A) and acetonitrile-0.1% formic acid (B). The gradient was: 95% A: 5% B (1 min), a linear gradient to 25% A: 75% B over 40 min, 2 min isocratic, before going back to the initial LC conditions in 18 min

LC-APCI-MS analysis of apocarotenoid aglycons was performed as previously described (Diretto et al., 2021). Non-polar metabolites were extracted from 5 mg of lyophilized, homogeneously ground tomato fruit tissues using 1 mL of 25% (v/v) methanol, 50% (v/v) chloroform, 25% (v/v) 50 mm-Tris-HCl, spiked with 50 μg/mL DL-α-tocopherol acetate as internal standard. After centrifugation, the organic hypophase was kept and the aqueous phase re-extracted with the same volume previously used of chloroform spiked with the internal standard. The pooled organic extracts were dried with a Speed Vac concentrator, and the residue was resuspended in ethyl acetate (100 μl). For each experimental point, 3 independent biological replicates, were analysed. For each biological replicate, at least one technical replicate was carried out. LC separations were performed using a C30 reverse-phase column (100 × 3.0 mm; 3 μm, YMC Europe). The solvent systems were methanol (A); 75% methanol (v/v), 25% water (v/v) and 0.2% ammonium acetate (B); tert-butyl-methyl ether (C). The gradient elution was as follows: 0–6 min 95% A, 5% B, and 0% C; 1 min 80% A, 5% B, and 15% C; 5 min 80% A, 5% B, and 15% C; 20 min 30% A, 5% B, and 65% C; 22 min 30% A, 5% B, and 65% C; 18 min 95% A, 5% B, and 0% C. Five microliters of each sample were injected and a flow of 0.25 and 0.8 mL was used throughout the LC semi-polar and non-polar runs, respectively. Mass spectrometry analysis was performed using a quadrupole-Orbitrap Q-extractive system (Thermo Fisher Scientific, USA), operating in positive/negative heated electrospray ionization (HESI) or atmospheric pressure chemical ionization (APCI) coupled to an Ultimate HPLC-DAD system (Thermo Fisher Scientific, Waltham, MA). For semi-polar metabolite analysis, mass spectrometer parameters were as follows: capillary and vaporizer temperatures 30 ℃ and 270 ℃, respectively, discharge current 4.0 KV, probe heater temperature at 370 ℃, S-lens RF level at 50 V. The acquisition was carried out in the 110/1600 *m/z* scan range, with the following parameters: resolution 70,000, microscan 1, AGC target 1e6, and maximum injection time 50. Full scan MS with data-dependent MS/MS fragmentation was used for metabolite identification. For non-polar analysis, APCI parameters were as follows: nitrogen was used as sheath and auxiliary gas, set to 20 and 10 units, respectively. The vaporizer temperature was 300 °C, the capillary temperature was 250 °C, the discharge current was set to 5.5 μA, and S-lens RF level was set at 50. All solvents used were LC-MS grade quality (CHROMASOLV® from Sigma-Aldrich). Metabolites were quantified in a relative way by normalization on the internal standard (formononetin and DL-α-tocopherol acetate) amounts. Targeted metabolite identification of apocarotenoid aglycons and glycosylated forms was performed by comparing chromatographic and spectral properties with reference spectra and literature data (if available), on the basis of the *m/z* accurate masses, as reported in the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic mass identification, or on the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) in the case of adduction detection, and considering MS/MS fragmentation patterns.

4.9. Statistical analysis

Student's *t*-test was used to determine significant difference between transgenic HU and RS means. Multiple pairwise mean comparisons were performed using the Tukey's pairwise *post-hoc* test. All statistical analyses were performed using the PAST (PAlaeontological Statistics) ver. 4.09 software (https://www.nhm.uio.no/english/research/resources/past).

CRediT authorship contribution statement

Caterina D'Ambrosio: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition. **Adriana Lucia Stigliani:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition. **José L. Rambla:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Sarah Frusciante:** Validation, Formal analysis, Investigation, Resources, Writing – review & editing. **Gianfranco Diretto:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition. **Eugenia M.A. Enfissi:** Validation, Formal analysis, Investigation, Resources. **Antonio Granell:** Resources, Writing – review & editing, Funding acquisition. **Paul D. Fraser:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Giovanni Giorio:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111575.

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