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Carotenoid Profiling and Biosynthetic Gene Expression in Flesh and Peel of Wild-Type and *hp-1* Tomato Fruit under UV-B Depletion

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ABSTRACT: Although light is recognized as one of the main factors influencing fruit carotenogenesis, the specific role of UV-B radiation has been poorly investigated. The present work is addressed to assess the molecular events underlying carotenoid accumulation in presence or absence of ultraviolet-B (UV-B) light in tomato fruits of wild-type and *high pigment-1* (*hp-1*), a mutant characterized by exaggerated photoresponsiveness and increased fruit pigmentation. Gene expression analyses indicated that in wild-type fruits UV-B radiation mainly negatively affects the carotenoid biosynthetic genes encoding enzymes downstream of lycopene both in flesh and peel, suggesting that the down-regulation of genes *CrtL-b* and *CrtL-e* and the subsequent accumulation of lycopene during tomato ripening are determined at least in part by UV-B light. In contrast to wild-type, UV-B depletion did not greatly affect carotenoid accumulation in *hp-1* and generally determined minor differences in gene expression between control and UV-B-depleted conditions.

KEYWORDS: *carotenoids, RT-PCR, tomato, Lycopersicon esculentum, UV-B depletion*

INTRODUCTION

Carotenoids are highly unsaturated compounds, synthesized by photosynthetic tissues, fruits, flowers and roots, whose structure consists in the repetition of isoprenoid units. Many studies have elucidated their important role in promoting human health due to their high antioxidant properties[1] and their involvement in some physiological function, such as sight.[2] Indeed, the consumption of these compounds via carotenoid-rich foods has proven to be inversely correlated to the incidence of certain chronic diseases.[3] In particular, lycopene and β-carotene displayed capacities in lowering the risk of specific types of cancer.[4]

Red-ripe tomato fruits, besides being a precious source of a number of health-protecting molecules such as tocopherols, ascorbic acid, and flavonoids, are particularly rich in carotenoids, especially lycopene which is the most abundant, but also β-carotene and colorless carotenes.

Carotenoid biosynthesis (reviewed by Hirschberg,[5] Figure 1) and accumulation, which occur in chloroplast and chromoplast, in tomato fruit shows important features along the ripening period due to the different roles played by these compounds in the green fruit or when the chloroplast turns to chromoplast. When the fruit is green and partially equiparable to a photosynthetic/autotrophic organ, the accumulation of lycopene is negligible, whereas its cyclization proceeds at a high rate in order to synthesize cyclic carotenoids, such as β-carotene or lutein that act as accessory pigments in photosynthesis. At the onset of ripening, due to the diminished activity of lycopene cyclases and the chlorophyll breakdown that accompanies lycopene accumulation, tomato fruit color turns to yellow and then to red. Beyond the genetic control on the expression of structural genes, there is some evidence that carotenoid accumulation could be regulated by the content of thylakoid and plastid lipid-associated protein called fibrillin that is thought to act as a structuring factor of membranes or plastoglobules.[6] Precursors for carotenoid biosynthesis are provided by the plastid-localized deoxy-xylulose 5-phosphate pathway, but there are data that also suggest a likely cytosolic origin for these precursors.[7]

UV-B radiation, despite being a minor component of sunlight, is known to affect many aspects of plant physiology, such as control of plant height and total mass production, but also to act at the molecular level on signal transduction[8] and gene expression.[9] The influence of UV-B radiation on carotenoids has been poorly investigated and mainly focused on the interaction between solar UV-B and the physiology of carotenoid metabolism in vegetative tissues. Photosynthetically accessory carotenoids have been found to be negatively affected by UV-B depletion in soybean [10] and in *Citrus aurantifolia*,[11] whereas no UVB-dependent difference in carotenoid concentration has been observed in quinoa (*Chenopodium quinoa*[12]). Our recent works showed how carotenoid are actually affected by the presence of UV-B radiation in the whole tomato fruit,[13] as well as in flesh and peel analyzed separately.[14] Moreover, by using tomato ethylene mutants, Becatti et al.[14] demonstrated that the UV-B effects on carotenoid metabolism occur through both ethylene-dependent and ethylene-independent mechanisms, which seem to act in an antagonistic way. The influence of UV-B radiation on fruit carotenoids has been reported to be cultivar-dependent also in apple fruits.[15]

The mechanism of UV-B perception and signal transduction has been elucidated in *Arabidopsis* and involves the UV RESISTANCE LOCUS 8 (UVR8) protein, CONSTITUTIVE

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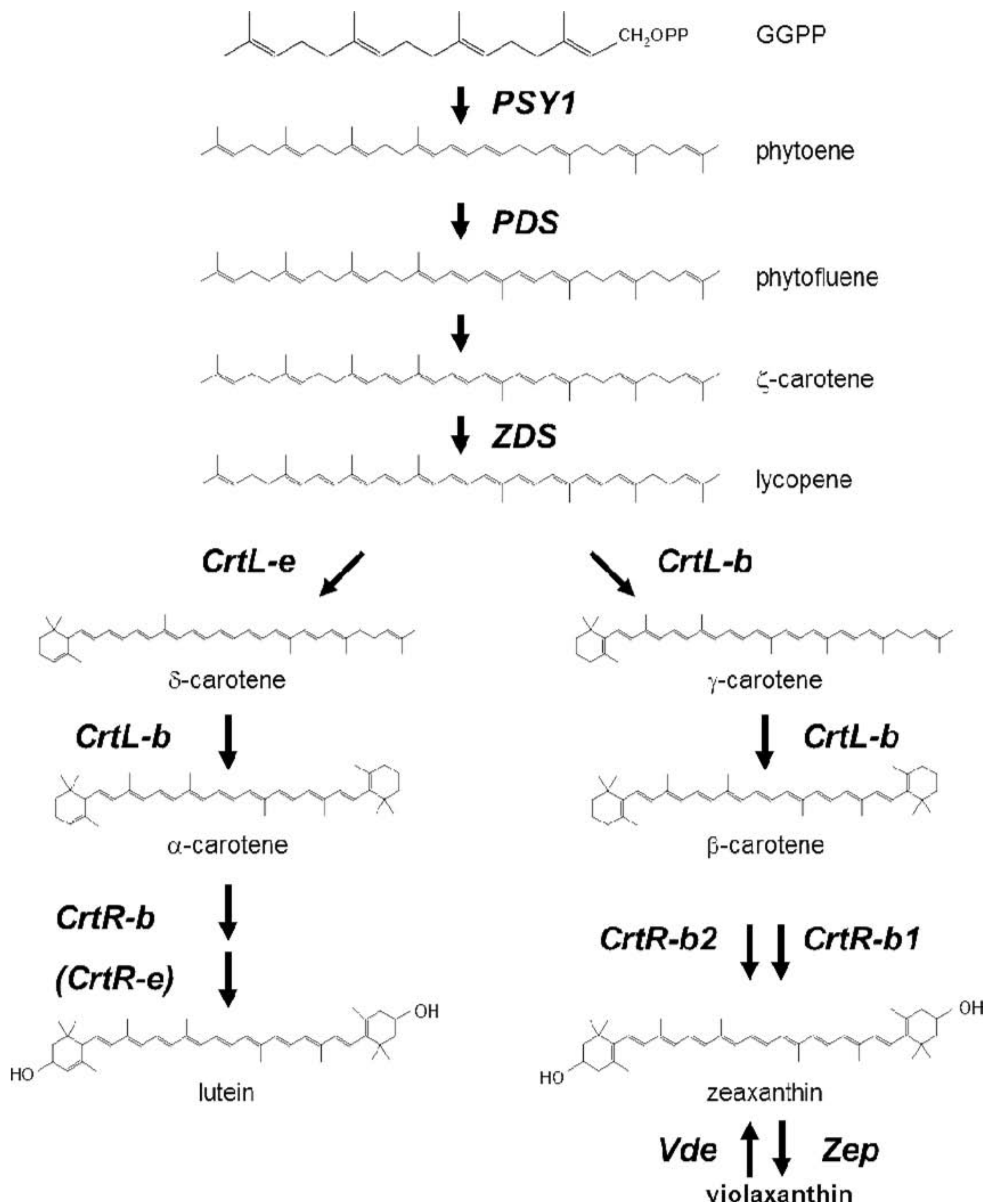

Figure 1. Carotenoid biosynthetic pathway. *CrtL-b*, lycopene *β*-cyclase; *CrtL-e*, lycopene *ε*-cyclase; *CrtR-b*, *β*-ring hydroxylase; *CrtR-e*, *ε*-ring hydroxylase; GGPP, geranylgeranyl pyrophosphate; *PDS*, phytoene desaturase; *PSY*, phytoene synthase; *Vde*, violaxanthin deepoxidase; *ZDS*, *ζ*-carotene desaturase; *Zep*, zeaxanthin epoxidase.

PHOTOMORPHOGENESIS 1 (COP1), and the bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5).[8] UVR8 itself has been recently identified to function as UV-B photoreceptor regulating *HY5* expression.[16] Recent data indicate that UVR8 dimers perceive UV-B probably by a tryptophan-base perception mechanism and undergo an instantaneous monomerization followed by the interaction with the multifunctional COP1 protein, which is necessary to relay the UV-B signal.[16] COP1, which is known as a suppressor of de-etiolation in darkness, acts as a positive regulator of responses to low levels of UV-B, being required for HY5 transcriptional activation in response to UV-B.[8,17]

Homologues of COP1 and HY5 have been described in tomato (LeCOP1LIKE and LeHY5) as negative and positive regulators of tomato fruit pigmentation.[18] Molecular characterization of *hp-1* and *hp-2* tomato mutants, which exhibit exaggerated light responsiveness, revealed that *HP1* and *HP2* genes encode tomato homologues of the light signal transduction proteins DDB1a and DET1, respectively, involved in the *Arabidopsis* CDD complex (COP10-DDB1a-DET1[18,19]). In *Arabidopsis*, CULLIN4 (CUL4) has been demonstrated to directly interact with either the CDD complex or the COP1-SUPPRESSOR OF PHYA (SPA) complexes in the absence of the CDD complex and to form a heterogeneous group of E3 ligases that regulate multiple aspects of the light regulation.[20]

Recent studies demonstrated that in tomato, HP1/LeDDB1 and HP2/LeDET1 are essential components of CUL4-based E3 ligase complex, in which LeDDB1 is associated with tomato CUL4 and DET1.[21]

The present work is addressed to investigate the molecular events underlying carotenoid accumulation in flesh and peel of wild-type and *hp-1* fruits during ripening in the presence or absence of UV-B light. Our analyses revealed that solar UV-B radiation exerts a negative modulation mostly on the carotenoid biosynthetic genes encoding enzymes downstream of lycopene synthesis in both flesh and peel of wild-type fruits. Consistent with this, in wild-type, UV-B depletion determined a more efficient conversion of phytoene/phytofluene and lycopene to lutein and β-carotene. In contrast to wild-type, UV-B depletion did not greatly affect carotenoid accumulation in *hp-1* and generally determined minor differences in gene expression between control and UV-B-depleted conditions.

■ MATERIALS AND METHODS

Plant Materials and Growth Conditions. Seeds of the *hp-1* tomato (*Lycopersicon esculentum*) mutant and the near isogenic corresponding wild-type cv. Money Maker, obtained from the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/), were sown in plug trays with a peat/perlite (3:1 v/v) medium. Seedlings were transplanted into pots containing a peat/pumice/commercial soil mixture (1:1:1 v/v). About 45 days after sowing, when the seedlings had reached the stage of four true leaves, the tomato plants were again transplanted into bigger pots (26 cm diameter) containing the same medium and 2 g of L-1 of 28N-8P-16K controlled-release fertilizer and irrigated daily. Pots were placed randomly under two different tunnels to avoid the border effect. Tunnels were located in an open field, and their longest sides were directed along the west−east direction to allow for uniform exposure of plants to sunlight. The experiment was carried out in the spring and summer of 2010 at Pisa, Italy (43°43′N, 10°23′E). The first tunnel was covered by polyethylene film transparent to the whole sunlight spectrum while the second tunnel was covered by polyethylene film stabilized with the UV-B absorber benzophenone (Agraria Di Vita, Pistoia, Italy) as previously described.[9]

Healthy fruits of comparable dimension were carefully harvested from sunny branches at the mature green (MG, 35−40 DPA), turning (TU, breaker +3), and red ripe (RR, breaker +7) stages, in accordance with the procedure reported by Grierson and Kader.[22] Peel and flesh were separated, frozen by liquid nitrogen, and stored at −80 °C until analysis.

Extraction and Quantification of Carotenoids. Carotenoids extraction followed the method reported by Tonucci et al.[23] with minor modifications. Peel and flesh were ground with liquid nitrogen and successively homogenized in an Ultra-Turrax with tetrahydrofuran (THF) stabilized with 0.01% butylated hydroxytoluene (BHT), 10% (P/P) magnesium carbonate, and 10% (P/P) Celite (Celite Filter Cel, Fluka). The combined extracts volume was reduced by two-thirds under vacuum at 35 °C on a rotary evaporator and then were portioned into dichloromethane (25 mL) and NaCl-saturated water (15 mL) in a separating funnel. The remaining carotenoids in the water layer were carefully re-extracted with dichloromethane until they completely passed into the organic layers. Another vacuum-drying was applied to reduce the organic layer to a small residue that was afterward filtered through a 0.22 μm filter and injected into the column (Phenomenex Prodigy LC-18 ODS, 250 × 4.6 mm, 5 μm with guard column Phenomenex AJO-4287 C-18 ODS). The analytical separation of carotenoid extracts was performed by Spectra SYSTEM P4000 HPLC equipped with a UV 6000 LP photodiode array detector (Thermo Fisher Scientific, Waltham, MA). Gradient elution was performed using solvent A (acetonitrile/hexane/methanol/dichloromethane, 4:2:2:2) and solvent B (acetonitrile) as mobile phases at a flow rate of 0.8 mL/min. The gradient program was as follows: 0−20 min, 100% A; 20−38 min, 60% A and 40% B; 38−45 min, 100% A.

Identification of the peaks in the HPLC chromatogram was made by comparison of UV−vis spectra and retention times of eluted compounds with pure standard for lycopene, β-carotene, and lutein at 450 nm, while phytofluene and phytoene peaks were identified respectively at 350 at 290 nm. Furthermore, the quantification of carotenoids was made, comparing their respective peak areas with the ones of standards at known concentrations, established by the molar extinction coefficient in acetone reported in the literature and corrected by the molar extinction coefficient relative at each compound. In the case of phytofluene and phytoene, the lack of standards compelled us to quantify their content as β-carotene equivalents.

RNA Isolation and Gene Expression Analyses. RNA was isolated from samples of 3 g of flesh and peel from wild-type and *hp-1* fruits as previously described.[9] About 5 μg of total RNA was reverse-transcribed using the RT Superscript™ II (Invitrogen, Carlsbad, CA) and, after first strand cDNA synthesis using an oligo dT as previously described,[24] the samples were diluted 50 fold and used as templates for real time RT-PCR analysis or semiquantitative RT-PCR.

Quantitative real time RT-PCR analysis was performed for the *PSY1*, *PDS*, and *ZDS* biosynthetic genes using SYBR Green with the Cfx96TMBioRad Real Time system in a final volume of 20 μL containing 5 μL of 50-fold diluted cDNA, 0.2−0.4 μM of each primer, and 10 μL of 2X SOS Fast EVA-Green Supermix (BioRad Laboratories, Hercules, CA). Oligonucleotides used as primers are indicated in Table 1. As a reference for normalization, we used the *LeEF1* gene, encoding the tomato ELONGATION FACTOR 1-α, because of its high and stable expression in mature tomato fruit[25] by using primers LeEF1-F4 and LeEF1-R3. Relative quantification was analyzed using Cfx Manager Software version 1.6 BioRad Laboratories). The protocol used was as follows: 95 °C for 2 min, 55 cycles of 95 °C for 15 s, and 60 °C for 30 s. A melt curve analysis was performed following every run to ensure a single amplified product for each reaction. Relative quantification of the target RNA expression level was performed using the comparative Ct method (UserBulletin 2, ABI PRISM7700 Sequence Detection System, Dec 1997; Perkin-Elmer Applied Biosystems) in which the differences in the Ct (threshold cycle) for the target RNA and endogenous control RNA, called ΔCt, were calculated to normalize for the differences in the total amount of cDNA present in each reaction and the efficiency of the reverse transcription. Finally, the target RNA expression level was obtained from the equation $2^{-\Delta\Delta Ct}$ and expressed relative to a calibrator (wild-type flesh of fruits at MG stage grown under control

Table 1. Primer Sets Used for Quantitative Real Time and RT-PCR Analysis

gene	primer name	sequences (5′ → 3′)
PSY1	PSY1-RT-F1	GAAAGGCGTGACAGAATTGA
	PSY1-RT-R1	TGCTCTCTTTGTGAAGTTGTTG
PDS	PDS-RT-F1	TGTTGGACGTAGCCAAAAG
	PDS-RT-R1	GCTTCACCTCGCACTCTTCT
ZDS	ZDS-RT-F1	TGCGTAAAAAGATCACTGCTG
	ZDS-RT-R1	TTGTGCGATGCCTAACTGA
CrtL-b	LeLCYB-F2	TCATGAGGAATCGAAATCCA
	LeLCYB-R2	AGTGGACCACCCATTGGTAT
CrtL-e	LeLCYE-F2	CATTTTCTGCACGAGGAGTT
	LeLCYE-R2	GGGTGTTCAATCGTAGCATC
CrtRb1	LeCrtRb1-F4	TGTGGCATGCTTCACTATGG
	LeCrtRb1-R4	TCAAAAATCACCCTTCCTCA
CrtRb2	LeCrtRb2-F1	CCTATTGCCAACGTGCCTTA
	LeCrtRb2-R1	TGTTTTGAATTATCGTTCTTCTGG
EF1	LeEF1-F3	TTGAGGCTCTTGACCAGATT
	LeEF1-F4	GTTGGTCGTGTTGAAATGG
	LeEF1-R3	AACATTGTCACCAGGGAGTG

conditions). Standard errors of Ct values were obtained from measurements performed in triplicate.

Semiquantitative RT-PCR analysis was performed for the *CrtL-b*, *CrtL-e*, *CrtRb1*, and *CrtRb2* biosynthetic genes using primers indicated in Table 1. As a control of cDNA concentration, we used primers specific for the *LeEF1* gene[25] (LeEF1-F3 and LeEF1-R3, Table 1). The amplifications were carried out within linear ranges (25 cycles for *CrtL-b* and *CrtRb1* and 30 cycles for *CrtL-e* and *CrtRb2*). Amplifications were performed on three replicates with similar results. The PCR products were transferred onto nylon membranes positively charged (Roche Diagnostics GmbH, Mannheim, Germany) and hybridized with gene-specific probes labeled using the DIG-High Prime kit (Roche). Probes were obtained by PCR using the four set of gene-specific primers reported in Table 1 and verified by cloning and sequencing. ImageJ (http://rsb.info.nih.gov/ij/index.html) was used to compare the density of bands on RT-PCR blots and normalize them compared to the *LeEF1* gene expression.

Statistical Analysis. Values shown in the figures are means of three replicates ± SE. At any ripening stage, carotenoid and RNA were extracted from peel and flesh collected from three groups of fruits each consisting of 15 berries. The effects of UV-B radiation, ripening stage, and their interactions on carotenoid levels were evaluated by two-way ANOVA and separation of means was performed by Fisher's least significant difference (LSD) test at the 0.05 significance level.

RESULTS

Carotenoid Content during Ripening in Flesh and Peel of Tomato Fruits. At the TU stage both the precursors phytoene and phytofluene and the colored carotenoids were more concentrated in control *hp-1* flesh than in the wild-type, while in RR fruits phytoene, phytophluene, and lycopene reached similar values in the two genotypes (Figure 2).

In wild-type and *hp-1* flesh, phytoene, phytofluene, and lycopene, which were virtually absent in MG fruits, started to accumulate from the TU stage and reached their maximum concentration in RR fruits (6-, 5-, and 9-fold higher than TU, in wild type, and 2-, 1.5-, and 1.25-fold higher than TU, in *hp-1*, respectively, Figure 2A–F). As ripening proceeded, the *β*-carotene concentration progressively increased in both genotypes (Figure 2G,H), whereas lutein showed only minor ripening-dependent variations (Figure 2I,J).

The content of colored carotenoids of *hp-1* peel in control conditions was always higher than in wild-type, apart from lycopene at the RR stage, whose level was similar in wild-type and *hp-1* (Figure 3E–J). However, differently from the behavior observed in the flesh, in RR, peel phytoene and phytofluene accumulated much more in wild-type than in *hp-1* fruits (Figure 3A–D).

In wild-type peel, phytoene, phytofluene, and lycopene behaved as in the flesh, starting to accumulate at the TU stage and further increased at full maturity (about 4.5-, 4-, and 6-fold higher than TU, in wild type, respectively, Figure 3A,C,E). Also in *hp-1* peel, the concentration of phytoene, phytofluene, and lycopene followed the same trend of accumulation (Figure 3B,D,F).

β-Carotene was found to increase in wild-type TU peel (3.5-fold), but no further accumulation was observed in fully ripened fruits (Figure 3G), while it progressively increased during the ripening process in *hp-1* peel (Figure 3H). Lutein showed an opposite behavior in the peel of the two genotypes, increasing in wild-type and decreasing in *hp-1* as ripening proceeded (Figure 3I,J).

Effect of UV-B Depletion on Tomato Fruit Carotenoid Content. In wild-type flesh, UV-B depletion determined a reduction in the final content (RR stage) of phytoene,

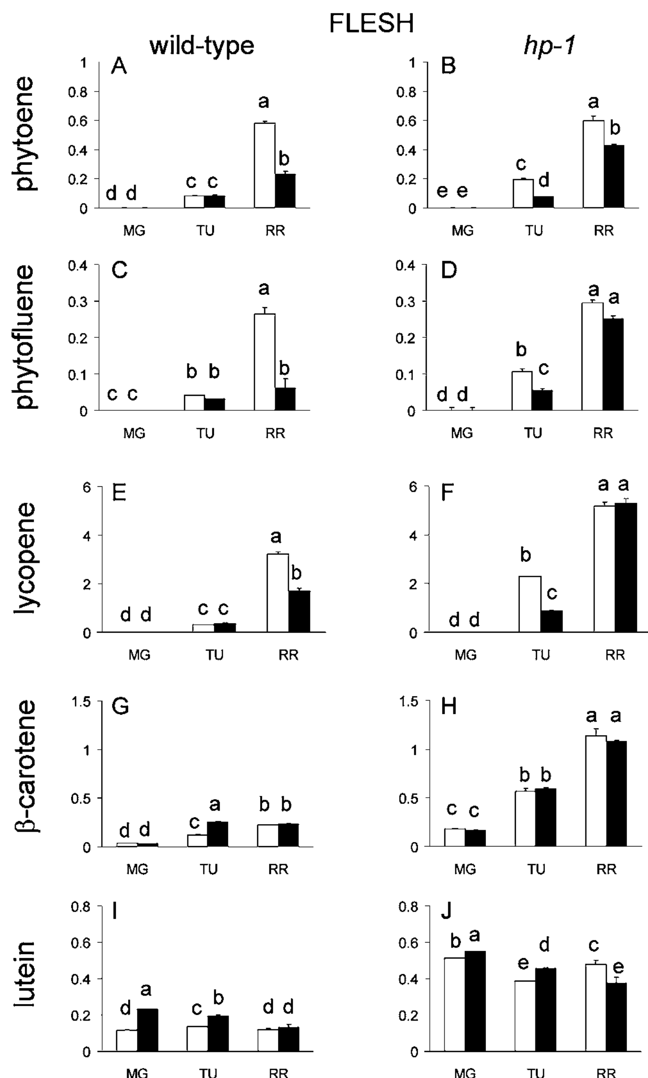

Figure 2. Carotenoid content in flesh of wild-type (A–I) and *hp-1* (B–J) fruits grown in presence (white columns) or absence of UV-B (black columns). Data are expressed as mg/100 g of fresh weight. Statistically significant differences were evaluated by two-way ANOVA ($P < 0.05$), and for each parameter, different letters indicate significantly different values according to LSD test. Abbreviations: MG, mature green; TU, turning; RR, red ripe.

phytofluene, and lycopene (−60%, −77%, and −47%, respectively, Figure 2A,C,E) whereas *β*-carotene and lutein were only transiently increased at the MG (lutein) and/or TU stage (*β*-carotene and lutein), but no difference at the RR stage was observed (Figure 2G,I).

UV-B depletion induced a significant decrease in phytoene concentration of *hp-1* flesh at both the TU (−60%) and RR (−28%, Figure 2B) stages. Despite a transient decrease at the TU stage, no significant change in phytofluene and lycopene final accumulation in *hp-1* flesh occurred as a consequence of UV-B depletion (Figure 2D,F). *β*-Carotene was not influenced by the presence–absence of UV-B radiation at any ripening stage (Figure 2H), while lutein concentration, after an initial slight increase at the MG and TU stage, was negatively affected by UV-B depletion (−28%, Figure 2J).

In wild-type peel, phytoene and lycopene content was slightly reduced in UV-B-depleted RR fruits (−14% and −10%, respectively, in comparison to control peel, Figure 3A,C,E),

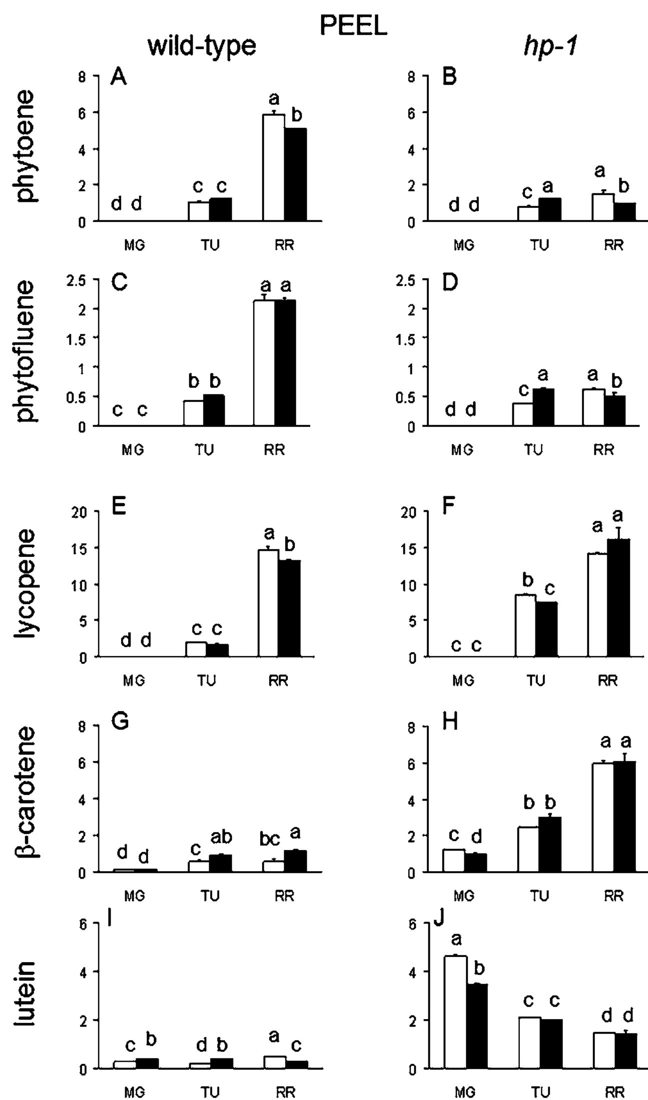

Figure 3. Carotenoid content in peel of wild-type (A–I) and *hp-1* (B–J) fruits grown in the presence (white columns) or absence of UV-B (black columns). Data are expressed as mg/100 g of fresh weight. Statistically significant differences were evaluated by two-way ANOVA ($P < 0.05$), and for each parameter, different letters indicate significantly different values according to LSD test. Abbreviations: MG, mature green; TU, turning; RR, red ripe.

whereas β-carotene was increased at the two last ripening stages (+65% and about 2-fold at the TU and RR stages, respectively, Figure 3G). In agreement with what occurred in the flesh, in wild-type peel, lutein content was transiently increased at MG (+43%) and TU (+92%) stages but then decreased in fully ripened fruits (−42%, Figure 3I).

In *hp-1* peel, despite a transient increase at the TU stage, the final concentration of phytoene and phytofluene was reduced by UV-B shielding (−37% and −19%, respectively, Figure 3B,D). No significant differences between control and UV-B-depleted fruits were observed at the RR stage as far as the other carotenoids are concerned, even if slight transient decreases occurred at the earlier ripening stages (Figure 3F,H,J).

Expression Patterns of the Carotenoid Biosynthetic Genes in Tomato Flesh and Peel during Ripening. Under control conditions, the expression level of carotenoid biosynthetic genes of wild-type fruits was generally higher in peel than in flesh (Figure 4A). In both flesh and peel of wild-type fruits, the *PSY1, PDS*, and *ZDS* genes, encoding enzymes upstream of lycopene synthesis, showed a similar expression pattern, because their transcript level increased during ripening (Figure 4A). On the contrary, the transcript level of the *CrtL-b* and *CrtL-e* genes, that encode lycopene cyclases, in wild-type flesh showed a peak of activation at the MG stage and then decreased in the subsequent stages, whereas in wild-type peel, both *CrtL-b* and *CrtL-e* showed an opposite expression pattern, because they were up-regulated during the late phases of ripening (Figure 4A). The gene encoding for the fruit-specific β-ring carotene hydroxylase, *CrtRb2,* was found to be expressed in both wild-type tissues at a relatively constant level during ripening, whereas the expression pattern of *CrtRb1* decreased at maturity in both tissues, but showed a higher expression level already at the MG stage in wild-type peel compared to wild-type flesh (TU stage) (Figure 4A). Furthermore, considering the number of PCR cycles, in both peel and flesh, the *CrtRb2* and *CrtL-e* genes displayed a lower transcript level as compared to *CrtRb1* and *CrtL-b*, respectively (see Materials and Methods).

Differences between gene expression levels of *hp-1* peel and flesh were less evident than in wild-type, because of the stimulating effect of *hp-1* mutation on the expression of all genes analyzed in comparison to wild-type fruits, particularly evident in *hp-1* flesh (Figure 4B). Similarly to wild-type, also in *hp-1* fruits the transcript levels of *PSY1*, *PDS*, and *ZDS* increased during ripening in both tissues, with *PSY1* showing activation already at the MG stage (Figure 4B).

The expression level of *CrtL-b* was found to decrease in the peel of fully ripened *hp-1* fruits (RR stage), while minor changes were observed in the flesh (Figure 4B). Similarly to wild-type, *CrtL-e* gene expression decreased in *hp-1* flesh of TU and RR fruits, while in the peel, after an initial decrease at the TU stage, gene transcription was reactivated in fully ripened fruits (Figure 4B).

As observed in the wild-type, also in *hp-1* fruits, considering the different number of PCR cycles, *CrtRb1* was expressed at levels higher than that of *CrtRb2* (see Materials and Methods). In flesh, the *CrtRb1* gene showed a reduced expression level at full maturity whereas in peel, after a decrease at the TU stage, a late reactivation was observed in RR fruits (Figure 4B). Minor ripening-dependent variations in gene expression were displayed by *CrtRb2* in *hp-1* fruits, as already observed in the wild type (Figure 4B).

Effect of UV-B Depletion on the Expression of the Carotenoid Biosynthetic Genes. Under UV-B depletion, in flesh of wild-type fruits, the *PSY1* and *ZDS* were only slightly down- and up-regulated, respectively, as compared to control flesh, whereas no appreciable difference was observed for *PDS* except for a slight increase at the TU stage (Figure 4A). On the contrary, both the *CrtL-b* and *CrtL-e* genes, which showed high transcript level at the MG stage and a lower transcript level in the subsequent stages in control fruits, were expressed at high level under UV-B depletion in all ripening stages considered (Figure 4A). *CrtR-b1* expression was stimulated by UV-B deprivation at any ripening stage, while *CrtR-b2* expression was repressed and reached the highest level at RR stage in comparison to control fruits (Figure 4A).

In wild-type peel of UV-B-depleted fruits, the *PSY1* gene showed a slightly reduced expression level at RR stage with the highest expression at the TU stage compared to control conditions (Figure 4A). *PDS* gene expression was substantially

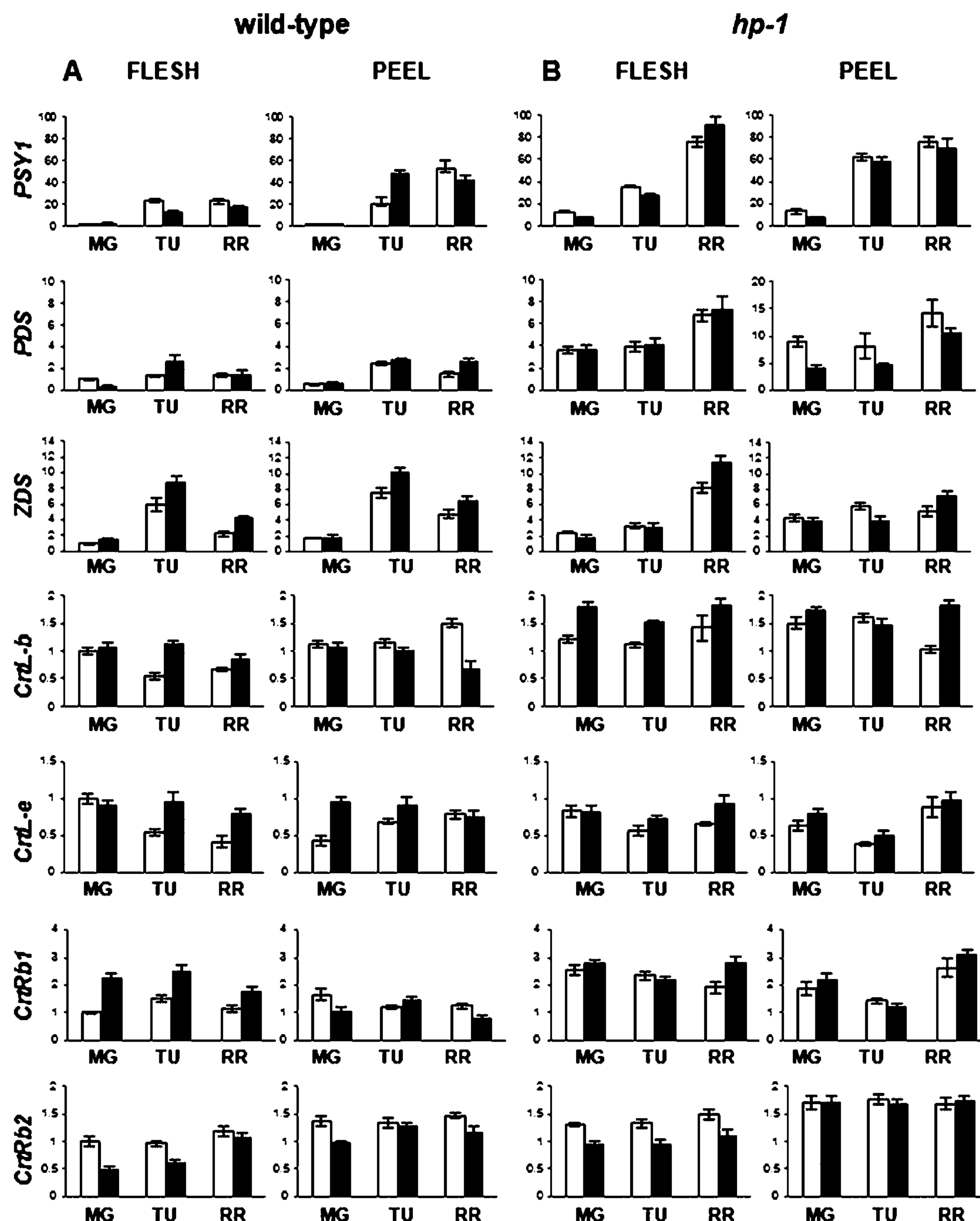

Figure 4. Relative expression levels of carotenoid biosynthetic genes in flesh and peel of wild-type (A) and *hp-1* (B) fruits grown in the presence (white columns) or absence (black columns) of UV-B. Transcript levels of *PSY1*, *PDS*, and *ZDS* were determined by quantitative real time RT-PCR using elongation factor1 (*LeEF1*) as endogenous control. Transcript levels of *CrtL-b*, *CrtL-e*, *CrtRb1*, *CrtRb2* were determined by RT-PCR, and density of bands on blots were normalized to the *LeEF1* gene expression by using ImageJ (see Materials and Methods). Bars represent standard errors of measurements performed in triplicate. *PSY1*, phytoene synthase1; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *CrtL-b*, lycopene cyclase-*β*; *CrtL-e*, lycopene cyclase-*ε*; *CrtRb1*, b-ring carotene hyroxylase1; *CrtRb2*, b-ring carotene hyroxylase2. MG, mature green; TU, turning; RR, red ripe.

unaffected by UV-B shielding, whereas the *ZDS* transcript level was enhanced by UV-B depletion at the TU stage (Figure 4A).

Interestingly, *CrtL-b* and *CrtL-e* displayed an opposite behavior: the first was slightly repressed by UV-B depletion and the second was activated and reached the highest level of expression already at the MG stage. On the other hand, *CrtR-b1* and *CrtR-b2* expression were only faintly negatively influenced by UV-B (Figure 4A).

In contrast to wild-type, the transcript analysis of *hp-1* revealed, in general, minor differences between control and UV-B-depleted conditions in both tissues (Figure 4). In particular, no effect of UV-B deprivation was evident on *PSY1* gene expression, while *PDS* and *ZDS* showed a lower transcript accumulation in UV-B-depleted peel (Figure 4B). In *hp-1* fruits ripened under the UV-B-shielded tunnel, *CrtL-b* expression was high also at the RR stage in peel. Similarly to wild-type peel under UV-B depletion, the expression of *Crtl-e* was already high at the MG stage, whereas only minor UVB-dependent changes were observed in *CrtR-b1* and no effect on *CrtRb2* expression level (Figure 4B).

■ DISCUSSION

The aim of this work was to investigate whether and how shielding of UV-B radiation may influence carotenoid metabolism in tomato fruit flesh and peel during ripening and to understand the molecular events underlying the phenotypic differences between the two genotypes and their response to UV-B radiation. The carotenoid metabolism is indeed a highly regulated process requiring the coordinated interplay between endogenous and environmental factors.

At any ripening stage, peel was found to accumulate higher amounts of carotenoids than flesh, in accordance with the observation of Toor and Savage[26] and similarly to what has been reported for flavonoids.[9,27] The tissue-dependent accumulation of carotenoids was coherent with the higher expression level of carotenoid biosynthetic genes detected at the peel level, as already reported for flavonoid biosynthesis.[9,27,28]

In wild-type flesh, the coordinated increased expression, during ripening, of *PSY1, PDS*, and partly of *ZDS* genes, encoding enzymes upstream of lycopene synthesis, together with the down-regulation of the *CrtL-b* and *CrtL-e* genes, that encode for lycopene cyclases, may account for lycopene accumulation at the RR stage, similarly to what previously reported.[29] A lower accumulation of β-carotene was observed, probably as a consequence of the reduced expression of *CrtL-b*.

The up-regulation of both *CrtL-b* and *CrtL-e* transcription in wild-type peel observed during the late phases of ripening is notably, because it is a first evidence that genes for lycopene cyclases follow a different kind of regulation in wild-type tomato surface tissue as compared to flesh. In wild-type peel, at the RR stage, despite the enhanced transcript level of *CrtL-b* compared to flesh, β-carotene, differently from lutein, did not accumulate over the concentration observed at TU stage. This result, together with the ripening-dependent increase in *CrtL-e* transcription, suggests a shift of the metabolic flux downstream of lycopene toward lutein rather than β-carotene.

The pattern of accumulation of carotenoids and of their precursors in *hp-1* compared to wild-type suggests that, in both *hp-1* flesh and peel, phytoene and phytofluene were more efficiently converted to lycopene, mainly in the flesh, and β-carotene in comparison to wild-type. The higher transcript level of *PSY1*, *PDS*, *ZDS*, and *CrtL-b* genes in *hp-1* peel, with respect to wild-type fruits, can account for the higher β-carotene and consequently the lower phytoene and phytofluene content in this tissue. On the other hand, the higher accumulation of lycopene and β-carotene observed in *hp-1* flesh, compared to wild-type, is not accompanied by a reduced content of the precursors phytoene and phytofluene, suggesting that, in this tissue, lycopene and β-carotene biosynthesis, although more effective than in the wild-type, was less efficient than in the peel. At the flesh level, the gene expression pattern of *CrtL-b* and *CrtL-e*, which did not change or decreased during ripening, respectively, may justify the reduced lutein accumulation in TU and RR fruits. Similarly, in *hp-1* peel, the down-regulation of *CrtL-b* and *CrtL-e* may explain the ripening-dependent reduction in lutein concentration, despite the late reactivation of *CrtL-e* gene transcription.

The molecular basis of the higher transcript level detected in *hp-1* fruits is still unknown, although differential expression of regulatory genes of the carotenoid biosynthetic pathway is likely to be involved.[30] In a previous work, we observed a higher and anticipated expression at the MG stage of *LeHY5* and *LeCOP1* in control *hp-1* fruits in comparison to wild type.[9] Their expression could account for the higher expression of carotenoid biosynthetic genes detected in *hp-1* fruits. An analysis of HY5 binding sites indeed demonstrated that in *Arabidopsis* phytoene synthase, lycopene cyclase-ε and β-carotene hydroxylase genes are putative targets of HY5.[31] It could be of interest to verify whether similar cis-elements are also present in promoters of tomato carotenoid genes.

The observation that the higher carotenoid content in *hp-1* is due to a higher expression of carotenoid biosynthetic genes is in contrast to a previous work where the enhanced carotenoid content in this genotype was related to the increased number of plastids and PSY1 activity but not with an increase in expression of the *PSY1* gene.[32] This discrepancy could depend on the different genetic backgrounds of the *hp-1* mutation analyzed, namely cv. Ailsa Craig in the paper of Cookson and co-workers[32] and cv. Money Maker in our case. A comparison of *hp-1* mutant in Money Maker and Ailsa Craig genetic background indeed showed interesting differences in carotenoid content and profile of the whole fruit. In particular, total carotenoids of red ripe fruits were less concentrated in Money Maker background (Ranieri et al., unpublished) than in the Ailsa Craig one.[13,32] Moreover, while Cookson[32] found a similar proportion of the individual carotenoids between *hp-1* and Ailsa Craig, and Long[33] only slight differences in *hp-1* carotenoid proportions as compared with Ailsa Craig, our data show a higher proportion of β-carotene (and to a lesser extent of lutein) in both flesh and peel of *hp-1* as compared with Money Maker. An influence of the genetic background on both carotenoid content and proportion of *hp-1* mutant therefore seems to occur, and its role on the different behavior shown by the PSY1 gene cannot be excluded as well. This hypothesis should however be confirmed by a detailed analysis of PSY1 gene transcription in fruits of *hp-1* mutant in the Money Maker and Ailsa Craig genetic background (and respective wild types) at different ripening stages.

Similarly to what has been previously reported by Becatti,[14] in the present study, carotenoid content was differently influenced by UV-B shielding depending on genotype, tissue, and ripening stage. The reduction in phytoene concentration in both tissues of RR wild-type fruits following UV-B depletion can be attributed to the minor reduction in *PSY1* gene transcription. Because phytoene synthase is considered the rate-limiting step in carotenoid synthesis in ripening tomato fruit,[34] such a reduction could be responsible, at least partially, for the downstream decrease in phytofluene (flesh) and lycopene (both tissues) concentration, despite the unchanged gene expression of *PDS* and *ZDS*, the latter even showing an enhanced transcription at the TU stage in both flesh and peel of UV-B-shielded fruits.

Gene expression analysis revealed that, in wild-type flesh, UV-B radiation plays a negative modulation on carotenoid biosynthetic genes downstream of *ZDS*, namely on *CrtL-b* and *CrtL-e*. The higher transcription of these genes induced by UV-B shielding, beside inducing an increased accumulation of β-carotene and lutein at TU stage, may explain the lower lycopene levels detected in fully ripened fruits. The results obtained suggest that the down-regulation of *CrtL-b* and *CrtL-e* during tomato ripening and the subsequent accumulation of lycopene are determined at least in part by UV-B light.

In wild-type peel, the observed increase in β-carotene concentration following UV-B depletion cannot be attributed to enhanced *CrtL-b* gene expression, which was instead found

to decrease, and neither to reduced transcription of *CrtR-b1* and *CrtRb2*, which were only slightly influenced by the different light regimens. Lycopene cyclization to β-carotene appears to be mostly under transcriptional regulation as demonstrated by the coordinated changes in gene expression, enzyme activity, and metabolite accumulation.[5,35] Although we cannot exclude that the absence of UV-B radiation may somehow induce post-transcriptional changes and/or alterations in enzyme activity, the lower β-carotene concentration observed in control wild-type peel despite the higher *CrtL*-b transcription may depend on UV-B-induced oxidation of β-carotene. Studying lipoxygenase-catalyzed oxidation of carotenoid extracts from ripe tomato fruits, Biacs and Daood[36] found that β-carotene was the most sensitive pigment. Moreover, this carotenoid is a strong absorber of UV light[37] which induces its oxidation, as demonstrated by in vitro UV-B irradiation of β-carotene test solution.[38]

The opposite gene expression pattern shown by both *CrtL-b* and *CrtL-e* in control and UV-B-depleted peel of wild-type fruits indicates a strict control played by UV-B radiation on these genes. Recently, Gruber[39] demonstrated that in *Arabidopsis*, REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2 act as potent repressors of UV-B signaling by direct interaction with UVR8. Both *RUP1* and *RUP2* are activated in response to UV-B light in a COP1-, HY5-, and UVR8-dependent manner. *RUP1* is the closest homologue of tomato *LeCOP1LIKE*, whose repression results in plants with exaggerated photomorphogenesis and high fruit carotenoid concentration.[18] We previously observed that *LeCOP1LIKE* was differently expressed in wild-type flesh and peel, showing the highest expression at the RR stage in wild-type flesh and at the MG stage in wild-type peel.[9] Interestingly, this expression pattern suggests that LeCOP1LIKE may act as negative repressor of *CrtL-b* and *CrtL-e*, because their transcripts increased at the MG stage in wild-type flesh and at the RR stage in wild-type peel. Consistent with this hypothesis, under UV-B depletion the expression of LeCOP1-LIKE is reduced at RR (flesh) and MG stages (peel), whereas the expression of both *CrtL-b* and *CrtL-e* is not reduced after the MG stage in flesh and it is anticipated at the MG stage in peel.

Interestingly, the different response of carotenoid biosynthetic genes to UV-B radiation in the two tissues is similar to that reported for flavonoid biosynthetic genes.[9] However, note that the activation of flavonoid gene expression in both tissues of wild-type cv. Money Maker fruits was generally delayed by UV-B depletion[9] and not anticipated as for carotenoid biosynthetic genes, indicating that UV-B radiation has a positive regulatory role on flavonoid but a negative one on carotenoid gene expression. Further studies need to be addressed to unravel the regulatory mechanisms underlying these differences.

Carotenoid concentration of *hp-1* fruits was less influenced by UV-B depletion than wild-type ones. This observation was in accordance with the results previously obtained in the entire fruit which, especially at RR stage, showed minor variations in terms of carotenoid content when ripened with or without solar UV-B light, while the commercial hybrid cultivar DRW 5981 displayed lower lycopene content in UV-B-depleted fruits.[13]

According to data on carotenoid and precursor concentration, *hp-1* gene expression generally showed minor differences between control and UV-B-depleted conditions. In UV-B-depleted peel, the lower transcript accumulation of the *PDS* and *ZDS* gene may account for the transient accumulation of the precursors phytoene and phytofluene and the concomitant transient reduction of lycopene levels at the TU stage. On the other hand, the reactivation of *CrtL-b* at the RR stage of *hp-1* peel may account for the reduction of phytoene and phytofluene at this stage, despite no significant difference in β-carotene level. Minor differences were also observed in *hp-1* flesh in agreement with the observation that UV-B depletion generally did not affect carotenoid accumulation in *hp-1* flesh and were similar to data reported for the effect of UV-B on flavonoid biosynthesis and accumulation in *hp-1*.[9]

In conclusion, our analyses revealed that in wild-type fruits the carotenoid content was higher in peel than in flesh and that this difference was determined by a general up-regulation of carotenoid biosynthetic genes in wild-type peel compared to flesh, including *CrtL-b* and *CrtL-e*, which are known to be down-regulated during ripening in flesh. We also observed that, in both *hp-1* peel and flesh, phytoene and phytofluene were more efficiently converted to lycopene and β-carotene than in wild-type, due to the higher transcript level of *PSY1*, *PDS*, *ZDS*, and *CrtL-b* genes in *hp-1* compared to wild-type. The data obtained from UV-B-depleted fruits showed that in wild-type fruits, solar UV-B radiation exerts a negative modulation mostly on the carotenoid biosynthetic genes encoding enzymes downstream of lycopene synthesis, mainly in the flesh, suggesting that the down-regulation of *CrtL-b* and *CrtL-e* and the subsequent accumulation of lycopene during tomato ripening are determined at least in part by UV-B light. Consistent with this, in wild type, UV-B depletion determined a more efficient conversion of phytoene/phytofluene and lycopene to lutein and β-carotene content. In contrast to wild type, the transcript analysis of *hp-1* revealed, in general, minor differences between control and UV-B-depleted conditions in agreement with the observation that UV-B depletion generally did not affect carotenoid accumulation in *hp-1*.

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Notes

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■ ABBREVIATIONS USED

UV-B, ultraviolet-B; *hp-1*, high pigment-1; BHT, butylated hydroxytoluene; DPA, days postanthesis; LSD, least significant difference; THF, tetrahydrofuran; MG, mature green; TU, turning; RR, red ripe *PSY1*, phytoene synthase1; *PDS*, phytoene

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desaturase; *ZDS*, ζ-carotene desaturase; *CrtL-b*, lycopene cyclase-β; *CrtL-e*, lycopene cyclase-ε; *CrtRb1*, b-ring carotene hyroxylase1; *CrtRb2*, b-ring carotene hyroxylase2.

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